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Biochemical variation among isolates of *Xanthomonas oryzae* pv. *oryzae* causing bacterial leaf blight in rice

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Abstract
Xanthomonas oryzae pv. *oryzae* is an important and destructive pathogen causing bacterial leaf blight in rice. Attempt were made to ascertain the variability in *Xanthomonas oryzae* pv. *oryzae* and five isolates collected from different locations showed biochemical variations. The tests conducted to know the biochemical properties, among the five isolates of *X. oryzae* pv. *oryzae* subjected for biochemical studies, all the isolates showed positive reaction for gelatin liquefaction, protein digestion, catalase test, KOH test, ammonia and hydrogen sulphide production. Isolates Xoo1, Xoo2 and Xoo3 showed positive reaction for starch hydrolysis. However, all the isolates showed negative reaction for indole production, voges-proskaure test, nitrate reduction, methyl red test and production of fluorescent pigment on King's B medium.

Keywords: *Xanthomonas oryzae* pv. *oryzae*, Rice, Biochemical, Variability

Introduction
Rice *(Oryza sativa* L.) is the staple food of more than 60 percent of world's population. About 90 per cent of all rice grown in the world is produced and consumed in the Asian countries. Bacterial leaf blight of rice caused by *Xanthomonas oryzae* pv. *oryzae* [1, 2] is a serious disease in south East Asia including India. The disease causes reduction in yield as high as 50 per cent in fields where the crop is severely infected and an early infection leading to kresek phase can cause total failure of the crop. The isolates of *Xanthomonas oryzae* pv. *oryzae* are known to vary widely in their pathogenicity to rice varieties. Well documented evidences are available to show that virulent strains of *Xanthomonas oryzae* pv. *oryzae* present in Asia are variable. The types of variation were claimed to be continuous based on the fact that the bacterial pathogen in east-Asia such as Japan causes less lesion on certain varieties when compared to the isolates of tropical Asian countries such as Indonesia or South India [3, 4]. The development of resistant varieties is the most economical and environmentally safe approach of disease management but for breeding long lasting resistant varieties and accurate knowledge of variation in the pathogen population is a prerequisite. Resistant varieties evolved against this disease; do not perform uniformly in all areas. It might be due to variability in the pathogen and pathogenic potential of the same pathogen. Hence, it becomes necessary to ascertain variability amongst the isolates of leaf blight causing pathogen in particular region. An attempt has been made to identify the variation in five isolates of *Xanthomonas oryzae* pv. *oryzae* collected from different rice cultivating locations in Karnataka.

Materials and methods:
The isolates of *Xanthomonas oryzae* pv. *oryzae* were collected from bacterial leaf blight infected rice samples in different places *viz.*, Shivamogga, Davanagere and Chikkamagalur districts during survey *Kharif* 2016. Rice leaf samples with typical leaf blight symptom were subjected for culture isolation by standard tissue isolation and streak plate method. Isolates with well-defined colonies were selected and designated as Xoo1, Xoo2, Xoo3, Xoo4 and Xoo5. The biochemical characters such as starch hydrolysis, gelatin liquefaction, H_2S production, catalase, protein digestion, indole production, ammonia production, methyl red test, nitrate reduction test, Vogues-Proskauer test and production of fluorescent pigment on King's B medium by the pathogen were studied as per the methods described [5, 6].

Starch hydrolysis
The starch agar medium was used to carry out the starch hydrolysis. Potato starch (10 g) was added to 1000 ml of nutrient agar. 20 ml of sterilized cool medium was dispensed in to each of the Petri plates. After solidifying, starch agar plates were spot inoculated with loopful culture of the bacterium and incubated for five days at 27 ± 1 ºC. After incubation period is over, the plates were flooded with Lugol's iodine solution and observations were drawn for starch utilization by the bacterium.

Gelatin liquefaction
Fifteen ml of freshly prepared and autoclaved nutrient agar added with 0.4 per cent gelatin was poured into the sterilized Petri plates. After the medium gets solidified, spot inoculation using a tooth prick on the surface of the medium was done. Plates were incubated at 27 ± 1ºC for three days. After that plates were flooded with 10 ml of acid mercuric chloride solution. Observations were made for the formation of clear zone around the growth of the bacterium.

H_2S Production
The peptone broth was prepared and sterilized. A loopful culture of 48 hr. old test bacterium is inoculated in to the slants containing the peptone broth. Filter paper discs (What man No. 42) impregnated with 10 per cent solution of neutral lead acetate was taken and air dried and then inoculated. The sterilized stripes were placed in to the inoculated test tubes. The inoculated tubes were incubated at 27 ± 1 ºC for 72 hrs. Observations were drawn for the H_2S production. Blackening of the stripes indicated the positive reaction.

