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Full Length Research Paper

Isolation and biochemical characterizations of the bacteria (*Acidovorax avenae* subsp. *avenae*) associated with red stripe disease of sugarcane

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Studies on *Acidovorax avenae* subsp. *avenae,* associated with red stripe disease of sugarcane was conducted in the Department of Plant Pathology, Pir Mehr Ali Shah Arid Agriculture University Rawalpindi during 2009 to 2010, in collaboration with Shakarganj Sugar Research Institute (SSRI), Jhang, Pakistan. Red stripe of sugarcane were recently observed on promising clones of sugarcane planted in autumn 2009 at Ashaba Research Farm of SSRI. Bacteria were isolated from diseased plants. These isolates yielded off white convex colonies on potato dextrose agar (PDA) media at 29°C with 1.7 to 1.9 mm diameter and were yellow on yeast extract dextrose chalk agar (YDC) media at 27°C with 1.8 to 2.0 mm diameter. The bacteria were rod shape measuring 0.5 to 0.6 × 1.4 to 1.6 µm on PDA and 0.6 to 0.7 × 1.5 to 1.7 µm on YDC. Bacterial culture was stored at different temperature levels for 150 days. Re-isolation of bacterial culture which was stored at 4°C showed best result on YDC at 27°C after 150 days, whereas it showed positive result after 120 days on PDA at 29°C. Bacteria were gram negative, citrate utilization was positive, oxidase was negative, catalase was positive and urease was negative. Morphological appearance and biochemical characterizations identified the bacteria as *A. avenae* subsp*. Avenae. In vitro* screening for the efficacy of various antibiotics to inhibit the growth of *A. avenae* subsp*. avenae* on YDC media showed that ampicillin and vancomycin were most effective. Artificial inoculation on sugarcane against red stripe disease was observed. Observations were made upto six weeks for disease development. Out of 27 varieties, 16 were found resistant, four moderately resistant, five moderately susceptible and two susceptible.

Key words: Sugarcane, yeast extract dextrose chalk agar (YDC), potato dextrose agar (PDA), *Acidovorax avenae* subsp*. avenae*, biochemical characterization, antibiotics.

INTRODUCTION

Sugarcane (*Saccharum officinarum* L.) is a crop of great agro-economic importance. It provides sugar, bio fuel, fiber, organic fertilizer and many by-products/co-products with ecological sustainability. Sugarcane growing countries of the world are lying between the latitude 36°N and 31°S of the equator extending from tropical to subtropical zones. Sugarcane is the second largest cash crop of Pakistan and it is planted on an area of 1.029 million hectares with a total annual cane production of 50.00 million tones. Sugarcane currently accounts for 0.7% of cropped area and 3.4% value added of the total crops (Anonymous, 2010).

In Pakistan, yield of sugarcane has declined drama-

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tically with the influence of different factors like salinity, irrigation, weed control, fertilizer, insect pests, fungal, viral and bacterial diseases. Amongst these, red stripe disease, incited by *Acidovorax avenae* subsp. *avenae* (Lee et al., 1925) has been one of the most serious and devastating diseases in Punjab province of Pakistan.

A. avenae subsp. *avenae* is the causal agent of several important plant diseases including bacterial stripe of rice (Kadota, 1996), bacterial stalk rot of corn (Summer and Schaad, 1977), bacterial leaf blight of oats (Manns, 1909) and red stripe of sugarcane (Martin and Wismer, 1989). Bacterium is characterized by narrow watery green stripes on the leaves which soon develop into sharply defined red stripe. Stripes normally start at the base of the leaves and vary in length and width. Infection may get into the apical growing point and kill the stalk. It is a widely distributed disease and has been reported from 50 sugarcane growing countries in the world (Agnihorti, 1983).

Recently, a high incidence of red stripe was observed in the promising clones at Shakarganj sugar research institute (SSRI), Jhang Pakistan. As a result, many clones had to be dropped from the breeding programme. Considering the wide spread of the disease in Pakistan, it was felt necessary to study the reaction of promising clones to red stripe through artificial inoculation with the bacterium *A. avenae* subsp. *avenae*. This manuscript records the first occurrence of red strip disease on sugarcane crop in Punjab province of Pakistan. In addition, the research paper covered the data on identification of causal organism and evaluation of resistance against the bacterium in commercial and promising sugarcane cultivars.

