ISOLATION AND CHARACTERIZATION OF XANTHOMONAS ORYZAE PV. ORYZAE ISOLATES FROM NORTH WEST FRONTIER PROVINCE (NWFP) PAKISTAN

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ABSTRACT

Xanthomonas oryzae pv. *oryzae,* the causal agent of bacterial leaf blight (BLB) of rice was characterized through pathogenecity and biochemical assays. Plant samples were collected from major agro-ecological rice zones including Lower Dir, Swat and Agricultural Research Station, Mingora in NWFP during 2002. The isolates obtained were subjected to pathogenecity test on six rice cultivars that varied significantly in terms of disease severity among each other. However, non-significant results were recorded when clip and pinprick methods of leaf inoculation were compared for pathogenecity test. The isolates were also subjected to different biochemical tests and were found to be negative for Oxidase, Lecithinase and Gram reactions. Results of biochemical tests like Tween 80 and Starch Hydrolysis, Anaerobic nature and Acid Production from Carbohydrates varied among the isolates. Only 20% isolates were similar in terms of their reactions to these tests. Based on biochemical responses it was established that despite the small sample size (n=15) genetic variability was detected in *Xathamonas oryzae* pv. *oryzue* isolates.

INTRODUCTION

Rice is grown in tropical and subtropical regions of the world and is a staple food for around 2.7 billion people worldwide (Salim *et al.,* 2003). Rice is ranked second in Pakistan in terms of area under cultivation and production after wheat crop (Agriculture Statistics of Pakistan, 2003-2004). Apart from being Pakistan's important crop, rice is also a major export item and contributed Rs. 36534.7 millions to the national exchequer during the fiscal year 2003-2004 (Agriculture Statistics of Pakistan, 2003-2004). Pakistan's Super Basnuui and Kernal rice are world famous for their invigorating aroma and taste.

The rice crop is susceptible to a number of diseases among which the bacterial blight of rice caused by *Xanthomonas oryzae* pv. *oryzac* (Swings *et al.,* 1990) has been an important constraint to rice production in Asia (Lozano, 1981). In Pakistan, this disease was reported first during 1977 (Mew and Majid, 1977). The bacterium can infect rice from seedling stage to mature plant and the disease is manifested by either 'leaf blight' or 'kresek' symptoms. During the leaf blight phase, causal organism enters the plant through wounds or through water pores located on the margins of upper part of the leaves, producing lesions, which are water soaked, yellow with irregular, wavy margins and progresses down the leaves. The lesion usually starts from the leaf margin near its tip. Bacterial ooze, which consists of small, yellowish, spherical masses, may sometimes be seen on the margins or veins of the freshly infected leaf under moist conditions. On the other hand, the Kresek phase is a systemic phase during which acute wilting of the seedlings take place. This symptom usually appears one or two weeks after transplanting. Leaves turn grayish green, wither suddenly and roll upwards. The Kresek phase was first reported from Indonesia and was later reported to occur in most of the rice growing areas in the tropics (Reitsma and Sehure, 1950). BLB under mild infection causes yield reduction ranging from 10-12% (Mew *et* al., 1993) whereas under severe condition, it can be as high as 50% (Ou, 1985). During milling. The grains from blighted plant are easily broken. The purpose of this study was to isolate and characterize different strains of the causal agent from NWFP using pathogenecity bioassay and biochemical tests. This is expected to provide first hand information on the pathogen and the database thus developed could be used in developing cultivars with durable resistance against the pathogen in future studies.

