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ISOLATION, CHARACTERIZATION, PRESERVATION AND PATHOGENICITY TEST OF *XANTHOMONAS ORYZAE* PV. *ORYZAE* CAUSING BLB DISEASE IN RICE

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Abstract

The present study was conducted to isolate the *Xanthomonas oryzae* pv. *oryzae* obtained from infected rice foliar samples collected from all agro ecological zones of Pakistan. Infected leaf samples when plated gave yellow, circular, smooth, convex and viscous bacterial colonies on (YDCA). Out of 123 isolates, 105 showed hypersensitive reaction on tobacco leaves (*Nicotiana rustica*). Six biochemical tests were conducted to characterize 17 isolates of pathogen. All isolates consistently gave similar results. Gram staining demonstrated that pathogen is a gram negative rod shaped, KOH test was performed in order to confirm gram staining results, further all isolates showed positive reaction and confirmed that they are gram negative. The egg yolk reaction was negative for the 7 isolates (Xoo 20, Xoo 36, Xoo51, Xoo 65, Xoo 75, Xoo 99 and Xoo 105). At 0.1 and 0.02 % concentration of TTC these seven isolates failed to produce colonies. In the oxidase test 7 isolates proved negative. In the starch hydrolysis above mentioned 7 isolates showed positive reaction. The Pathogenicity test of 7 isolates tested on eight rice varieties (Basmati 385, IRRI 26, Basmati 386, Dilroosh 97, JP 5, Super Basmati, Basmati 2000 and Ks 282). Analysis showed that out of eight tested varieties IRRI 6 was significantly resistant whereas the varieties Basmati 385, Basmati 386, Jp5, Super Basmati and Basmati 2000 were significantly susceptible and var. Dilroosh 97 and Ks 282 were moderately resistant.

Introduction

Bacterial leaf blight was first time reported in Pakistan in 1977 (Mew & Majid, 1977). It is a very serious disease causing million of tones of grain losses annually. In Pakistan the incidence of BLB has increased in recent years especially in Kaller belt (Fig. 1) that is famous for producing high quality rice (Akhtar *et al*., 2003; Ali *et al.*, 2009; Bashir *et al*., 2010).

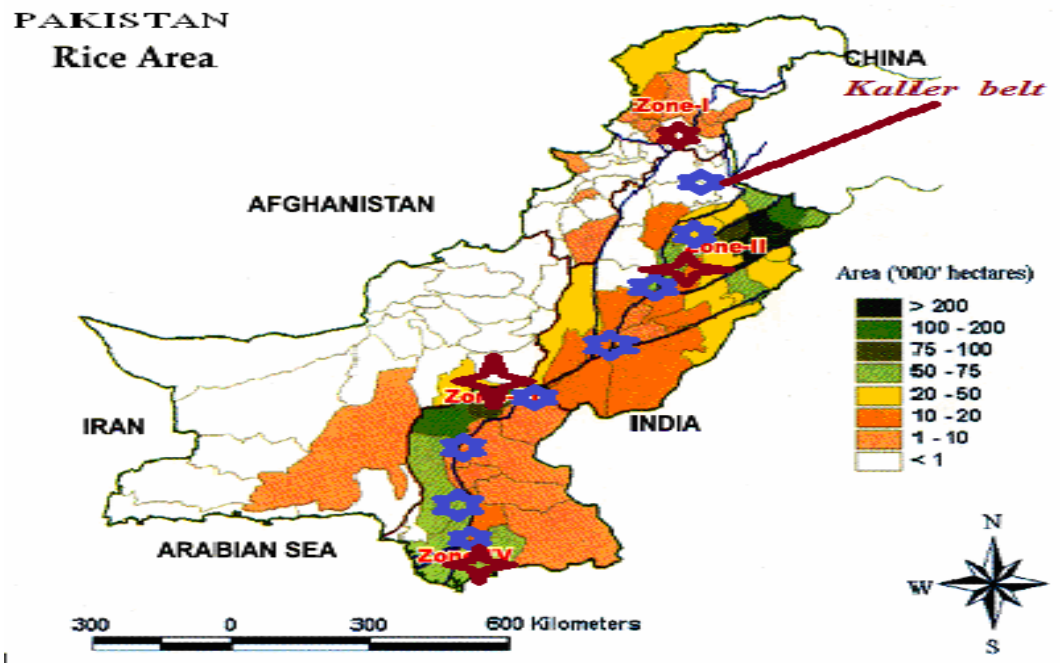

Fig. 1. Bacterial Leaf Blight occurring area of Pakistan.

