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Biochemical Characterization of *Xanthomonas campestris pv. oryzae* Causing Leaf Blight Disease in Rice Plant

Dr. Priyanka Chatterjee

Author's Affiliation
Assistant Professor, Department of Botany, R.N. College, Hajipur, Vaishali, Bihar 844101, India

***Corresponding Author:**
Dr. Priyanka Chatterjee
Assistant Professor, Department of Botany, R.N. College, Hajipur, Vaishali, Bihar 844101, India
E-mail:
priyanka.chatterjee41@gmail.com

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Abstract

Xanthomonas campestris pv. oryzae is an important and destructive pathogen causing bacterial leaf blight disease in rice plant. The different tests were conducted in this research work to know the biochemical properties of the pathogen by the three rice isolates KRH-2, Swarna & Jurly and also investigation of resistant variety of rice germplasm. Pathogen was subjected to various biochemical tests, the results indicated that H_2S (hydrogen sulphide) production test +ve in cysteine & -ve in peptone, Starch hydrolysis in KRH-2 & Swarna rice isolates showed +ve reaction whereas, negative results were obtained for Starch hydrolysis in Jurly rice isolate, Fluorescent test and Gram staining showed negative reaction whereas, positive reaction were observed in Catalase test, Liquefaction of Gelatin, KOH Solubility test, Protein digestion test.

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INTRODUCTION

Rice is the most important staple food for a large part of the world's including India. It is a nutritious food, providing about 90 percent of calories from carbohydrates, proteins, vitamins and other minerals.

The pathogen *Xanthomonas campestris pv. oryzae* causes typical symptoms on infected rice such as leaf blight which appears on leaves of young and matured rice plants, after planting out as pale green to grey green water soaked lesions on margin and lower portion of matured young leaves. For the identity of the pathogen, in this research work the morphological features & biochemical properties were investigated in detail in three local rice isolates of KRH-2, Swarna and Jurly of the pathogen and were compared with the authentic culture of the bacterium *Xanthomonas campestris pv. oryzae*. Therefore in an every rice growing areas it is an urgent need for the protection, cultivation in pollution free environment and conservation of

resistant varieties of rice for current and future generation.

MATERIALS AND METHODS

In the present study biochemical characters such as Hydrolysis of starch, Fluorescent test, Gram staining, Hydrogen sulphide production, Gelatin liquefaction, KOH solubility test, Catalase reaction test & Protein digestion test by the pathogen were studied as per the methods described. For this work three rice isolates KRH-2, Swarna & Jurly as well as different chemicals & media were taken.

Starch hydrolysis affected by the bacterium

Maximum numbers of bacteria hydrolyze starch due to amylase activity. This test was conducted on yeast extract nutrient agar (YNA) medium with both potato starch and soluble starch added separately. According to Dye, the method employed and medium was taken.

The medium contains following ingredients:

Yeast extract (Difco)	5.0g
Peptone	5.0g
Beef extract powder (Lab-Lemco)	5.0g
Distilled Water	1L.
PH	6.8

The one case potato starch (Difco) 10g (1%) and in another soluble starch 2g (0.2%) were taken. The medium was then autoclaved and plated. Starch plates were streaked with 48h SPA grown (0.1 OD at 600 nm = 10^6 CFU/ml) bacterial suspension of the three different rice isolates of the pathogen separately and incubated for 7 days at 27°C. After that the plates were flooded with iodine solution. Starch stained blue black. However a clear zone present shows that amylase activity has occurred and hydrolysis of starch has taken place. Blue colour was formed on the surface of the medium where starch remained unhydrolysed. The iodine solution was prepared previously containing iodine 1.0g, potassium iodide 2g and distilled water 300 ml. The potassium iodide was first dissolved in water and then iodine was added to it.

Gelatin liquefaction affected by the bacterium

Bacteria having proteolytic enzymes usually degrade gelatin and cause its breakdown. This results in the loss of gelling properties of gelatin. Bacteriological grade gelatin was used as 12% solution. As per recommendation of Fahy and Hayward, the medium used was dehydrated nutrient gelatin.

This medium comprises the following ingredients:

Beef extract (Lab-Lemco)	3.0g
Peptone	5.0g
Bacteriological grade gelatin	120g
Distilled Water	1L.

This medium is suitable for most bacteria. The ingredients were dissolved by steaming and the medium was dispensed in 5ml aliquots in tubes. The tubes were autoclaved at 110°C for 15 min. The tubes were then stab inoculated with three different rice cultivars isolates of the bacterium separately and incubated for 7days at 20°C temperature. The tubes were examined after 5, 7 and 14 days for liquefaction of gelatin. Liquefaction was observed after keeping the tubes for some time at 4°C temperature. The type of liquefaction developed in the gelatin tube was also noted.

H_2S Production test

The Peptone & Cysteine broth were prepared and sterilized. The ability to produce H_2S from sulphur containing compounds in the medium is of differential value for certain genera of bacteria. Peptone in the medium is more frequently used but cysteine gives better and consistent results. According to the Dye YS broth medium was used.

