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SOME BIOCHEMICAL CHARACTERIZATION AGAINST *XANTHOMONAS ORYZAE* PV. *ORYZAE* CAUSE BACTERIAL LEAF BLIGHT OF RICE

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ABSTRACT

In current studies enzymes production and antibiotics sensitivity of some strains of *Xanthomonas oryzae* pv. *oryzae* tested in *in-vitro* conditions. Results of biochemical tests like catalase, amylase (starch hydrolysis) and growth on Yeast Dextrose Calcium Carbonate Agar (YDCA) varied among the isolates. The results regarding catalytic and amylase activity showed that out of 19 isolates, 18 isolates (95%) were Catalase positive and 1 isolate (5%) Catalase negative. 16 strains (84%) were amylase positive and 4 strains (21%) were amylase negative. SHB-5, SHB-12, SHB-116 showed maximum inhibition zone against streptomycin where as SHB-18, SHB-19 showed minimum inhibition.

Keywords: *Xanthomonas oryzae* pv. *oryzae*, biochemical tests, rice *(Oryza sativa),* Bacterial leaf blight (BLB) disease, Sindh

INTRODUCTION

Xanthomonas oryzae pv. *oryzae* (*Xoo*), belongs to c-subdivision of the Proteobacteria. *Xoo*, a major bacterial pathogen that cause bacterial leaf blight disease (BLBD) of rice crop (*Oryza sativa* L.) (Ishiyama, 1922; Swings *et al*., 1990). The BLBD disease is affecting crop throughout world in climatic conditions of irrigated, deep water, temperate, rain fed and tropical rice areas (Mew, 1987). In past, severe epidemics of the disease have been reported such as during 1884-5 the disease caused 25-30% in Japan, 50-60 in India, Indonesia, and 10-81 in Philippine (Ahmad and Singh, 1975). However, In Pakistan, the disease was first time observed in 1977 at Rice research Institute, Kala Shah Kaku on rice varieties Palman, Basmati 198 and IRRI-6 (Mew and Majid, 1977; Ahmad and Majid, 1980). Later the disease has spread to other major rice growing provinces like Khayber Phukhtoon Khaw (KPK), Punjab and Sindh (Akhtar and Sarwar, 1986; Akhtar and Akram, 1987). The epidemics of this disease was reported in 1997, 2002, 2006, 2007 and 2008 due the wide spread of susceptible variety for cultivation (Khan *et al*., 2009). Since last few years, the incidence and severity of BLB in Sindh province has been increasing (Akhtar *et al.*, 2003, Bhutto *et al.*, 2018). If untreated seeds grown in the field then seed-borne pathogen reduce the crop yield up to15-90% (Zafar et al., 2014: Bhutto *et al.*, 2018: Jatoi. *et al.*, 2016b). The bacterium enters through hydathoodes or by wounds on the roots or leaves and its infection and symptoms are observed at tillering stage which depend on two phases leaf blight and kresek phase (Ou, 1985; Akhtar *et al*., 2008). The disease is recognized with appearance of yellow lesions and leaf blade shows wavy margins which later on extend to leaf sheath; then the lesions turn whitish straw in color followed by the ooze of the bacterial from infected leaves (Mew, 1987; Jatoi *et al*., 2016a). All commercial varieties have shown highly susceptibility to the disease (Cheema *et al*., 1998; Khan *et al*., 2000, Akhtar *et al*., 2008; Ali *et al*., 2009).

Therefore, in this study efforts are taken for biochemical characterization of *Xoo* and their response against antibiotic.

MATERIALS AND METHODS

Collection of diseased samples: Before collection of the samples, a survey was conducted to collect information about the diseased crops in farmer fields at Hyderabad, Tando Muhammad Khan, Thatta and Badin District of Sindh Province. The diseased leaves samples were collected from different localities after every 10-20 kilometers. Bacterial blight was identified on the basis of visual observation of 10 leaves of infected plants selected randomly from rice field.

Isolation and Purification of BLB pathogen: The infected rice leaf (28×7 mm) was cut into pieces with a sterile scalpel. Ethanol at concentration of 75% was used to sterilize the leaf pieces for three minutes and then washed with sterilized distilled water. Six to seven infected leaf pieces were transferred to nutrient agar medium and incubated at 25±2 °C for 72 hours. The cultures were purified by streaking method and were preserved in Sterilized Distilled Water (SDW) and silica gel as reported by (Wilson *et al*., 1993).