MATERIALS AND METHODS

Isolation

Ninety samples of rice at panicle initiation stage, showing typical bacterial blight symptoms were collected comprising of eight samples from Lower Dir, 36 from Agricultural Research Station, Mingora and 46 from Swat regions of NWFP. Diseased leaf pieces (2x7 mm) were surface sterilized with 1% Clorox and transferred to Petri plates containing Yeast-Extract-Caleium-Carbonate (YDC) agar medium using three replicates and incubated at 25 -27^{o}C for 72 hrs (Wilson *et al.* 1967). Yellow, mucoid, doom shaped colonies with entire margins developed that were further sub-cultured. Cultures were preserved for longer duration (at 4^{0}C) by pipetting 0.5ml of an inoculated heavy nutrient

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broth solution into a tube of sterile, sandy-loam soil and tightly capped the tubes.

Hypersensitivity Reaction and Pathogenecity Test

Approximately 10^8-10^9 cfu/ml of freshly cultured bacteria from YDC plates were injected with a hypodermic syringe into the abaxial surface of three sections/tobacco leaf of cultivar Virginia, for routine hypersensitivity test. Inoculation was done at 5-6 leaf stage. Sterile distilled water was used as control. Complete collapse of tissue after 24 h followed by necrosis was recorded as positive reaction (Klement and Goodman, 1967). Fifteen isolates showing typical colony characteristics and strong hypersensitivity reaction were selected for further testing. Six rice cultivars including 1RR1 6, KS-282. .JP 5, Basmati 385, Dilrosh 97 and Kashmir Basmati 385 were used for pathogenecity test. Seedlings of each cultivar were grown on moist sterilized filter papers in Petri plates, maintained in a growth chamber at .U) 30-35^{o}C (' (100% RH). Two weeks old seedlings were transplanted lo small plastic pots (diameter 13cm) containing loamy soil and placed in green house. At pre-tillering stage, plants were again transplanted to bigger plastic pots (diameter 27 cm). These cultivars were subjected to pathogenecity test using pinprick and clip inoculation methods. Pinprick method consisted of pricking three leaves/cultivar with the inoculum loaded needle (10 -10^8 cfu/ml) whereas during clip method, a pair of sterile scissors was dipped in the inoculum (10^7-10^8 cfu/ml) and three leaves per plant were cut with it approximately 2-3 cms from the tip. Controls were treated with sterile distilled water for both the methods. Inoculated plants, wrapped in moist plastic bags were then transferred to green house where bags were removed after three days. Twelve to fourteen days after inoculation, lesion sizes were measured along both the leaf axes with a scale. CRD three factorial test was used to determine differences between the two inoculation methods for each cultivar separately at 5% level of probability. The effect of isolates on mean lesion size was determined with CRD two factorial test. Means showing significant difference were separated by Least Significant Difference (LSD) test.

Biochemical Characterization Tests

YDC cultures (24 hrs old) were used for the nine biochemical tests. Gram staining (Gerhardt, 1981) consisted of subjecting a thin bacterial film on a glass slide to aqueous Crystal Violet, Iodine, Ethanol and Safranin solutions for various periods of time and washing with tap water. Gram negative stain red whereas Gram positive bacteria give blue-blue black color. Gram staining results were confirmed with reaction to Potassium hydroxide (3% KOH) test (Ryu, 1940). During this lest, a loopful of bacteria was stirred in 3% KOI 1 and any change in the viscosity was recorded. Gram negative bacteria forms thread like slime when picked with a tooth pick while gram positive bacteria disperses and forms no slime. Starch hydrolysis test consisted of treating the inoculated basal medium of powdered agar and dissolved starch with Lugol's iodine and recording the absence or presence of clear zones in stained media (Cowan, 1974). Similarly Tween 80 hydrolysis was determined with Sierra's method (Sierra, 1957). Development of milky white precipitate around the colonies in the basal media consisting of peptone, NaCl, $CaCl_2$, agar and Tween 80 confirmed Starch hydrolysis. Dye's medium (Dye, 1968) was used for acid production from 10% w/v aqueous solution of glucose, fructose, sucrose and galactose that were filter-sterilized through Millipore injection before adding to the Dye's medium. Acid production was confirmed with the appearance of yellow colour in the inoculated tubes. MC Clung and Tuabe procedure was employed for recording-Lecithinase activity (MC Clung and Toabe, 1947). Production of turbid /zone of free fats around the colonies in petri plates containing egg yolk incorporated into molten agar were considered positive. Tolerance to Tetrazolium salts was tested at 0.02% and 0.1% concentrations. Different concentrations of Tetrazolium salt were added to nutrient agar and bacterial growth was recorded (Lelliott and Stead, 1987).

