

Biochemical Characterization of *Xanthomonas Oryzae* Pv. *Oryzae* (Xoo) Populations from Kallar Belt of Punjab, Pakistan

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Abstract: *Xanthomonas oryzae* pv. *oryzae* (Xoo) is an important pathogen of rice causing leaf blight BLB disease, resulting heavy yield losses in fine Basmati rice cultivars in Kallar Belt of Punjab Pakistan. High variability of the pathogenicity existed in the rice growing areas. Comprehensive survey studies were carried out throughout the Kallar Belt of Punjab during summer 2011-12, for the collection of diseased samples. Isolation of bacteria from 105 collected samples only (71%) 75 bacterial growth were obtained and 35 (33.33%) were identified as Xoo based on colony morphology and biochemical assays. These 35 populations were clustered in 3 groups in dendrogram. This study revealed the biochemical diversity among the populations of Xoo in the Kallar Belt, which is resulting in diversity of pathogenicity, its surviving ability and level of pathogenic threats to our Basmati fine rice cultivars.

Keywords: *Xanthomonas Oryzae* Pv. *Oryzae*, BLB, Kallar Belt, Basmati Rice.

1. INTRODUCTION

Rice (*Oryza sativa*) is one of the most important food crop of the world including Pakistan. It provides food to more than 2.7 billion people around the world. Almost 90% of total rice is produced and consumed in Asian countries. In Pakistan, it is second staple food after wheat and second major export commodity after cotton. Production area range is 2.7 million hectares with production of 6.7 million tones. It accounts for 3.1% of value addition in agriculture and 0.7% of GDP [1]. Per hectare rice production is very low when compared to Korea 122%, China 107%, Indonesia 61%, Japan 67%, Bangladesh 38% and India 09% higher than Pakistan. Major yield limiting factors are insect pests and diseases.

Bacterial leaf blight (BLB) of rice caused by *Xanthomonas oryzae* pv. *oryzae* (Xoo) are getting prime economic importance due to heavy yield losses [2]. [3] first time reported the disease from Pakistan, followed by [4]. In Pakistan, BLB is an atrocious disease to rice farmers and became one of the main yield limiting factors in fine rice Basmati varieties as well as coarse varieties. The diseases resulted yield losses upto 15-20% on mild attack but severe attack results may exceed 50% [5] - [7].

The disease incidence increased in recent year especially in "Kallar belt" which is a famous for producing premium quality of scented basmati rice [8] - [10]. BLB is a vascular bacterial disease which infects rice plant from seedling to mature plant stage.

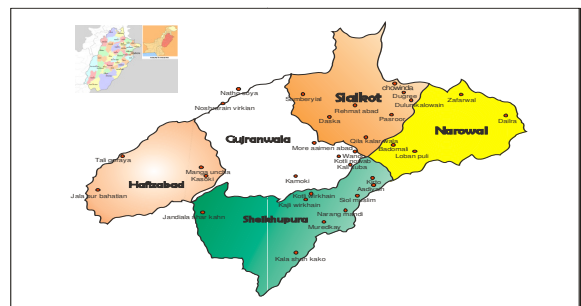


Fig1: Map of 35 Xoo isolates

X. oryzae pv. *oryzae* is a rod-shaped gram-negative bacterium that enters rice leaves through stomata, wounds, or hydathodes [11]. The bacterial multiplication takes place in epitheme, the tissue connecting the hydathodes to the xylem, to which they subsequently move and further multiply to infect the plant [12]. Irrigated paddy fields and high nitrogen application aggravated by warm, humid, and wet conditions results in severe epidemic of the disease. Symptoms of disease usually appear at tillering stage. Diseased plants show foliar blight or kresek, water-soaking or gray yellowish, irregular, wavy margin lesions, progressing down the leaves, which often dry and turn chlorotic, leaves often curve inward. In severe infections, bacterial ooze comes out of cracks or hydathodes. The lesions usually start from the leaf margin near the leaf tips. The disease incidence increases with plant growth and is on its peak at flowering and grain filling stage. In past 17 isolates of Xoo had been characterized from Pakistan [13] including Kallar belt and KPK, however variability of the pathogenicity was found [14]. Due to frequent attack on fine rice in Punjab it was found imperative to know the pathogenic potential of the organism found in the area. Present studies were designed to know the pathogenicity potential and variability of the pathogen in Kallar Belt.