Biochemical characterization: The biochemical characterization of all the isolates was carried out as per the procedure outlined in Maheshwari and Dubey (2006). The tests performed are detailed below.

Gram staining: Bacterial smear was prepared in a drop of water on clean glass slide and heat fixed by gently over the flame on the sprite Lamp. One to two-drop of crystal violate was applied over the smear and left for 1 min and then washed with sterile distilled water. Then few drops of Gram's iodine solution were applied for 1 min and then washed with 95% alcohol. Finally, safranin was applied for 30 seconds and smear was washed with sterile distilled water. After the slide air dried and examined under light microscope under oil immersion lens by using one drop of immersion oil. The Gram positive bacterial cells appeared violet while gram negative bacteria turned pink to red (Vincent, 1970).

Potassium hydroxide (KOH) test: Potassium hydroxide test was conducted to confirm the results obtained during gram staining test with procedure described by Suslow *et al*., 1982. For this purpose, the fresh bacterial growth was harvested from an agar plate with help of tooth pick and placed on glass slide in a drop of 3% KOH solution. Then the growth was stirred into KOH for 10 seconds maximum of 1 minute using a quick circular motion of hand (Ryu, 1940). Slowly lifting the loop frequently raise the loop 1 cm off surface to test if the mixture is becoming viscous and has the Gram negative: Mixture becomes viscous and "strings out". Gram positive: After 1 minute the mixture is not viscous and does not string out.

Catalase test: Bacterial smear was placed on a microscopic slide containing a drop of hydrogen peroxide to perform this test. Growths were categorized as Catalase-positive or negative either bubbles or froth were produced or not.

Amylase test (starch hydrolysis): Starch hydrolysis was tested for isolated strains to determine the capability of using carbon source like starch as described by De Oliverira (2007). The medium was inoculated with bacterial strains in signal Petri plate four bacteria were steak with help of lop and incubated at 25±2°C for 172 hours. After full growth of the deferent bacterial strains the drop of the Iodine was applied on the surface and again incubated 25±2°C for 24 hours. A color change i.e blue color formation indicates that the bacteria negative of starch and other not formation in blue color were recorded as positive.

Growth on (Y D C A) media: The yeast extract granulates 5g, Dextrose 10g, Calcium carbonate 10g and Ager technical 7g, these ingredients mix with each other and autoclave at 120 ^{0}C for 15 minutes. Isolated strains were steak in Petri plate, in signal plate four bacteria were steak different bacterial lines and incubated at 25±2 °C for 172 hours. The strains which were grown on media it means positive and which not grow on (Y D C A) media it means negative.

Antibiotic sensitivity assay: Antibiotic sensitivity testing was conducted using the cup assay method. Petri plates containing sterilized nutriaent ager media were inoculated with bacterial suspension through spread plate method. Six holes were prepared in plates containing Nutrient Agar medium with the help of cup borer. 10 ul of Streptomycin (100 ppm) was poured on each cups of pre-inoculated plates. All the plates were incubated at 28±2 °C for up to 8 days. The inhibition zone around each cup was recorded in mm with the help of scale. Plates without any amendment of antibiotic were kept as control

RESULTS

Identification of bacterial isolates: Identification was done using manual and books. Bacterial strains isolated were tentatively identified as belong to genus *Xanthomonas oryzae* pv. *oryzae* (Table. 1)

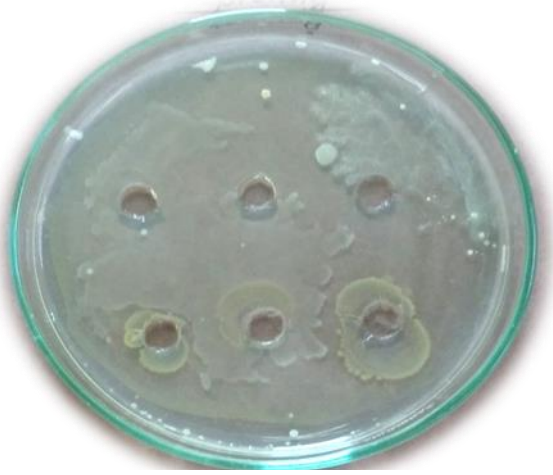
Biochemical characterization: Gram staining: On the basis of Gram reaction out of 19 strains, most of strains such as SHB-1, SHB-3, SHB-4, SHB-5, SHB-6, SHB-7, SHB-8, SHB-9, SHB-11, SHB-12, SHB-13, SHB-14, SHB-15, SHB-17, were found gram negative. Whereas other 5 remaining strains SHB-2, SHB-10, SHB-16, SHB-18, and SHB-19, were Gram negative. This shows that there is 73% contribution of Gram negative to total collected strains and

remaining contribution of 26% was of Gram positive strains.

