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Characterization of *Ralstonia solanacearum* isolates using biochemical, cultural, molecular methods and pathogenicity tests

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Abstract
Bacterial wilt of tomato caused by *Ralstonia solanacearum* is one of the most destructive disease of tomato. A study was undertaken to characterize five isolates of *Ralstonia solanacearum* (from different regions of Uttrakhand (Pantnagar, Dehradun, Bilaspur and Kicha) and near Delhi) by biochemical, cultural, molecular methods and pathogenicity tests. The colonies of *R. solanacearum* on Nutrient Agar (NA) medium were smooth, circular, raised and dirty white. Colonies of *R. solanacearum* formed on Casamino Peptone Glucose (CPG) medium were white or cream-colored, irregularly round, fluidal and opaque, the colonies formed on TZC medium were white with pink centers. All the five isolates were gram negative, oxidase positive, catalase positive, reduced nitrate to nitrite, hydrolyzed Tween 80 and oxidized glucose. Molecular characterization of the suspected isolates was done. Total five suspected isolates showed the 281-bp amplification with the specific primers RS-F-759 and RS-R-760. This indicates the isolated bacterial cultures were *R. solanacearum* culture. The pathogenicity of test bacterium was established by Koch's postulate under glasshouse condition and it was proved to be pathogenic.

Keywords: Bacterial wilt, *R. solanacearum*, pathogenicity, Koch's postulate, biochemical, cultural

Introduction
Tomato is susceptible to more than 200 diseases. Losses of yield due to diseases may be as high as 70 percent to 95 percent. Among diseases, bacterial wilt is usually the most damaging causing about 60-70 percent yield loss [1]. The characteristic symptom of bacterial wilt in normal grown up tomato plants is the rapid and complete wilting [2]. Identification is necessary for the taxonomy and efficient management of any disease inciting microorganism. When an unknown bacterium is isolated in the laboratory, it is usually identified by a combination of information from microscopic observations, i.e. morphology and arrangement of cells; gram staining; cultural (growth) characteristics on cultural medium, biochemical and physiological characterization. Molecular tools are available to identify the organism, but classical approaches combined with it give most authentic results. The investigation is aimed to study the cultural and morphological characteristics of *Ralstonia solanacearum*, on differential media and molecular detection by PCR technique. The rapid identification of a plant pathogen, allows for the appropriate control measures to be applied prior to the further spread of the disease or its introduction. The most frequent external symptoms of the infected plants are wilting, stunting and yellowing of the foliage [3, 5]. Other symptoms are leaves bent downward showing leaf epinasty, adventitious roots growing in the stems, and the observance of narrow dark stripes corresponding to the infected vascular bundles beneath the epidermis. Although the disease usually progresses until complete wilting and collapse of the plant, expression of the symptoms and rate of disease development may vary depending on host susceptibility and the aggressiveness of the pathogenic strain. The most frequent internal symptoms are progressive discoloration of the vascular tissue, mainly the xylem, at early stages of infection, and of portions of the pith and cortex, as disease develops, until complete necrosis [3]. Slimy viscous ooze typically appears on transverse-sectioned stems at the points corresponding to the vascular bundles [4]. In order to optimize the efficiency of prophylactic measures, powerful tools for the identification and detection of the bacterium in diverse substrates (plant, seeds and soil) are required. DNA-based methods have provided powerful tools to identify and detect microorganisms with high sensitivity and specificity. The PCR-based detection method using *R. solanacearum* specific primer offers a rapid and sensitive method for unambiguous detection of this pathogen in soil, seed and infected tomato plant materials [6].

Material and Methods

A total of 5 isolates were collected from diseased tomato plants from different location in Uttarakhand (Kicha, Pantnagar, Bilaspur and Dehtadun) and Delhi in 2015 and 2016 from the first fortnight of September to second fortnight of November. The collected plant samples were packed in polythene bags and kept at 4ºC for isolation of causal organism.

