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## Physiological and biochemical characterisation of *Xanthomonas oryzae* pv. *oryzae* inciting bacterial leaf blight of rice and proving of its pathogenicity

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### Abstract

Bacterial blight caused by *Xanthomonas oryzae* pv. *oryzae* is a major disease of rice throughout the world including Konkan Region of Maharashtra known for causing serious losses of the crop. Five different isolates of *Xanthomonas oryzae* pv. *oryzae* were collected from different regions of Maharashtra and subjected to different physiological and biochemical tests. All the isolates were found Gram positive and further KOH solubility test was conducted to re-confirm Gram staining of all isolates. All isolates exhibited positive results for starch hydrolysis test, gelatin liquefaction test, acid production from carbohydrates test, gas production test and catalase activity test. However isolates Xoo<sub>1</sub> and Xoo<sub>2</sub> showed negative results to hydrogen sulphide production test but Xoo<sub>3</sub>, Xoo<sub>4</sub> and Xoo<sub>5</sub> were positive for hydrogen sulphide test. pathogenicity tests were varied significantly in terms of disease severity among each other and further confirmed with different biochemical tests.

**Keywords:** *Xanthomonas*, biochemical, Gram staining, carbohydrates, pathogenicity

### Introduction

Rice is the basic food crop in India and being a tropical plant, it flourishes comfortably in hot and humid climate. Rice is mainly grown in rainfed areas that receive heavy annual rainfall. Rice crop is prone to number of bacterial diseases among which bacterial leaf blight caused by *Xanthomonas oryzae* pv. *oryzae* is a serious problem and threat to rice production in both tropical and temperate rice growing regions due to its high epidemic potential. The disease occurs in the host plant at the seedling, vegetative and reproductive stages but bacterial leaf blight infection at the tillering stage causes severe blighting of leaves resulting in yield loss up to 75% depending on weather, location and particular rice cultivar used (Shivalingaiah and Umesh, 2011) <sup>[7]</sup>. The bacterial leaf blight disease caused by *Xanthomonas oryzae* pv. *oryzae* causes typical vascular wilt damage due to partial or total blighting of the leaves or due to complete wilting of the affected tillers leading to unfilled grains. In the wilt phase the crop may dry up completely before the seed maturation. The bacterium was considered to be localised to Maharashtra until it broke out in an epidemic form in the Shahabad district of Bihar in 1963 (Srivastava, 1972) <sup>[8]</sup>. Initially water soaked yellowish stripes appear on the leaf blades starting at the leaf tip, later increasing in length with a wavy margin. On the leaf surface a milky or opaque dewdrop can be noticed during early morning time. Lesions turn yellow to white as the disease advances. Severely infected leaves tend to dry quickly. Lesions later turn grey in colour. As the infection progresses the lesions over the entire leaf blade which may turn white or straw coloured. Later leaves wilt and roll up. Under severe conditions the entire crop wilts completely. The aim of the present experiment is to characterize the isolates collected from different parts of Maharashtra physiologically and biochemically.

### Materials and methods

The isolates collected from five different regions of Maharashtra were freshly cultured and were subjected to different physiological and biochemical tests under *in vitro* conditions.

#### A. Physiological studies of *Xanthomonas oryzae* pv. *oryzae* Gram staining

The Gram staining test was carried out as per (Aneja, 2003) <sup>[11]</sup> using fresh pure cultures of the test bacterial isolates. Thin smears were made on separate glass slides, air dried and heat fixed.

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Smears were treated with crystal violet for 30 seconds and washed with distilled water. Again treated with Gram iodine solution for 60 seconds and washed with 95% ethyl alcohol. The smear was washed with distilled water, drained and air dried. Safranin was applied for 30 seconds (counter-staining) and washed with distilled water. The stained slides were air dried. The slides were examined under stereoscopic microscope for confirmation of Gram reaction.

#### **KOH (Potassium hydroxide) test**

A loopful of fresh week old culture of the test bacterium was transferred onto to a slide and stirred with a drop of 3% aqueous KOH in circular motion with the help of the toothpick for 5 to 60 seconds. Toothpick was then raised to few centimeters from the glass slide and observed for formation of strands of viscid material for confirmation of the Gram reaction (Jonit *et al.*, 2016) [3].

