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Morphological and Biochemical Characterization of *Xanthomonas oryzae* pv *oryzae* isolates from Punjab, Pakistan

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ABSTRACT

A study on the loss of rice production due to bacterial leaf blight of rice has been reported. A total of 23 bacterial isolates were collected from eight districts of Punjab Pakistan and were characterized on the basis of various morphological and biochemical features. After microscopic study, it was concluded that all Xoo isolates were gram-negative, non endospore-forming bacteria and with encapsulated cells. Growth behavior of all the isolates was checked at two different pH levels i.e., 7.0 and 5.5. The number of bacterial colonies recorded at pH 7.0 was significantly higher than the colonies grown at pH 5.5. Growth of bacteria at two different temperatures i.e. 25℃ and 40℃ was checked and that it was found that most of the isolates showed maximum growth at 25℃ while at 40℃ isolates showed poor or no growth. Biochemical characterization of Xoo isolates were conducted which showed positive results for methyl red test and indole test while oxidase test was negative for entire set of collected isolates. The 23 isolates inoculated on test tubes exhibited the blackening of medium confirming to be positive against Hydrogen Sulfide (H_2S) test. The two isolates (8a-KSK, 1-S) that showed a different behavior might also help to check the diverse behavior of pathogen and is helpful in detecting the new genetic approaches to develop host plant resistance.There is an alarming situation in these areas and there is a dire need to adopt the management practices to control this disease.

Keywords: Morphological, Biochemical, *Xanthomonas oryzae,* Rice, Pakistan.

INTRODUCTION

Rice (*Oryza sativa* L) is staple food of more than half of the world's population and has been used as a model plant for research purpose (Kawahara *et al*., 2013). It is the second-largest export commodity of Pakistan after textile. Rice is considered as a second largest cereal and feed for about 2.7 billion people around the world. The important crops of Pakistan are wheat, rice, maize, sugarcane and cotton and accounts for 25.6 percent share in overall agriculture and 5.3 percent of GDP. About ninety percent (90%) of rice is cultivated in Asia and among them Pakistan is the major exporter of basmati (Saqib *et al*., 2018). New diseases arise when agriculture moves toward higher productivity. However, it is unlucky that such a major crop is under the attack of several viral, fungal and bacterial diseases**.**

Many biotic and abiotic stresses are major threats to the low production of rice. Low rice production may be attributed to various constrains, but bacterial leaf blight (BLB) is very destructive and cause severe yield losses in rice crop. It is a major pathogen of rice (*Oryza sativa* L.) causing bacterial leaf blight (BLB) disease (Feng *et al.*, 2009). In Pakistan, the symptoms of BLB were first recorded in 1977 (Dinh *et al.*, 2008) and in 1987 its occurrence was recorded from all over the Punjab (Khan *et al.*, 2014). Recently an increase in BLB disease incidence is reported in the most important rice-growing areas including Kallar Tract that is well known for the cultivation of high-quality rice (Jabeen *et al.*, 2011; Ali *et al.*, 2009). In general, the disease favors temperatures at 25−34°C, with relative humidity above 70%. With the passage of time the

Author's Contribution: N. R., Conceived the idea of the study and performed the experiment; **M. T. M.,** Designed and supervised the experimental work; **M. A.,** Supported in write up and review the manuscript; **M. A.,** Helped I recording the data; **M. S.,** Helped in data analysis; **M. S.,** Helped in organizing the experimental material; **A. B.,** Helped in data analysis and recording the observation.

disease is increasing day by day and significantly reducing both the quality and quantity of the crop. The pathogen is seed-borne (Reddy *et al.*, 1974; Singh *et al.*, 1990) and has been considered as an important quarantine organism in many countries. *X. oryzae* pv *oryzae*, belongs to c-subdivision of the Proteobacteria (Feng *et al.*, 2009). The BLB pathogen enters the rice leaf through hydathodes cells on the leaf sheath and spread to the plant through xylem and developed the emerging roots on it for causing the BLB disease (Ou, 1985; Curtis, 1943; Noda and Kaku, 1999). It is a major pathogen of rice (*Oryza sativa* L.) causing bacterial leaf blight (BLB) disease (Feng *et al*., 2009) and causing huge yield losses in South Asian countries since the widespread cultivation of dwarf high-yielding cultivars. Stubbles of infected plants serve as the primary inoculum (Ashfaq *et al*., 2017). In general, the disease favours temperatures at 25−34°C, with relative humidity above 70%. Authentic isolation, handling and identification methods may be very useful for controlling the disease for further spreading and causing yield losses. For developing a successfully integrated disease management package the first step is to know the characteristics of the pathogen.