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

The discolored vascular tissues of plant samples were cut into small pieces and they were kept in the glass beaker containing clean water taking care that the infected tissue is in contact with the water surface. After five minutes the water in the glass beaker becomes turbid due to oozing of bacterial cell from the cut ends of the diseased tissue and thus confirming the bacterial nature of the wilted plants. After the water in the test tube becomes turbid due to oozing of bacterial cells from cut ends of diseased tissue, the bacterial suspension was serially diluted in 9 ml sterile distilled water. One hundred microliter of the diluted bacterial suspension was poured onto the surface of solidified Nutrient Agar (NA) medium [8] in sterilized petri plates. The bacterial suspension was spread on the surface of TZC medium with a sterilized spreader. The inoculated plates were incubated at 28ºC for 48 hours. To isolate the pathogen from soil, the soil samples were serially diluted and the pathogen was isolated using NA medium. At the end of the incubation period, the plates were observed for the development of both the virulent and avirulent colonies of *R. solanacearum*.

Morphological and biochemical characterization

Colony characters on culture medium

Casamino acid peptone glucose (CPG) medium

The bacterial isolate was transferred onto CPG medium by streaking bacterial suspension of 10^4 cfu/ml and incubated at 27ºC for 24 h. After incubation, colonies of the normal (virulent type) are white or cream colored, irregularly-round, fluidal and opaque.

Triphenyl tetrazolium chloride (TTC or TZC) medium

The bacterium was isolated by streaking a loopful of flowing ooze containing the bacterium on to sterile TTC (Triphenyl tetrazolium chloride) agar plate [8] and plates were incubated at 30ºC for 48 to 72 h. After incubation the virulent *R.solanacearum* colonies (irregular shape, white to cream color, slimy with pink color in the centre) were selected. Dark red colonies were considered to be a negative reaction. The experiment was replicated thrice.

Biochemical characterization

Gram Staining

The Gram's staining reaction were performed using crystal violate. The purple dye, crystal violet, is picked up by the cell wall of both Gram-positive and Gram-negative bacteria. The cells are then dipped into an iodine solution. When ethanol (the decolorizing agent) is applied, the ethanol dissolves the lipids in the outer membrane of the cell wall of Gram-negative bacteria causing the CV-I complex to leave the cells. Gram negative cells, therefore, appear colourless after the ethanol wash. As Gram-negative cells are colourless after the ethanol wash, they are counterstained with safranin, a pink or reddish-coloured dye. Therefore, Gram-negative cells appear pink after the Gram stain procedure.

KOH test

Placed approximately 50µl of 3% (w/v) KOH on a clean glass slide. Aseptically transferred bacterial cells from an agar plate to the drop of KOH with a sterile toothpick. Agitated the cells with the toothpick. The viscosity of the drop increased and a string of "goo" easily picked up with the tip of the toothpick, the cells are gram-negative. If the viscosity does not greatly increase, the cells are gram-positive. The experiment was replicated thrice.

Oxidase test

Oxidase activity was detected by the method of Kovacs [9]. Freshly grown (24 to 48 hrs) culture from nutrient agar with 1% glucose were patched onto a filter paper moistened with a fresh oxidase reagent (1% w/v aqueous solution of tetramethyl-para-phenylene diamine dihydrochloride) using a wooden stick.

Catalase test

Catalase test was performed according to methods described by He *et al.* [10]. Only 1 ml of a 3% solution of hydrogen peroxide was added to a Petri dish and a loop of fresh culture grown on CPG agar medium was added into the solution. Release of bubble from the culture was recorded as catalase positive [11].

Nitrate reduction test

This test was accomplished as suggested by Hayward [12] using the medium of Fahy and Hayward [13]. It was a semi soft agar medium containing (g/l): peptone 10g, NaCl 5g, KNO_3 2 g and agar 3g, which was boiled to dissolve the agar. The pH was adjusted to 7.0 with concentrated NaOH, and the medium dispensed into test tubes and autoclaved. Tubes were stab inoculated with a loop of a test strain and then filled with sterile melted 3% water agar. Control test tubes were not inoculated with strains. Good growth in 5 days at 30ºC was taken as indicative of nitrate reduction into nitrite [11].