It comprises following components:-

$NH_4H_2PO_4$	0.5g
K_2HPO_4	0.5g
$MgSO_4, 7H_2O$	0.2g
NaCl	5.0g
Yeast extracts	5.0g
Distilled Water	1L.
To this was added	
Cysteine hydrochloride	0.1g

The medium was dispensed in 5ml aliquots in tubes and autoclaved. It was then inoculated separately with three isolates of rice cultivars of the bacterial pathogen. The cultures were shaker incubated and the results were recorded after 7

days. To test positive H_2S production a lead acetate strip was suspended over the inoculated medium and held by a bung. The lead acetate papers were prepared first. Filter paper strips 50x10 mm size were soaked in saturated lead acetate solution and then dried. The immersion was done in 5% lead acetate solution. The dried strips were sterilized at 121°C in an autoclave for 15 min. The paper was inserted in between plug and glass so that the lower end of the paper remained above the liquid level. The tubes were incubated at 30°C. As mentioned, slopes with lead acetate paper were examined after 7 days of incubation. The positive results were taken when the lead acetate paper became black. This was due to the formation of black lead sulphide on paper strip after H_2S has reacted with it.

Catalase Activity

For determining catalase activity 10 vol. hydrogen peroxide solution was prepared. A loopful of solid growth taken from 24h GYCA grown local rice isolated and lyophilized culture were separately added into a drop of hydrogen peroxide solution on clear glass plates. They were examined for the production of gas bubbles. Productions of bubbles indicate positive catalase activity.

KOH Solubility test:-

This test was done to reaffirm the results of Gram staining. In this test 1 or 2 drops of 3% KOH (Potassium hydroxide) were placed on a glass slide. A few colonies out of 48h SPAgrowth culture of the bacterium was picked up on an inoculating loop. This was stirred in a KOH broth for 5 to 10 Sec. The inoculating loop was then raised from the drop. When the KOH solution became viscous while raising the loop a thread of slime follows for 0.5 to 2 cm distance or more. This positive reaction is for Gram negative bacteria. However, if there is no slime and instead a watery suspension that does not follow the loop, the reaction is taken as negative and confirmatory for Gram positive bacteria.

Action in milk or Protein digestion method

This was observed in purple milk (reconstituted powdered skim milk-1 litre + bromocresol purple 0.04g) sterilized by steaming on three successive days. The method was modified from that of smith et. al and results were confirmed on milk agar plates. Accordingly this nutrient agar (Difco) plates were prepared. Over this agar surface was poured a layer of yeast extract nutrient agar (YNA) containing nutrient agar + 0.5% (w/v) yeast extract to which 15% (v/v), sterile milk has been added aseptically. Plates were dried for 2h at 45°C. Spot inoculations were made with two cultures per plate and incubated for 6 days. Clearing of the milk was due to proteolysis in agar milk plates showing positive action of milk. Acid reaction in milk was also tested.

Fluorescent production test

King's B medium is commonly used for fluorescent pigment production, not all fluorescent pseudomonads readily produce pigment on the medium, but they may do so on Caesamino-sucrose gelatin agar or Oxoid Pseudomonas F medium including Nutrient agar medium also.

King's B Medium	
Proteose peptone (Difco no.3/Oxoid L46)	20.0 g
Glycerol	10.0g
K_2HPO_4	1.5g
$MgSO_4.7H_2O$	1.5g
Agar	15.0g
Distilled water	1 Litre

Adjust pH to 7.2 & sterilize by autoclaving at 121°C for 15 minutes.

Green fluorescent pseudomonads produce a diffusible yellow, green or brownish pigment, which fluorescence green or blue in ultraviolet light.

GRAM Staining

The Gram staining was done to determine whether the bacterium is Gram positive or Gram negative. On the basis of Skerman this procedure was followed. A 24h SPA grown culture was smeared on a tannic acid treated slide. It was air dried and heat fixed by gentle flaming. After the slide was cooled it was flooded with 2% crystal violet (Crystal violet 2.0 g, ammonium Oxalate 1.0 g, distilled water 100 ml) for 1 min. It was then washed for 3-4 sec in a gentle stream of tap water. It was flooded with

iodine solution (Iodine 1.0 g and potassium iodide 2.0 g was mixed and grinded in mortar then dissolved slowly in 100 ml of distilled water) for 1 min. Afterwards the slide was washed again with water. It was decolorized by applying 95% ethanol drop by drop on the smear held at an angle and kept against the white filter paper back ground until no more color runs out from the lower end usually the de colorization is done within 10-20 seconds. It was counter stained with safranine (2.5% Solution in 95% ethanol diluted to 1:10 with distilled water). The slide was observed under oil immersion of the microscope. Blue colour indicated Gram positive. Here the crystal violet stain was absorbed. Red colour indicated Gram negative. Here the bacterial cell wall failed to retain the crystal violet stain after decolorization. Instead the safranine stain was taken up.

