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Isolation, bio-chemical and molecular characterization of *R. solanacearum*, causal agent of bacterial wilt of tomato

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Abstract
Bacterial wilt of tomato caused by *Ralstonia solanacearum* is one of the most destructive diseases of tomato in major tomato growing regions of our country. A research work was undertaken characterize the causal agent of bacterial wilt of tomato by bio-chemical and molecular characterization. *R. solanacearum* colonies on Nutrient Agar (NA) medium were dirty white, smooth, round and elevated. *R. solanacearum* colonies grown on TZC media were white with pink centers, while colonies grown on Casamino Peptone Glucose (CPG) medium were white or cream-colored, irregularly shaped, fluidal, and opaque. *R. solanacearum* was Gram negative, catalase positive, KOH positive and also gave positive reaction for starch hydrolysis, methyl red test and H2S production but gave negative reaction for gelation liquification. Molecular characterization of pathogen was carried out by using bacterial universal primers and showed amplification at 1500-bp. Sequence analysis reveled that that pathogen was *R. solanacearum*. Under glasshouse conditions, Koch's postulate was used to determine the test bacterium's pathogenicity, and it was shown to be pathogenic.

Keywords: *R. solanacearum*, bacterial wilt, bio-chemical and molecular characterization and Koch's postulate

Introduction
There are more than 200 diseases that can affect tomato plants. Disease-related yield losses might range from 70 to 95 percent. Bacterial wilt is typically the most harmful disease, causing 60–70% yield loss [1]. Rapid and complete wilting is the defining feature of bacterial wilt in normally grown tomato plants [2]. The taxonomy and effective management of any disease-causing bacterium require identification. A combination of microscopic observations, such as the morphology and cell arrangement, Gram staining, cultural (growth) features on cultural medium, and biochemical and physiological characterization are typically used to identify an unknown bacterium when it is isolated in a lab [2]. Although the organism can be identified using molecular technologies, traditional methods paired with them produce the most reliable findings. The purpose of the experiment is to examine the morphological and cultural traits of *R. solanacearum* using different medium and PCR molecular detection. Rapid detection of a plant pathogen enables the application of the necessary controls before the disease spreads further or is introduced. Wilting, stunting, and yellowing of the leaves are the most frequent external symptoms of infected plants [3]. Other symptoms include adventitious roots sprouting in the stems, leaves bent downward exhibiting leaf epinasty, and the observation of narrow dark stripes matching to the infected vascular bundles beneath the epidermis. Although the disease often worsens until the plant completely wilts and collapses, the severity of the symptoms and the rate at which they manifest might vary depending on the host and how aggressive the pathogenic strain is. Internal symptoms that occur most frequently include gradual darkening of vascular tissue, primarily the xylem, in the early stages of infection and sections of the pith and cortex as the disease progresses until full necrosis [3]. On transverse-sectioned stems, slimy, sticky ooze generally develops where the vascular bundles meet [4]. Powerful technologies for the identification and detection of the bacteria in a variety of substrates (plant, seeds, and soil) are needed to maximize the effectiveness of preventive actions. High sensitivity and specificity tools for identifying and detecting microbes have been made possible by DNA-based techniques. For the unambiguous detection of this pathogen in soil, seed, and diseased tomato plant materials, the PCR-based detection approach using a

particular primer for *R. solanacearum* is fast and sensitive [6].

Material and Methods

Isolation of *R. solanacearum* from bacterial wilt affected tomato plant

Tomato plants exhibiting the typical wilting symptoms were collected from field. The oozing test was used to make a preliminary diagnosis of the disease. The bacterium was isolated by dilution plate technique on Casamino Acid peptone agar using TZC (triphenyl tetrazolium chloride) medium after extracting the ooze in sterile distilled water obtained in test tube. Following the removal of the epidermal layer, a sterile surgical blade was used to aseptically cut small sections of the affected vascular tissue. The diseased tissue fragments were surface sterilized in 1% sodium hypochlorite for a minute, and any remaining sodium hypochlorite was rinsed away in two to three rounds of sterile water. After that, the infected tissues were suspended in sterile water that had been drawn from a test tube and had turned turbid due to the release of bacterial cells from the infected tissue. A sterile spreader was used to apply 1000 □l of the serially diluted bacterial suspension to the surface of the solidified sucrose peptone agar medium. 48 hours were spent incubating the inoculated plates at 29±1 ºC. The formation of virulent colonies in well-spaced rows was monitored on the plates [7, 8].