Tween 80 hydrolysis

Fatty acid esterase activity was tested by streaking the bacteria onto a nutrient agar medium containing calcium chloride and Tween 80, a polymer consisting of polyoxy-ethylene-sorbitanmonooleate [11]. The medium contains: peptone, 10g; $CaCl_2$ dihydrochloride, 0.1g; NaCl, 5g; Agar, 15g; distilled water, 1litre,; with the pH adjusted to 7.4. Tween 80 was autoclaved separately and added with 10ml/l and mixed before plating. Incubation was made at 30ºC for up to 7 days [13]. An opaque zone of crystals around a colony was recorded as positive reaction for hydrolysis of Tween 80 [11].

H_2S production

This was detected according to Sands [11] by using a medium which constitutes (g/l): $NH_4H_2PO_4$ 0.5 g; K_2HPO_4 0.5g; $MgSO_4.7H_2O$ 0.2g; NaCl 5g; yeast extract 5g; cysteine hydrochloride (anhydrous) 0.1gm and dispended in 5ml aliquots into tubes and autoclaved. Lead acetate impregnated paper strips were prepared by dipping 5mm strips into 5% lead acetate, dried and autoclaved. These were hung above inoculated media using cotton plugs and black discoloration of the lead sulphide was monitored as an indicator for H_2S production.

Starch Hydrolysis

Nutrient agar plates containing 0.2% soluble starch (w/v) were streaked by the tested isolates and incubated at 30ºC

until heavy growth occurred. Then plates were flooded with IKI solution (iodine, 1g; potassium iodide, 2g; distilled water, 100ml). A clear zone around a colony was recorded as positive reaction [11].

Gelatin Hydrolysis

For this test, nutrient agar with 0.4% (w/v) gelatin was poured into petri dishes, cooled and dried overnight. The following day strains were inoculated on to each plate and incubated at 30ºC for 3 days. When good growth was observed, the plate surfaces were flooded with 5ml of mercuric chloride solution ($HgCl_2$ 12g; distilled water, 80 ml; concentrated HCl, 16 ml) [11]. A clear zone surrounding bacterial growth indicates positive reaction for the test [14].

Citrate Utilization

The citrate test performed by inoculating 100 µl of a 10^4 cfu/ml of *R. solanacearum* into plates containing the Simmon's Citrate Agar medium, where sodium citrate is the only source of carbon and energy. Then all plates were incubated at 37 °C for 48 hrs. Bromothymol blue is used as an indicator from sodium carbonate an alkaline product, which changes the colour of indicator from green to blue and this constitutes a positive test.

Oxidation of glucose

The basal medium described by Hayward [12] was used. The pH was adjusted to 7.1 with 40% (w/v) NaOH solution before adding the agar. Five ml of glucose solution was added to 45 ml of molten cooled Hayward's basal medium and 10 ml volume of the resulting amended medium were dispended into test tubes [12]. Hayward's medium without glucose served as control. A suspension of each strain grown on CPG medium for 48 h at 30ºC was prepared by inoculating 300µl of sterile water with a wire loopful of cells [15]. The test tubes of Hayward's medium were inoculated with 30µl of the prepared suspensions incubated at 30ºC and check for acid production (yellow color) at various intervals up to 5 weeks [12, 10].

Pathogenicity test and artificial inoculation technique

Pathogenicity test and standardization of Inoculation techniques under artificial conditions

Soil was prepared by the integration of farm yard manure, sand and clay in 1:1:1 ratio. This mixture was sterilized with 37% formalin by 1:9 ratio (1 part formalin and 9 parts soil). Soil mixture was covered with polyethylene sheet and placed for 3-4 days in sunlight. Afterwards the sheet was removed and soil was exposed to sunlight for about 5 days to release fumes. Then in this soil tomato seeds of susceptible cultivar Arka Vikas were grown in pots. Twenty one days (4-5 leaf stage) old seedlings were used for performing pathogenicity test. Bacterial cell suspension was prepared from 24 hr old culture of *Ralstonia solanacearum* and adjusted to $1x10^7$ cells/ml (cfu) by adding sterilized distilled water and 0.7% (v/v) of tween-40 (surfactant). Twenty one days old plants were inoculated with the bacterial suspension of all five isolates. After the pathogenicity of all the five isolates have been proved, Dehradun isolate was used for evaluating different inoculation techniques. Twenty one days old tomato plants were than inoculated with the bacterial suspension of Dehradun isolate between 4-7 pm, by five different methods viz., soil drenching method, leaf clipping method, stem inoculation method, leaf whorl method and cotton wool method. For control only sterilized water was used. Immediately after inoculation plants were placed in moist chamber for 48-72 hours and then transferred in glass house having a temperature of about 30±1ºC and relative humidity >90%. The symptoms expressed were studied and re-isolation of the pathogen was made. Experiment was conducted using completely randomized design (CRD) with three replications. The inoculation test, as above was repeated once more to confirm the result. Disease assessment was done based on percentage of plants showing bacterial wilt symptoms in relation to total inoculated plants after one week of inoculation [16].

