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The Pharma Innovation

ISSN (E): 2277-7695
ISSN (P): 2349-8242
NAAS Rating: 5.23
TPI 2023; 12(10): 27-31

www.thepharmajournal.com
Received: 02-08-2023
Accepted: 07-09-2023

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Isolation and biochemical characterization of *Ralstonia solanacearum* of chilli

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Abstract
Ralstonia solanacearum, a devastating phytopathogenic bacterium, poses a significant threat to global agriculture, particularly in crops like chilli (*Capsicum annum*). This study aimed to isolate and biochemically characterize *Ralstonia solanacearum* strains associated with chilli plants. Chilli plants exhibiting typical symptoms of bacterial wilt, including wilting, leaf yellowing, and vascular discolouration, were sampled from multiple regions. Isolation of the bacterium was achieved through culture-dependent techniques on selective media. Subsequently, the isolated strains were subjected to a comprehensive biochemical characterization, including Gram staining, KOH test, Catalase test, Starch hydrolysis test, Lipase activity on Tween 80 Agar, Hugh-Leifson test, Gelatin liquefaction test, Trypton Glucose Extract Agar test, Triphenyl Tetrazolium Extract Agar test. The result revealed the successful isolation of *Ralstonia solanacearum* strains from affected chilli plants.

Keywords: *Ralstonia solanacearum*, biochemical characterization, isolation, chilli

Introduction
Chilli (*Capsicum annuum*) is an economically significant crop worldwide, valued for its culinary and nutritional contributions. However, the cultivation of chilli faces a persistent threat in the form of bacterial wilt, a devastating disease caused by *Ralstonia solanacearum*. As a diverse species complex, *R. solanacearum* has developed an extreamly broad host range throughout the world, including > 450 host species representing 54 plant families (Wicker *et al.*, 2009) [30]. Race 1 strains can be found in humid areas throughout the world and attack many solanaceous crops i.e. pepper, tomato and eggplant (Shahbaz *et al.*, 2015) [25]. This study focuses on the isolation and biochemical characterization of *Ralstonia solanacearum* in chilli. The identification of this pathogen was also carried out in Brazil from bell pepper (Garcia *et al.*, 2013) [7] and its presence in hot and sweet pepper was reported by Begum *et al.* (2012) [32]. Through isolation and biochemical analysis, this research aims to provide valuable insights into the physiological and genetic traits of *Ralstonia solanacearum* in chilli, paving the way for targeted strategies in disease control and the protection of this essential crop.

Material and Methods
Collection of diseased samples
The leaves and stems showing typical symptoms of bacterial wilt disease were collected from the multiple regions. Then these infected samples were collected in paper envelope and carried out to laboratory for further study. The infected samples were stored in refrigerator for preservation and used for isolation.

Isolation of *Ralstonia solanacearum*
Isolation was carried out under aseptic condition using laminar flow clean air bench.
1. The plants showing characteristic symptoms were collected and subjected to isolation.
2. Infected stem segments were cut from collar region of wilted plants.
3. Then vascular portion were cut with the help of a sterilized scalpel up to few ‘mm’ along with healthy portion and disinfected with 2% sodium hypochlorite solution for one minute and repeatedly washed with sterile distilled water thrice to remove the traces of sodium hypochlorite.
4. Cut portion directly placed on Nutrient Agar plates incubated at 28-30 °C for 2-3 days.
5. Smooth circular, raised and dirty white colonies were again streaked on NA plates and the process was repeated till purified bacterial cultures were obtained with the homogeneity in colony morphology.

The cultures were maintained by adopting subsequent sub culturing at periodical and regular intervals on TZCA (2,3,5 – triphenyl tetrazolium chloride) media plates (Hugh and Leifson, 1953) [13]. Three-day old cultures were used for further studies (Chaudhary, R. and H. Rashid, 2011) [4].

Characterization of pathogen

Different biochemical tests were performed to characterize the *Ralstonia solanacearum*

Gram's staining

1. A loopful of bacterial suspension was transferred in the center of slide, with help of wire loop.
2. The drop was smeared over slide and air dried.
3. Then dried smear was fixed by passing the slide 3-4 times rapidly over the flame.
4. The smear was flooded with crystal violet for 30 seconds, washed in the tap water.
5. Then the smear was immersed in potassium iodide/Lugol's iodine solution for 30 seconds washed in tap water then decolorized with 95% alcohol and rinsed with water.
6. Counterstained with safranin for 10 seconds, again washed with tap water and air dried.

Drop of cedar wood oil was placed on the slide and examined the smear under oil immersion lense (Schaad, 1980) [33].