Catalytic and amylase activity: The results regarding catalytic and amylase activity showed that out of 19 isolates, 18 isolates (94%) were Catalase positive and 1 isolate (6%) Catalase negative, whereas 16 (84%) and 4 strains (21%) were amylase positive and negative, respectively.

Growth on (Y D C A): Among 19 strains, 14 strains i. e SHB-1, SHB-2, SHB-3, SHB-6, SHB-7, SHB-9, SHB-10, SHB-11, SHB-12, SHB-13, SHB-14, SHB-15, SHB-18 and SHB-19, were positive on Y D C A, while remaining 5 strain such as SHB-4, SHB-5, SHB-8, SHB-16, SHB-17, were negative on Y D C A. This result showed YDCA positive were *Xoo* strains (Table. 2).

Antibiotic sensitivity assay: All the tested strains of *Xoo* showed different level of reaction against Streptomycin. None of them was found to show complete resistance at 100 ppm up to 8 days interval. Moreover, Streptomycin response against tested strains resulted formation of maximum inhibition zone i.e 8,9 and10 mm after eight days of incubation against strains SHB5, SHB-12, SHB-16 and minimum inhibition zone i.e 3 mm against SHB-18 and SHB-19 (Table. 3).



Zone Produced by Streptomycin

Catalase Tested Slide

Table. 1Outline of bacterial isolates *Xanthomonas* spp.

Strain Name	Host	Origin	Tentative identification
S.H.B-1	Rice plant	Hyderabad	*Xanthomonas*
S.H.B-2	Rice	Hyderabad	N.D
S.H.B-3	Rice	Hyderabad	*Xanthomonas*
S.H.B-4	Rice	Tando-M.Khan	*Xanthomonas*
S.H.B-5	Rice	Thatta	*Xanthomonas*
S.H.B-6	Rice	Thatta	*Xanthomonas*
S.H.B-7	Rice	Thatta	*Xanthomonas*
S.H.B-8	Rice	Thatta	*Xanthomonas*
S.H.B-9	Rice	Badin	*Xanthomonas*
S.H.B-10	Rice	Badin	N.D
S.H.B-11	Rice	Badin	*Xanthomonas*
S.H.B-12	Rice	Tando-M.Khan	*Xanthomonas*
S.H.B-13	Rice	Tando-M.Khan	*Xanthomonas*
S.H.B-14	Rice	Tando-M.Khan	*Xanthomonas*
S.H.B-15	Rice	Badin	*Xanthomonas*
S.H.B-16	Rice	Badin	N.D
S.H.B-17	Rice	Badin	*Xanthomonas*
S.H.B-18	Rice	Badin	N.D
S.H.B-19	Rice	Hyderabad	N.D

Table. 2 Biochemical characterization of bacterial blight strains

Strains	Gram reaction	Catalase (Test)	Amylase Test (Starch Hydrolysis)	Y D CA (Test)
S.H.B-1	-	+	+	+
S.H.B-2	+	+	+	+
S.H.B-3	-	+	+	+
S.H.B-4	-	+	-	-
S.H.B-5	-	+	+	-
S.H.B-6	+	+	+	+
S.H.B-7	+	+	-	+
S.H.B-8	+	+	+	-
S.H.B-9	-	+	+	+
S.H.B-10	+	+	-	+
S.H.B-11	-	+	+	+
S.H.B-12	-	+	+	+
S.H.B-13	-	+	+	+
S.H.B-14	-	-	-	+
S.H.B-15	-	+	+	+
S.H.B-16	+	+	+	-
S.H.B-17	-	+	+	-
S.H.B-18	-	+	+	+
S.H.B-19	+	+	+	+

