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Characterization of *Ralstonia solanacearum* causing bacterial wilt of potato

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Abstract
Bacterial wilt or brown rot of potato caused by *Ralstonia solanacearum*, considered as a destructive disease of potato crops. In the present research work, the bacterial wilt infected potato plant samples were collected from different location of Samastipur district of Bihar (India). Twenty strains of *R. solanacearum* were isolated and identified based on different biochemical characteristics. The strains of *R. solanacearum* showed fluidal, irregular and creamy white with pink at the center on TZC medium after 48 h of incubation were selected. The strains of *R. solanacearum* showed a positive result for the pathogenicity test and produced brown rot symptoms in potato tubers. The results of biochemical studies showed that all the 20 isolates were gram-negative, rod-shaped and positive for potassium hydroxide test, oxidase test, catalase test, nitrate reduction test, production of hydrogen sulfide and citrate utilization test but they showed negative reaction for indole test. Based on the isolation study (colony characteristics on TZC media), ooze test, pathogenicity test, and different biochemical tests, the identity of potato wilt pathogen was established as *R. solanacearum*. Race characterization showed that strains of *R. solanacearum*, causing bacterial wilt disease in potato, belong to race 3. The result of biovar showed that the strain of *R. solanacearum* collected from different locations of Samastipur districts of Bihar belong to bv2 (80%) & bv2T (20%).

Keywords: *Ralstonia solanacearum*, bacterial wilt, potato

Introduction
Bacterial wilt or brown rot, caused by *Ralstonia solanacearum*, considered as the destructive disease of potato crops. This pathogen *R. solanacearum* limits the potato production worldwide where it causes severe crop losses in tropical, subtropical, and warm temperate regions (Elphinstone, 2005) [8]. It causes disastrous yield loss in major crops like potato, tomato, eggplant, banana, chili, tobacco, ginger, etc. It has a vast host range of ~ 450 plant species across 54 families (Wicker *et al*., 2007) [23]. Kishun, 1985 [14] reported that the incidence of bacterial wilt is 10 to 100% in India during the summer season. *Ralstonia solanacearum* is a gram- negative, rod-shaped and belonged to the class β-proteobacteria. This pathogen is soil-borne, aerobic, nonsporulating, and the size varies between 0.5-0.7 x 1.5-2.0 µm. The disease was first recorded in India from the Pune district of Maharashtra (Cappel, 1892) [5] and the bacterial nature of the disease was described by Butler (1903).
It is essential to study the races and biovars populations of *R. solanacearum* to develop a pathogen and geographically targeted integrated disease management strategy against the disease. The strains of *R. solanacearum* group into 5 races based on their ability to infect different host plants. Buddenhagen *et al*. (1962) [4] categorised the *R. solanacearum* into three races. Race 1 is pathogenic on different solanaceous crops having a wide host range such as tomato, potato, chilli, brinjal and others. Race 2 infects only triploid banana and Heliconia. Race 3 infects potato and sometimes tomato. Race 4 was reported by Aragaki and Quinon (1965) [2] which infects ginger, and race 5 is pathogenic on mulberry which was reported by He *et al*. (1963). *R. solanacearum* was grouped into six biovars based on the
utilization of alcohols (mannitol, dulcitol, and sorbitol) and disaccharides (lactose, maltose, and cellobiose) (He *et al*., 1983) [11].
The present study was undertaken to characterize different isolates of *R. solanacearum* causing bacterial wilt in potato collected from different locations of the Samastipur district of Bihar (India).

Materials and Methods
Isolation and purification of *R. solanacearum*
Disease specimens of potato showing typical symptoms of bacterial wilt were collected from different locations of Samastipur districts of Bihar (India). Wilted samples were surface

sterilized with 70% alcohol. The diseased plant part was cut from the basal node of the plant and placed in 5 ml of sterile water. It was kept for 10 minutes during which the water turned turbid due to oozing of bacteria (fig. 1a) from the stem and the bacterial ooze was used for isolation of the *R. solanacearum*. Isolation of the bacterium was done following the spreading method on TZC (triphenyl tetrazolium chloride) agar media. A single isolated bacterial colony was picked from TZC agar plates and streaked on pre-sterilized CPG slant to obtain a pure culture of the isolates.