Inoculation techniques

Soil drenching

In the soil drenching method, 5.0ml of bacterial suspension was inoculated to each of the seedlings by drenching the soil around the root zone with the help of micro pipette. Before inoculation, the roots were slightly severed by inserting a sharp knife 1.0cm away from the stem. Root severing was done to ensure bacterial penetration through roots.

Leaf clipping method

Leaf clipping method is carried out by dipping sterile scissors in the bacterial suspension and clipping the leaves. Three to four leaves were clipped per seedling by giving horizontal cut.

Stem inoculation method

Bacterial suspension was inject-inoculated with a 21G hypodermic needle into the vicinity of a growing point of 21 days old plants as described by earlier investigators [17-19]. All the plants in front row were only inoculated. Control plants were inject-inoculated with sterilized water only.

Leaf-Whorl inoculation method

Leaf-Whorl inoculation method was adapted from Hartman and Kelman [16] used in corn without causing injury. All the plants in each row were artificially inoculated by spraying the bacterial suspension in leaf whorls (2ml/whorl) with the help of atomizer without causing any injury between 5-7 pm after 21 days of sowing as night temperature and humidity are conducive for infection. Care was taken not to disturb the plants after inoculation so that maximum inoculum was retained in the leaf-whorls. Plants sprayed with sterilized water served as control.

Cotton wool method

Non-absorbent cotton wool was dipped in bacterial cell suspension and rubbed gently on the both surfaces of leaves to expose the maximum stomatal opening. Cotton wool dipped in sterilized water was used to rub the leaves of control plants.

Molecular characterization of the pathogen

All isolates that produced typical symptom in tomato assay and typical *R.solanacearum* colonies on TTC agar subsequently were subjected to PCR for conformation. Extraction of total genomic DNA from bacterial cells was performed using the "CTAB method" [20]. DNA concentration and purity of each isolate was determined by quantifying absorbance at 260 and 280 nm. Absorbance of DNA samples dissolved in TE buffer was recorded against TE buffer as a blank using UV-VIS spectrophotometer. DNA concentration was calculated from optical density using following formula.

$$\text{Concentration in } \mu g/\mu l = \frac{OD_{260} \times 50 \times \text{Dilution factor}}{1000}$$

DNA polymerase chain reaction procedure described by Choi *et al.* [21] was used by following reaction mixture (20µl) which consisting of 1.25µl of 10X PCR buffer, 0.37µl of 25mM $MgCl_2$, 2.0 µl dNTPs mix (10mM each of dATP, dCTP, dGTP, dTTP), 0.1µl Taq DNA polymerase, 0.8µl of forward and reverse primer, 2µl of genomic DNA and 14.18 µl of sterilized double distilled water. The reaction mixture was vortexed and centrifuged at 12000 rpm for 2 min. Amplifications were performed in Biored Master Cycler gradient. Amplified products were separated on 1.2 per cent agarose gel in 1X TAE buffer at 100V. The gel was stained with 0.5µg/ml Ethidium bromide solution and visualized by illumination under UV light in gel doc system. The size of amplification products were determined by comparison to low range DNA ruler plus marker. The amplification reactions were carried out in 0.2 ml PCR tubes using a bio-red thermal cycler with RS-F-759 and RS-R-760 (forward and reverse) primers corresponds to the upstream region of *lpxC* gene of *R. solanacearum* (Euro fins Technologies, Bangalore). The amplification consists of a 5 min. initial denaturation step at 94 ºC followed by denaturation at 94 ºC for 1 minute. Annealing of the primer was done from 53 ºC to 60 ºC for 1 min, followed by an extension period for 1 minute at 72 ºC. The reactions were subsequently subjected to 30 additional cycles after reaching the final annealing temperature. This was followed by a final extension at 72 ºC for 5 min.