2. MATERIAL AND METHODS

Collection of diseased samples

An extensive survey was carried out and rice leaves showing BLB diseased symptoms were collected from major rice growing districts of province Punjab during rice season 2011. Total 105 samples of BLB had collected based on random sampling method, as rice plant at panicle initiation to mature stages, as the disease usually developed well at these plant stages, from surveyed fields representing the disease symptoms. Disease leaves were detached and put into the paper envelope. These envelopes were labeled explaining variety, location and sampling date and these samples were taken into the laboratory and kept in the refrigerator for further process.



Fig- 2: Pure culture of *Xanthomonas oryzae* pv. *oryzae*



Fig- 3: Attack of bacterial leaf blight in Kallar belt

Isolation of bacteria from disease infected rice leaves

Collected samples from different rice growing areas of Punjab province were brought to Phyto-bacteriology laboratory, Crop Diseases Research Institute (CDRI), National Agricultural Research Center (NARC),

Islamabad for isolation. Infected leaves were cut into pieces from advancing portion of lesions. The leaves pieces were surface sterilized with 2% Clorox for 2 minutes followed by three washings with sterilized distilled water. The sterilized leaf pieces were plated on medium and were placed at 28-30 °C in an incubator [16]. Plates were seen daily until growth of bacterial. The bacterial colonies having morphological characteristic such as short, rod shape with round end, gram negative, aerobic and non-spore forming and produce yellow, circular, smooth and viscous colonies on potato yeast dextrose calcium carbonates medium if bacterial colonies are similar to *Xanthomonas oryzae* pv. *oryzae* (Xoo) morphological characteristics were picked with the help of sterilized loop and purified culture was obtained by streaking on different media in plates.

Confirmation of Xanthomonas oryzae pv. oryzae (Xoo)

Bacterial culture was re-inoculated on the fine variety Super Basmati for confirmation of *Xanthomonas oryzae* pv. *oryzae* (Xoo) in glasshouse of insectary, Bio-Control Lab NARC Islamabad. The bacterial inoculum suspension was prepared from 24 hours old culture of Xoo. After that 25-30 days old seedling of local susceptible variety name super basmati were used as confirmatory host grown in plastic pots using Clip method [15] with sterilized surgical scissors dipped in bacterial suspension of 10⁹ CFU/ml. The inoculated plants were put in the glass house at 28-30°C with 70% relative humidity. The symptoms were observed on regular basis so that the symptom of bacterial leaf blight was appeared or not. After recording the observations foliar samples will be plated again on PSA media and bacterial colonies will be compared with mother culture.

Biochemical characterization of Xoo isolates

The isolates of Xoo were characterized according to their reaction to certain biochemical test. Following tests were performed.

1-Gram Staining Test

Gram staining is the base for the identification of gram positive and negative bacteria. Thin layer of bacterial culture was smeared on the slide, and gently heated. The bacterial cells were stained with 0.5% crystal violet solution for 30 sec and washed the slide with tap water for one minute. Now slide was flooded with decolorized agent 95% ethanol for 30sec. The slide was again rinsed with tap water and finally counters stained by Safranin for 10 sec. The bacterial cells slide was eventually washed with water, dried with tissue paper and observed under microscope at 10X and 40X magnifications. If the stain isolates showed the reddish pink color which means gram negative bacteria if isolates retain purple to blue cooler it means gram positive bacteria.

2-KOH Test

KOH is another test for conformation gram negative or gram positive. One drop of 3% aqueous solution of potassium hydroxide (KOH) was placed on clean surface of slide. Picked up freshly prepared culture of Xoo isolates

aseptically and stirred circularly into the solution for 10 seconds. Gram negative suspension will become viscous, thick and form thread like structure.

3-Starch Hydrolysis Test

Starch hydrolysis test is used for characterization of Xoo isolates. 25gram starch agar powder was suspended in one liter distilled water and mixed thoroughly. Heated with continuous agitation and boiled the media until powder of starch was completely dissolved in water. Maintain the media pH at 7.5 ± 0.2 , autoclaved at 125°C for 15 minutes. After autoclaved media was poured into sterilized Petri plates. After 24 hours when media was solidified, these media plates were inoculated with bacterial Xoo isolates and incubate at $28-30^{\circ}\text{C}$ for several days for maximum bacterial growth. Then these inoculated plates were flooded with lugol's iodine solution. Hydrolysis or breakdown of starch was indicated by the presence of clear zones in the blue stained media.