Catalase test
A loopful of 48 hr sold slant growth of the bacterium was smeared on a slide and was covered with few drops of hydrogen peroxide (H_2O_2). The reaction will be positive if gas bubbles are produced.

Potassium hydroxide (KOH) solubility test
A loop full of bacteria was aseptically removed from culture plates with tooth pick, placed on glass slide in a drop of KOH (3 %) solution and stirred for ten second using a quick circular motion of hand, then the tooth pick was raised a few centimeter's above the slide and observed for the formation of viscid strand represent the bacterium as Gram-negative.

Protein digestion
For agar plate test, reconstituted powdered skim milk was sterilized and is mixed with sterile melted yeast extract nutrient agar(YENA) to obtain a10 per cent concentration and poured over the surface of nutrient agar in Petri plates. The plates were dried, spot inoculated and observed for a clear zone around the colonies after 3,5and 7 days.

Indole production
Five ml of the medium containing tryptophan 10g, L-tryptophan 1g and distilled water was taken in test tube and sterilized by autoclaving at 15lbs. at 121 ºC for 15 min. Inoculated test tubes were incubated at 27 ºC on a shaker for five days. After incubation 0.5ml of Kovacs indole reagent was added and shaken well. A positive test is indicated by the development of dark red color in the surface layer.

Ammonia production
Test tubes containing 8 ml of sterile nutrient broth were inoculated and one tube kept uninoculated as control and incubated at 27 ºC for 48hrs, after incubation cotton stopper from the tubes were removed and strip of red litmus paper was inserted inside the wall of the tube and stopper was placed as earlier to hold the strip of litmus paper in place. No change in the color indicated the negative result.

Voges-Proskauer test
Test was performed in glucose phosphate broth (0.5% glucose, 0.5% peptone and 0.5% K_2HPO_4). Cultures were shaken incubated at 27 ºC for 5 days. 1 ml of culture was added to a test tube containing 0.6ml of naphthol and 0.2 ml of 40 per cent KOH and shaken vigorously. Change in color indicated positive reaction.

Nitrate reduction test
Eight ml media was taken in test tube (KNO_3 3g, yeast extract 3g, agar 3g and distilled water 1 liter) and sterilized for 15min at 121º C, tubes were stab inoculated and incubated for 4 days. To each tube 1 ml of 0.6 per cent solution of dimethyl-naphthylamine and 1 ml of 0.8 per cent solution of sulphanilic acid was added. Positive reaction indicated development of red color.

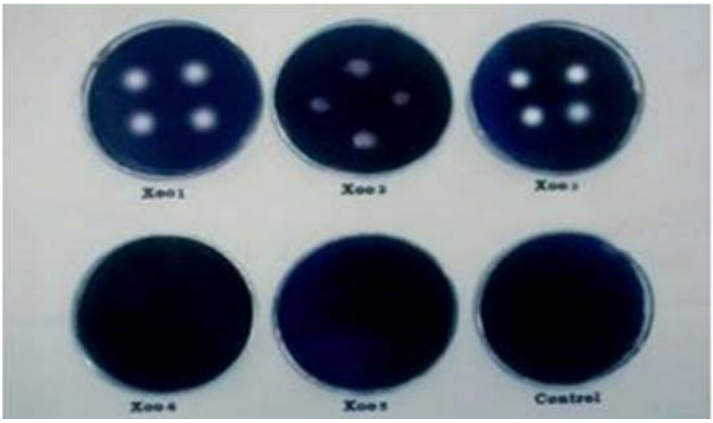
Methyl red test
Methyl red indicator (0.1g methyl red dissolved in 300 ml of 95% ethanol and made up to 500 ml with distilled water) was added to test culture; change in color indicates the positive reaction.