MATERIALS AND METHODS

Sample Collection

Diseased plant materials were collected from the different areas of Punjab including Rice Research Institute (RRI) Kala Shah Kaku, Sahiwal, Okara, Khanewal, Multan, Yousaf Wala and Shorkot, Vehari, Hafizabad, Sheikhupura and Kasur. The samples with BLB were recognized through visual observation of yellow to white water-soaked stripes at the margins of infected leaves (Isaka, 1970).

Isolation and Purification of *Xoo* from Infected Samples

The diseased samples were cut in to small pieces and sterilized with the 70% ethanol, Sodium Hypochloride and distilled water one by one and were placed on filter paper. Eppendorfs were taken that were half-filled with distilled water. Half of the diseased samples were macerated in this distilled water and were left for half an hour so that the bacterial ooze can come out. The surface-sterilized samples were direct inoculated on Nutrient Agar (NA) plates in aseptic conditions. The resulting suspension in eppendorf was streaked on the nutrient agar plates and was incubated at 25-28˚C for 24 hours to obtain the colonies of *Xanthomonas oryzae* pv *oryzae* (Reddy & Ou, 1974). After the incubation of one night, yellow colonies were picked and streaked on the fresh nutrient agar plates. The plates were incubated for 24 hours at 30˚C. The pathogen was further identified on the basis of different morphological characters.

Morphological Characterization of Bacteria

Morphological characteristic of all samples of *Xanthomonas oryzae* from different locations were observed. In morphological characteristic colony color, texture, margins and elevation were seen. For colony color, all the bacterial plates were observed that either it is yellow, light yellow or yellowish-white. The texture of all bacterial colonies was observed slimy or rough. To observe texture all the bacterial colonies were examined that either it is slimy or rough. Margins of colonies were observed to know that either they are smooth or irregular. For elevation, plain, convex and concave characteristics were observed from bacterial colonies (Shankara *et al*., 2017).

Staining Techniques and Growth at Different pH and Temperature

Staining Techniques

A drop of water with the bacterial film was spread and heat-fixed on a clean slide. This smear was flooded with crystal violet, washed with tap water and then treated with iodine and then gently washed with ethanol 2-3 times and stained with safranin for one minute. Slide was rinsed with tap water and air-dried (Gerhardt, 1981; Jabeen *et al*., 2012). Slides with smear were covered with tissue paper and placed with wire gauze on a beaker half-filled with the boiling water and flooded with malachite green. After 2-3 minutes the slides were removed and after removing the tissue the slides were rinsed with water. The smear was stained with safranin for two minutes and rinsed with tap water and air-dried. A small drop of the crystal violet was placed on one side of slide. Using sterile techniques, a loop full of bacteria was added to crystal violet drop, smearing it into the dye. Another slide was used to drag the dye-cell mixture into a thin film along with the first slide and let it stand for 5-7 minutes. The slides were flooded with H_2SO_4 solution and the slides were washed with it 2-3 times. The slides were air dried. The slides were observed under the microscope for further study.

Growth at Different pH and Temperature

The pH of NA media was set using pH meter at 5.5 and 7.0 in two flasks. The media of both flasks was autoclaved. The plates with both pH were streaked one by one by taking the inoculum from pure plates. Incubate the plates at 30℃ for 24 hours and check for the positive and negative growth on both pH. The 46 nutrient agar media plates were prepared for the two experiments in aseptic conditions and left overnight to check contamination. The plates were labeled and streaked according to the samples. The 23 plates were placed at 25℃ and the replicates of these plates were placed at 40℃. Left the plates for 24 hours and checked for the relative growth at both temperatures (Jones *et al*.,1989; Jarial and Shyam, 2005). The 2mg of NaCl was dissolved in 100ml distilled water. The solution was autoclaved before use. The 23 autoclaved test tubes were half-filled with this 2% NaCl solution. A loop full of bacteria from every pure plate was inoculated in the test tubes. The test tubes were placed at 30℃ for 24 hours.