For determining anaerobic growth of the pathogen, a basal medium consisting of peptone. NaCl, agar, bromothymol blue was prepared and an aliquot of sterile aqueous glucose solution was added to it aseptically. Two tubes for each isolate were inoculated over-laying one with sterile liquid paraffin. A colour change from blue to yellow in both tubes was considered as positive for anaerobic growth (Hugh and Leifson, 1953). Kovacs Oxidase test (Kovacs, 1956) was carried out using a filter paper impregnated with 1% tetramethyl-p-phenylene diamine dihydrochloride solution and rubbing a loopful of inoculum on it. The appearance or absence of color on these filter papers was recorded.

RESULTS AND DISCUSSION

Isolation

Out of the ninety samples processed, 78 bacterial isolates were recovered from 46 samples whereas; no bacteria were recovered from the remaining 44 samples. Xanlhomonads are reported to produce colonies that are yellow, convex, mucoid and shiny in texture (Figure A and B). Such colonies were consistently observed and tentatively identified as the causal organism. Bacteria could lose pathogenecity when maintained by subculturing. Lyophilization is regarded as the most appropriate method for long term preservation, in the absence of which xanthomonads are preserved in soil or in a medium over-lain with oil (Lelliott and Stead, 1987). In present studies, soil preservation was preferred over all other methods since it is readily available, easy to handle and bacteria were recovered with minimal contamination.

Hypersensitivity Reaction (HR) and Pathogenecity Test

Hypersensitivity reaction can only be induced by pathogenic bacteria, which is manifested by tissue collapse and necrosis within 24-48 hrs (Lelliott and Stead, 1987). A total of 49 isolates out of the 78 initially recovered were positive for hypersensitivity reaction whereas the remaining 29 isolates were recorded negative. All HR-positive isolates were recovered from Swat region while all isolates from Lower Dir were HR-negative where mainly Shoga variety was cultivated. Among the cultivars, isolates obtained from JP 5 yielded the maximum number of HR-positive reaction (Table I). The positive HR response of 49 bacterial isolates was helpful in screening of these isolates as potential pathogens (Figure C).

The comparison of both the inoculation methods at 5% level of significance yielded non--significant results suggesting that both the methods are equally effective to initiate leaf blight lesion under green house conditions. It is a common practice in tropics to clip off leaf tips of rice with scissors or shears, before transplanting. This could provide entry ports for the pathogen and might be one of the causes of 'Kresek' phase of BLB. The findings of this study are in support of Mew *et al.* (1981) who compared needle prick, clip and spray inoculation methods but could not find any differences in lesion length or disease score.

Among the isolates that were compared individually on six different cultivars in terms of mean lesion sizes, 12 showed significant results (Table II). Non-significant results were recorded for isolates Xo-103, Xo-108 and Xo-112 Ly. This suggests their inability to cause infection on cultivars under test. A range of lesion sizes was observed on the cultivars tested. The isolates showed more aggressiveness on cv. Ksh Bas 385.

