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Cultural, biochemical and physiological studies of *Ralstonia solanacearum* causing wilt of brinjal

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Abstract

Bacterial wilt caused by *Ralstonia solanacearum* is a lethal disease of brinjal in tropical, subtropical and warm temperate regions of the world. In present investigation, isolation of the biochemical and physiological characterization of *R. solanacearum* was conducted. The results of the various cultural and biochemical characters revealed that the bacterium isolates were rod shaped, Gram negative, non-capsulated. They appeared dull white with light pink colored center on TZC medium and the colonies were highly fluidal producing copious slime. The bacterium was positive for nitrate reduction test and negative for starch hydrolysis and gelatin liquification. Physiological studies *viz.*, Effect of temperature and effect of pH on the growth of *R. solanacearum* on CPG agar medium was studied. The temperature of 30 °C was found optimum for the growth of pathogen as the highest population (196.66 x 10⁵cfu/ml) were recorded at this temperature level followed by 25 °C (114.66 x 10⁵cfu/ml) and 35 °C (103.33 x 10⁵cfu/ml) and the maximum number of bacterial colonies were recorded at pH 7 (154.00 x 10⁵cfu/ml).

Keywords: Biochemical, physiological studies, *Ralstonia solanacearum*

Introduction

Bacterial wilt of brinjal and other solanaceous vegetables caused by *Ralstonia solanacearum* (Smith) Yabuchi (Yabuchi *et al.*, 1995) ^[16] is the most destructive disease in the tropical, subtropical and temperate regions of the world, causing heavy economic loss. The bacterial wilt disease is wide spread, affecting many solanaceous vegetable crops in India, especially in Karnataka. The major hosts affected by this disease in India include tomato, potato, brinjal, Chilli, ginger, groundnut, tobacco, banana and other floricultural plants. In India, brinjal is cultivated in an area of 4,74,400 ha with a production of 76,61,510 tonnes and in Karnataka it is grown in an area of 22,481 ha with production of 4,49,620 tonnes (Anon., 2004) ^[1].

Ralstonia solanacearum is an aerobic non-spore forming, Gram-negative, plant pathogenic bacterium. It is motile with tuft polar flagella. It colonises the xylem, causing bacterial wilt in a very wide range of potential host plants. Bacterial wilt of tomato, pepper, eggplant and Irish potato caused by *R. solanacearum* was among the first diseases that Erwin Frank Smith proved to be caused by a bacterial pathogen. It belongs to Kingdom: Bacteria; Phylum: Proteobacteria; Class: Beta- Proteobacteria; Order: Burkholderiales; Family: Ralstoniaceae and Genus: Ralstonia.

They were found to be motile with lophotrichous flagellum, non-spore forming, gram negative and cell size ranged from 1.3 to 1.02 x 1.02 to 1.78 µm (Khan, 1974) ^[6]. Bhide (1948) ^[2] reported that *R. solanacearum* was positive for nitrate reduction, citrate utilization, utilization of inorganic nitrogen and were negative for gelatin liquefaction, indole production, starch hydrolysis and production of hydrogen sulphide. Temperature is an important environmental factor that affects *R. solanacearum* multiple plant patho systems and their interactions with their hosts (Hayward, 1991). An increase of temperature to a range of 30 to 35°C is associated with an increase in severity of the disease caused by *R. solanacearum* in several hosts.

Understanding host-pathogen interactions, cultural, biochemical studies and physiological characters *viz.*, temperature and pH on disease caused by *R. solanacearum* development may offer information to advance breeding and disease management strategies.

Material and Methods

In the present investigation, laboratory studies were conducted at the Department of Plant Pathology, University of Agricultural Sciences, Gandhi Krishi Vignana Kendra, Bangalore. The details of materials used and methodology followed during the course of investigation were described.

The plant and soil samples from wilt affected brinjal plants showing typical symptoms of bacterial wilt were collected from infested fields of Department of Horticulture, University of Agricultural Sciences, Bangalore.

