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Cultural and Biochemical Characterization of *Ralstonia solanacearum* Causing Bacterial Wilt in Tomato

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Cultural characteristics *viz.,* colony count, colony colour and colony shape of *Ralstonia solanacearum* on different period of time were studied *in vitro* using eight culture media. Highest average colony count was recorded on triphenyl tetrazolium chloride agar (65.33), white fluidal colonies with spiral pink centre and the minimum average colony count was recorded on yeast extract chalk agar (41.50) with irregular yellow color colonies. The bacterium showed positive reaction for solubility in KOH, production of catalase, motility test, hydrolysis of starch & casein and negative reaction for gram staining.

Keywords: *Ralstonia solanacearum*, culture media, biochemical characteristics and *Solanum lycopersicum*.

Tomato (*Solanum lycopersicum* L.) is one of the most widely grown fruit vegetable in the world; India ranks second in the area as well as in production of Tomato. Tomato is one of the most important "protective foods" because of its special nutritive value. China is the largest tomato producing country in the world, followed by India and USA. In India, the area under tomato cultivation was 880 thousand hectare with production of 18227 thousand MT and productivity of 20.7MT/ha. The Maharashtra state is the fourth largest tomato producer in the India with an area of 50 thousand hectare, production of 1050 thousand MT and productivity 21MT/ha. Other leading tomato producing states are Andra Pradesh, Karnataka and Orrisa.

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In the tropics, tomato production is severely constrained by disease and insect pests. Tomato crop is being affected by many fungal, bacterial, viral and nematode diseases. Among these diseases, bacterial wilt caused by *Ralstonia solanacearum* is one of the most economically important and devastating disease of tomato crop. The disease was first reported from Asia and South America (Smith, 1880). This disease is of common occurrence whenever solanaceous crops *viz* tomato, brinjal, potato and chilli etc are grown and is more severe under weather conditions of high temperature and high humidity, congenial for disease development [7]. In India, the losses due to bacterial wilt varied from 31.47 to 81.7 % and 36.88 to 91.06 % in fruit number and weight respectively [3].The plant mortality and losses in fruit yield due to bacterial wilt ranged from 10 to 100 and 10.83 to 92.62were observed by earlier worker[4].

The present article deals with cultural characteristics on various culture media and biochemical characteristics of *R. solanacearum.*

MATERIALS AND METHODS

Isolation of the pathogen

The soil samples and tomato crop wilted plants samples collected were subjected to isolations on selective synthetic media. The discolored vascular tissues of tomato plants were cut into small pieces and kept in glass beaker containing distilled water, maintaining infected tissue in contact with water surface. After five minutes water in glass beaker turned turbid due to oozing of bacterial cells from cut ends of diseased plants tissue and thus conforming the bacterial nature of the disease.

The small pieces of discolored vascular tissue measuring 4-5cm length were cut from the discolored stem and surface sterilized with mercuric chloride (0.1%) for 30 seconds. Then these were subjected to sequential washing in sterile water to remove traces of mercury chloride, if any. These surface sterilized bits were then suspended in 10ml sterile water in test tube for ten minutes, later water in test tubes turned turbid due to oozing of bacterial cells from cut ends of the diseased tissue. The bacterial suspension thus obtained was then diluted serially in sterile distilled water. One ml of the bacterial suspension was poured onto solidified surface of selective medium Triphenyl tetrazolium chloride agar (TZC) in sterilized glass petriplates (90mm) and incubated at 28° for 48 hours. The soil sample was serially diluted in distilled water and isolated the bacterium on TZC medium and incubated. After completion of incubation period, the plates were observed for development of the colonies of *R. solanacearum*, and identified virulent and avirulent colonies [2].The suspension was then streaked onto triphenyl Tetrazolium chloride (TTC) medium and incubated at 28±2ÚC for 2-3 days. Single bacterial colonies were transferred to fresh TTC plates. Creamy white colonies with pink centre occurred on TTC medium. The creamy white colonies were transferred to 5 ml of sterile distilled water 16 cm^3 ependorf tubes and represented stock suspensions.