There are 2 distinct types of symptoms, leaf blight phase and kresek phase in bacterial blight of rice (Ou, 1985; Akhtar *et al*., 2008). The leaf blight phase appears on leaf blade especially in temperate regions. The disease symptoms appear after maximum tillering stage. It starts from the lower portion of the plant and proceeds gradually towards the upper leaves. The acute symptoms are seen on susceptible cultivars when they are grown under heavy fertilization. The upper half of the blade or the whole blade turns pale before drying up. The kresek phase was first reported as a distinct disease in Indonesia. This phase is characterized by systematic infection. The

symptoms usually appear one to two weeks after transplanting, the leaves become grayish green, suddenly wither and roll up. According to Watanabe (1975) kresek phase sometime occurs on mature plant as well.

The topographic and climatic conditions greatly influence disease incidence and development. The causal organism is affected by discrete sunshine and dryness. The disease is mostly prevalent in area with monthly rainfall of more than 200 mm. The temperature for lesion development is reported to be 25-30^{o}C. Acidic soil is assumed to be an important factor causing the disease in the early days (Maruyama, 1909). Heavy fertilizer application is one of the most important cultural practices affecting the development of bacterial blight in rice. From the three elements of fertilizer, nitrogen is mostly responsible for encouraging disease development (Cha, 1982). The planting density of rice also affects the severity of disease, being more severe under wider spaced conditions at constant nitrogen level.

In review of the severity and significant damage caused by this destructive disease (BLB) worldwide, the scientists focused their attention on its control and management by using resistant varieties, for this the first purpose is the isolation and characterization of casual organism. In the present study we are tried to achieve this, the isolation, chemical characterization and pathogenicity test of *Xanthomonas oryzae* pv. *oryzae* which was the casual organism of Bacterial Leaf Blight disease in rice.

Material and Methods

Sample collection: A comprehensive survey of various agro-ecological zones in Pakistan was conducted for the collection of leaf samples of rice suspected to be infected with Bacterial Leaf Blight. The samples were used for isolation and characterization of bacterium. The recovered isolates were selected for preservation.

Isolation: Infected leaf pieces of rice (28×7 mm) were excised with sterile scalpel. The leaf surface was sterilized with 1% clorox for three minutes and then washed with sterile distilled water (SDW). Leaf pieces (6-7) of rice leaves after drying on sterile blotting paper were transferred to yeast extract dextrose calcium carbonate (Y D C) agar medium and incubated at 25–28^{o}C for 72 h. The cultures were suspended in SDW, for short term preservation and in silica gel for long term preservation (Wilson *et al.*, 1993).

Diagnostic tests

Hyper-sensitivity test: To determine the pathogenic nature of the isolates, hypersensitivity reaction was studied on tobacco (*Nicotiana rustica)* plants by injection infiltration technique developed by (Klement & Goodman, 1967).

Biochemical Characterization of *Xanthomonas Oryzae*

Gram staining: A thinly spread air-dried bacterial film was fixed on clean glass slide by a light flame The specimen was treated with 0.5% aqueous crystal violet for 30 seconds and afterwards washed with running tap water for one minute, rinsed in water and decolorized with 95% ethanol. The specimen was again rinsed with tap water and counter-stained with safranin for approximately 10 seconds. It was eventually washed with water and observed under microscope at X10 and 40 magnifications (Gerhardt, 1981).

Potassium hydroxide (KOH) test: Gram staining results were confirmed by potassium hydroxide test (KOH) 3% (Suslow *et al*., 1982). The bacteria were aseptically removed from Petriplates with tooth pick, placed on glass slide in a drop of 3% KOH solution and stirred for 10 second using a quick circular motion of hand (Ryu, 1940).