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One hundred microliter of the diluted bacterial suspension was poured onto the surface of solidified Triphenyl Tetrazolium Chloride agar (TZC) medium (Kelman, 1954) [5]. The bacterial suspension was spread on the surface of TZC medium with a sterilized spreader. The inoculated plates were incubated at 28 °C for 48 hours. At the end of the incubation period, the plates were observed for the development of both the virulent and avirulent colonies of *R. solanacearum*. The virulent colonies are irregularly shaped, fluidal, dull white colonies with slight red center. Whereas, avirulent colonies were small, round, convex, butyrous with large red pigment and narrow bluish margins as described by Kelman (1954) [5]. The identification of the bacteria isolated from brinjal was based on the morphological, cultural, biochemical characteristics and pathogenicity studies.

Morphological and cultural characters

The morphological characteristics such as cell shape, gram reaction and capsule staining were carried out as per the methods described by Kelman (1954) [5] and Schaad (1992) [10].

Biochemical characters

Some of the biochemical tests *viz.* Gram staining, starch hydrolysis, nitrate reduction test and gelatin hydrolysis in the department of Plant Pathology.

Effect of Physical factors on the growth and multiplication of *R. solanacearum*

Temperature requirement

The effect of temperature on the growth of the bacterial isolates was studied in different temperatures. The bacterial cultures were multiplied separately in Casmino acid Peptone Glucose (CPG) broth. The cultures were prepared by inoculating a loopful of bacterial cultures from stock culture to 100 ml CPG broth contained in 250 ml conical flask and incubated for 48 hours at 28 °C. Bacterial cultures (50 µl) diluted to a concentration of 5 x 10⁵cfu/ml was poured on to the surface of CPG agar medium taken in sterilized petri dishes. The inoculated plates were incubated at different temperatures *viz.*, 5 °C, 10 °C, 15 °C, 20 °C, 25 °C, 30 °C and 35 °C for 48 hours.

pH requirement

The effect of hydrogen ion concentration on the growth of the bacterial isolates was studied by adjusting the pH of the CPG agar. The pH was adjusted to 4, 5, 6, 7, 8, 9 and 10. Colonies were counted and recorded. Data was analyzed statistically.

Results and Discussion

Isolation of *R. solanacearum* from bacterial wilt affected brinjal plants

Wilted brinjal plants showing typical symptoms of bacterial wilt *viz.* the lower leaves turning pale yellow, loss of turgidity of the leaves, followed by drooping of the leaves and sudden wilting of plants and brown discoloration of vascular bundles of the infected plants were collected from Department of Horticulture, UAS, Bangalore.

Tentative diagnosis of the disease was made by ooze test. The ooze test was conducted by placing longitudinal section of diseased vascular tissue from the identified plant in a glass beaker containing clean water, within a few minutes; fine milky exude clouded the water streaming out from the margin of the cut end which revealed the presence of bacteria in the discoloured vascular tissue.

Isolation was made from the bacterial ooze obtained from the infected discoloured tissue of the plants by serially diluting the bacterial suspension in sterile distilled water and planting on TZC medium (Kelman, 1954) [5].

The pathogen isolation by serial dilution technique yielded *R. solanacearum* colonies which were fluidal, dull-white colored with slight pink to red colored centers. The results of morphological studies revealed that the pathogen was Gram-negative rod and non-capsulated. Several workers have confirmed the morphological of *R. solanacearum* as rod shaped and non-capsulated (Smith, 1896; Norman and Yuen, 1988) [16, 8].

Identification of the pathogen isolated from wilted plants collected from bacterial wilt infested brinjal plants

The bacteria isolated from brinjal were identified as per standard morphological procedures *i.e.*, on the basis of morphological, cultural, biochemical and pathogenic characteristics.

Morphological and Cultural characters

The results of the various morphological and cultural characters were studied. The bacterium isolates were rod shaped, Gram negative, non-capsulated. They appeared dull white with light pink colored center on TZC medium and the colonies were highly fluidal producing copious slime.

Biochemical characters

The bacterium was positive for nitrate reduction test and negative for starch hydrolysis and gelatin liquification.

The pathogen colonies on TZC medium was highly fluidal with copious slime and appeared white with light pink colored centre. Biochemical characteristics *viz.*, positive to nitrate reduction test; negative to starch hydrolysis and gelatin liquification also confirmed the identity of the pathogen as *R. solanacearum* (E. F. Smith, Yabuchi *et al.*, 1995) [16]. Khan *et al.* (1974) [6] and Shobha (2002) [11] observed similar colony characters in brinjal isolate on TZC medium and further also identified that, the bacterium as *R. solanacearum* based on standard morphological procedures. These results are in accordance with other reports of Kelman, 1954 [5]; Buddenhagen *et al.*, 1962; Singh and Hussain (1991) [3, 12].