KOH test

Two drops of Potassium hydroxide was placed on a glass slide. A colony of culture was picked up from the medium with the help of inoculating needle and mixed with KOH drops for 10 seconds and raised the needle for 0.5 to 2 cm to form thread which was treated as positive test (Suslow *et al*., 1982) [28].

Catalase test

A loopfull of 24 hours old culture of the test bacterium was placed on the clean glass slide and to this a drop of 3 percent hydrogen peroxide (H2O2) was mixed and allowed to react for few minutes and observed for the production of gas bubbles (Schaad, 1980) [33].

Lipase activity on Tween 80 Agar test

Bacterial culture was inoculated on Tween 80 agar plates and incubated 7 days at 28+20C. The plates were examined daily for the presence of a dense precipitate around the bacterial growth (Sierra, 1957) [27].

Hugh-Leifsin test

Whether an organism is oxidative or fermentative can be determined by using Hugh and Leifson's medium, commonly called as OF medium which contain tryptone and bromothymol blue (An indicator). One of the sugars, such as glucose, xylose, mannitol, lactose, sucrose and maltose is added to the medium which serves as the fermentable carbohydrate. An organism is inoculated to two tubes of each OF medium. Once inoculated, one tube is overlaid with mineral oil or melted paraffin producing an anaerobic environment. The other tube is left open to the air. Growth of microorganisms in this medium is either by utilizing the tryptone which results in an alkaline reaction (dark blue colour) or by utilizing glucose, which results in the production of acid (Turning bromothymol blue to yellow). Oxidative utilization of the carbohydrate will result in acid production (Yellow) in the open tube only. Fermentative utilization of the carbohydrate will result in acid production (yellow) in both the open and closed tubes. Acidic changes in the overlaid tubes are considered to be a result of true fermentation, while acidic development in the open tubes are due to oxidative utilization of the carbohydrate present. (Chaudhary, R. and H. Rashid, 2011) [4]

Gelatin liquefaction test

Bacterial culture were inoculated through stab of a nutrient gelatin tube and incubated for 7 days, uninoculated tubes serves as control and observed for liquefaction. Deep gelatin inoculated tubes that remain liquified produce gelatinase and show positive test for gelatin hydrolysis and those tubes that remain solid demonstrate negative reaction for gelatin hydrolysis.

Tryptone Glucose Extract Agar test

A loop full of 72 hours old bacterial culture was streaked on the TGA media plates and incubated at 28+20C temperature for 72 hours. After the incubation period observations were recorded for the growth of the bacterium as mucoid, white colonies.

Triphenyl Tetrazolium Agar test

A loop full of 72 hours old bacterial culture was streaked on the TZCA media plates and incubated at 28+20C temperature for 72 hours. After the incubation period observations were recorded for the growth of the bacterium as mucoid, drop-shaped white colonies with pink, half-moon-shaped centre.

Starch hydrolysis test

Bacterial culture was inoculated on starch agar plates and incubated for 7 days. After incubation, the plates were flooded with Lugol's iodine solution. Presence of amylase was indicated by the appearance of clear zone around the inoculated colony. The clear zone indicated that the starch was partially hydrolysed to dextrin.

Result and Discussion

Isolation of pathogen from diseased specimen

The isolation of bacterial wilt of chilli caused by *Ralstonia solanacearum* was carried out on nutrient agar media. The diseased stems and roots samples were rinsed and washed in running tap to remove the attached soil particles water and used for isolation. Samples were disinfected with 0.1 per cent aqueous solution of Sodium Hypochlorite (NaOCl) for two minutes. Then these surface sterilized bits were washed by giving three changes of sterile distilled water to remove traces of Sodium hypochlorite. These bits were placed in sterile test tube containing 2.5 ml sterile distilled water and crushed with the help of sterilized glass rod and loopful of bacterial suspension was streaked on solidified Nutrient Agar medium in zigzag manner by streaking method under aseptic condition in laminar air flow chamber. Isolations from the affected stems and roots formed smooth circular, raised and dirty white colonies on nutrient agar medium. Then these bacterial colonies were transferred on to fresh nutrient agar plates by streak plate method. The pathogen was purified through frequent sub culturing and purified growth or culture was maintained on fresh nutrient agar slant and preserved in refrigerator for further studies.

