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Biochemical and physiological characterization of *Ralstonia solanacearum* causing bacterial wilt of tomato

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Abstract

Bacterial wilt, caused by *Ralstonia solanacearum* (Smith) is a destructive and prevalent soilborne disease that limits tomato production in the tropics, subtropics, and warm temperate regions of the world. The present studies physiological, biochemical and pathogenicity tests were conducted to characterize *R. solanacearum*. The bacterium showed positive reaction in simple staining as purple coloured rod shaped cells, in potassium hydroxide solubility test the bacterium formed thick mucoid slime thread on reacting with KOH solution, in gelatin liquification the bacterium inoculated test tubes failed to solidify in comparison to control, among test conducted for utilization of sugars *viz*., maltose, lactose, cellobiose, sorbitol, mannitol and dulcitol the change in colour was observed from green to yellow, in dole and H_2S production test also showed positive reaction as there was turbid growth around stab and formation of black precipitates was observed. In case of starch hydrolysis, absence of colourless halo around the bacterial streak was observed and in gram staining the bacterium appeared as pink coloured rod-shaped cells which indicated the negative reaction. The pathogenicity test was conducted by three methods *viz*., soil drenching method, stem inoculation method and seedling root dip method containing bacterial suspension of 10^8 cfu/ml to study the virulent behaviour of *Ralstonia solanacearum*. Among all methods the initial symptoms of disease development were observed within 5-6 days of inoculation. The initial symptoms produced were flaccid appearance of leaves due to loss of turgidity which further progressed as drooping of leaves and complete wilting of plants. However, the drooping of leaves was observed in 15 days by stem inoculation method, followed by 14 days by seedling root dip inoculation method and 12 days by drench inoculation method.

Keywords: Bacterial wilt, Tomato, biochemical, physiological, characterization, *Ralstonia solanacearum*

Tomato (*Solanum lycopersicum* L.) is one of the most widely grown and economically significant solanaceous vegetable crop throughout the world after sweet potato and potato. In terms of nutrition, tomato plants are good source of iron, vitamins, minerals and antioxidants that are beneficial to human body. The major component of red tomatoes is lycopene, a main carotenoid in tomato. Among the pharmacological activities of lycopene and other phenolic compounds includes anticancer, anti-inflammatory, antidiabetic, antioxidant, vasodilator and cardio protective effects (Zhu *et al*., 2020).

India ranks second in the area as well as in production of tomato with production of 20.573 million grown in 8,12,000 hectares with an average yield of 25.3 MT/ha (Anonymous, 2020). In Himachal Pradesh tomato is cultivated in an area of 13,185 hectares with production of 5,39,540 tonnes (Anonymous, 2021). Bacterial wilt incited by *Ralstonia solanacearum* (synonyms *Pseudomonas solanacearum* and *Burkholderia solanacearum*) is one of the devastating diseases of tomato crop in the tropical and subtropical areas of the world (Wei *et al*., 2018).

Ralstonia solanacearum is a soil borne, rod shaped, gram negative, β-proteobacterium which is pathogenic on more than 200 plant species belonging to 54 different botanical families including wide range

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of economically important crops such as eggplant, potato, tobacco, tomato and non-solanaceous crops such as peanut, banana and ginger (Genin and Denny, 2012). In India, the prevalence of bacterial wilt in tomato was first reported from Solan district of Himachal Pradesh (Gupta *et al*., 1998) where tomato is one of the major crops generally grown in summer season. Bacterial wilt was first noticed in Kangra valley of Himachal Pradesh in 1981 and remained sporadic in nature till 1985. Since then, the disease has become endemic in the mid-hill and sub-humid zone of Himachal Pradesh, comprising Kangra and Mandi districts (Sood and Singh, 1993). It causes substantial losses, varying from 2 to 90 % in different agroclimatic conditions, seasons, cultivars and strains of pathogen thus a major constraint on tomato production. Therefore, the present study was conducted to study the virulent behaviour of *Ralstonia solanacearum* based on cultural, physiological and biochemical characteristics.

Materials and Methods

The plants showing typical symptoms of vascular discoloration caused by *R. solanacearum* were collected and brought to bacteriological research laboratory in the Department of Plant Pathology. The stem of wilted plants showing discolored vascular tissues was cut into small pieces of 3-4 mm size using a sterilized scalpel blade. The infected bits were then surface sterilized in 1.0 per cent sodium hypochlorite for 30 seconds followed by three subsequent washings of sterile water to remove traces of sodium hypochlorite. The infected bits were then suspended in a test tube containing sterilized distilled water for 10 minutes. The oozing of the bacterial cells from the tissue took place, turning the water in the test tube milky.

