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Physiological and biochemical characterization of *Ralstonia solanacearum* inciting bacterial wilt of brinjal

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Abstract

Ralstonia solanacearum causes bacterial wilt of solanaceous crop plants including brinjal, a most devastating in humid tropic. Ten different isolates of the causal bacterium *R. solanacearum* were isolated from naturally wilted brinjal plants collected from different locations of Dapoli tahsil and were characterized. Gram's staining and Potassium hydroxide solubility test revealed that all isolates of *R. solanacearum* were Gram negative in reaction and positive reaction for motility test. The all isolates of *R. solanacearum* were positive in acid production, gas production, starch hydrolysis, hydrogen sulphide production, cellulose decomposition and catalase activity. In case of methyl red test appearance of red colour indicates strong acid forming ability of the bacterium, while retaining of normal colour of the medium indicates alkalinity and negative reaction.

Keywords: Bacterial wilt, brinjal, biochemical, characterization, *Ralstonia solanacearum*

Introduction

Brinjal or eggplant (*Solanum melongena* L.) is an important solanaceous crop of sub-tropics and tropics region. The name brinjal is popular in Indian subcontinents and is derived from Arabic and Sanskrit whereas the name eggplant has been derived from the shape of the fruit of some varieties, which are white and resemble in shape to chicken eggs. In India, brinjal it is one of the most common, popular and principal vegetable crops grown throughout the country except higher altitudes. It is a versatile crop adapted to different agro-climatic regions and can be grown throughout the year.

India is the second largest brinjal producing country followed by China. In India, the total area under brinjal was 669 thousand hectares with production of 12,400 thousand MT and with productivity of 18.9 MT/ha during 2016-17. In Maharashtra, area under brinjal was 22.14 thousand hectares with annual production of about 433.28 thousand MT and with a productivity of 19.57 MT/ha. during 2016-17 (Anonymous, 2017). Pune is the leading brinjal growing district in Maharashtra with 3.59 thousand hectares area and production of 71.80 thousand MT. In *Konkan* region of Maharashtra, total area under brinjal was 1.21 thousand ha. with production of 20.02 thousand MT and productivity of 16.54 MT/ha. during the year 2016-17 (Anonymous, 2018) [2]. Brinjal is primarily consumed as cooked vegetable in various ways. It is low in calories and fats, contains mostly water, some protein, fibre and carbohydrates. Bitterness in brinjal is due to the presence of saponins and glycoalkaloids (Mariola *et al.*, 2013) [13]. Brinjal is also known for its medicinal properties and has also been recommended as an excellent remedy for liver complaints and diabetic patients (Tiwari *et al.*, 2009) [22]. Brinjal crop is known to be infected by a number of diseases caused by fungi, bacteria, viruses and phytoplasma which adversely affect the yield and quality of produce. Bacterial wilt caused by *Ralstonia solanacearum* (Smith) Yabuuchi is deemed to be one of the most important plant diseases in tropical agriculture. It affects a wide range of economically important solanaceous crops such as potato, tomato, brinjal and chilli. Bacterial wilt is said to be causing 10 to 90% crop losses around the world. In India, the disease is highly devastating and causes losses up to 80-90% (Katakya *et al.*, 2017) [9]. The pathogen is well established in the soils of *Konkan* region and it poses a major threat to the crop in the region during *Rabi* and summer season. As the crop is one of the major vegetable crops in the region and sometimes completely lost due

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to bacterial wilt, it was felt necessary to carry out the studies against this disease to find out effective disease management to minimize the losses caused by the disease.

Materials and Methods

The brinjal plants showing typical wilting symptoms were carefully uprooted and brought to the laboratory. Each plant was critically observed by ooze test taking sections of roots in clean water to confirm the presence of bacterium in the form of ooze. All the physiological and biochemical tests viz., simple staining, Gram's staining, KOH solubility test, motility test, acid production test, gas production test, starch hydrolysis, production of hydrogen sulphide (H₂S), cellulose decomposition, catalase test and methyl red test were carried out under *in vitro* conditions as per standard methods.