Biochemical Characterization Tests

Biochemical tests showed that all isolates were negative for Oxidase and Gram reactions (Table III). Lecithinase activity was found in all isolates except Xo-112 Dy. Variable results were observed for the remaining biochemical tests. Both positive and negative isolates for Starch hydrolysis have been reported for this pathogen. According to Swings *et al.* (1990), the pathogen can hydrolyze starch after a week's incubation. On the contrary Guvera and Marsella (1999) did not find this feature in their isolates. Both the cases of Starch hydrolysis were observed in the present study. Only 20% isolates were similar to each other in their biochemical responses and contrast to the remaining 80% that differed among themselves. These 20% isolates were collected from different areas of Swat. Isolates Xo-128C, Xo-139A (i) w, Xo-143A (ii) and Xo-153 A were although similar but differed in their response to Starch hydrolysis and Anaerobic growth test. Based on the biochemical responses it could be established that despite small sample size (n = 15) genetic variability was detected in *Xanthomonas oryzae* pv. *Oryzae*

Though rice is not a major crop of NWFP, it has the potential of increasing its yield tremendously if proper varieties and cultural practices are adopted. A number of cultivars other than Basmati and IRRI type are largely grown in the province. In Swat and Lower Dir mixing of Shoga variety with JP 5, which is, outdated and susceptible to bacterial blight is the main cause of high disease incidence in these areas.

CONCLUSION AND RECOMMENDATIONS

i. The isolates varied in terms of disease severity among, themselves and on the cultivars tested.
ii. The isolates were most aggressive on cv. Ksh Bas 385 than any other cultivar.
iii. There was no significant difference between pinprick and clip method of Inoculation in terms of lesion size.
iv. All the isolates tested negative for Gram reaction, Oxidase and Egg yolk reaction. Isolates, however, varied in their response to other biochemical tests.

v. In future more isolates collected on wider scale need to be sampled and tested to verify existing data and develop a wider database of the pathogen.

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Figure A. colonies of *Xanthomonas oryzae pv. oryzae* growing on YDC medium.

Figure B. Isolating *Xanthonomas oryzae pv. oryzae* from rice samples growing on YDC medium.

Figure C. Hypersensitivity reaction on tobacco leaf.

Table I. ***Characterization of isolates of Xanthomonas oryzae pv. oryzae by hypersensitivity test.***

S.no	Isolates	Location	Cultivar/line/ germplasm	Hypersensitivity response
1	Xo –97	Lower Dir	Shoga	–
2	Xo –98	Lower Dir	Shoga	–
3	Xo –103 A	Mingora	Breeding material	+
4	Xo –105	Mingora	C-Test No.2 entry no 3	+
5	Xo –107	Mingora	B- Test No.2 entry no 4	+
6	Xo –108	Mingora	Advance line C-Test No.10	+
7	Xo –109	Mingora	Breeding material	+
8	Xo –110	Mingora	C-Test No.6 entry no 6	+
9	Xo –111	Mingora	11 ron entry no 12	–
10	Xo –112	Mingora	NURYT entry no 3	+
11	Xo –112 Ly	Mingora	NURYT entry no 3	+
12	Xo –112 Dy	Mingora	NURYT entry no 3	+
13	Xo –113	Mingora	C-Test No.11 entry no 3	–
14	Xo –114	Mingora	Breeding material	–
15	Xo –116	Mingora	Advance line C-Test No.5	–
16	Xo –117	Mingora	Segregating material	–
17	Xo –118	Mingora	Breeding material	–
18	Xo –119	Mingora	Bas 385	–
19	Xo –121	Mingora	Breeding material	–
20	Xo –125	Mingora	JP5	–
21	Xo –127 (i) y	Hingodehra	JP5	+
22	Xo –127 (ii)	Hingodehra	JP5	+
23	Xo –128 B (i)	Hingodehra	Bas-385	+
24	Xo –128 B (ii)	Hingodehra	Bas-385	+
25	Xo –128 B(iii)	Hingodehra	Bas-385	+
26	Xo –128 A (i)	Hingodehra	Bas-385	+
27	Xo –128 A (ii)	Hingodehra	Bas-385	+
28	Xo –128 C	Hingodehra	Bas-385	+
29	Xo –129	Matatotkai	JP5	–
30	Xo –134	Mata	JP5	+
31	Xo –135	Mata	JP5	+
32	Xo –136	Matatotkai	JP5	–
33	Xo –139 A (i) w	Nrngonlai	JP5	+
34	Xo –139 A (i) y	Nrngonlai	JP5	+
35	Xo –139 A (ii)	Nrngonlai	JP5	+
36	Xo –140	Farhatabad	JP5	–
37	Xo –140 A (i)	Farhatabad	JP5	+
38	Xo –140 A (ii)	Farhatabad	JP5	+
39	Xo –140 B (i)	Farhatabad	JP5	+
40	Xo –142 A (i)	Farhatabad	C-Test No.2 entry no 4	+