Starch hydrolysis test: Powdered nutrient agar was dissolved in water by progressive heating. Starch was then dissolved in distill water separately and added to the molten agar with constant stirring. An aliquot of this basal medium was dispensed in conical flask and sterilized at 11^{o}C for 10 minutes. The medium was then poured in Petri plates into which each isolate was transferred aseptically and incubated at 27^{o}C for seven days. After scraping the superfluous growth, the plates were flooded with Lugol' S iodine, which was prepared by dissolving iodine along with potassium iodide in distilled water, and stirred for several hours until dissolved completely (Cowan, 1974).

Egg yolk reaction: Egg yolk emulsion was prepared from a fresh egg, washed well in soap solution, rinsed and surface sterilized with 70% ethanol for 5 minutes.

The egg was then flamed, broken aseptically, yolk separated in to sterile graduated cylinder and diluted to 40 %v/v with sterile water. An aliquot of egg yolk was incorporated into molten nutrient agar (cooled to 55^{o}C) prior to pouring in to the plates. The medium was spot cultured and incubated for 3 days at 27^{o}C (McClung & Toabe, 1947).

Tetrazolium salt tolerance test: Nutrient agar was prepared and dispensed in flasks and sterilized at 121^{o}C for 15 minutes. Aqueous 1% triphenyl tetrazolium chloride (TTC) solution was aliquoted (filter-sterilized) to the molten agar at 55^{o}C to give a concentration of 0.02%. similarly 0.1% concentration was also prepared. The medium was then dispensed in plates and inoculum was added to the medium held at two different concentrations whereas nutrient agar alone served as control. Presence or absence of growth was recorded since most xanthomonads were inhibited at 0.02% TTC but completely inhibited at 0.1% concentration.

Oxidase test: A 24 h old bacterial colony on nutrient agar, supplemented with 1% glucose was used in this assay. A loopful of the inoculum was rubbed on filter paper impregnated with 15 % (w/v) freshly prepared aqueous solution of Tetramethyl-p-phenylene diamine dihydrochloride (Kovaes, 1956).

Preservation: Yellow viscous bacterial colonies of *Xanthmonas oryzae* that subsequently developed were subcultured on peptonesucrose agar (PSA) medium and grown at 25^{o}C for 2 days (Devadath, 1970). For long-term Preservation, the bacterial cells suspended in 10% (w/v)

on sterile soil, similarly on silica gel granules and skim-milk containing 0.05% L-glutamic acid was stored at 0°C until needed.

Results and Discussion

Isolation of *Xanthomonas Oryzae*: A survey of various agro-ecological zones of Pakistan was conducted in 2002-2003, during which infected leaf samples of rice having Bacterial Leaf Blight symptoms were collected from kaller belt of Pakistan (Fig. 1). After 48-72 h of incubation at 28°C, infected leaf samples when plated gave yellow, circular, smooth, convex and viscous bacterial colonies on yeast extract (dextrose calcium carbonate agar medium (YDCA) (Figs. 2 & 3) In XoS medium the samples gave light yellow, mucoid, round and smooth bacterial colonies (1 mm in diameter) while whitish, mucoid and smooth colonies were observed on Wakimoto medium. One hundred and twenty three isolates of *Xanthmonas oryzae* were recovered on the basis of colony morphology for further studies (Table 1).

Fig. 2. Isolation of Xoo bacterium on YDC medium.

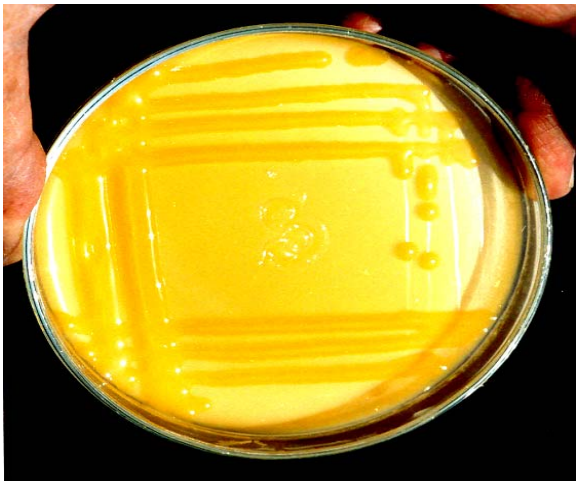

Fig. 3. Isolation of Xoo colonies on YDC medium.

Table 1. List of recovered isolates and their hypersensitive reaction.