Biochemical tests for Bacterial Identification

Different biochemical tests are very useful for bacterial identification. For this purpose, the 23 autoclaved test tubes were half-filled with the autoclaved solution of hydrogen per oxide. A loop full of inoculum from the pure bacterial plate was taken and dissolved in the tube. After a while bubbles will produce in the tube (Bradbury, 1986). Test tubes were half-filled with semi-solid SIM agar media. The inoculum was taken from pure plates with an inoculating needle and inoculates in the test tube by piercing in the media. Test tubes were placed at 30℃ for 24 hours. Results were checked by the presence or absence of black precipitation. Another test a loop full of inoculum was streaked on the media. The test tubes were an incubated at 30℃ for 24 hours and clear bacterial growth was observed on the surface. A drop of kovac's reagent was dropped in each test tube and waited for one minute. The result was checked by the appearance of yellow and red color which shows negative and positive results respectively (Ding *et al*., 2008). Five ml of an autoclaved MR-VP media was poured into each test tube. A loop of inoculum was taken and mixed in the broth media in the test tube. The test tubes were incubating at 30℃ for 24 hours and results were observed in broth media. The 0.5ml of methyl red was dropped in each test tube for examination and checked the results by the appearance of yellow and red color which shows negative and positive results respectively (Swings *et al*., 1990). This test was carried out using filter paper with 1% tetramethyl-p-phenylenediamine dihydrochloride solution and rubbing the inoculum material on it. The observation was recorded due to the appearance or absence of colour on the filter paper (Kovacs, 1956).

RESULTS

Morphological Characterization of *Xoo*

The diseased sample material placed on NA plates were observed for bacterial growth after 24 hours of incubation at 24℃. In morphological characters colony color, texture, margins and elevation were observed. Different isolates were observed having off white, pink and yellow color (Table I). The colonies were medium to small in size and circular in shape with smooth margins and texture. Elevation of all the colonies was observed convex.

Table I: Result of morphological characters of different isolates of *Xanthomonas oryzae* pv *oryzae*

S. No.	Strain Code No.	Colony Color	Texture	Margin	Elevation
1.	1-b KSK	Yellow	Slimy	Smooth	Convex
2.	2-b KSK	Yellow	Slimy	Smooth	Convex
3.	4 KSK	Yellow	Slimy	Smooth	Convex
4.	5 KSK	Off white	Slimy	Smooth	Convex
5.	7-a KSK	Yellow	Slimy	Smooth	Convex
6.	7-b KSK	Yellow	Slimy	Smooth	Convex
7.	8-a KSK	Pink	Rough	Irregular	Convex
8.	8-b KSK	Yellow	Slimy	Smooth	Convex
9.	1-I	Yellow	Slimy	Smooth	Convex
10.	1-S	Yellow	Slimy	Smooth	Convex
11.	2-P	Yellow	Slimy	Smooth	Convex

12.	3-S	Yellow	Slimy	Smooth	Convex
13.	4-S	Yellow	Slimy	Smooth	Convex
14.	5-I	Yellow	Slimy	Smooth	Convex
15.	5-S	Yellow	Slimy	Irregular	Convex
16.	6-P	Yellow	Slimy	Irregular	Convex
17.	7-S	Yellow	Slimy	Smooth	Convex
18.	8-I	Off white	Rough	Irregular	Convex
19.	8-S	Yellow	Slimy	Irregular	Convex
20.	9-S	Yellow	Slimy	Smooth	Convex
21.	10-I	Yellow	Slimy	Smooth	Convex
22.	10-S	Yellow	Slimy	Irregular	Convex
23.	11-S	Yellow	Slimy	Irregular	Convex

Staining behaviour of *Xoo*

Gram staining results revealed that all 23 isolates were gram-negative with pink or red color as rod-shaped and with single polar flagellum. Spore staining showed that the isolated pathogen was a non spore-forming bacterium. Capsule Staining showed that all isolates were encapsulated indicated by clear zones in the surrounding of the cells (Figure 1 and Table II). It was observed that the growth (number of colonies) of the pathogen was increased with rise in pH from 4.0 to 7.0.