Cultural characteristics

A total of 8 culture media were used to study their effect on colony count, colony color and shape of the colony on the different culture media. All the 8 test media were sterilized in autoclave at 15 lbs / $inch^2$ pressure for 20 min and cooled media were poured (@ 20 ml/plate) in sterilized glass Petri plates (90 mm dia) and allowed to solidify at room temperature. The 0.1ml of 48 hours old bacterial culture taken with micropipette, placed at the center of surface solidified media, spread uniformly to obtain well separated bacterial colonies. The inoculated plates were incubated at 28±2°C for 72 hours. Observations were taken on the colony characters like color, number of colonies and shape of the colonies.

Biochemical characteristics

Several biochemical tests *viz.*, Gram staining reaction, Potassium hydroxide solubility test, Catalase test, Starch hydrolysis, Motility test and Casein hydrolysis test were performed for confirmation of *R. solanacearum* isolates as described earlier worker.

Gram staining

A loop full of the bacterium suspension was smeared on clean glass slide, air fixed by gentle heating on flame of the spirit lamp. Aqueous crystal violet solution (0.5%) spread over this smear for 30 seconds and then washed with running tap water for a minute. This stained smear was later flooded with Grams iodine solution for one minute and rinsed in tap water. Later decolorized with 95% of ethanol until color runoff, washed with water and treated with Safranin as counter stain about 10 seconds, washed with water, air /blot dried and observed under research microscope at 100X using oil immersion technique.

Potassium hydroxide (KOH) solubility test

A drop of 3 per cent potassium hydroxide was placed on clean glass slide and to this 48 hr old bacterial culture was mixed with clean inoculation loop and stirred for 10 sec and observed for slime threads. When raised the wire loop, if strands of viscid material seen, then the bacterium is gram-negative.

Catalase test

A loopful of 24 to 48 hrs old culture of the test bacterium was placed on a clean glass slide and to this a drop of 3% hydrogen peroxide (H_2O_2) was mixed and allowed to react for few minutes and observed for the production of gas bubbles.

Starch hydrolysis test

The medium employed is referred to as starch broth and contains (peptone 10 g, beef extract 5 g, starch soluble 2 g, agar 20 g, distilled water 1000 ml). Sterilized the medium by autoclaving

and poured into sterilized Petri plates and on solidification of the medium, streaked pure culture of the test bacterium and incubated for 96 hrs at 28^0 C. Then flooded these plates with lugol's iodine and allow to reaction for few minutes. Reddish colored zones indicate negative reaction and appearance of yellowish, clear zones around the bacterial growth indicate positive reaction.

Motility test

The autoclaved and cooled motility agar medium contains (peptone 1 g, sodium chloride 5 g, agar-agar 4 g, distilled water 1000 ml) was poured in glass Petri plates and allowed to solidify. On this solidified medium loopful of pure culture (48 hrs old) of test bacterium streaked and incubated for 48 hrs. The motile bacteria forms spreading colony on the soft motility agar media.

Casein hydrolysis test

Autoclaved and cooled skim milk agar medium was poured in glass Petri plates and allowed to solidify. On the solidified medium a loopful of culture (48 hrs old) of the test bacterium was streaked and incubated for 48 hrs in an inverted position. A clear area/zone around the bacterial growth indicates positive reaction to casein hydrolysis, while absence of clear zone indicates negative reaction.

RESULTS AND DISCUSSION

Isolation of the pathogen

Typical virulent colonies of *R. solanacearum* developed within 48 hours. The virulent colonies appeared well-separated, irregular fluidal, dull white colored with slight pink centre and non-virulent colonies appeared dark red on TZC media. Similar findings were reported by earlier workers[1].

The 44 isolates of *Ralstonia solanacearum*, causing bacterial wilt of potato and reported that all the isolates produced creamy or off white colored colonies on NA medium after 24 hr incubation at 28°C were observed by previous worker[1].

The bacterium produced milky white ooze containing bacterial cells and their extra cellular polysaccharide in sterile distilled water. On TZC medium, the bacterial growth was dull white, fluidal, irregular or round colonies with light pink centers. The prevalence of races and biotypes of *Ralstonia solanacearum* in India and studied the cultural characteristics of bacterial wilt affected plants showed ooze when isolated on SMSA medium were reported by earlier worker[7].