### **B. Biochemical characteristics of *Xanthomonas oryzae* pv. *oryzae***

#### **Starch hydrolysis**

The ability of bacterium to hydrolyse starch was studied by growing on nutrient agar medium containing 1% soluble starch. The sterilized liquefied NA was poured into sterilized Petri plates and allowed to solidify. The plates were then inoculated with individual isolate aseptically and incubated at room temperature for 5 days. The surface of the plates was then flood with iodine solution and pour off the excess iodine solution. Formation of clear zones around the bacterial growth indicates the hydrolysis of starch (Mew and Mishra 1994) [4].

#### **Gelatin hydrolysis**

Gelatin agar medium (yeast extract 3 g/l, peptone 5 g/l gelatin 120 g/l) was prepared and allowed the solid to stand in water for 15 min. and dissolved by heating. Such was then dispensed into the test tube to a depth of 5cm and sterilized by autoclaving at 121°C for 15 min. These nutrient gelatin deep tubes were stab inoculated with bacterial isolates and incubated for 7-14 days. Liquefaction of the medium was recorded every 2-3 days. On the final day, tubes were cooled at 5°C for 30 minutes before reading the results, if the test culture solidifies remove the culture and incubate in a tilted position at room temperature or at 30°C in an incubator for 30 min. If form slant indicates test is +ve and no form slant only stab indicates -ve test (Aneja, 2003) [1].

#### **Production of hydrogen sulphide**

Nutrient broth with additional 3% peptone were poured in test tubes and sterilized in an autoclave at 15lbs psi for 20 minutes. These deep broth tubes were inoculated with the bacterial isolates. Filter paper stripes which were soaked in supersaturated solution of lead acetate and dried. These were inserted into the tubes such a way that they do not touch the medium. The tubes were inoculated for 7 days. The blackening of the stripes indicates production of hydrogen sulphide gas (Aneja, 2003) [1].

#### **Acid production test**

Medium [NH<sub>4</sub>H<sub>2</sub>PO<sub>4</sub> (0.5 g), K<sub>2</sub>HPO<sub>4</sub> (0.5 g), MgSO<sub>4</sub>.7H<sub>2</sub>O (0.2 g), NaCl (5 g), yeast extract (1 g), agar (12 g) water (1 L), bromocresol purple (1.5% alcohol solution 0.7 ml)] was dispensed in test tubes and autoclaved at 121°C for 15 minutes. A 10% aqueous solution of glucose, fructose, sucrose and galactose was prepared, filter-sterilized through millipore injection and added to the molten base. Each isolate

was then transferred aseptically into a tube, incubated for 7 days at room temperature and checked for production of yellow color indicated production of acid (Jonit *et al.*, 2016) [3].

#### **Gas production test**

For this test nutrient broth containing 2% glucose was prepared and poured in the test tubes containing inverted Durham's tube. These were autoclaved and inoculated with bacterial isolates separately and incubated at room temperatures for 7 days. Gas production was indicated by a bubbles in the inverted Durham's tube (Aneja, 2003) [1].

#### **Catalase test**

A fresh culture was taken and a drop of hydrogen peroxide was put onto it. The production of bubbles give the positive results (Aneja, 2003) [1].

### **C. Pathogenicity test *Xanthomonas oryzae* pv. *oryzae***

Artificial inoculation of the pathogenic bacterium was carried out to prove the pathogenicity using leaf clip inoculation technique under glass house condition. The bacterial inoculum of 10<sup>7</sup> CFU/ml was prepared and seedlings of 30 days old were artificially clip inoculated. The inoculated plants were observed for the development of symptoms. After the symptom development, the bacterium was re-isolated from the artificially inoculated seedlings to prove the Koch's postulates and compared with the original culture (Shankara *et al.*, 2016) [6].