4-Tween 80 hydrolysis test

The hydrolytic activity of bacterial Xoo isolates were done on Tween 80 media. This media has been prepared by adding peptone, $\text{NaCl}\cdot 2\text{H}_2\text{O}$, plant agar in distilled water, pH was maintained at 7.2-7.4 then autoclaved at 125°C for 15 minutes, Tween 80 was mixed at end to the sterilized media. Media was poured into autoclaved Petri plates. After 24 hours these plates were inoculated with Xoo fresh culture and incubate at $28-30^{\circ}\text{C}$ for seven days. Positive reaction of milky white precipitation was formed around the colonies.

5-Gelatin hydrolysis test

This gelatin media (consisting of peptone, beef extract and gelatin) was prepared by gently mixing of all ingredients in 1L deionized water and heating until complete dissolving of the ingredients, poured 3-5ml into culture tubes. Then these tubes were autoclaved at 125°C for 15 minutes. After sterilization these tubes were cooled until for inoculation. 24 hours old bacterial Xoo isolates were used for stab inoculation in tube media and these incubated at $28-30^{\circ}\text{C}$. After 7-14 day tubes were placed into the refrigerator at 4°C for 30 sec before recording of the results. If the gelatin remains liquid it means the result is positive and if the gelatin is solid it showed that the result is negative. A negative result or non hydrolyzed gelatin was only considered when gelatin was remained solid during 7 days inoculation.

6-Oxidase test

Xoo fresh colony culture on nutrient agar was used by putting 1-2 of 1% Tetra-methyl-p-phenylenediamine dihydrochloride. Flooding and inverting of plates were avoided during the assay. Development of dark purple color was within 5 to 10 sec indicated the positive sign of Xoo presence; delayed positive when the color was changed during 1 to 2 minutes.

7-Lecithinase activity test

Bacterial activity of Xoo isolates confirms the lecithinase activity of Egg yolk. Egg was washed in soap solution, rinsed and surface sterilized with 70% ethanol. Then egg was broken and separate the yolk in breaker and

diluted in sterilize water by 1:1 ratio. At the same time nutrient agar media was prepared and autoclaved at 125°C for 15 minutes. Now lecithinase plates were prepared by adding 10ml of egg emulsion to 100ml cooled at 55°C , nutrient agar media and pouring the mixer into Petri plates. The plates were spot inoculated and incubates at $28-30^{\circ}\text{C}$ for 3days. These plates were observed for white opacity, surrounding the Xoo colony which extends beyond the edge of growth. The white opaque edge was very clear and very even it means result is positive.

8-Anaerobic Growth Test

Media was prepared by adding the Peptone, NaCl , KH_2PO_4 , agar and Bromothymol blue in 1 liter distilled water and pH was maintained at 7.1. Took 5ml of this basal media into test tubes and autoclaved at 125°C for 15 minutes. Similarly an aliquot of 0.5 ml of 10% sterilized glucose solution was aseptically added into each test tube. Two tubes were used for each Xoo isolate. Then these two inoculated test tubes were over laid with 5ml of liquid paraffin. At the end these tubes were incubated at $28-30^{\circ}\text{C}$. Change of blue color into yellow means result are positive for anaerobic growth.

9-Tetrazolium Salt Tolerance Test

Nutrient agar (29gm) was dissolved in 1liter distilled water and autoclaved at 121°C for 15minutes. Meanwhile 1% solution of triphenyl tetrazolium chloride (TTC) was prepared and added into molten agar at 55°C and 0.1% concentrated media was prepared and poured into petri plates. After 24 hour these plates were inoculated with Xoo culture. If growth was inhibited it means result was positive.

10-Acid formation from Glucose, Fructose and Galactose

Xoo isolates were subjected to acid formation test for characterization. Media having $\text{NH}_4\text{H}_2\text{PO}_4$, K_2HPO_4 , $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$, NaCl , yeast extract and agar in 1litre distilled water. Bromocresol 1.5% (0.7ml) was dispensed in test tube and all material was autoclaved at 121°C for 15minutes. An aqueous solution of glucose, fructose and Galactose (10% w/v) was prepared separately and added to molten basal medium and formed a final concentration of 1%. Xoo isolates were transferred aseptically into the tube and incubated at 28°C and checked for acid production after 2, 4 and 6 days. Yellow color indicated production of acid and positive reaction.