Production of fluorescent pigment on King's B medium
Test was conducted by use of technique described [7]

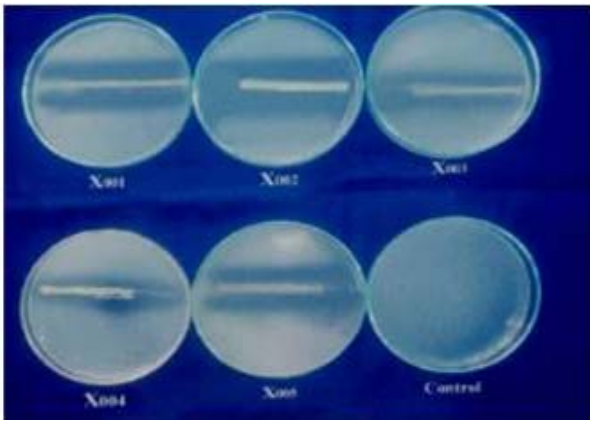
Results and discussion
The tests for biochemical properties revealed that the isolates showed similar reaction in most of the tests though some variations were seen (Table. 1). Productions of hydrogen sulphide, hydrolysis of starch were noticed in all the isolates except Xoo 4 and Xoo5 confirming the observations of [8, 9] who identified that, the bacterium liquefy the gelatin, produces H_2S and positive for catalase reaction. But can't hydrolyse the starch. However, [10, 11] obtained positive reactions for hydrolysis of starch (Plate 1.).
All the isolates showed positive reaction for ammonia production, hydrolysis of starch, H_2S production, liquefaction of gelatin, catalase test and KOH test (Plate. 2 and Plate. 3). The results are in line with findings of [12] who reported that all the isolates were positive to ammonia production, hydrolysis of starch, liquefaction of gelatin, catalase test and KOH test.
All isolates showed negative response for some of biochemical properties such as indole production, voges-proskaure test, nitrate reduction, methyl red test and production of fluorescent pigment on King's B medium. Similar behavior of isolates of pathogen was observed in findings of [13] he reported that among the five isolates of *Xanthomonas oryzae* pv. *oryzae* subjected for biochemical studies all the isolates showed negative reaction for indole production, voges-proskaure test, nitrate reduction, methyl red test and production of fluorescent pigment on King's B medium.

Table 1: Biochemical characteristics of five isolates of *Xanthomonas oryzae* pv. *Oryzae*

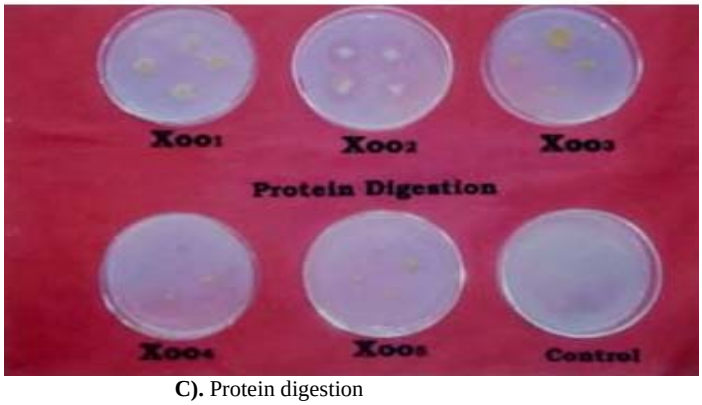
Sl. No.	Biochemical character	Isolates				
		Xoo1	Xoo2	Xoo3	Xoo4	Xoo5
1	Starch hydrolysis	+	+	+	-	-
2	Gelatin liquefaction	+	+	+	+	+
3	Protein digestion	+	+	+	+	+
4	Production of ammonia	+	+	+	+	+
5	Production of indole	-	-	-	-	-
6	Production of hydrogen sulphide	+	+	+	+	+
7	The Voges-Proskaure test	-	-	-	-	-
8	Nitrate reduction	-	-	-	-	-
9	Methyl red test	-	-	-	-	-
10	Production of fluorescent pigment on king's B medium	-	-	-	-	-
11	Catalase test	+	+	+	+	+
12	KOH test	+	+	+	+	+