Pathogenicity test

The test was conducted under pot culture conditions. Disease-free potato tubers were collected and sown in the pot. 30 days old healthy potato plants were used for inoculation. The concentration of the suspension was maintained at about108CFU/ml. for the experiment, the stem inoculation method was followed. The plants were observed for the symptoms at regular intervals.

Artificial inoculation of *R. solanacearum* on potato tuber

Potato tuber was inoculated with different isolates of *R. solanacearum* to produce brown rot symptom on the tuber. Potato tubers were cut into many thin slices. After that, two moist blotter papers were placed in the sterilized Petri plate. The sterilized slide was placed on blotter paper and then surface sterilized potato slice was placed on the slide. Potato slice was inoculated with *R. solanacearum* by touching at the vascular region. Then it was incubated at 28o±10C and observed for the symptom.

Characterization of *Ralstonia solanacearum*

Biochemical tests for the identification of *Ralstonia solanacearum*

Several biochemical tests viz. gram staining (Schaad *et al*., 2001) [19], potassium hydroxide test (Chaudhry and Rashid, 2011) [6], oxidase test (Sharma, 2018) [20], catalase test (Chaudhry and Rashid, 2011) [6], nitrate test (Fahy and Persley, 1983) [9], indole test (Vanitha *et al*., 2009) [22], production of hydrogen sulphide (Pawaskar *et al*., 2014) [17] and citrate utilisation test (Sinha, 2016) [21] were carried out by using standard protocol for confirmation of *R. solanacearum* isolates.

Races identification

The races of *R. solanacearum* were identified by inoculating the *R. solanacearum* on a wide host range (Buddenhagen & Kelman, 1964) [3]. Potato (Solanum tuberosum), Tomato (Solanum lycopersicum), eggplant (Solanum melongena) and chili (Capsicum annuum) were used as a test plant to determine the host range of *R. solanacearum*. The stem puncture technique was made for tomato, potato, brinjal and chili for inoculation of *R. solanacearum*. In this technique, bacterial suspension of potato and tomato isolate was prepared and adjusted to inoculum density of 108CFU/ml and wilt symptoms were observed daily.

Determination of biovars

Biovar characterization was done based on the utilization of different carbohydrates (sucrose, lactose, and maltose) and alcohols (mannitol, sorbitol, and dulcitol). Biovar test was performed with the help of KB009 HiCarbohydrateTM Kit (HiMedia Laboratories Pvt. Limited), which contains the above-mentioned disaccharide and sugar alcohols (Hayward, 1964) [10] to characterize the bacterial isolate. Twenty isolates of *R. solanacearum* were taken to differentiate biovar. The suspension was prepared by mixing a loop of 48 h old *R. solanacearum* culture in 5 ml of sterile distilled water to 108CFU/ml. Each well in the kit was loaded with 50µl of the bacterial suspension by surface inoculation method. Control well was

loaded with sterile distilled water. Then it was incubated at temperature 35±10C. The change of color of carbohydrate/alcohol disc from red to yellow was considered positive and no change of color was considered as negative. The observation of changing color from red to yellow was recorded after 18 h of incubation up to 21 days.

Results and Discussion

Isolation and identification of bacterial wilt pathogen *R. solanacearum*

Isolation of *R. solanacearum* was made on the Kelman TZC medium. Typical colony characters like fluidal, irregular slimy, creamy white colored with pink to red-colored center appeared on 2, 3, 5-tetrazolium chloride medium (TZC medium) after 48 hours of inoculation (fig. 1b). A total of twenty isolates of *R. solanacearum* were isolated from potato collected from Samastipur district of Bihar (India). Sharma (2018) [20] reported typical virulent colonies of

R. solanacearum which appeared as an irregular, fluidal, white, virulent colony with pink center on TZCA (tetrazolium chloride agar) medium.

Pathogenicity test

The healthy potato plants were used for pathogenicity test by inoculation into the stem above the soil region. The result of the pathogenicity test showed that all isolates of *R. solanacearum* were able to produce wilt symptoms in potato plants. The symptoms of wilting were started after 12 days of inoculation. Initial symptoms observed as wilting of the inoculated plant and stunting of growth. Most of the isolates were found highly virulent on potato and produced wilt symptoms like drooping and loss of turgidity in leaves. Some of the isolates induced mild symptoms where leaves were pale green. The findings of the present study are supported by Ahmed *et al*., (2013) [1], they reported that all strains of *R. solanacearum* isolates collected from a potato from Bangladesh were able to cause wilt symptoms in potato plants.