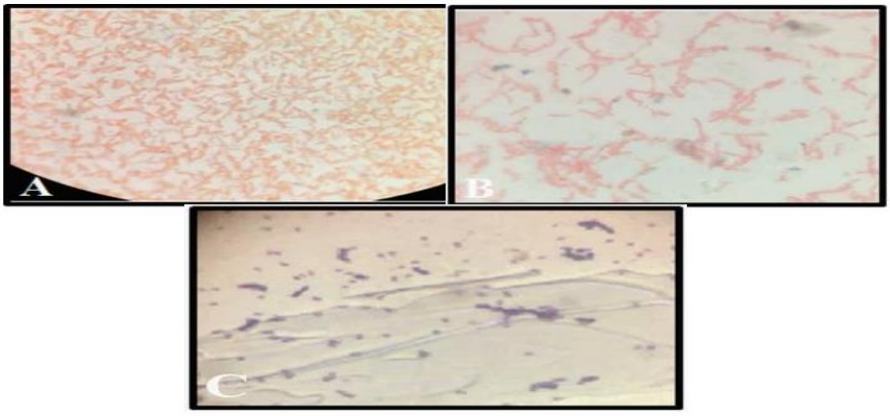

Figure 1: Staining of different isolates of *Xanthomonas oryzae* pv *oryzae*. (A), (B) Gram Staining, (C) Capsule Staining

Table II: Result of various staining methods of different isolates of *Xanthomonas oryzae* pv *oryzae*

S. No.	Strain Code	GRAM STAINING				SPORE STAINING	CAPSULE STAINING
		Gram Type	Cell Shape	Cell Color	Cell Arrangement		
1.	1-b KSK	-	Bacilli	Pink	Chains	Absent	Present
2.	2-b KSK	-	Bacilli	Pink	Diplo	Absent	Present
3.	4 KSK	-	Bacilli	Pink	Tetra	Absent	Present
4.	5 KSK	-	Bacilli	Pink	Clusters	Absent	Present
5.	7-a KSK	-	Bacilli	Pink	Chains	Absent	Present
6.	7-b KSK	-	Bacilli	Pink	Chains	Absent	Present
7.	8-a KSK	-	Bacilli	Pink	Diplo	Absent	Present

8.	8-b KSK	-	Bacilli	Pink	Diplo	Absent	Present
9.	1-I	-	Bacilli	Pink	Tetra	Absent	Present
10.	1-S	-	Bacilli	Pink	Chain	Absent	Present
11.	2-P	-	Bacilli	Pink	Diplo	Absent	Present
12.	3-S	-	Bacilli	Pink	Cluster	Absent	Present
13.	4-S	-	Bacilli	Pink	Cluster	Absent	Present
14.	5-I	-	Bacilli	Pink	Chains	Absent	Present
15.	5-S	-	Bacilli	Pink	Diplo	Absent	Present
16.	6-P	-	Bacilli	Pink	Diplo	Absent	Present
17.	7-S	-	Bacilli	Pink	Cluster	Absent	Present
18.	8-I	-	Bacilli	Pink	Chain	Absent	Present
19.	8-S	-	Bacilli	Pink	Diplo	Absent	Present
20.	9-S	-	Bacilli	Pink	Diplo	Absent	Present
21.	10-I	-	Bacilli	Pink	Chain	Absent	Present
22.	10-S	-	Bacilli	Pink	Cluster	Absent	Present
23.	11-S	-	Bacilli	Pink	Chains	Absent	Present

Growth at pH 5.5 and 7.0

Growth of all 23 isolates was observed at both pH 5.5 and 7.0 after 24 hours of incubation at 27˚C. Results indicated that at pH 7.0, the positive growth of all the isolates was recorded except the three isolates '5-KSK', '7-b KSK', '10-S' that showed weak positive growth. The growth at pH 5.5 falls in three different categorize that were positive, weak positive and negative (Table III). The maximum isolates that showed positive results at pH 5.5 were 7.0 that include '4-KSK', '1-I', '2-P', '6-P', '7-S', '9-S' '5-1'. Eleven isolates (1-b KSK, 8-a KSK, 8-b KSK, 1-S, 3-S, 4-S, 5-S, 8-I, 8-S, 10-I, 11-S) were observed for weak positive growth. Negative growth at this pH was recorded for 5 isolates i-e.,'2-b', '7-a KSK', '7-b KSK', '5 KSK', '10-S'.