S. No.	Provinces	No. of recovered isolates	Hypersensitivity reaction	
			HR^+	HR^-
1.	Punjab	62	52	10
2.	Sindh	16	10	6
3.	NWFP	45	43	2
4.	Balochistan	Nil	Nil	Nil
5.	Total	123	105	18

HR^+ = Showing hypersensitivity
HR^- =No hypersensitive

The causal bacterium was isolated from green leaves with yellow BLB lesion and not from torn and rotten tissue that are usually overgrown by microorganism. The bacterial exudates from fresh lesion are better isolation material as compared to infected tissue because of less contamination. This was also observed by Isaka (1970). Similarly, Di *et al.*, (1991) reported that recovery of *Xanthmonas oryzae* colonies from infected leaves sample is easy rather than infected seeds, due to the presence of other strains of bacteria and fungi in high population in seeds.

The media used for isolation were yeast extract dextrose calcium carbonate agar medium (YDCA), Wakimoto semi synthetic agar (WSSA) and nutrient agar (NA). The xanthomonad produced yellow, mucoid, dome shaped, shiny co lonies on YDC medium. The bacterium when grown on WSSA medium produced viscous, smooth, circular colonies. On NA (Nutrient agar) medium the bacterium produced light yellow, circular dome shaped colonies. The yellow color and mucoid colonies is cultural characteristics of Xanthomonads and is due to the production of extracellular polysaccharides slime (EPS in media containing sugar).

After, 48-72 incubation at 28°C, infected leaf samples when plated gave yellow, circular, smooth, convex and viscous bacterial colonies on yeast extract dextrose calcium carbonate agar medium (YDCA) (Figs. 2 & 3). In XoS medium the samples gave light yellow, mucoid, round and smooth bacterial colonies (1 mm in diameter) while whitish, mucoid and smooth colonies were observed on Wakimoto medium. One hundred and twenty three isolates of *Xanthmonas oryzae* were recovered on the basis of colony morphology for further studies.

Hypersensitivity reaction: Out of 123 recovered isolates of *Xanthomonas oryzae* pv. o*ryzae* only 105 isolates showed typical hypersensitive reaction on tobacco leaves (*Nicotiana rustica* (Fig. 4). After 24 to 48 hr of injection the injected leaf area became necrotic (Fig. 5) and in 3-4 days the treated tissue was entirely dry and yellow Out of 105 isolates only 17 showing high hypersensitive reaction (tissue completely dry and become yellow) were further selected for biochemical studies. (Table 2) The hypersensitivity reaction (HR) is a rapid method to detect pathogenicity of bacteria. It is also used to determine viability of strains. The test isolates showed typical hypersensitivity reaction on tobacco leaves inoculated by injection infiltration technique. The leaf tissue infiltrated with high concentration of virulent strains developed yellow discoloration necrosis after 48 hours, while avirulent strains did not exhibition-necrosis and delayed reaction behavior. This is confirmed through various studies Klement & Goodman 1967). The biochemical tests resulted in further screening of isolates and seven isolates (Xoo20, Xoo 36 Xoo51, Xoo65, Xoo75, Xoo99, Xoo105) were selected for pathogenicity studies.

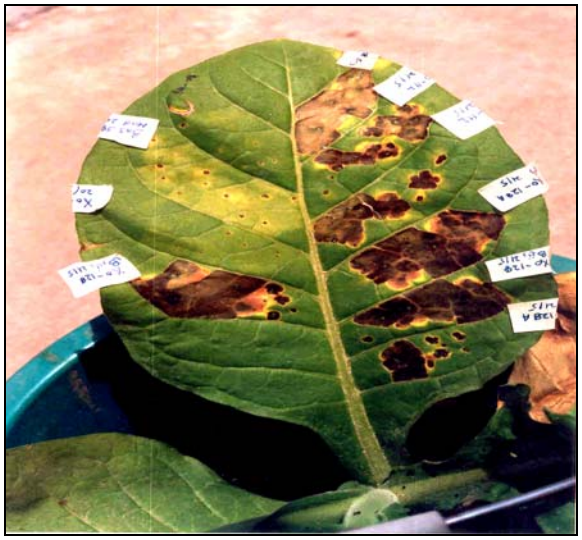

Fig. 4. Hypersensitive reaction on *Nicotiana rustica*.