Table –I continued

S.no	Isolates	Location	Cultivar/line/ germplasm	Hypersensitivity response
41	Xo –142 B (i)	Farhatabad	Breeding material	+
42	Xo –142 B (ii)	Farhatabad	Breeding material	+
43	Xo –143 A (ii)	Farhatabad	JP5	+
44	Xo –143 A (iii) w	Farhatabad	JP5	+
45	Xo –143 A (iv)	Farhatabad	JP5	+
46	Xo –143 B (i)	Farhatabad	JP5	+
47	Xo –143 B (ii)	Farhatabad	JP5	+
48	Xo –143 B (iii)	Farhatabad	JP5	+
49	Xo –144 B (i)	Farhatabad	B- Test entry no 2	+
50	Xo –146 B (i)	Kanju	JP5/Shoga	+
51	Xo –146 B (iii)	Kanju	JP5/Shoga	+
52	Xo –146 A (ii)	Kanju	JP5/Shoga	+
53	Xo –146 AB (i)	Kanju	JP5/Shoga	+
54	Xo –146 A (N)	Kanju	JP5/Shoga	+
55	Xo – 146 B (iii)	Kanju	JP5/Shoga	+
56	Xo –147	Kanju	Shoga	–
57	Xo –148	Kanju	Shoga	–
58	Xo –152	ARS, Swat	V_5R_1C- Test-I	–
59	Xo –153 A (iii)	ARS, Swat	Breeding material	+
60	Xo –153 B (i)	ARS, Swat	$V_{10}R_1$ C-Test- I	+
61	Xo –154	ARS, Swat	V_8 R_1 C- Test- I	–
62	Xo –154 A (ii)	ARS, Swat	V_8 R_1 C- Test- I	+
63	Xo –154 B	ARS, Swat	V_8 R_1 C- Test- I	+
64	Xo –156	ARS, Swat	V_1R_1 C-Test-I	–
65	Xo –156 A	ARS, Swat	V_1R_1 C-Test-I	+
66	Xo –157	ARS, Swat	V_{11} R_1 C-Test – I	–
67	Xo –157 A	ARS, Swat	V_{11} R_1 C-Test – I	+
68	Xo –163	ARS, Swat	Kohsin	–
69	Xo –166 A	ARS, Swat	NURYT- II S.No 3 R_1	+
70	Xo –170	ARS, Swat	Data planting trial DV No7	–
71	Xo –171	ARS, Swat	Breeding material	–
72	Xo –172	ARS, Swat	Data planting trial DV3	–
73	Xo –177 A	ARS, Swat	Advance 2002 $R_1V_5$385	+
74	Xo –180	ARS, Swat	Advance 2002 V_4 R_1	–
75	Xo –181	ARS, Swat	Advance 2002 V_3 R_1	–
76	Xo –182	ARS, Swat	Advance Trial 2002 V_1R_1	–
77	Xo-182 (i)	ARS, Swat	Advance Trial 2002 V_1R_1	–
78	Xo –184	ARS, Swat	Mineralization Trail JP 5	–

Table II. ***Lesions size (cm) of various isolates of Xanthomonas oryzae pv. oryzae on six rice cultivars, collected from different agro-ecological zones of NWFP.***