Isolation of causal organism

The plant showing wilt symptoms were collected from Botany farm and nearby places around Dapoli and brought to the laboratory for further studies. The soil adhering on roots was

washed under tap water. The roots which were positive in ooze test were cut into bits. Such root bits used for isolation. The root bits were surface sterilized in 0.1 percent mercuric chloride and washed thrice in distilled sterilized water to remove the traces of HgCl₂. These root bits were kept in a test tube containing sterile distilled water for 5 to 10 minutes to facilitate the bacterial ooze flows to form the bacterial suspension. A loopful of the suspension was transferred with the help of sterilized inoculation needle and streaked on solidified nutrient agar medium poured in Petri plates. Inoculated plates were incubated in BOD incubator at 28 ± 2 °C until the growth of associated organism was observed.

The isolation of the pathogen from soil was carried out by serial dilution of the soil samples and plated on nutrient agar medium. The slants with pure culture were preserved in refrigerator at 4 °C and maintained in pure form for further studies.

Same methodology was used for collection of different isolates of the causal bacterium from different locations. Total 10 isolates of *R. solanacearum* were collected (Table 1).

Table 1: Isolates of *Ralstonia solanacearum* collected from different locations.

Sr. No.	Isolate No.	Isolated from plant/ soil	Place of Collection
1	RS1	Plant	Wakawali
2	RS2	Plant	Dapoli
3	RS3	Soil	Wakawali
4	RS4	Plant	Gavhe
5	RS5	Plant	Gavhe
6	RS6	Plant	Mauje Dapoli
7	RS7	Plant	Tetawali
8	RS8	Plant	Vanaushi (Dumadev)
9	RS9	Plant	Bondivali
10	RS10	Soil	Dapoli

Simple staining

Fresh cultures of all isolates were separately diluted in sterilized water to so as to get the thin smear on the glass slide. A layer of smear was gently applied on slide and allowed to air dry. Heat fixing of smear was done by gently passing the reverse surface of the slide through burner. Bacterial smear heat fixed on slide was then flooded with methylene blue for one minute. Slides were then washed gently by passing through gentle flow of water air dried and observed under low power and high power to record morphological characters.

Gram staining

Gram staining procedure was performed as per standard method. Hi-media Gram staining kit was used for staining of culture. The 24 hrs. old culture was mixed separately in sterilized water to get suspension. A thin smear of suspension was prepared on a clean slide and allowed to air dry. Heat fixing of smear was done by passing over a flame. The smear was flooded with (0.5%) crystal violet solution for two minute and then washed under gentle stream of water, dried. It was further treated with Gram's iodine solution for 1 minute, washed and air dried. The smeared portion of the slide was then decolorized with 95% ethanol. Again, washed the slide, air dried and then counter-stained with safranin for 30 seconds. The slides were washed with water, air dried and observed under low power, high power and oil immersion lens of microscope.

Koh solubility test

A loopful of bacterial culture from one-week old colony was placed on a glass slide and mixed with a drop of 3 percent

aqueous KOH solution for 10 seconds with the help of a toothpick. The toothpick was then raised a few centimeters from the glass slide and observed for formation of strands of viscid material for confirmation of Gram reaction. This test was carried out for the cultures of all isolates. Thread like slime formation indicates Gram negative reaction.

Motility test

Motility test agar semisolid medium was prepared (peptone - 10 g; Beef Extract -3 g, sodium Chloride - 5 g; Agar - 4 g; TTC- 0.05 g and 1 liter distilled sterile water) in test tubes separately for each isolate and autoclaved at 1.054 kg/cm² (121 °C) for 20 minutes. Then such tubes were cooled in standing position to prepare stab. Two days old colony on motility test agar medium was picked up with the sterilized needle and stabbed into the medium in test tube at a depth of 1 cm. The needle was removed in the line of insertion without disturbing the medium. Tubes were incubated at 28 °C until growth. A cloud of growth away from the line of insertion indicates motility positive reaction.

Acid production test

Acid production ability of the bacterium was tested by inoculating the culture on nutrient broth (pH - 7.0) containing 2 percent glucose. The tube was incubated for 24 hrs. at room temperature (28 ± 2 °C). The production of acid was tested by adding few drops of methyl red indicator. This test was performed for all the isolates. A distinct pink or red colour indicates the presence of acid.

Gas production test

The ability of the culture to produce gas was tested by growing the organism in nutrient broth containing 2 percent glucose. The test was performed for all the isolates separately. About 5 ml medium was poured in the test tube containing inverted Durham's tube and plugged with cotton. The test tube was then sterilized in an autoclave. After cooling the tube at room temperature 1 ml of bacterial suspension was poured in to the tube. Then the tube was incubated at ambient temperature (28 ± 2 °C) for seven days. An air bubble in the inverted Durham's tube indicates gas producing ability.