Morphological and biochemical characterization

Colony characters on culture medium

Casamino acid peptone glucose (CPG) medium

By streaking a 10^4 cfu/ml bacterial suspension over CPG medium, the bacterial isolate was then cultured for 24 hours at 27 ºC. The usual (virulent type) colonies are opaque, fluidal, white or cream in colour, and irregularly shaped after incubation [8].

Triphenyl tetrazolium chloride (TTC or TZC) medium

A loopful of flowing ooze containing the bacterium was streaked onto a clean TTC (Triphenyl tetrazolium chloride) agar plate [8] and the plates were incubated at 30 ºC for 48 to 72 hours. The virulent *R. solanacearum* colonies (oddly shaped, white to cream in colour, slimy, with pink colour in the centre) were chosen after incubation. Colonies that were dark red were viewed negatively.

Biochemical characterization

Gram Staining

The Gram's staining reaction were performed using Gram's staining kit. On a clean slide, a thinly dispersed bacterial smear was first created. The slide was heated to repair it after air drying. The dry smear was submerged in crystal violet solution for a minute and briefly rinsed with tap water. It was then cleaned, blotted dry, and submerged in iodine solution once again for a minute. The slide was then washed and blot dried after being decolored with 95 percent ethyl alcohol, which was applied drop by drop until no more colour leaked from the smear. After around 10 seconds of counter staining with safranin, the slides were cleaned and viewed under a microscope [8].

KOH test

Using a toothpick, a loop of bacteria was aseptically removed from culture plates, placed on a glass slide, and quickly stirred in a KOH (3%) solution for ten seconds. The tooth pick was then raised a few centimeters above the slide and examined for the formation of viscid strands, which indicate the bacterium as Gram-negative [9].

Catalase test

On a slide, a loop of the bacterium's 48-hour slant growth was applied, and a few drops of hydrogen peroxide were added (H_2O_2). Gas bubbles will result in a favourable reaction [10]

H_2S production test

The peptone broth was prepared and sterilized. The slants holding the peptone broth received an inoculation of a loopful culture of 48-hour-old test bacteria. Filter paper discs (What man No. 42) were taken, air dried, and then inoculated after being impregnated with a 10% solution of neutral lead acetate. The sterilized strips were inserted into the inoculation test tubes with the free end hanging inside the tube and one end of the strip being held by the cotton plug. The infected tubes underwent a 72-hour incubation period at 27±1 ºC. A conclusion was reached regarding the H_2S production. The favourable response was evidenced by the stripes' blackening [10].

The composition of the medium used for H_2S production is given below.

Sl. No	Components	g/L
1	Peptone	10.0
2	NaCl	5.0
3	Distilled water	1000 ml
4	pH	7.0

Starch Hydrolysis

Potato starch (10 g) was added to 1000 ml of nutrient agar. The mixture was autoclaved for 30 minutes at 1.1 kg per cm^2 pressure to sterilize it. Each of the Petri plates received 20 ml of sterile cold medium (The total of four plates was dispensed with the medium). After setting up, starch agar plates were spot-inoculated with the bacterium's loopful culture and kept at 27±1 ºC for five days. After the incubation period, the plates were flooded with Lugol's iodine solution and checked to see if the bacterium had used the starch. For the preparation of Lugol's iodine solution, 5 g of iodine and 10 g of potassium iodide (KI) was dissolved in 10 ml of distilled water and then the volume of solution was adjusted to 100 ml by adding the distilled water [11].

Gelatin liquification

0.4 percent (4 g/1000 ml) gelatin was added to 15 ml of freshly made, autoclaved nutritional agar before being applied to the sanitized Petri plates. Spot inoculation was carried out utilizing a tooth prick on the medium's surface following the medium's solidification. For three days, plates were incubated at 27±1 ºC. Plates were flooded with 10 ml of an acid mercuric chloride solution following the incubation period ($HgCl_2$, 12 g; distilled water, 80 ml; concentrated HCl, 16 ml). The development of a distinct zone around the bacterium's growth was taken was positive reaction [12].