MATERIALS AND METHODS

Isolation and growth attribute of pathogen

Studies on *A. avenae* subsp. *avenae,* associated with red stripe of sugarcane were conducted during 2009 to 2010 in the Department of Plant Pathology, Pir Mehr Ali Shah Arid Agriculture University, Rawalpindi, with the collaboration of Shakarganj Sugar Research Institute, Jhang-Pakistan. The newly red stripe infected leaves were sterilized by dipping in 0.1% calcium hypochlorite solution and washed several times by sterilized distilled water. For isolation of the bacterium, infected leaves were plated on Potato dextrose agar (PDA) as described by Iqbal et al. (1995) and yeast extracts dextrose chalk agar plates (YDC) by following the procedure of Dye (1968). Isolates were incubated at 25, 27, 29, 35 and 40°C for the study of temperature and time for high recovery and survival rate of the bacteria on both medium. Single colony of bacteria was sub cultured on PDA and YDC medium by streaked method to obtain pure culture and appraisal between medium for colony appearance, colour, colony diameter and size of bacteria (µm) (Dye and Kemp, 1977; Wilkie and Dye, 1974). Growth at 4 and 41°C was determined by incubating cultures on YDC and PDA for maximum of one month. Plates were examined weekly and scored positive when single isolated colonies were observed. Plates were incubated at 41°C and wrapped in parafilm and placed in a moisture chamber to prevent media from dehydration (Ramundo and Claflin, 1990). Bacteria were stored for four to five months at each temperature level including 04, 08 and 10°C. Reduction of metabolic rate, high recovery maintenance and survival rate of the bacteria were observed at these temperature levels (Goszczynska et al., 2000). Bacteria were re-isolated from preserved agar plates on both medium at specific incubation temperature for high recovery of the bacterial colonies.

Characterizations of the pathogen

A series of tests were performed to characterize the *A. avenae* subsp. *avenae* on the basis of physiological and biochemical properties. Gram staining reaction was observed by applying dye crystal violet (0.5% aq., w/v) for 1 min, Lugol's iodine (containing 2 g potassium iodine and 1 g iodine in 25 ml distilled water) for 1 min, decolorizer (ethanol) for 30 s and the counter stain safranine for 10 s on fixed smear of bacterial growth on clean glass slide. Arginine dihydrolysis activity was determined by stabbing a culture into Thornley's medium (Thornley, 1960). Acid production from carbohydrate (glucose, lactose, sucrose, mannitol and maltose) and carbon sources (sucrose and glucose) was tested using the oxidation/fermentation (O/F) basal medium (Hugh and Leifson, 1953). Acid production was scored positive on colour change from green to yellow throughout the length of the tube. Bacteria for oxidase reaction, aesculin hydrolysis, urease production, gelatin utilization, H_2S production, nitrate reduction, citrate utilization, catalase activity, fluores pigment on KB media, indole production, starch hydrolysis and Tween 80 hydrolysis were obtained according to the manual of Goszczynska et al. (2000).

Resistance to antibiotics

The resistance of bacterium *A. avenae* subsp. *avenae* to antibiotics and inhibition of other saprophytic bacteria from sugarcane leaf samples was tested. Four antibiotics (ampicillin, novobiocin, penicillin and vancomycin) with the concentration of 25, 50 and 75 µg/ml were used by using paper disk method (Song et al., 2000). The strain was cultured in YDC broth for 24 h and the concentration of bacterial suspension was adjusted to 5×10^7 cfu/ml. The bacterial suspension was sprayed onto the surface of the medium. After 20 min, 0.1 ml absorbable paper disc was dipped in each antibiotics solution with different concentration and the disc was placed on the agar with sterile forceps and gently pressed down to ensure contact. The plates were incubated at 27°C for overnight. Growth of *A. avenae* subsp. *avenae* and reduction of saprophytic bacteria was observed (Song et al., 2000).