Cultural Characteristics

Colony count

The results (Table 1 and Fig 2B) revealed that the average colony count recorded with all the test media was ranged from Average colony count recorded with the test media was ranged from 41.50 (Yeast extract chalk agar) to 65.33 (Triphenyl tetrazolium chloride). However, it was significantly highest average colony count (65.33) was recorded on Triphenyl tetrazolium chloride Agar (Table 1). This was followed by Casamino peptone glucose agar (59.16), Potato dextrose agar (55.58), Yeast extract peptone agar (51.00), Yeast extract milk agar (48.41), Nutrient agar (47.58) and Yeast extract agar (46.79) these three were at par and SMSA (43.25). Whereas Yeast extract chalk agar was found least suitable with minimum average colony count (41.50) of the test pathogen. The results are in confirmatory with earlier worker[7].

Colony colour

The results (Table 1) revealed that the pink centered white fluidal colonies were devoloped on Triphenyl tetrazolium chloride agar and SMSA media; cream or off-white colored colonies on Casamino peptone glucose agar, Yeast extract agar and Potato dextrose agar; creamy white and dull white colonies on Nutrient agar, Yeast extract peptone agar and Yeast extract milk agar and yellow colored colonies on Yeast extract chalk agar were developed of the bacterium *R. solanacearum*. Similar findings are reported by previous worker [7].

Colony Shape

The results (Table 1.) revealed that irregular shaped, smooth, highly fluidal colonies were developed on Triphenyl tetrazolium chloride, Casamino peptone glucose, Potato dextrose agar and SMSA media; while, round small colonies developed on rest of the test media *viz*., Nutrient agar, Yeast extract milk agar, Yeast extract agar, Yeast extract peptone agar and Yeast extract chalk agar.

Typical dull white, highly fluidal and pink centered colonies of virulent *R solanacearum* and those of non virulent as dark red colonies were reported by many worker .[6]

Biochemical Characteristics

The results (Table 2) revealed that the

Table 1. Effect of culture media on growth and cultural characteristics *R. solanacearum*

S. No	Treatment	Mean count */ Plate				Avg. (No.)	Color	Shape
		24hrs	48hrs	72hrs	96hrs			
T_1	Triphenyl tetrazolium chloride agar (TZC)	56.67 (48.83)	64.00 (53.13)	68.00 (55.55)	72.67 (58.48)	65.33 (53.93)	Pink centered	Irregular –round
T_2	Casamino peptone glucose agar (CPG)	46.67 (43.09)	54.33 (47.48)	66.67 (54.74)	69.00 (56.17)	59.16 (50.28)	Cream White	Irregular –round
T_3	Yeast extract milk agar (YEMA)	42.67 (40.79)	46.00 (42.71)	50.67 (45.38)	55.00 (47.87)	48.41 (44.09)	Dull white	Round small
T_4	Yeast extract peptone agar (YPA)	39.00 (38.65)	48.00 (43.85)	56.00 (48.45)	61.00 (51.35)	51.00 (45.57)	Dull white	Round small
T_5	Potato dextrose agar (PDA)	43.33 (41.17)	50.67 (45.38)	61.00 (51.35)	67.33 (55.14)	55.58 (48.20)	Cream white	Irregular
T_6	Nutrient agar (NA)	37.33 (37.66)	43.33 (41.17)	52.00 (46.15)	57.67 (49.41)	47.58 (43.61)	Dull white	Round small
T_7	Yeast extract agar (YEA)	33.33 (35.26)	45.67 (42.52)	53.33 (46.91)	56.33 (48.64)	46.79 (43.16)	Cream white	Round small
T_8	Yeast extract chalk agar (YEA)	32.33 (34.65)	37.67 (37.86)	45.00 (42.13)	51.00 (45.57)	41.50 (40.11)	Yellow	Round
T_9	SMSA	31.67 (34.25)	39.33 (38.84)	47.00 (43.28)	55.00 (47.87)	43.25 (41.12)	Pink centered	Irregular – round
	S.E. ±	0.62	0.64	0.68	0.57	0.62	—	—
	CD (P= 0.01)	1.86	1.90	2.02	1.70	1.87		

Table 2. Biochemical characterization of *Ralstonia solanacearums*

S. No.	Biochemical tests	Reaction
1	Gram staining	Gram negative
2	Potassium hydroxide test (KOH)	Positive
3	Catalase test	Positive
4	Starch hydrolysis	Positive
5	Casein hydrolysis	Positive
6	Motility test	Positive

bacterium *R. solanacearum* which showed positive reactions for potassium hydroxide solubility test, catalase test, starch hydrolysis test, motility test and casein hydrolysis test and showed negative reaction for gram staining. Staining results were observed under microscope for negative reddish pink staining. Similar results were also reported by earlier author [1 & 5].

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