Artificial inoculation of *R. solanacearum* on potato tuber

Potato tuber was inoculated with different isolates of *R. solanacearum* to produce brown rot symptom and the results revealed that all isolates were able to produce brown rot symptom in potato tuber. The symptoms of brown rot were observed after 24 h of incubation. After 72 h all the isolates produced brown rot symptom on potato tuber which is presented in table 1 and fig. 2a. Further, the pathogen *R. solanacearum* was confirmed by ooze test (fig. 2b). The result was confirmatory to an earlier report given by Ahmed *et al*. (2013) [1]. They reported that all strains of *R. solanacearum* isolates collected from potato from Bangladesh were able to produce brown rot symptoms in potato tubers.

Biochemical tests Gram staining

All the tested *R. solanacearum* isolates showed pink to light red colored colonies under the microscope indicating all the isolates were negative for gram reaction. As the bacterial isolate was gram-negative it retained the color of safranin and while seen 100 x objectives with oil emersion it was like very

minute rods which were pink in color (fig. 3a). Similar findings were made by Murthy and Srinivas (2012) [15] who reported that *R. solanacearum* isolates were negative for gram's reaction.

Potassium Hydroxide Test

The gram-negative test of *R. solanacearum* was also confirmed by the potassium hydroxide solubility test. The result of the potassium hydroxide test showed that the solution was viscous enough to stick to the loop causing a thin strand of slime, which was recorded as positive (fig. 3b). Popoola *et al*. (2015) [16] reported that strains of *R. solanacearum* produced slime thread which is an indication of gram-negativity.

Oxidase test

A purple color appeared within 30 seconds after touching and spreading a well isolated *R. solanacearum* colony on the oxidase disc recorded as oxidase-positive. This indicated that all the tested isolates (20) were positive for an oxidative test (fig. 3c). Dhital *et al*. (2000) [7] who reported that all the strains of *R. solanacearum* were positive for oxidase.

Catalase test

Air bubbles were produced within 60 seconds was recorded as a positive reaction for the catalase test. All the isolates (20) were recorded positive for catalase test (fig. 3d). Therefore, the test bacterium *R. solanacearum* is strictly aerobic. Dhital *et al*., (2000) [7] who reported that all the strains of *R. solanacearum* were positive for catalase test.

Nitrate test

The color of the nitrate medium was changed to pink to red was recorded as positive for the nitrate reduction test. All the twenty isolates of *R. solanacearum* showed positive for nitrate reduction test (fig. 3e). The intensities of pink or red color were varied with the different isolates of *R. solanacearum* which is presented in table 2. Kataky *et al*. (2017) [13] reported that isolates of *R. solanacearum* were positive for the nitrate test.

Indole test

There was no formation of pink to red color. This indicated that all the twenty isolates of *R. solanacearum* were negative for indole production test (fig. 3f). Murthy and Srinivas (2012) [15] reported that *R. solanacearum* isolates were negative for the indole test.

Production of hydrogen sulfide

All the isolates showed blackening at the lower end of strips within 72 h. This indicated that all the twenty isolates of *R. solanacearum* were positive for hydrogen sulfide production test (fig. 3g). The time duration for the development of color varied with the different isolates of *R. solanacearum* which is presented in table 3. Kataky *et al*. (2017) [13] reported that isolates of *R. solanacearum* were positive for H2S production.

Citrate utilization test

The result showed a positive reaction when there was a color change of the medium from green to royal blue showing the potentiality of the pathogen to utilize citrate. All the isolates of *R. solanacearum* showed a positive reaction (fig. 4). Murthy and Srinivas (2012) [15] reported that *R. solanacearum* isolates were positive for citrate utilization test.

Race identification

Races of *R. solanacearum* were identified, based on the ability of the isolates to infect different host plants. All the 20 isolates of *R. solanacearum* from potato were virulent to potato plants and able to cause wilt symptoms in potato plants and unable to produce wilt symptoms on eggplant, chili, tomato plants under artificial inoculation conditions. Those isolates produced wilt symptom in potato plant belonged to race 3, due to a narrow host range. Ranjan and Singh (2015) [18] reported that those strains, caused wilt symptoms in tomato, potato and tobacco were placed under race 1 and those strains fail to produce the wilt symptom in tobacco, only showed chlorosis on the inoculated leaf of tobacco plants were placed under race 3.