Artificial inoculation

27 commercial and promising cultivars were tested for resistance to *A. avenae* subsp. *avenae* during 2010 at Shakarganj Sugar Research Institute (SSRI) research farm Ashaba. The bacterium suspension (5×10^7 cfu/ml) was prepared from freshly grown pure culture of *A. avenae* subsp. *avenae*. Inoculation was made by introducing the inoculum into the growing points with a plastic syringe (Iqbal et al., 1995; Patil, 2004). 14 weeks old plants from each clone were selected for inoculation out of which, 25 plants were inoculated with bacterial suspension and 25 with sterile distilled water as the control group. Observations for the development of red stripe were started at an interval of 7 days after inoculations and continued upto 6 weeks. Red stripe infected plants were counted out of the total inoculated plants. Percentage of infected plants was also calculated. During study period, average maximum, minimum temperature, relative humidity and rainfall remained 40 ± 2°C, 26 ± 2°C, 78% and 65 mm, respectively. Bacterial red stripe symptoms were evaluated on 0 to 5 disease

Table 1. Bacterial red stripe symptoms.

Rating scale	% infection	Grade
0	No visible symptoms	
1	0-10	Resistant
2	11-30	Moderately resistant
3	31-50	Moderately susceptible
4	51-70	Susceptible
5	71 and above	Highly susceptible

Table 2. Growth attribute on different medium.

Attribute	Medium			
	PDA		YDC	
Colony appearance	Convex		Convex	
Colony colour	Off white		Yellow	
Size of bacteria	0.5-0.6 × 1.4-1.6 µm		0.6-0.7 × 1.5-1.7 µm	
Growth at 04°C	-		-	
Growth at 41°C	+		+	
Growth Temperature/72 h	**Appearance**	**Diameter (mm)**	**Appearance**	**Diameter (mm)**
25°C ±	Shy	0.5-0.7	Good	1.0-1.5
27°C ±	Good	1.0-1.5	Excellent	1.8-2.0
29°C ±	Excellent	1.7-1.9	Excellent	0.9-1.5
35°C ±	Good	1.2-1.7	Good	1.3-1.5
40°C ±	Shy	0.6-0.9	Satisfactory	0.8-1.0

Storage						
	Temperature (°C)	**Days**	**Re-isolate at 29°C**	**Temperature (°C)**	**Days**	**Re-isolate at 27°C**
		90	+		90	+
	04	120	+	04	120	+
		150	-		150	+
		90	+		90	+
	08	120	+	08	120	+
		150	Shy		150	+
		90	+		90	+
	10	120	+	10	120	+
		150	-		150	Shy

rating scale as can be seen in Table 1.

RESULTS AND DISCUSSION

Isolation and growth on different media at different temperature levels

Isolation and growth attribute of *A. avenae* subsp. *avenae* was analysed on PDA and YDC media at different temperature levels (Table 2). Bacterium showed same colonial appearance (convex) with different colors on both media. On PDA medium, bacterial colony was off white coloured with 0.5 to 0.6 × 1.4 to 1.6 µm bacterium size, while on YDC, it showed yellow appearance with 0.6 to 0.7 × 1.5 to 1.7 µm size. There was no growth at 4°C, but quick growth was observed at 41°C on both media. Red stripe associated bacteria has already been shown (Ramundo and Claflin, 1990; Hseu et al., 2008).

At different temperature levels, bacterial growth pattern was also different on both media after 72 h. Bacterium diameter was also affected at different temperature levels. On PDA media, *A. avenae* subsp. *avenae* showed excellent growth with 1.7 to 1.9 mm diameter at 29°C but on YDC media, it showed first-rate growth with 1.8 to 2.0 mm and 0.9 to 1.5 mm diameter at 27 and 29°C,

Table 3. Biochemical characteristics of *A. avenae* subsp. *avenae* from red stripe of sugarcane.