Growth at Temperature 25℃ and 40˚C

The growth of all isolates at both temperatures was observed. The isolates almost showed the same growth habits at both temperatures with a little difference. Results indicated that at 25℃, only 8 isolates showed weak positive growth while the remaining ones were observed for positive growth. At 40℃ nine isolates were observed for weak positive growth and the remaining ones were positive. Results for this test are shown in table 3. On the other hand, biochemical characterization was described in table IV and different tests were performed for the identification of bacteria along with the entire set of isolates. Six biochemical tests were conducted to characterize the pathogen on the infected samples of BLB collected from different areas of Punjab.

Table III: Growth of different isolates of *Xanthomonas oryzae* pv *oryzae* at pH 5.5 and 7.0 and at temperature 25°C and 40°C

S. No	Strain Code No.	Growth at pH 5.5	Growth at pH 7.0	Growth at 25°C	Growth at 40°C
1.	1-b KSK	+ weak	+	+	+
2.	2-b KSK	-	+	+	+
3.	4 KSK	+	+	+	+ weak
4.	5 KSK	-	+ weak	+	+
5.	7-a KSK	-	+	+ weak	+
6.	7-b KSK	-	+ weak	+ weak	+
7.	8-a KSK	+ weak	+	+ weak	+ weak
8.	8-b KSK	+ weak	+	+	+
9.	1-I	+	+	+	+
10.	1-S	+ weak	+	+	+ weak
11.	2-P	+	+	+ weak	+weak
12.	3-S	+ weak	+	+ weak	+ weak
13.	4-S	+ weak	+	+ weak	+ weak
14.	5-I	+	+	+	+
15.	5-S	+ weak	+	+	+

16.	6-P	+	+	+	+ weak
17.	7-S	+	+	+	+
18.	8-I	+ weak	+	+	+ weak
19.	8-S	+ weak	+	+	+
20.	9-S	+	+	+ weak	+ weak
21.	10-I	+ weak	+	+	-
22.	10-S	-	+ Weak	+ weak	+
23.	11-S	+ weak	+	+	+

Table IV: Biochemical tests of different isolates of *Xanthomonas oryzae* pv *oryzae*

Sr. No	Strain Code No.	Catalase Test	Motility Test	Hydrogen Sulfide Test	Indole Test	Oxidase Test	Methyl Red Test
1.	1-b KSK	+	Present	+	-	-	-
2.	2-b KSK	+	Present	+ weak	-	-	-
3.	4 KSK	+	Present	+	-	-	-
4.	5 KSK	+	Present	+	-	-	-
5.	7-a KSK	+ strong	Present	+	-	-	-
6.	7-b KSK	+ strong	Absent	-	-	-	-
7.	8-a KSK	+ strong	Present	+ weak	-	-	+
8.	8-b KSK	+ strong	Present	+	-	-	-
9.	1-I	+	Present	+	-	-	-
10.	1-S	+	Present	+	-	-	+
11.	2-P	+	Present	+	-	-	-
12.	3-S	+	Present	+	-	-	-
13.	4-S	+	Present	+ weak	-	-	-
14.	5-I	+	Present	+	-	-	-
15.	5-S	+	Absent	+ weak	-	-	-
16.	6-P	+	Absent	+ weak	-	-	-
17.	7-S	+ strong	Present	+ weak	-	-	-
18.	8-I	+	Present	+	-	-	-
19.	8-S	+	Present	+	-	-	-
20.	9-S	+	Absent	+ weak	-	-	-
21.	10-I	+	Present	-	-	-	-
22.	10-S	+	Absent	+	-	-	-
23.	11-S	+	Present	+ weak	-	-	-