Fig. 5. Necrotic area on *Nicotiana rustica*.

Table 2. Comparison of biochemical tests for the selected isolates of *Xanthomonas oryzae* pv. o*ryzae*.

S. No.	Selected Isolates	Bio-chemical tests						
		Gram reaction	KOH Test	Egg yolk reaction	Tetrazolium Tolerance Test		Starch Hydrolysis	Oxidases Test
					0.10 %	0.02 %		
1.	Xoo 6	-	+	+	+	-	-	-
2.	Xoo 7	-	+	+	-	-	+	-
3.	Xoo 20	-	+	-	+	+	+	-
4.	Xoo 36	-	+	-	+	+	+	-
5.	Xoo 51	-	+	-	+	+	+	-
6.	Xoo 60	-	+	+	+	+	-	-
7.	Xoo 65	-	+	-	+	+	+	-
8.	Xoo 69	-	+	-	-	+	+	-
9.	Xoo 72	-	-	+	-	-	+	-
10.	Xoo 74	-	+	-	-	-	+	-
11.	Xoo 75	-	+	-	+	+	+	-
12.	Xoo 76	-	+	-	+	-	-	-
13.	Xoo 98	-	+	-	+	-	-	-
14.	Xoo 99	-	+	-	+	+	-	-
15.	Xoo 100	-	+	-	+	-	-	-
16.	Xoo 102	-	-	+	-	-	+	-
17.	Xoo 105	-	+	-	+	+	+	-

Biochemical test: A total of 6 biochemical tests were conducted to characterize 17 of isolates of pathogen recovered from BLB infected samples collected from four provinces of Pakistan. Result of Gram staining demonstrated that pathogen was a Gram negative rod, producing red color when counter stained with safranin.

All 17 isolates consistently gave similar results (Table 2). KOH test was performed in order to confirm Gram staining results. The selected isolates showed positive reaction and confirmed that they were Gram negative. The egg yolk reaction was negative for the 7 isolates (Xoo 20, Xoo 36, Xoo51, Xoo 65, Xoo 75, Xoo 99 and Xoo 105). At 0.1 and 0.02% concentration of TTC these 7 isolates failed to produce colonies. In the oxidase test the 7 isolates proved negative since they failed to produce the desired color. In the starch hydrolysis above mentioned 7 isolates showed positive reaction. On the basis of biochemical tests only 7 out of 17 isolates were similar while others were variable, hence these were selected for pathogenicity studies.

Preservation: Best method for short term preservation *of Xanthmonas oryzae* isolates was peptone sucrose agar (PSA) medium store at 4^{o}C for week and for long-term preservation on sterile soil

Pathogenicity Test For *Xanthomonas Oryzae* Isolates: Analysis showed that out of 8 tested varieties IRRI 6 was significantly resistant whereas the varieties Basmati 385, Basmati 386, Jp5, Super Basmati & Basmati 2000 were significantly susceptible and Dilroosh 97 and Ks 282 were moderately resistant .The pathogenicity test was used to characterize plant pathogenic bacteria (Khan and Hingorani 1970). In the present study the pathogenicity test was carried out on a series of 7 HR positive isolates (Xoo 20,xoo 36, Xoo 51, Xoo 65, Xoo 75, Xoo 99, Xoo 105) of *Xanthomonas oryzae* pv. *oryzae*. The isolates were selected on the basis of strong hypersensitivity reaction and colony morphology (Table 3).

Table 3. The Measurment of bacterial blight lesion length (cm) on Potted Plants of 8 varieties of rice.

S. No.	Rice varieties	*Xanthomonas oryzae* isolates						
		X_{00} 20	X_{00} 36	X_{00} 51	X_{00} 65	X_{00} 75	X_{00} 99	X_{00} 105
1.	Bas 385	S	S	S	S	S	S	S
2.	IRRI 6	S	S	R	S	S	R	S
3.	Bas 386	S	S	S	S	S	S	S
4.	Dilroosh 97	MR	MR	S	S	R	MR	S
5.	JP 5	S	S	S	S	S	S	S
6.	KS 282	S	MR	S	S	S	MR	MR
7.	Super Bas	S	S	S	S	S	S	S
8.	Bas 200	S	S	S	S	S	S	S

S: Susceptible, R: Resistance, MR: Moderately resistance

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