The pathogenicity test was carried out to prove Koch's postulates. Tomato nursery was raised by sowing the seeds in pro-trays and filled with coco-peat, vermiculite and perlite in the ratio 3: 1: 1 respectively. The plastic pots (23×20×26 cm) containing sterilized soil were used for transplanting. Twenty-five to thirty days old healthy seedlings having 3-4 true leaves were used for transplanting in pots and having two seedlings per pot. The pots were filled with sand and potting medium in the ratio of 1:3 respectively. The potting mix was composed of humus and soil in the ratio 1:2 respectively. For the pathogenicity test a set of three (30 day old) tomato plants were inoculated by following soil drenching method, stem inoculation method and seedling root dip technique with the bacterial suspension containing 10^8 cfu/ml.

The pathogen *R. solanacearum* was characterized according to the guidelines described in the Bergey's Manual of Systematic Bacteriology (Garrity, 2001).

Simple staining- A drop of crystal violet (0.5% aqueous) was added to the bacterial smear and kept for 1 minute followed by rinsing in a gentle stream of running tap water. The slide was blotted dry and then microscopic observations were recorded.

Gram staining- The slide was flooded with aqueous crystal violet stain (0.5% aqueous) for 30 seconds and followed by washing with water and decolorized with 95 per cent ethanol. Then, the slide was flooded with counter stain safranin for 30 seconds and washed in a gentle stream of running tap water and decolorized with 95 per cent ethanol. The slide was rinsed with water, blotted dry and examined under the microscope.

Potassium hydroxide (KOH) solubility test- A drop of 3 per cent aqueous KOH was placed on slide and a single colony of the pathogen was removed using a cooled sterilized wire loop and mixed into KOH until an even suspension was obtained. The loop was then lifted from the slide and observations were recorded.

Starch hydrolysis-The ability of bacterium to hydrolyse starch was studied on nutrient agar media containing 1.0 per cent soluble starch. The liquefied nutrient agar was poured to Petri plates and allowed to solidify. After inoculating plates, it was incubated for seven days at 28 ± 1°C. The plates were then flooded with Lugol's iodine solution (Iodine 1g, potassium iodide 2 g and distilled water 300 ml).

Production of hydrogen sulphide- Sulphide indole motility medium was prepared and autoclaved. After autoclaving 10 ml of medium was poured in test tubes and allowed to solidify. The bacterial colony of *R. solanacearum* was picked and test tubes were inoculated followed by incubation for 24-36 hrs. Then 2 to 3 drops of Kavoc's reagent were added into inoculated test tubes to detect the H_2S production.

Gelatin liquification- Gelatin agar medium was prepared and 10 ml of medium was dispensed in test tubes. The test tubes were then allowed to solidify in upright position. The solidified test tubes were then

inoculated with a loopful of *R. solanacearum* by stabbing 4-5 times in centre of medium. The inoculated test tubes were then incubated at 28 ± 1°C for 72 hrs. along with control. After incubation test tubes were kept at 4°C until the control test tubes were solidified and observations were recorded.

Results and Discussion

The pathogen *R. solanacearum* was characterized according to the guidelines described in the Bergey's Manual of Systematic Bacteriology (Table 1). After conducting ooze test, bacterial growth was observed after streaking a loopful of bacterial cell suspension on Triphenyl Tetrazolium Chloride (TZC) medium after 24-36 hrs. The well separated irregular round, dull white, fluidal colonies, with light pink centre were observed (Plate 1) When stem pieces of such plants were suspended in distilled water, a milky white stream of ooze emerged from the tissue (Plate 1b). According to Kelman (1954), triphenyl tetrazolium chloride (TZC) medium is used to distinguish *R. solanacearum* from other bacteria during isolation. French *et al.*

Table 1: Physiological and biochemical tests for characterization of *Ralstonia solanacearum* (+) positive result, (-) negative result