11-Acid formation from Ribose, Maltose and Lactose

Xoo isolates were subjected to acid formation test for characterization. Media having $\text{NH}_4\text{H}_2\text{PO}_4$, K_2HPO_4 , $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$, NaCl , yeast extract and agar in 1litre distilled water. Bromocresol 1.5% (0.7ml) was dispensed in test tube and all material was autoclaved at 121°C for 15minutes. An aqueous solution of ribose, maltose and lactose (10% w/v) was prepared separately and added to molten basal medium and formed a final concentration of 1%. Xoo isolates were transferred aseptically into the tube and incubated at 28°C and checked for acid production after 2, 4 and 6 days. Yellow color indicated production of acid and positive reaction.

3. RESULTS

Biochemical Characterization of Xanthomonas oryzae pv. oryzae

All 35 isolates showed hypersensitive reaction (HR) on tobacco plant. All the Xoo isolates were gram negative and rod shape confirmed by gram staining technique and further confirmation was done by KOH test (Fig-5).

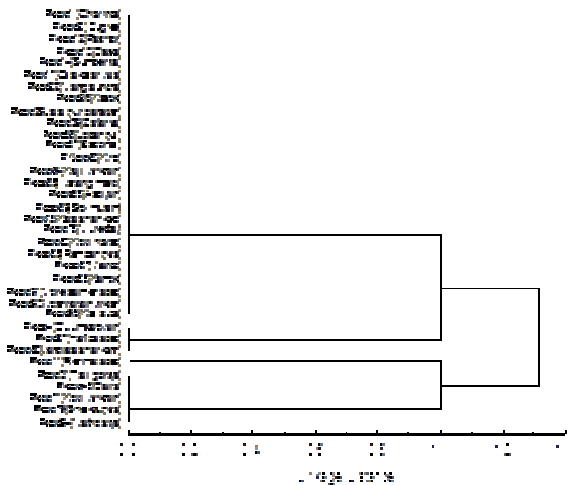


Fig-4: Dendrogram of 35 *Xanthomonas oryzae pv. oryzae* based on biochemical test



Fig-5: KOH Test

The starch hydrolysis test (Fig-6) for positive to all 35 isolates except 6 isolates.



Fig-6: Starch Hydrolysis test

similarly Tween- 80 hydrolysis test (Fig-7) showed the positive result to all 35 isolates.

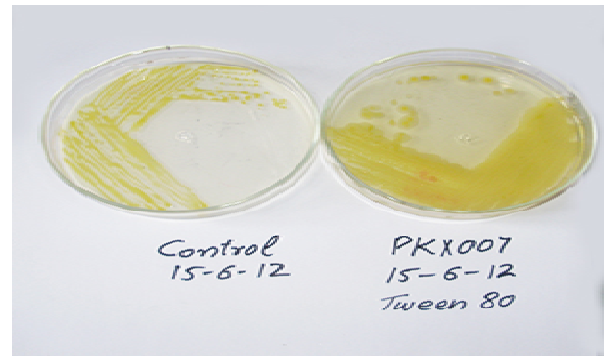


Fig-7: Tween 80 test

Acid formation from Glucose, Fructose and Galactose (Fig-8), all the isolates show that Xoo have the ability to produced acid.



Fig-8: Acid formation test

All isolates are unable to produced acid from ribose, maltose and lactose. During Gelatin hydrolysis, all isolates liquefied gelatin except 8 isolates. In the Oxidase test all isolates were showed negative results. All isolates were negative to lecithinase test. All isolates were obligate aerobic bacteria and at 0.1% concentration of TTC all isolates were failed to produce color (table 1).