Test	Stain from sugarcane
Gram positive	-
Acid produced from	
Glucose	+
Lactose	+
Sucrose	+
Mannitol	+
Maltose	+
Fluores pigment on KB	-
Oxidase reaction	-
Aesculin hydrolysis	-
H_2S production	+
Tween 80 hydrolysis	+
Arginine dihydrolysis	-
Nitrate reduction	+
Urease production	-
Catalase activity	+
Citrate utilization	+
Gelatin utilization	-
Indole	-
Starch hydrolysis	s
Carbon sources utilized for growth	
Sucrose	-
Glucose	+

Indicator:(+) Positive reaction; (-) negative reaction; (s) slight reaction.

respectively while on 25, 35 and 40°C, bacterial growth was less with small diameter. It was also reduced on PDA media at 27°C. Growth temperature of red stripe similar to those was reported by Hayward (1962).

Re-isolation from stored bacterial culture was also observed. Bacteria were stored for 90, 120 and 150 days at each temperature level including 04, 08 and 10°C. Re-isolated culture of *A. avenae* subsp. *avenae* on PDA and YDC media were observed at 29 and 27°C, respectively. On YDC media, it showed satisfactory growth from stored culture, but the stored bacterium on PDA at 4 and 10°C for 90 and 120 days showed positive re-isolation result, but no growth was observed after 150 days storage. Although at 8°C after 150 days storage, only shyness appeared and after 90 and 120 days storage it showed positive re-isolation result.

Phenotypic characterization

Isolate of *A. avenae* subsp. *avenae* was tested for some confirmatory physiological and biochemical tests (Table 3). The isolate was tested for gram reaction. It showed a negative gram stain. Acid production from carbohydrate (glucose, lactose, sucrose, mannitol and maltose) and carbon source like glucose was positive while acid was not produced in the case of sucrose. Bacteria showed negative result in fluores pigment on KB media, oxidase reaction, aesculin hydrolysis, gelatin utilization, urease production, arginine dihydrolysis activity and Indole production and positive result was observed in H_2S production, Tween 80 hydrolysis, nitrate reduction, catalase activity and citrate utilization but in the case of starch hydrolysis, slight reaction was observed. These results are also in accordance with Goszczynska et al. (2000), Martin and Wismer (1989), Alvarez (2004), Hseu et al. (2008), Harighi (2007) and Ramundo and Claflin (1990).

Resistance to antibiotics when compared with other saprophytic bacteria

With different concentration of antibiotics, the growth of *A. avenae* subsp. *avenae* and reduction of saprophytic

Table 4. Growth of *A. avenae* subsp. *avenae (Aaa)* on YDC medium with different concentration of antibiotics (µg/ml).

Antibiotic	Concentration of antibiotic	Strain	
		Acidovorax avenae* subsp. *avenae	**Other bacteria**
Ampicillin	25	+	+
	50	+	a
	75	+	-
Novobiocin	25	+	+
	50	-	+
	75	-	+
PenicillinG	25	a	+
	50	-	+
	75	-	-
Vancomycin	25	+	-
	50	a	-
	75	-	-

Indicator: (+) Growth; (a) slight shyness; (-) no growth.

bacteria on YDC medium was also observed (Table 4). The antibiotics ampicillin, novobiocin, penicillinG and vancomycin were used at 25, 50 and 75 µg/ml by using paper disk method (Song et al., 2000). The bacterium showed best results with ampicillin and vancomycin at 75 and 25 µg/ml, respectively when compared with other saprophytic bacteria.