Biovar characterization

The biovars of *R. solanacearum* isolates were identified by the utilization of disaccharides and hexose alcohols. The oxidation reaction was indicated by the change of color. The results revealed a change of color red to a yellow color indicating the oxidization of disaccharides and alcohols by *R. solanacearum* isolates (fig. 5). From the observation, it was clear that the 16 isolates of *R. solanacearum* isolated from potato plants from different locations of Samastipur districts of Bihar (India) belonged to biovar2 whereas 4 isolates of *R. solanacearum* isolated from potato plant belonged to biovar 2T. Ranjan and Singh, (2015) [18] also reported that bv1, bv2, bv2T, and bv3 were present in India, cause bacterial wilt in potato plant

Table 1: Brown rot symptom produced after inoculation with *R. solanacearum* isolates

Isolates of *R. solanacearum*	Duration of symptom expression
SP-1	48 h
SP-2	72 h
SP-3	48 h
SP-4	48 h
SP-5	72 h
SP-6	24 h
SP-7	48 h
SP-8	72 h
SP-9	24 h
SP-10	48 h
SP-11	48 h
SP-12	48 h
SP-13	72 h
SP-14	48 h
SP-15	24 h
SP-16	48 h
SP-17	48 h
SP-18	72 h
SP-19	72 h
SP-20	48 h

Table 2: Result of nitrate test for different isolates of *R. solanacearum*

Isolates of *R. solanacearum*	Developed colour after 72 h
SP-1	Light red
SP-2	Pink
SP-3	Pink
SP-4	Pink
SP-5	Pink
SP-6	Deep red
SP-7	Deep red
SP-8	Light red
SP-9	Deep red
SP-10	Light red
SP-11	Light red
SP-12	Deep red
SP-13	Pink
SP-14	Pink
SP-15	Light red
SP-16	Light red
SP-17	Deep red
SP-18	Deep red
SP-19	Deep red
SP-20	Deep red
Control	Light yellow

Table 3: Result of hydrogen Sulphide production from different isolates of *Ralstonia solanacearum*

Isolates of *R. solanacearum*	Duration after inoculation		
	24h	48h	72h
SP-1	-	+	+
SP-2	-	-	+
SP-3	-	-	+
SP-4	-	+	+
SP-5	-	-	+
SP-6	-	+	+
SP-7	-	-	+
SP-8	-	-	+
SP-9	-	+	+
SP-10	-	+	+
SP-11	-	+	+
SP-12	-	+	+
SP-13	-	-	+
SP-14	-	-	+
SP-15	-	-	+
SP-16	-	+	+
SP-17	-	-	+
SP-18	-	+	+
SP-19	-	+	+
SP-20	-	+	+
Control	-	-	-

Table 4: Characterization of *Ralstonia solanacearum* isolates from wilted potato plant

Isolates of *R. solanacearum*	Cultural character		Pathological test		Biochemical test					Race determination	Biovar determination
	Gram's reaction	KOH test	Pathogenicity test	Potato brown rot test	Oxidase test	Catalase test	Indole production test	Hydrogen sulphide production test	Citrate utilization test		
SP-1	-	+	+	+	+	+	-	+	+	3	2
SP-2	-	+	+	+	+	+	-	+	+	3	2
SP-3	-	+	+	+	+	+	-	+	+	3	2
SP-4	-	+	+	+	+	+	-	+	+	3	2T
SP-5	-	+	+	+	+	+	-	+	+	3	2
SP-6	-	+	+	+	+	+	-	+	+	3	2
SP-7	-	+	+	+	+	+	-	+	+	3	2
SP-8	-	+	+	+	+	+	-	+	+	3	2
SP-9	-	+	+	+	+	+	-	+	+	3	2
SP-10	-	+	+	+	+	+	-	+	+	3	2T
SP-11	-	+	+	+	+	+	-	+	+	3	2
SP-12	-	+	+	+	+	+	-	+	+	3	2
SP-13	-	+	+	+	+	+	-	+	+	3	2
SP-14	-	+	+	+	+	+	-	+	+	3	2
SP-15	-	+	+	+	+	+	-	+	+	3	2
SP-16	-	+	+	+	+	+	-	+	+	3	2T
SP-17	-	+	+	+	+	+	-	+	+	3	2T
SP-18	-	+	+	+	+	+	-	+	+	3	2
SP-19	-	+	+	+	+	+	-	+	+	3	2
SP-20	-	+	+	+	+	+	-	+	+	3	2