Purification of bacterial culture

Well sundered colonies of *Ralstonia solanacearum* isolated from the infected stems and roots were further purified by streaking on the surface of TZCA medium and preserved in slants. The bacterial colonies on TZCA medium were irregular, fluidal, white, virulent colony with pink center. According to Kelman (1954) [17], triphenyl tetrazolium chloride (TZC) medium is used to distinguish *R. solanacearum* among other bacteria during isolation. Also when TZC medium is used with *R. solanacearum*, it shows the difference between avirulent colonies that look dark red from the fluidal virulent that are white with pink center (Klement, 1990; Rahman *et al*., 2010) [34, 19]. In the present study, the colonies were fluidal whitish with a pink center, indicating virulent species of *R. solanacearum*.

Biochemical characterization of the collected isolate

The isolate of *Ralstonia solanacearum* was subjected to the biochemical tests for identification. The tests were performed for comparing the characteristics depicted in Manual for Phytobacteriology (Introduction to Practical Phytobacteriology) compiled by T. Goszczynska, J. J. Serfontein and S. Serfontein. The results are presented in Table 1.

Table 1: Biochemical tests of isolate

Tests	Results
Gram's staining	+
KOH test	+
Catalase test	-
Starch hydrolysis test	-
Lipase activity on Tween 80 Agar	+
Hugh – Leifson test	-
Gelatin liquefaction test	-
Trypton Glucose Extract Agar test	+
Triphenyl Tetrazolium Chloride Agar test	+

*+ = Positive test, - = Negative test

1. Gram's staining

Microscopic examination of Gram stained *Ralstonia solanacearum* mount elucidated that the test bacterium did not retain violet color of primary stain (crystal violet) but cells appeared pink in color due to counter staining with the safranin. Hence the test bacterium was Gram negative and rod shaped, which is the characteristics of the plant pathogenic bacteria. The results of staining reactions revealed the cells *R. solanacearum* were short straight rods and Gram negative in reaction. Chaudhry and Rashid (2011) [35] reported similar staining reaction of *R. solanacearum.*

2. Potassium hydroxide (KOH) test

Formation of slime threads or loop is an indication of being gram-negative because gram negative bacteria have relatively fragile cell walls, bounded by an outer membrane. This is readily disrupted by exposure to 3% KOH releasing the viscous DNA. The isolate of *Ralstonia solanacearum* was showed to form mucoid thread after added KOH and found positive test isolate of *Ralstonia solanacearum.* Chaudhry and Rashid (2011) [35] also reported similar results for *R. solanacearum* grown on nutrient agar medium.

3. Catalase test

Catalase mediates the breakdown of hydrogen peroxide (H_2O_2) into oxygen and water. To find out if a particular bacterial isolate was able to produce catalase enzyme, a small inoculum of bacterial isolate was mixed into hydrogen peroxide solution (3%) and observed for the rapid elaboration of oxygen bubbles. Catalytic activity of the isolate of *Ralstonia solanacearum* was found to be negative, since the culture did not generated bubbles of oxygen within 30 seconds of addition of H_2O_2. Production of gas bubbles gives a clue for presence of aerobic and facultative anaerobic bacteria (Schaad, 1980) [33].

4. Starch hydrolysis test

The isolate of *Ralstonia solanacearum* not produced colourless zone around bacterial growth on starch agar medium flooded with Lugol's iodine and showed negative for starch hydrolysis test. Similarly, Bhide (1959) [36] reported that *P. solanacearum* was negative for starch hydrolysis.

5. Lipase activity on Tween 80 Agar

The isolate showed positive for lipase activity on Tween 80 agar by forming a dense precipitate around the bacterial growth.

6. Hugh-Leifson test

The tubes containing OF medium when inoculated with organism, no changes were observed in tubes, indicating absence of oxidation and fermentation as there was no colour change. Thus Hugh-Leifson test showed negative results for the isolate.

7. Gelatin liquefaction test

Bacterial culture were inoculated through stab of a nutrient gelatin tube and incubated for 7 days. The tubes remained solid demonstrated negative reaction for gelatin hydrolysis, indicating that bacteria is not capable to hydrolyse the gelatin.

8. Tryptone Glucose Extract Agar test

After streaking bacteria on Tryptone Glucose Extract Agar plate, showed growth of bacterium as smooth, mucoid and white colonies, indicating positive results for TGA test.

9. Triphenyl Tetrazolium Chloride Agar test

After streaking bacteria on Triphenyl Tetrazolium Chloride plate, showed growth of bacterium as mucoid, drop-shaped white colonies with pink, half-moon-shaped centre. When TZC medium is used with *R. solanacearum*, it shows the difference between avirulent colonies that look dark red from the fluidal virulent that are white with pink center (Klement, 1990; Rahman *et al*., 2010) [34, 19].

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