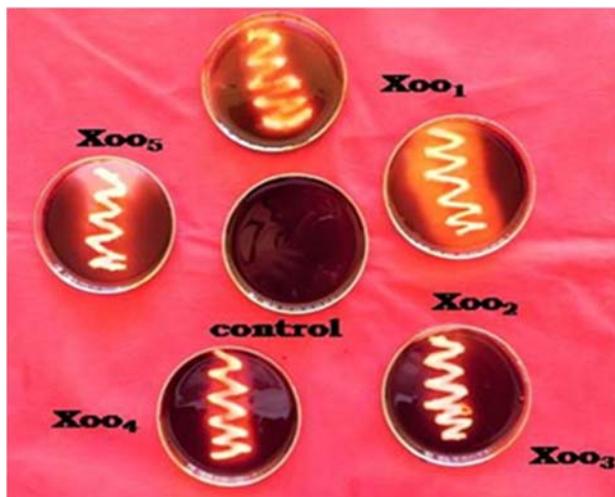
### **Results and discussion**

In Physiological and biochemical characterization studies of different isolates of the test bacterium *Xanthomonas oryzae* pv. *oryzae* it was observed that all isolates tested exhibited Gram negative reactions. Positive results were obtained for potassium hydroxide (3%) solubility test, starch hydrolysis, gelatin liquefaction test, acid production, gas production test and catalase activity test. But in case of hydrogen sulphide test the isolates Xoo1 and Xoo2 gave negative results whereas Xoo3, Xoo4, Xoo5 showed positive results (Table 1). All the biochemical characters under present study were co-related with the characters reported by Shankara *et al.* (2016) [6], where all the isolates of *Xanthomonas oryzae* pv. *oryzae* were subjected to different biochemical tests and were found positive for 3 per cent KOH test, gelatin liquefaction, catalase test while negative for starch hydrolysis and oxidase tests. Five isolates namely, Xoo7, Xoo16, Xoo22, Xoo27 and Xoo50 were found negative for H<sub>2</sub>S production test. Jonit *et al.* (2016) [3], also observed that most of the isolates of *Xanthomonas oryzae* pv. *oryzae* showed positive reaction for catalase test. However, there were two isolates which showed negative reaction against catalase [Xoo-19 and Xoo-20]. Jabeen *et al.*, (2012) [2] conducted biochemical tests to characterize 17 isolates of *Xanthomonas oryzae* pv. *oryzae*. Gram staining demonstrated that the pathogen was a Gram negative rod, producing red colour when counter stained with safranin. KOH test was confirmed and gave positive result. In the starch hydrolysis all the 7 isolates showed positive reaction. The findings of the present investigation are also in close conformity with those reported by Shivalingaiah and Umesh (2011) [7]. A clear zone of hydrolysis was formed around the bacterial colonies indicating positive for starch hydrolysis, the bacterium also indicated positive reaction for gelatin hydrolysis.

In the experiment of testing the pathogenicity symptoms were produced after 15 days of clip inoculation with the formation of water soaked lesions from the margin and progressed all along the leaf blade in wavy pattern. The region adjoining the healthy part showed water soaked blighted lesions extended rapidly to cover large areas of the leaf blade, turned white and later became greyish. The bacterium was re-isolated, which resembled the original culture of *Xanthomonas oryzae* pv. *oryzae*. Thus, the Koch's Postulates were proved which confirmed the pathogenicity of the test bacterium. The results obtained are in agreement with those of Shankara *et al.*, (2016) [6]. The results presented indicated that pathogenicity test for isolates obtained on BPT-5204 that varied significantly in terms of disease severity among each other and further confirmed with colony morphological features and biochemical tests. Patel (2008) [5] reported that clip inoculation revealed that the pathogen (Xoo) inoculated did infected leaves and produced blight symptoms from the cut end of leaves. The microscopic observation of blighted leaf tip piece gave out bacterial oozing and the reisolation from such blighted leaf tip yielded the typical bright yellow bacterial colonies. The results are also in close conformity with the results of Tolba and El-Sharkawy (2011) [9].

**Table 1:** Physiological and biochemical characterization of isolates of *Xanthomonas oryzae* pv. *Oryzae*

Physiological characters	Reactions to the test				
	Xoo1	Xoo2	Xoo3	Xoo4	Xoo5
Gram staining					
3% KOH test	+	+	+	+	+
Biochemical characters					
Starch hydrolysis test	+	+	+	+	+
Gelatin hydrolysis test	+	+	+	+	+
Hydrogen sulphide test	-	-	+	+	+
Acid production test	+	+	+	+	+
Gas production test	+	+	+	+	+
Catalase test	+	+	+	+	+



**Fig 1:** Starch hydrolysis test

### Conclusion

From the study it is concluded that all the isolates of rice BLB causing bacterium *Xanthomonas oryzae* pv. *oryzae* collected from different locations of Maharashtra were Gram negative with positive 3% KOH solubility test. Except isolate Xoo3 all the isolates studied were positive in starch hydrolysis test, gelatin hydrolysis test, hydrogen sulphide test, acid production test, gas production test and catalase test. Isolate

Xoo3 only was negative for hydrogen sulphide test but positive for other tests. Similarly, all the isolates were pathogenic to rice and produced typical symptoms of BLB when leaf clip inoculated.

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