Pathogenicity test of *R. solanacearum*

Inocula for pathogenicity tests were prepared with 48 h old cultures grown on TZC medium at 29±1 ºC. Colonies were suspended in sterilized water to get $1x10^8$ cfu/ml using a spectrophotometer transmission at A_{600} = 0.1 and were used

for inoculation immediately after preparation. The soil mixtures used in the research were steam sterilized for 2 h. Fourteen days old seedlings of Arka Vikas (Susceptible check cultivar) were grown in the pot containing sterilized soil mixture as one plant per pot and maintained under greenhouse conditions. Root inoculation was done preferably at third true leaf stage or slightly older root zone was damaged using a sterilized scalpel and 50 ml of inoculum culture was introduced to each plant. Control plant was applied with sterilized water [13].

Molecular identification of *R. solanacearum*

Identification of *R. solanacearum* was made primarily by partial sequencing of 16S rRNA genes. Genomic DNA of the pathogen was extracted using the phenol-chloroform method. PCR was performed using the forward primer 8F-5′AGAGTTTGATCCTGGCTCAG 3′ and reverse primer 1391R-5′GACGGGCGGTGWGTRCA 3 [14].

Results and Discussion

Isolation of *R. solanacearum* from bacterial wilt affected tomato plant

The biochemical behaviour and cell and colony shape of plant bacteria have traditionally been used to identify them. Using a sharp knife or blade, a stem portion is removed from the plant with vascular discoloration. From the xylem's severed ends, milky white strands containing bacteria and extracellular polysaccharide will stream. Cross-sections of stem and tuber typically exude white bacterial exudates. The colonies of *R. solanacearum* on Nutrient Agar (NA) medium were smooth round, elevated, and filthy white, according to the findings of cultural research. The colony's optical feature was opaque and averaged 3 millimeters in size. In 24 hours, the bacterium became visible on the surface of the nutritional broth as a thin pellicle. When incubated at 28 °C for 48 hours, all isolates displayed morphological similarity. According to the results of the bacterium's growth in nutrient broth, the bacterium was visible in the form of a thin pellicle on the nutrient broth's surface after 24 hours. Later, the growth became a little thicker, and the medium turned murky. Additionally, there was a putrid odour. After 12–15 days, the growth had begun to turn yellow and the pellicle had begun to split. The outcomes are consistent with those from Patel *et al* [15]. The development of *R. solanacearum* from diverse locations in nutrient broth was described by Rangaswami and Sannegowda [16] as turbid with ring formation, pellicle formation, and flocculation (Fig 1).

Morphological and biochemical characterization

Colony characters on culture medium

Casamino acid peptone glucose (CPG) medium

R. solanacearum colonies that developed on CPG medium were opaque, fluid, spherical, and white or cream in appearance. The results closely match those of Stanford and Wolf [17], who described *R. solanacearum* colonies as being white, wet, shiny, round, elevated, and smooth. Similar observations about *R. solanacearum* colony characteristics were also made by Khetmalas [18] and Tahat and Sijam [19].

Triphenyl tetrazolium chloride (TTC or TZC) medium

On TTC or TZC media, white colonies with pink centres were produced (Fig 1). These outcomes support the conclusions of Hugh and Leifson [20].

Biochemical characterization

Test bacteria was screened for characterization up to species level based on the most often suggested set of biochemical tests to distinguish presumed *Ralstonia*. The cells of *R. solanacearum* were short, straight rods and Gram negative in reactivity, according to the results of staining tests. *R. solanacearum* showed similar morphological and staining reactions, according to several researchers [21]. When treated with 3% KOH on a glass plate, the bacterial culture of *R. solanacearum* generated strands of viscid materials, confirming the bacterium's status as Gram negative. Similar outcomes were also reported for *R. solanacearum* cultivated on nutritional agar medium by Chaudhry and Rashid [22]. The bacterium gave positive results for starch hydrolysis test, demonstrating that it was able to hydrolyze starch. *R. solanacearum* was reported as having positive starch hydrolysis by Anonymous [23]. *R. solanacearum* was able to produce hydrogen sulphide gas, indicating that the bacterium was capable of doing so and it also gave positive results for methyl red test but gave negative result for gelatin liquification (Table 1and Fig 2).

Pathogenicity test of *R. solanacearum*

The pathogenicity of *R. solanacearum* was determined by inoculating a bacterial suspension ($1x10^8$ cfu/ ml) into susceptible tomato (Arka Vikas) plants at preferably the third true leaf stage. Artificially inoculated plants started expressing wilt symptoms within 15 days. Re isolation yielded colonies which were similar to the ones isolated from diseased tomato plants. The results were in agreement with the earlier work [24] where the pathogen 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.