RESULT AND DISCUSSION

Different types of biochemical test revealed that from Table 1 and Plates 1, 2 & 3 this bacterium is a rod shaped and also all the three rice isolates KRH-2, Swarna & Jurly of the bacterium caused positive results in Catalase reaction, Liquefaction of gelatin, KOH solubility test, Protein digestion and H_2S (hydrogen sulphide) production test +ve in cysteine & -ve in peptone, Starch hydrolysis in KRH-2 & Swarna rice isolates showed +ve whereas, negative results were obtained for Starch hydrolysis in Jurly rice isolate, Fluorescent test and Gram reaction i.e they are Gram –ve bacteria. In all these respect "Jurly" was found most resistant variety whereas "Swarna" rice isolate was moderate susceptible and susceptible or weaker variety was observed in "KRH-2" rice isolate. Several studies of this research work by the biochemical test, it has been completely revealed that, it was a disease of bacterial leaf blight of rice and caused by *Xanthomonas campestris pv. oryzae* (Ishiyama, 1922).

Table 1: Biochemical characterization of *Xanthomonas campestris pv. oryzae*

Sl. No.	Biochemical Test	Result
1.	Starch hydrolysis	+ve in KRH-2 & -ve in Jurly
2.	Gelatin liquefaction	+
3.	H_2S Production	+ve in Cysteine & -ve in Peptone
4.	KOH Solubility test	+
5.	Catalase test	+
6.	Protein digestion	+
7.	Gram reaction	_
8.	Fluorescent colour test	_

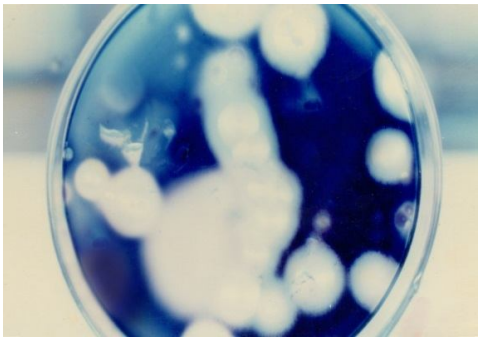

Plate1: Starch hydrolysis

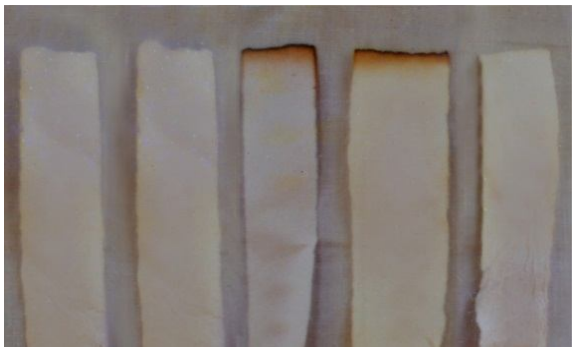

Plate 2: H_2S Production by the three rice isolates

Plate 3: Gelatin liquefaction affected by the three rice isolates

DISCUSSION

Xanthomonas campestris pv. oryzae was detected from rice seeds and also from plant material and its identity was confirmed by morphological, physiological and biochemical tests, hypersensitive and pathogenicity tests (Umesha, 2011).Six biochemical tests were conducted to characterize 17 isolates of pathogen. All isolates consistently gave similar results. Gram staining demonstrated that pathogen is a gram negative rod shaped, KOH test was performed in order to confirm gram staining results, further all isolates showed positive reaction and confirmed that they are gram negative (Batool, 2012). KOH solubility test is now being used with greater frequency for confirmation of Gram reaction (Isaka. 1982). Starch was also utilized by several cultures of additional *Xanthomonas* spp. isolated from different hosts from India (Dye, 1963). The bacterium *X. c. pv. oryzae* the causal organism of bacterial leaf blight of rice, was found to produce H_2S. In several other *Xanthomonas* species non-production of H_2S (Srivastava, 1968) from cysteine was found. Gelatin liquefaction is caused by *X. c. pv. cerealis*, Starch hydrolysis by *X. c. pv. cerealis* was recorded (tsuboki. 1980). Slight starch hydrolysis was reported for Indian isolate of *X. c. pv. oryzae* (Srivastava, 1968). The cucurbit isolates failed to show proteolytic breakdown of milk leading to formation of clear zones. This feature distinguishes it from *Xanthomonas* species especially *X. cucurbitae* which has strong protease activity leading to clearing of milk (Dye, 1962). The results obtained by them reported that the *Xanthomonas* pathogen utilizes xylose, glucose, mannose, galactose, sucrose, lactose and positive tests for KOH string assay, H_2S, catalase, arginine dehydrolase test and raffinose but not maltose, glycerine and salicin. It readily hydrolyses the starch and liquefies the gelatin (Khan, 2013 & Nagar, 2014).

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