Clustering of Xoo isolates on the basis of biochemic-al test

The results of biochemical test are subjected to cluster analysis based on using software version Statistica7. The data was incorporated in the form of '+' in the case of positive test whereas negative test was denoted as '-' and three groups were constructed (fig 4). Group1 includes 6 isolates having negative to starch hydrolysis test and positive to all other. Group 2 have the 3 isolates that showed the negative reaction against gelatin liquefaction test and positive reaction against all other tests. Similarly group 3 has 26 isolates for which the entire test were positive. On the basis of the morphological, hypersensitivity test, Koch 'postulates and biochemical test, we identified the *Xanthomonas oryzae pv. oryzae* bacterial pathogen which caused the bacterial leaf blight disease in rice crop. All 35 Xoo isolates produced the bacterial leaf blight disease symptoms during Koch 'postulates. Based on these biochemical responses we

developed 4 groups of Xoo isolates during clustering which previously reported as 7[17].

4. DISCUSSION

Although rice is a major crop of this area but yield potential is low as compared to other rice growing countries because BLB disease is a major thread in this area. [17] reported that bacterial leaf blight disease was more prevent in rice zone 2 (Punjab province) as compared to other rice zones. This disease is caused by bacteria and very difficult to control by chemicals. Therefore resistant variety is the easiest and cheapest way to control the disease. This study will be help out in management of this disease.

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Table 1: Phenotypic characteristics of 35 *Xanthomonas oryzae* pv. *oryzae* isolates tested

s.no	Names of isolates	Gram staining test	KOH test	Starch hydrolysis Test	Tween80 Hydrolysis test	Gelatin liquefaction test	Oxidase test	Leithinase activity test	Anaerobic growth test	Salt tolerance test	Acid formation from glucose, fructose, and galactose	Acid Formation from ribose, maltose and lactose
1	Pkxoo1	-	+	+	+	+	-	-	-	-	+	-
2	Pkxoo2	-	+	+	+	+	-	-	-	-	+	-
3	Pkxoo4	-	+	+	+	-	-	-	-	-	+	-
4	Pkxoo11	-	+	-	+	+	-	-	-	-	+	-
5	Pkxoo12	-	+	+	+	+	-	-	-	-	+	-
6	Pkxoo13	-	+	+	+	+	-	-	-	-	+	-
7	Pkxoo14	-	+	+	+	+	-	-	-	-	+	-
8	Pkxoo17	-	+	+	+	+	-	-	-	-	+	-
9	Pkxoo22	-	+	+	+	+	-	-	-	-	+	-
10	Pkxoo26	-	+	+	+	+	-	-	-	-	+	-
11	Pkxoo27	-	+	+	+	-	-	-	-	-	+	-
12	Pkxoo29	-	+	+	+	+	-	-	-	-	+	-
13	Pkxoo31	-	+	-	+	-	-	-	-	-	+	-
14	Pkxoo39	-	+	+	+	+	-	-	-	-	+	-
15	Pkxoo48	-	+	-	+	-	-	-	-	-	+	-
16	Pkxoo56	-	+	+	+	+	-	-	-	-	+	-

17	Pkxoo57	-	+	+	+	+	-	-	-	-	+	-
18	Pkxoo63	-	+	+	+	+	-	-	-	-	+	-
19	Pkxoo64	-	+	+	+	+	-	-	-	-	+	-
20	Pkxoo65	-	+	+	+	+	-	-	-	-	+	-
21	Pkxoo68	-	+	+	+	+	-	-	-	-	+	-
22	Pkxoo69	-	+	+	+	+	-	-	-	-	+	-
23	Pkxoo71	-	+	-	+	-	-	-	-	-	+	-
24	Pkxoo75	-	+	+	+	+	-	-	-	-	+	-
25	Pkxoo78	-	+	+	+	+	-	-	-	-	+	-
26	Pkxoo79	-	+	-	+	-	-	-	-	-	+	-
27	Pkxoo80	-	+	+	+	-	-	-	-	-	+	-
28	Pkxoo83	-	+	+	+	+	-	-	-	-	+	-
29	Pkxoo85	-	+	+	+	+	-	-	-	-	+	-
30	Pkxoo87	-	+	+	+	+	-	-	-	-	+	-
31	Pkxoo88	-	+	+	+	+	-	-	-	-	+	-
32	Pkxoo91	-	+	+	+	+	-	-	-	-	+	-
33	Pkxoo93	-	+	+	+	+	-	-	-	-	+	-
34	Pkxoo94	-	+	-	+	-	-	-	-	-	+	-
35	Pkxoo95	-	+	+	+	+	-	-	-	-	+	-

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