Test	Reactions	Observations
a) Simple staining	+	Appearance of bacterium as purple colored rod cells
b) Gram staining	-	Appearance of bacterium as pink colored rod cells
c) KOH solubility tests	+	Appearance of thick mucoid string
d) Starch hydrolysis	-	Bacterial streak does not exhibit colorless halo
e) Gelatin liquification	+	No solidification of inoculated test tubes
f) H_2S production	+	Appearance of turbid growth around stab and formation of black precipitates
g) Utilization of sugars		
i. Maltose	+	Change of color from green to yellow
ii. Lactose	+	Change of color from green to yellow
iii. Cellobiose	+	Change of color from green to yellow
iv. Sorbitol	+	Change of color from green to yellow
v. Mannitol	+	Change of color from green to yellow
vi. Dulcitol	+	Change of color from green to yellow

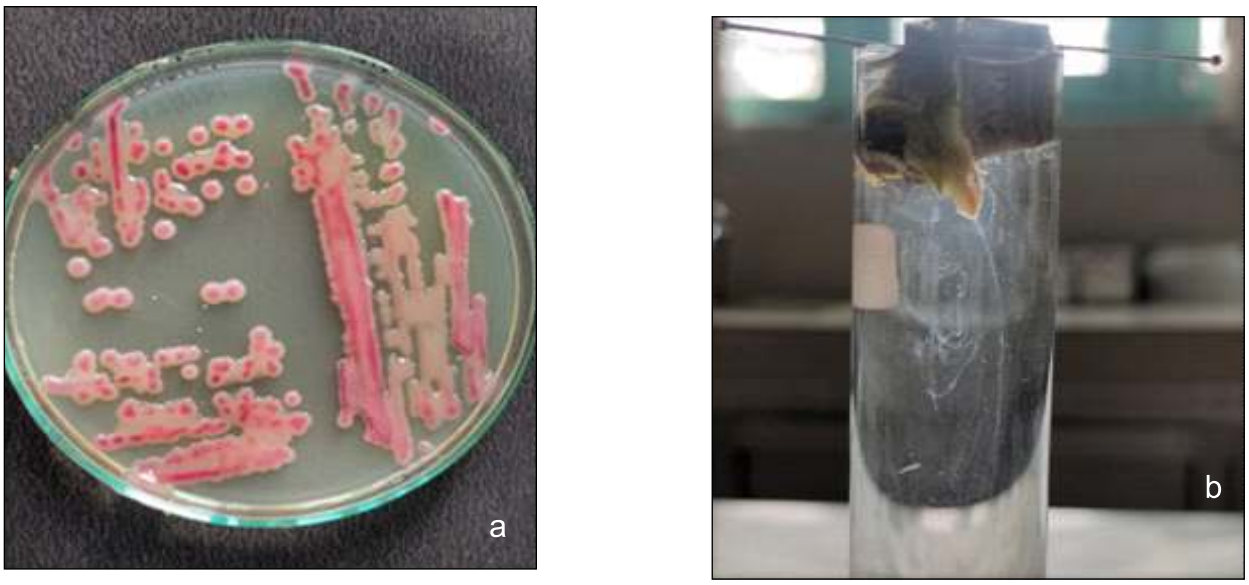

Plate 1.Isolation of R. solanacearum on TZC medium (a) and bacterial ooze test (b)

(1995) also classified the virulent coloniesof *R. solanacearum* as elevated, fluidal and either entirely white or with a pale red centre in contrary avirulent mutant colonies were butyrous and deep red. Khasabulli *et al*. (2017)and Chauhan *et al*. (2022) also reported similar results for the bacterium growth as dull white colour, fluidal, irregularly round colonies with light pink centre.