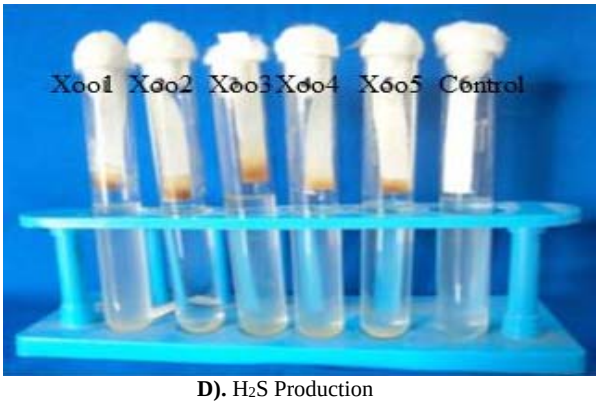
A). Starch hydrolysis



B). Gelatin liquefaction

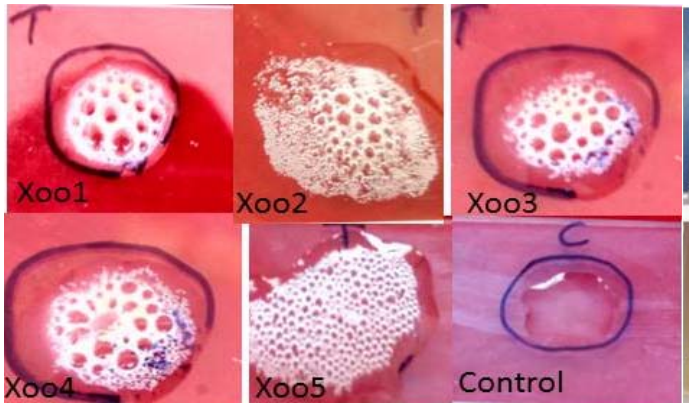


C). Protein digestion

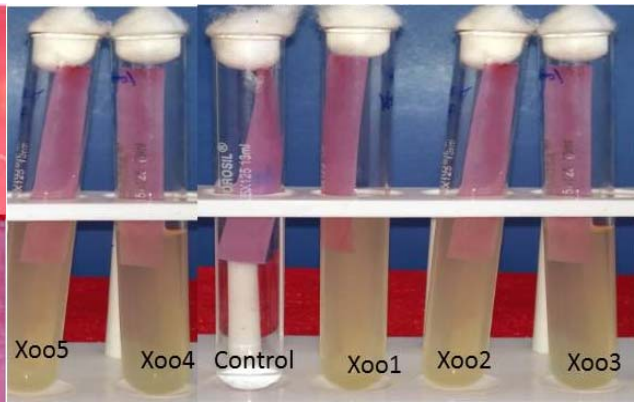


D). H_2S Production

Plate 1: Biochemical characteristics of five isolates of *Xanthomonas oryzae* pv. *oryzae* (a-d)

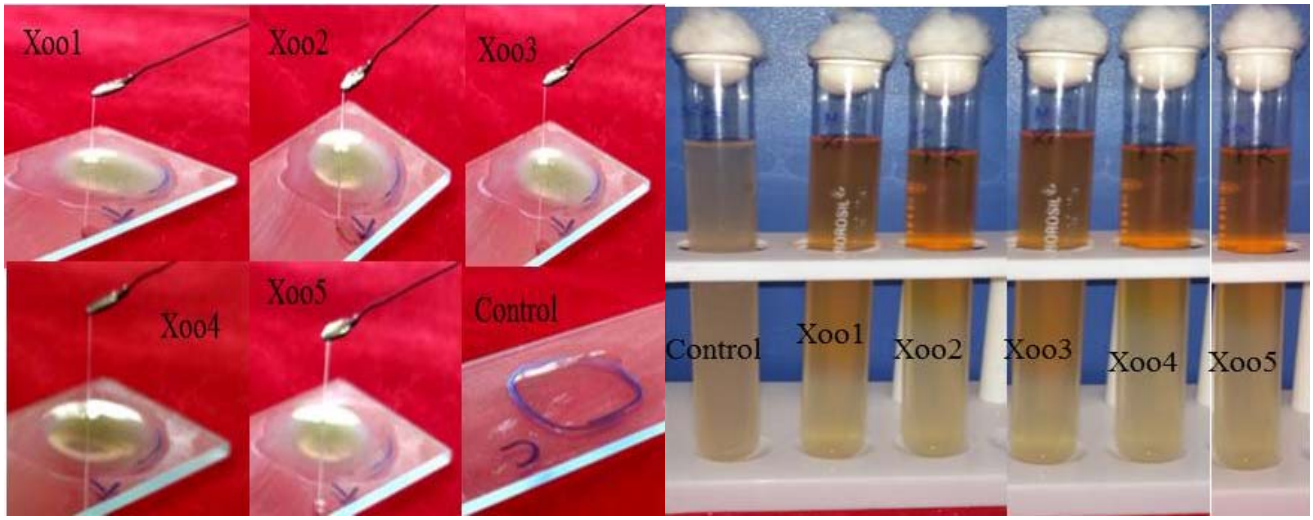


A). Catalase test



B). Ammonia production

Plate 2: Biochemical characteristics of five isolates of *X. oryzae* pv. *oryzae* (a-b)



A). KOH test **B).** Methyl red test

Plate 3: Biochemical characteristics of five isolates of *X. oryzae* pv. *oryzae* (a-b)

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