Starch hydrolysis

The ability of bacterium to hydrolyse starch was studied by growing on starch agar plates i.e., nutrient agar containing one percent soluble starch (soluble starch - 2 g/l; peptone - 5 g/l; beefs extract - 3 g/l; agar-agar - 20 g/L). The medium was prepared and autoclaved at 121 °C for 20 minutes. The sterilized liquefied medium was then poured into sterilized Petri plates and allowed to solidify. The plates were then inoculated by streaking with individual culture aseptically, labelled with separately for each isolate and sealed to avoid chances of contamination. These plates were then incubated in inverted position for 7 days at room temperature (28 ± 2 °C). Then the plates were flooded with Lugol's iodine solution (prepared by mixing 1g iodine and 2 g potassium iodide in 300 ml distilled water, stirred for until dissolved completely) and observed for appearance of clear zone around the bacterial growth which indicates starch hydrolysis.

Production of hydrogen sulphide

The ability of the culture to produce hydrogen sulphide was tested by inoculating the tubes of nutrient broth containing 3 percent additional peptone. The tubes were sterilized at 1.054 Kg/cm² (121 °C) for 20 minutes. Each tube was labelled with the culture code and then inoculated with the respective culture. Filter paper strips were soaked in super saturated solution of lead acetate, dried and inserted in the tubes by taking care that the strips do not touch the medium. The tubes were then plugged with cotton. The tubes were incubated for seven days at room temperature (28 ± 2 °C). Blackening strips indicates the production of hydrogen sulphide gas.

Cellulose decomposition

Nutrient broth was preparing and dispensed in test tubes. Then tubes were inoculated with separately each of the isolated culture. The sterilized filter paper strips one in each were immersed in the medium. Each tube were plugged with cotton and incubated at room temperature (28 ± 2 °C) for 30 days. Non-inoculated tubes containing filter paper strips served as control. Decomposition of the cellulose is indicated by maceration of the filter paper strips at the site of growth of the bacterium.

Catalase test

Sterilized nutrient agar medium was poured into sterilized Petri plates and allowed to solidify. A fresh bacterial culture (48 hrs. old) was streaked on to solid medium in plates and was incubated at 28-30°C for 2 days. After incubation plates were flooded with 3% hydrogen peroxide (H₂O₂) solution. Bubble formation indicates a positive reaction, while no bubble shows negative reaction to catalase activity. This test was performed for all the cultures.

Methyl Red (MR) test

Methyl red broth (buffered peptone- 7.0 gm; glucose- 5.0 gm; dipotassium hydrogen phosphate- 5.0 gm; Distilled water- 1000 ml) was prepared and dispensed in test tubes and sterilized. Each test tube inoculated separately with culture code. The test tubes were incubated at room temperature for 2 days. After 2 days few drops of methyl red indicator was added to each test tube and shaken gently to homogenize. The tubes were kept under observation for 4 more days. Appearance of red colour indicates strong acid forming ability of the bacterium, while retaining of normal colour of the medium indicates alkalinity and negative reaction.

Result and Discussion

The bacterium *R. solanacearum* was isolated on nutrient agar medium from infected brinjal roots and soils and its pathogenicity was confirmed. The results of staining reactions revealed that the cells of *R. solanacearum* were straight rods scattered singly or in pairs. These results are in an agreement with the findings of Umesha *et al.* (2005) [23], Singh *et al.* (2010) [21], Selastin *et al.* (2014) [19] and El-Habbaa *et al.* (2016) [7].

Biochemical tests such as Gram's staining, KOH test, motility test were carried out and the results revealed that all the ten isolates of *R. solanacearum* were Gram negative in reaction and were positive for KOH test and motile. Rahman *et al.* (2010) [17], Chakravarty and Kalita (2011) [4], Katakya *et al.* (2017) [9] and Sharma (2018) [20] reported similar results. Murthy and Srinivas (2012) [15] and Mohamed *et al.* (2014) [14] reported that all the tested isolates had a positive reaction for motility.