Results and Discussion

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

The identification of plant bacteria has traditionally been based on the biochemical behaviour and the cell and colony morphology of these organisms. A stem section is cut from the plant with vascular discoloration using a sharp knife or blade. Milky white strands containing bacteria and extracellular polysaccharide will stream from the cut ends of the xylem. Typically, stem and tuber cross-sections ooze whitish bacterial exudates. The results of cultural studies revealed that the colonies of *R. solanacearum* on Nutrient Agar (NA) medium were smooth circular, raised and dirty white. The optical feature of the colony was opaque and measured around the average of 3 mm in size. The bacterium was visible in the form of thin pellicle on the surface of nutrient broth in 24 hrs. All isolates were morphologically similar in their appearance when incubated at 28°C for 48 h. The results of growth of the bacterium in nutrient broth indicated that bacterium was visible in the form of thin pellicle on the surface of nutrient broth in 24 hrs. Later on, the growth became a little thick and medium became turbid. There was also putrefactive odour. The growth after 12-15 days was little yellowish and breaking of pellicle was also seen. The results are in conformation with findings of [22-24]. Similarly, Rangaswami and Sannegowda [25] reported growth of *R. solanacearum* from various places in nutrient broth as turbid with ring formation, pellicle formation and flocculation.

Morphological and biochemical characterization

Colony characters on culture medium

Casamino acid peptone glucose (CPG) medium

Colonies of *Ralstonia solanacearum* formed on CPG medium were white or cream-colored, irregularly round, fluidal and opaque (Table-1). The findings are in close conformity with Stanford and Wolf [26] who described the colonies of *R. solanacearum* as white, wet, shining, circular, raised and smooth. Khetmalas [27] and Tahat and Sijam [28] also recorded similar observations regarding colony characters of *R. solanacearum.*

Triphenyl tetrazolium chloride (TTC or TZC) medium

The colonies formed on TTC or TZC medium were white with pink centres (Table-1). These results are in agreement with the findings of [29, 8, 30, 9, 31-38].

Biochemical characterization

On the basis of a most widely recommended set of biochemical test to differentiate presumptive *Ralstonia*, test bacterium was screened for characterization up to species level. The results of staining reactions revealed that the cells *R. solanacearum* were short straight rods, measuring 1.5–3.12µ X 0.25–2.5µ and Gram negative in reaction Several researchers [39, 27, 40, 41, 24] reported similar morphological and staining reaction of *R. solanacearum.* KOH test of *R. solanacearum* confirmed the bacterium as Gram negative as it produced strands of viscid materials on treating bacterial culture with 3 % KOH on glass slide. Chaudhry and Rashid [41] also reported similar results for *R. solanacearum* grown on nutrient agar medium. Starch hydrolysis test of the bacterium showed that the bacterium was unable to hydrolyse starch. Anonymous [42] reported *R. solanacearum* as negative in starch hydrolysis. Similarly [43, 10, 27, 44, 24] also reported *P. solanacearum* to be negative in starch hydrolysis. However [23] found the positive results from *P. solanacearum* isolated from brinjal. Hydrogen sulphide gas production test of *Ralstonia solanacearum* showed that the bacterium was negative in H_2S production. This result is in agreement with the report of [43, 23, 44, 24] while [39, 10] reported *P. solanacearum* to be positive in gas production. All the tested isolates were oxidase positive, catalase positive, reduced nitrate to nitrite, hydrolyzes Tween 80 and oxidizes glucose. These results are in accordance with the findings of Ocho [45].