Molecular identification of *R. solanacearum*

Isolation of genomic DNA was made by Phenol: Chloroform method and confirmed by running on 1 per cent agarose gel electrophoresis as explained in 'Material and Methods.' The genomic DNA of the isolates was seen as a single and no smear was observed in any sample. It indicates that the isolated DNA is of good quality. This DNA was utilized as a template for the PCR amplification. The obtained genomic DNA was used as a template and subjected to PCR using 8F (forward primer) and 1391R (reverse primer). 2 per cent agarose gel electrophoresis of amplicons with primers yield around 1500 bp product. PCR products of amplified regions were sequenced using 8F and 1391R. The BLAST program was used to determine the species identification and the plant pathogenic bacteria were identified as *R. solanacearum*. The sequences obtained were deposited in National Center for Biotechnology Information (NCBI) GenBank database, Mary Land, USA. The results are in confirmation with the earlier [25].

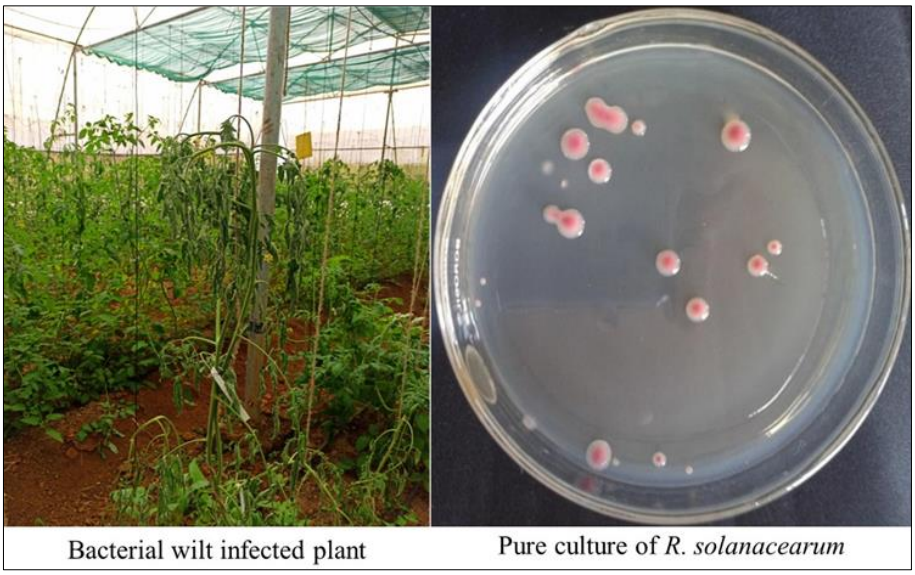

Fig 1: Symptoms of bacterial wilt of tomato and pure culture of *R. solanacearum*

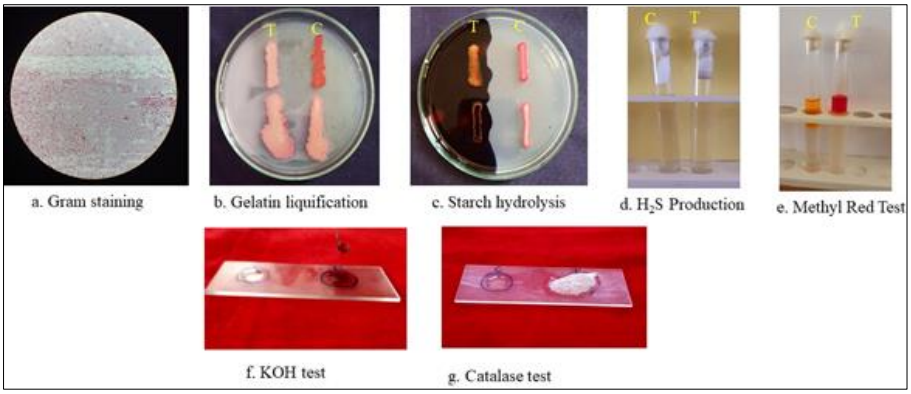

Fig 2: Bio-chemical characterization of *R. solanacearum*

Table 1: Biochemical characterization of *R. solanacearum*

Sl. No	Biochemical tests	*R. solanacearum*
1	Gram staining	Negative
2	KOH test	Positive
3	Catalase test	Positive
4	Starch hydrolysis	Positive
5	Gelatin liquefaction	Negative
6	H_2S production	Positive
7	Methyl red test	Positive

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