The bacterium was able to produce acid in medium containing two percent glucose. All the ten isolates showed positive results for acid production test. The results are in agreement with Rath and Addy (1977) [18] who reported that, *Pseudomonas solanacearum* was positive in acid production with dextrose. Khetmalas (1984) [11] and Pawaskar *et al.* (2014) [16] also found that, *P. solanacearum* isolated from groundnut and chilli, respectively produced acid when grown in glucose containing medium. Similarly, Chakravarty and Kalita (2011) [4] and Zhang *et al.* (2011) [26] concluded that *R. Solanacearum* produces acid from glucose and sucrose.

All the ten isolates of *R. solanacearum* tested in present study were able to produce gas in a medium containing 2 percent glucose. These results are in confirmation with Rath and Addy (1977) [18] who reported that *P. solanacearum* produce gas from glucose, dextrose and salicilin. Khetmalas (1984) [11] reported *P. solanacearum* to be positive in gas production in presence of glucose. Pawaskar *et al.* (2014) [16] and Katakya *et al.* (2017) [9] also reported that *R. solanacearum* produces gas in glucose containing medium.

In respect of hydrolysis of starch, all the isolates were positive. This confirmed the findings of Das and Chattopadhyay (1955) [6] and Katakya *et al.* (2017) [9] who found that, *P. solanacearum* was positive to starch hydrolysis. However, the results of Bhide (1948) [3], He *et al.* (1983) [8], Khetmalas (1984) [11] and Chakravarty and Kalita (2011) [4] are in congruous to present results as they opined that *P. solanacearum* has negative reaction to starch hydrolysis.

All the isolates produced hydrogen sulphide gas after 7 days of incubation as revealed by blackening of strips. This result are in agreement with the reports of Rath and Addy (1977) [18], He *et al.* (1983) [8], Murthy and Srinivas (2012) [15], Mohamed *et al.* (2014) [14] and Katakya *et al.* (2017) [9] who reported *P. solanacearum* to be positive in H₂S production.

But the conclusion of Bhide (1948) [3] and Das and Chattopadhyay (1955) [6] disagree with present findings.

As far as cellulose decomposition ability of the test isolates is concerned, it was positive for all the ten isolates of bacterium as there was maceration of the filter paper strips at the site of growth of the bacterium. The results of Khetmalas (1984) [11] and Pawaskar *et al.* (2014) [16] were negative for cellulose decomposition.

The bacterium was positive for catalase test. When a drop of hydrogen peroxide (3%) was added to respective culture, air bubbles were released. All the ten isolates gave a positive response to catalase test. The results of Williamson *et al.*

(2002) [24], Chaudhary and Rashid (2011) [5], Murthy and Srinivas (2012) [15], Maji and Chakrabarty (2014) [12], Mohamed *et al.* (2014) [14] and Katakya *et al.* (2017) [9] are in concurrence with the results of present study.

Methyl red test differentiates between the organisms producing high acidity and those producing lower acidity initially which reverts towards neutrality. Among ten isolates, RS1, RS2, RS3, RS8 and RS10 showed positive reaction to this test while RS4, RS5, RS6, RS7 and RS9 showed negative reaction; which is in conformity with earlier reports of Chakravarty and Kalita (2011) [4] and Zhang *et al.* (2011) [26].

Table 2: Physiological and biochemical tests of *Ralstonia solanacearum* isolates

Tests	<i>Ralstonia solanacearum</i> isolates									
	RS1	RS2	RS3	RS4	RS5	RS6	RS7	RS8	RS9	RS10
Simple staining	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
Gram staining	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
KOH solubility test	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
Motility test	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
Acid production	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
Gas production	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
Starch hydrolysis	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
H ₂ S production	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
Cellulose decomposition	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
Catalase test	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
Methyl Red test	+ve	+ve	+ve	-ve	-ve	-ve	-ve	+ve	-ve	+ve

+ve = Positive test; -ve = Negative test

Conclusion

From the results (Table 2) of the present investigation it was concluded that the bacterium *R. solanacearum* was small straight rod shaped and Gram negative in reaction. The bacterium isolates showed positive reaction to all test conducted in *in-vitro* condition *viz.*, simple staining, KOH solubility test, motility test, acid production test, gas production test, starch hydrolysis, production of hydrogen sulphide (H₂S), cellulose decomposition, catalase test. Whereas, isolates RS1, RS2, RS3, RS8 and RS10 were positive for methyl red test and rest were negative.

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