Table 1: Morphological and biochemical characterization of isolates of *Ralstonia solanacearum* isolated from infected tomato plants

S. No.	Characters	Isolates				
		Pantnagar	Kichha	Bilaspur	Dehradun	Delhi
1.	Gram staining	-	-	-	-	-
2.	KOH test	+	+	+	+	+
3.	Catalase oxidase	+	+	+	+	+
4.	Oxidation test	+	+	+	+	+
5.	Simmon citrate medium	+	+	+	+	+
6.	Oxidation of glucose	+	+	+	+	+
7.	Tween 80 hydrolysis	+	+	+	+	+
8.	Starch hydrolysis	-	-	-	-	-
9.	Gelatin hydrolysis	-	-	-	-	-
10.	Nitrate reduction	+	+	+	+	+
11.	H_2S production	-	-	-	-	-
12.	CPG medium	+	+	+	+	+
13.	TZC medium	+	+	+	+	+

+ = Positive reaction, - = Negative reaction

Pathogenicity test and artificial inoculation technique

Results presented in Table2 revealed that all methods produced bacterial wilt symptoms with variable and non-uniform pattern, except soil drenching in which all inoculated plants showed most typical, reliable and uniform disease development. The disease mainly affected tomato plant

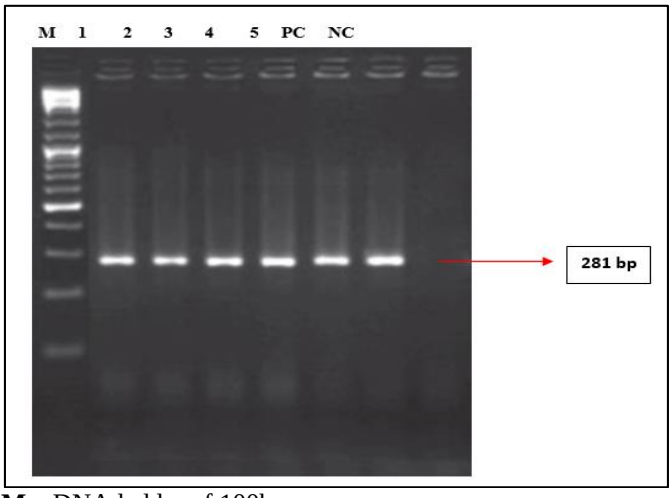
showing wilting and necrosis symptoms. Typical symptoms were first observed in soil drenching method, the first symptom appear on youngest leaves which are the first to be affected on 3rd day of inoculation. Wilting occurs quickly without any symptoms in the plant. There will not be any symptoms of rotting of roots or stem at the base unlike in fungal attack, but browning of the vascular systems in roots and lower portion of stem is observed. As the disease advanced, all the plants collapsed on 5th day of inoculation. However, symptoms started appearing after 4th day of inoculation in other methods. Highest bacterial wilt severity (91.33%) with typical wilting symptom and uniform type disease development was found in case of soil drenching method followed by leaf clipping method (52.00%), stem inoculating method (39.33%), leaf whorl method (26.66%) and cotton wool (20.00%) method of inoculation over control. The results are in accordance with the findings of Artal *et al.* [46] who reported that the inoculation through soil drenching recorded significantly highest bacterial wilt incidence.

Table 2: Percent disease severity in artificial inoculation methods after 7 days of Inoculation

S. No.	Methods of artificial inoculation	Disease severity (%)
1.	Soil drenching method	91.33
2.	Leaf clipping method	52.00
3.	Stem inoculation method	39.33
4.	Leaf whorl method	26.66
5.	Cotton wool method	20.00
6.	Control	0.00
	S.Em±	3.16
	CD at 5%	8.26

Molecular characterization of *R. solanacearum* isolates

The specific primers RS-F-759 and RS-R-760 corresponds to the upstream region of *lpxC* gene of *R. solanacearum*, used to identify *R. solanacearum* at the species level and amplify a predicted 281-bp DNA fragment. The specific primers used for the detection of the isolated *R. solanacearum*, amplified an expected band of 281-bp as reported by Opina *et al.* [47] and Vanitha *et al.* [7]. Total 5 suspected isolates showed the 281-bp amplification with the specific primers. This indicates that the isolated bacterial cultures were *R. solanacearum* culture (Fig.1).



M – DNA ladder of 100bp
1 – DNA amplification of Dehradun culture
2 – DNA amplification of Delhi culture
3 – DNA amplification of Bilaspur culture
4 – DNA amplification of Kichha culture
5 – DNA amplification of Pantnagar culture
PC – Positive control (DNA amplification of *R.solanacearum* culture from ITCC)
NC – Negative control (DNA amplification of *Erwinia chrysanthemi* from pure culture)

Fig 1: This fig indicates that the isolated bacterial cultures were *R. solanacearum* culture

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