Effect of temperature and pH on the growth and multiplication of *R. solanacearum*

Temperature

The effect of temperature on the growth of *R. solanacearum* was studied as per the procedure described in material and methods. The results are presented in Table 1. The effect of varied temperature levels on the growth of *R. solanacearum* was significant. The temperature of 30 °C was found optimum for the growth of pathogen as the highest population (196.66 x 10⁵cfu/ml) were recorded at this temperature level followed by 25 °C (114.66 x 10⁵cfu/ml) and 35 °C (103.33 x 10⁵cfu/ml). The growth of bacterium at these two temperature levels differed significantly and also found superior to other temperature levels tested. However, the pathogen grew at temperature of 20 °C, but it failed to grow at the lowest extreme temperatures of 5 °C to 15 °C.

Wang and Lin (2005) reported that temperature of 30 – 35 °C is conducive for the disease occurrence of bacterial wilt whereas, soil temperature < 20 °C was not found suitable for

the disease. Further, Prior *et al.*, (1996) [9] observed that *R. solanacearum* rapidly moves through the plant at temperature above 25 °C, thus the temperature of >28 °C is ideal for the growth of the bacteria both in plant and on nutrient medium as evident in the present study. These results are in conformity with the results of Hayward, (1991) [4] that the temperature between 30 and 35°C significantly increases wilt severity in tobacco and other hosts. Also, in another study *R. solanacearum* caused significantly greater disease in resistant tomato cultivars at 32.2 °C than at 26.6 °C (Krausz and Thurston, 1975) [7].

pH requirement

This experiment was carried out to know the effect of pH on the growth of *R. solanacearum*. The effect of pH on the growth was studied at varied pH levels from 4 to 10 as mentioned in material and methods and the results obtained are presented in Table 2.

Table 1: Effect of temperature on the growth of *R. solanacearum* on CPG agar medium

Sl. No.	Temperature (°C)	Colony Number (10 ⁵ cfu/ml)
1	5.00	0.00 (1.00) *
2	10.00	0.00 (1.00)
3	15.00	0.00 (1.00)
4	20.00	34.33 (6.85)
5	25.00	114.66 (11.70)
6	30.00	196.66 (15.02)
7	35.00	103.33 (11.16)
S.Em ±		0.14
CD @ 1%		0.60
CV (%)		4.11

*Transformed values

Table 2: Effect of pH on the growth of *R. solanacearum* on CPG agar medium

Sl. No.	pH	Colony Number (10 ⁵ cfu/ml)
1	4	0.00 (1.00)*
2	5	11.66 (4.40)
3	6	52.33 (8.23)
4	7	154.00 (13.41)
5	8	46.00 (7.78)
6	9	8.33 (3.89)
7	10	0.00 (1.00)
S.Em ±		0.15
CD @ 1%		0.63
CV (%)		5.26

*Transformed values

The data revealed that, number of bacterial colonies increased with increase in pH to certain range from 5 to 7 and thereafter colony numbers decrease with increase in pH indicating 6 and 7 were the optimum pH for maximum growth of the pathogen *R. solanacearum*. The maximum number of bacterial colonies was recorded at pH 7 (154.00 x 10⁵cfu/ml). The next best pH levels, which significantly supported the good growth, were 6 (52.33 x 10⁵cfu/ml) and 8 (46.00 x 10⁵cfu/ml). The least growth of the pathogen was observed at the lowest and the highest pH levels of 5 (11.66 x 10⁵cfu/ml) and 9 (8.33 x 10⁵cfu/ml) respectively. However no growth of the pathogen was recorded at pH level of 4 and 10.

Vincent and Mew (1997) reported that, growth of *R. solanacearum* was suppressed at pH 3, 10, and 11, and strongly reduced at pH 4 and 9. At pH 5 and 8, growth reduction was weak or it did not occur at all. Normal growth of all strains occurred at pH 6.

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