Isolates/ cultivars	Xo-103	Xo-107	Xo-108	Xo-110	Xo-112 Dy	Xo-112 Ly	Xo-127 (i) y	Xo-128 C	Xo-135 A	Xo-139 A (i) w	Xo-140 B (i)	Xo-143 A (ii)	Xo-146 A (N)	Xo-146 B (iii)	Xo-153 A
JP 5	0	0 b*	10.18	0 b	23.05 a	14	22.16 a	14.96 ab	2.40 b	0 c	3.18b	26.50 a	20.91 a	24.90 a	11.70 b
IRRI 6	0	13.7 a	5.16	0 b	14.71 b	8.33	2.25 c	13.55 bc	11.60 ab	22.30 a	14.25a	13 b	10.3 ab	7 b	17.86 a
Basmati-385	3.50	0 b	1.50	1.20 b	16.85ab	12.66	21.06 b	18.16 a	8.91 ab	8.28 bc	12.45a	5.81 b	6.75 b	12.33 ab	9.75 bc
Ksh Bas-385	4.11	13.40 a	10.46	8.28 a	20.78ab	11.90	23 a	18.25 a	9.06 ab	13.16 ab	13.20a	12.96 b	13.61ab	6.60 b	11.76 b
Ks-282	0	1.45 b	7.08	2.16 ab	8.46 c	10.56	6 c	7.21c	14.58 a	10.33 bc	16.50a	12.61 b	6.28 b	5.75 b	0d
Dilrosh 97	0	0 b	2.41	0 b	22.01 a	9.85	17.36 b	8.73 bc	4.43 bc	6.01bc	3.20b	17.18 ab	14.50ab	11.75 b	5.78cd
cv.	29%	28%	24%	22.6%	21%	14%	17.29%	29.55%	19.4%	30.3%	18.1%	23.3%	20%	18%	16.7%
LSD		6.75		6.54	6.21		4.45	6.73	10.06	10.96	13.8	13.29	12.48	16.66	5.83

5% level of probability was used

*Means followed by different letters in a column are significantly different.

*Underlined isolates showed non-significant results.

*Colony colour Ly=light yellow Dy = Dark yellow W = white Y = Yellow

*Alphabets A = Replicate 1 B =Replicate 2 C = Replicate 3

Roman numerals (i)= colony number 1 (ii) = Colony number 2 (iii) = colony number 3

Table III. ***Biochemical characterization of various isolates of Xanthomonas oryzae pv. oryzae, collected from agro-ecological zones of NWFP during 2002.***

S.No	Isolates	Gram reaction	KOH test	Starch Hydrolysis	Tween 80 hydrolysis	Acid From *Carbs*	Egg Yolk reaction	Tetrazolium Tolerance test 0.1%	Tetrazolium Tolerance test 0.02%	Anaerobic growth test	Oxidase Test
1	Xo- 103	–	+	+	+	+	–	–	–	–	–
2	Xo-107	–	+	–	+	+	–	+	+	+	–
3	Xo-108	–	+	+	+	+	–	–	–	–	–
4	Xo-110	–	–	+	+	+	–	–	+	+	–
5	Xo-112 Ly	–	+	–	+	+	–	–	–	+	–
6	Xo-112 Dy	–	–	+	+	+	+	–	–	–	–
7	Xo-127 (i) y	–	+	–	–	+	–	–	–	–	–
8	Xo-128 C	–	+	+	+	+	–	–	–	–	–
9	Xo-135 A	–	+	–	–	+	–	+	+	+	–
10	Xo-139 A (i) w	–	+	+	+	+	–	–	–	+	–
11	Xo-140 B (i)	–	–	–	+	–	–	–	–	–	–
12	Xo-143 A (ii)	–	+	+	+	–	–	–	+	–	–
13	Xo-146 A (N)	–	+	+	–	+	–	–	–	–	–
14	Xo-146 B (iii)	–	+	–	–	–	–	–	–	+	–
15	Xo-153 A	–	+	–	+	–	–	–	+	–	–

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