The results of gram staining reactions showed that the cells of *R. solanacearum* were small rod shaped, pink coloured when observed under microscope. The bacterium showed negative reaction for gram staining. Schaad (2001) alsotested colony of *R. solanacearum* from TTC plates and mixed with a few drops of water on a glass slide and gram stained. He observed staining results under microscope as negative reddish pink rod-shaped cells. Similar results were also reported Shahbaz *et al*. (2015). When single colony of pathogen was mixed with a drop of 3 per cent aqueous KOH solution for 5 seconds, it formed slime thread of culture suspension when loop was lifted from glass slide. It was observed that the test bacterium gave a positive result for the KOH test, as it formed thick mucoid string on reacting with KOH solution (Plate 2 a). According to Suslow *et al*. (1982) the KOH technique is far simpler and quicker than the conventional Gram strain method, which uses dyes for distinguishing between Gram-positive and Gram-negative bacteria. The results agree with those described by Vanitha *et al*. (2009).The bacterium R. solanacearum showed negative reaction in starch hydrolysis test. The results are in conformation with as reported by Zhang *et al*. (2006) and Nouri *et al*. (2009). The bacterium R. solanacearum was tested for the indole production and motility which revealed positive results for motility, cultures showed turbid growth around the stab (Plate 2 b). Tripathi (2004) reported positive results of *R. solanacearum* in the production of indole. The bacterium was tested for utilization of sugar and results revealed the change of colour from green to yellow indicating the oxidization of sugars by bacterial isolates. However, the control plates of different sugar and sugar alcohol remain unchanged. Similar results were also reported by Kumar *et al*. (1993). The results obtained from pathogenicity tests revealed that the initial symptoms of disease development were observed within 5-6 days of inoculation in drench inoculation, seedling dip inoculation and stem inoculation. The initial symptoms produced were flaccid appearance of leaves due to loss of turgidity which further progressed as drooping of leaves and complete wilting of plants. However, the drooping of leaves was observed in 15 days by stem inoculation, followed by 14 days by seedling root dip inoculation and 12 days by drench inoculation (Fig.1). *Vasse et al*. (2000) conducted two independent

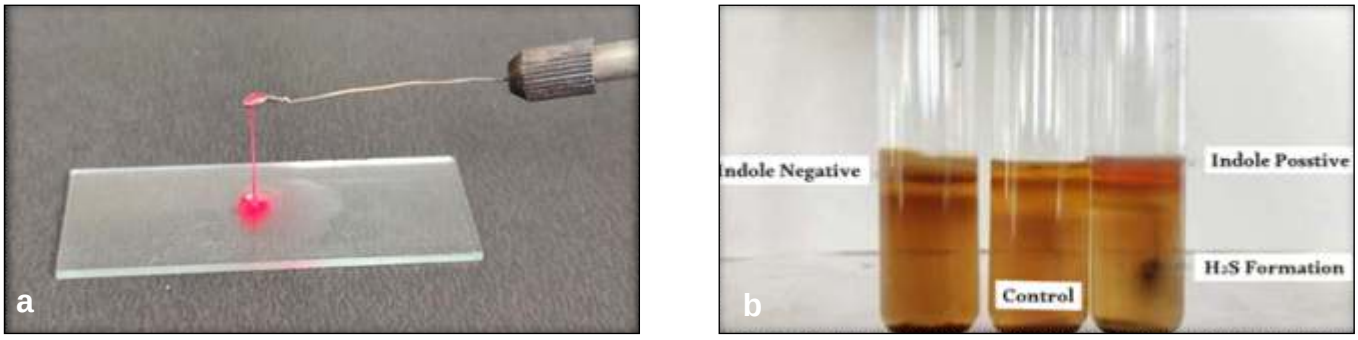

Plate 2. Potassium Hydroxide Solubility (KOH) test (a) Indole and H_2S production (b)

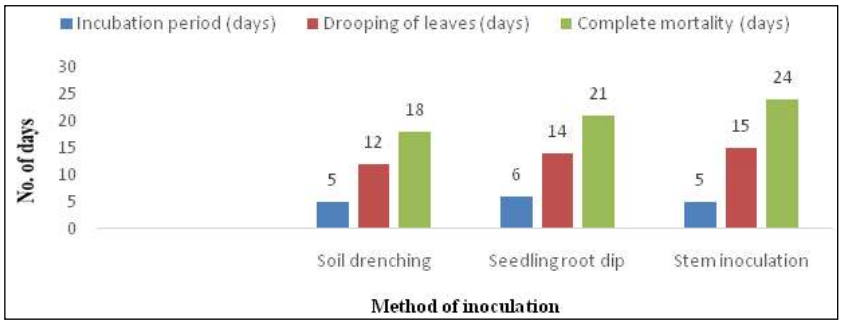

Fig.1. Pathogenicity test of R. solanacearum

pathogenicity tests, with 20 tomato plants per experiment. The wilting symptoms along with bacterial colonization were observed in tomato plants within 10 or 18 of post inoculation. Kumar and Sood (2003) also observed wilting of plants within 12 and 14 days of inoculation by suspension drenching and seedling root dip method.

Conflict of interest: The authors declare that there is no conflict of interest in this research paper.

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