DISCUSSION

Bacterial leaf blight caused by *Xanthomonas oryzae* pv *oryzae* is the most destructive disease and it has the potential to incur severe yield losses to the most important cash crop of Pakistan i.e., rice. The colony colour of most of the isolates was yellow whereas two isolates (5 KSK, 1-8) off white and one were pink (8-a KSK). Most of the isolated raised slimy colonies and some of the isolates produced rough colonies with smooth and irregular shapes and having convex elevation appeared on the nutrient agar medium. Similar results were reported by (Han *et al*., 2005; Suresh, 2012). Growth behavior of bacterium at both temperatures that most of the isolates showed maximum growth at 25℃ while at 40℃ some isolates showed poor or no growth. No growth occurred in the presence of 2% NaCl (Thimmegowda, 2006).

It was observed that all the isolate showed almost same growth habits at different temperatures with little bit differences. The positive growth of all the isolates was observed at pH 7 with respect to pH 5.5. It was indicated that the growth of isolates increasing at a high pH level (Shankara *et al*., 2017). According to the gram staining test the bacteria were found rod-shaped, pink colour with spore staining absent and capsule staining present for the entire set of isolates. Similar studies were reported by (Rukshana *et al*., 2012; Ashfaq *et al*., 2017) regarding gram negative, rod shaped bacteria that producing red colour when counter stained with safranin.

Under glass house, condition pathogenicity test was conducted by applying artificial inoculum by using clip inoculation technique for further confirmation of pathogenic bacteria. After 15 days

of inoculation symptoms appeared and the pathogen was re-isolated which resembles the original culture of Xanthomonas oryzae pv oryzae (Ghasemie *et al*., 2008). There is a need to develop rice varieties having genetic resistance against the diseasetoreduce its attack and obtain maximum crop production.This is the most economical way of controlling diseases by using resistant rice cultivars.This will be very useful for the scientists to plan future strategies to check the disease incidence and imply the control measures and develop new rice varieties with host genetic resistance (Ashfaq *et al*., 2017).

All the bacterial isolates showed positive results to the Catalase test that was indicated by the bubble formation in hydrogen per-oxide solution after the bacterial inoculation as in agreement with the findings of (Bradbury, 1984; Jonit, 2016). A positive and negative motility test is indicated by a diffuse zone of growth flaring from the stab line of inoculation. *Xanthomonas* is a polar flagellum bacteria and all of the 23 isolates showed positive results to motility test. A positive Hydrogen Sulfide (H_2S) test is denoted by a blackening of the medium along the line of inoculation. All of the 23 isolates inoculated test tubes exhibited the blackening of medium, confirming to be positive for H_2S test.

Some bacteria have the ability to break down the amino acid tryptophan with the formation of indole that gives red color. But indole test was found negative for all the *Xoo* isolates after adding Kovac's reagent as they were not able to break down the amino acid same as results obtained by Ding *et al*, (2008). The production of sufficient acid detected by using the methyl red (MR) test during the fermentation of glucose as shown by a change in the color of the methyl red indicator which is added at the end of the period of incubation (Swings *et al*., 1990). Methyl red test showed negative response for 21 isolates as the similar results obtained in the findings of Gilligan, (2003), while two isolates showed positive results.

According to the oxidase test all the tested isolates were found negative to produce the desired colour after rubbing the culture on the strip and remained yellow that is the indication of a negative oxidase test and were in agreement with the results of (Bradbury, 1986; Mew, 1992; Mew and Misra, 1994). In the present study, based on morphological characters and some biochemical tests, it was observed that genetic variability occurred in almost all the isolates of *Xanthomonas oryzae* pv oryzae.

CONCLUSION

Keeping in view the economic importance of this problem, the present research work was conducted to check the diversity of pathogen in different rice areas of Punjab. The two isolates that showed a different behavior might also help to check the diverse behavior of pathogen in (8a-KSK, 1-S) and is helpful in detecting the new genetic approaches to develop host plant resistance.Developing disease resistance rice varieties will be very useful approach for sustainable productivity *w*hich in turn improve the financial status of the farming community and reduce poverty. As a result of this study, knowledge about the pathogenicity of the existing isolates has been generated.Host plant resistance is an important component of an integrated management programme against the diseases that can be achieved with the help of present study.

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