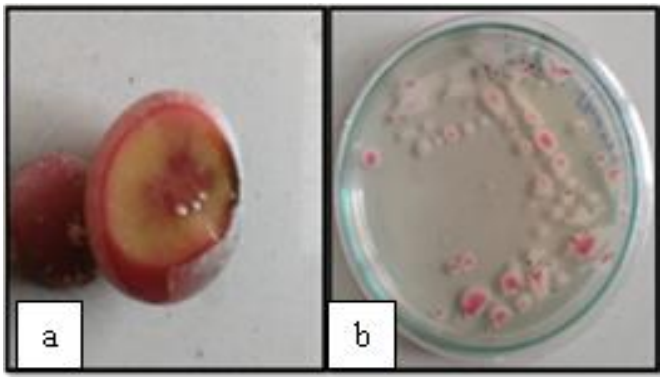

Fig 1: a) bacterial oozing from bacterial wilt infected potato tuber b) isolation of *R. solanacearum* on TZC media

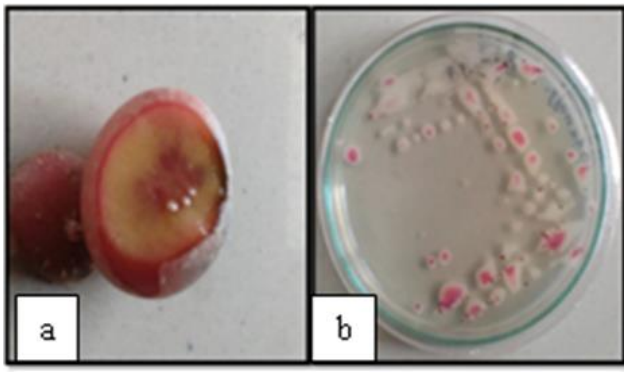

Fig 2: a) Potato tuber inoculated with *R. solanacearum* isolates producing brown rot symptom b) Ooze test from potato tuber inoculated with *R. solanacearum* isolates

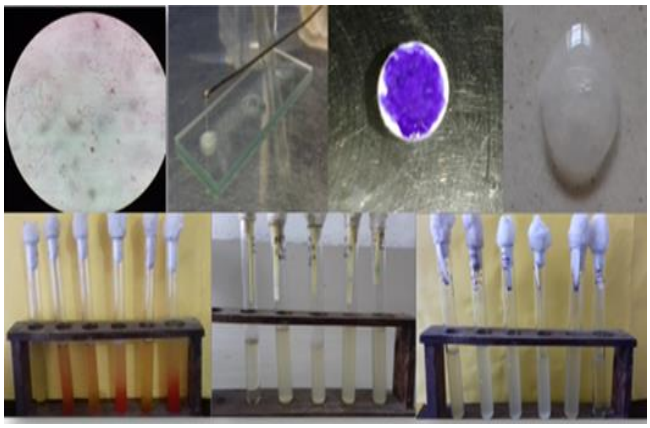

Fig 3: a) Gram staining b) KOH test c) Oxidase test d) Catalase test e) Nitrate test f) Indole test g) Hydrogen sulphide production test (from left to right)

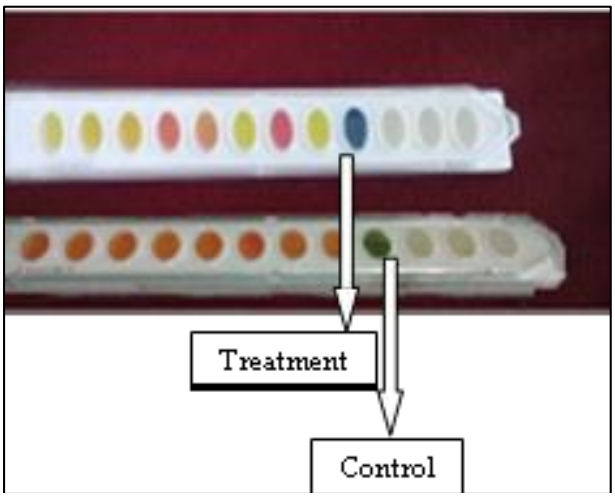

Fig 4: Citrate utilization test

Fig 5: The result of biovar test of strains of *R. solanacearum*

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