Table. 3 Response of Bacterial isolates against streptomycin

Strains	Zone Diameter Produced by Streptomycin at Different Days					
	3 DAYS		5 DAYS		8 DAYS	
S.H.B-1	2 mm	0.0±	3 mm	0.0±	6 mm	2.0±
S.H.B-2	0 mm	0.0±	2 mm	1.0±	4 mm	0.0±
S.H.B-3	2 mm	0.0±	4 mm	1.0±	5 mm	0.0±
S.H.B-4	3 mm	1.0±	4 mm	0.0±	4 mm	0.0±
S.H.B-5	4 mm	0.0±	7 mm	3.0±	10 mm	0.0±
S.H.B-6	2 mm	0.0±	2 mm	0.0±	3 mm	0.0±
S.H.B-7	4 mm	1.0±	5 mm	0.0±	7 mm	0.6±
S.H.`B-8	2 mm	0.0±	3 mm	0.0±	5 mm	2.0±
S.H.B-9	3 mm	1.0±	4 mm	0.0±	5 mm	1.0±
S.H.B-10	2 mm	0.0±	4 mm	1.0±	5 mm	0.0±
S.H.B-11	1 mm	0.0±	3mm	2.0±	5 mm	0.0±
S.H.B-12	3 mm	1.0±	6 mm	2.0±	9 mm	1.0±
S.H.B-13	2 mm	0.0±	3 mm	1.73±	4 mm	0.00±
S.H.B-14	3 mm	1.0±	5 mm	1.15±	6 mm	0.00±
S.H.B-15	4 mm	0.0±	4 mm	1.15±	6 mm	1.00±
S.H.B-16	2 mm	0.0±	5 mm	1.15±	8 mm	2.00±
S.H.B-17	3 mm	1.0±	4 mm	1.15±	5 mm	0.00±
S.H.B-18	0 mm	0.0±	2 mm	2.31±	3 mm	0.00±
S.H.B-19	0 mm	0.0±	2 mm	2.31±	3 mm	0.00±

DISCUSSION

Bacterial leaf blight has been found very destructive disease in rice growing regions of world including Pakistan. Its frequency is increasing in Punjab as well as in Sindh province from last 3 year. To tackle the disease, current studies resulted with following findings:

All bacterial isolates in present studies were identified as *Xanthomonas oryzae* pv *oryzae*. Our procedure of identification is reliable and coincides with the results of (Muneer *et al.*, 2007). Rafi *et al*., (2013) and Joint (2016) reported formation of light yellow, mucoid, circular dome shaped and smooth colonies on nutrient Agar. The yellow color and mucoid colonies is cultural characteristics of *Xanthomonads* that was due to the production of extracellular polysaccharides (EPS in media containing sugar). More over they stated that *Xanlhomonads* are reported to produce colonies that are yellow,

mucoid, convex, and texture in shiny. Such colonies were consistently observed and tentatively identified of as *Xanthomonas oryzae* pv. *oryzae.* These bacterial strains also need identification on molecular level for authentication.

14 strains were identified as gram positive and 5 strains as gram negative. All strains except one were found catalase negative. This is also clue for identification of *Xanthomonas oryzae pv oryzae* (*Xoo)*. Joint (2016) also considered all his catalase positive strains as *Xoo* and discarded catalase negative from his studies. In our study different results were recorded for other biochemical tests like amylase and YDCA. Starch hydrolysis positive results are found at incubition period of a week, but it is not common feature of *Xoo* (Swings *et al.,* 1990). Similarly, we found that some strains of *Xoo* are starch hydrolysis positive and other negative.

Streptomycin at concentration of 100 ppm was used to observe the sensitivity of *Xoo* strains and was found to form zones of inhibitions up to 8 days of incubation. Similarly, Zhang *et al*., (2011) found growth of 11 *Xoo* isolates grew well in Streptomycin amended medium at 100 µg ml^{-1}. Khan *et al*., (2012) evaluated efficacy of different antibiotics for controlling *Xoo*. They concluded that, Chloramphenicol, Amplicillin and Dihydrostreptomycin were most effective for controlling *Xoo* (Fitt *et al.,* 1992). The present study showed that 100 ppm concentration was found to be the best among all treated concentration for the controlling of *Xanthomonas Oryzae* pv. *Oryzae*.

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