Pathogenic infection on different clones after artificial inoculation

Table 5 represents that all clones except NSG-555, CPF-237, NSG-59 and CPSG-25 showed red stripe infection from the 1st to 6th weeks. In some varieties, red stripe infection remained constant from the 1st to 6th weeks, whereas in others, this infection gradually increased. Variety HoSG-315 showed 33% infection in the 1st and 2nd week and 36% from the 3rd to 6th week. CSSG-2402 showed 52% infection in the 1st week, 53% in the 2nd, 3rd and 4th week and 55% in the 5th and 6th week. Infection appeared in variety CPSG-437; 33% in the 1st week, 34% in the 2nd and 3rd week and 36% in the 4th, 5th and 6th week. Variety SPF-238 showed 13% in the 1st week, 16% from the 2nd to 4th week and 17% in the 5th and 6th week. The clone CP77-400 showed 14% red stripe infection in the 1st week, 17% in the 2nd and 3rd week and 19% from the 4th to 6th week. 4% infection was observed from the 1st to 6th weeks in the clone SPSG-79. The clone HSF-240 showed 5% infection from the 1st to 3rd week and 7% from the 4th to 6th week. In the case of SPF-213 clone, the bacterial infection was observed as 12% from the 1st to 6th weeks. Clone CSSG-668 showed 4% infection from the 1st to 3rd week and 5% from the 4th to 6th week. The clone CSSG-676 showed 3% infection in the 1st and 2nd week, 7% from the 3rd to 5th week and 8% in the 6th week. Infection appeared in NSG-311 as 4% from the 1st to 3rd week and 5% from the 4th to 6th week. The variety GT-11 demonstrated 13% bacterial infection in the 1st and 2nd week, 16% from the 3rd to 5th week and 17% in the 6th week. The clone CPSG-3481 showed infection of 3% in the 1st week, 5% in the 2nd and 3rd week and 7% from the 4th to 6th week. The variety CPSG-2923 showed 3% infection throughout the observations. The variety CSSG-212 showed less infection; 1% in the 1st week which increased up to 3% in the 2nd and 3rd week and 5% from the 4th to 6th week. The clone CPSG-104 showed 3% infection in the 1st week, 5% in the 2nd and 3rd week, 7% infection in the 4th, 5th and 6th week. 4 % infection was observed in the first three weeks which increased up to 8% in the 4th and 5th week and 9% in the 6th week in HoSG-1257 clone. The variety CSSG-239 demonstrated 4% bacterial infection in the first 2 weeks, 5% in the 3rd and 4th week and 7% in the 5th and 6th week. In the first two weeks, red stripe infection was observed as 4 and 5% from the 3rd to 6th week in CPSG-2713 variety. The clone NSG-49 showed 33% infection in the 1st week, 35% in 2nd to 5th week and 36% in the 6th week. The clone CPSG-2453 showed 32% infection throughout the observation. The clone US-114 showed as high as 53% infection in the 1st week and as high as 57% infection in the remaining weeks. Clone CP-NIA-82-223 showed 34% infection in the 1st 2 weeks, 35% in the next 2 weeks and 36% infection in the last 2 weeks. Similar observations

Table 5. Varieties showing red stripe infection.

Variety	% infection					
	1st week	2ndweek	3rd week	4th week	5th week	6th week
HoSG-315	33	33	36	36	36	36
CSSG-2402	52	53	53	53	55	55
CPSG-437	33	34	34	36	36	36
NSG-555	0	0	0	0	0	0
SPF-238	13	16	16	16	17	17
CP77-400	14	17	17	19	19	19
SPSG-79	4	4	4	4	4	4
CPF-237	0	0	0	0	0	0
HSF-240	5	5	5	7	7	7
SPF-213	12	12	12	12	12	12
CSSG-668	4	4	4	5	5	5
CSSG-676	3	3	7	7	7	8
NSG-311	4	4	4	5	5	5
GT-11	13	13	16	16	16	17
CPSG-3481	3	5	5	7	7	7
NSG-59	0	0	0	0	0	0
CPSG-2923	3	3	3	3	3	3
CSSG-212	1	3	3	5	5	5
CPSG-104	3	5	5	7	7	7
HoSG-1257	4	4	4	8	8	9
CPSG-25	0	0	0	0	0	0
CSSG-239	4	4	5	5	7	7
CPSG-2713	4	4	5	5	5	5
NSG-49	33	35	35	35	35	36
CPSG-2453	32	32	32	32	32	32
US-114	53	57	57	57	57	57
CP-NIA-82-223	34	34	35	35	36	36

Table 6. Reaction of varieties to red stripe disease.

Variety	% infection	Grade	Number of variety
NSG-555, SPSG- 79, CPF-237, HSF-240, CSSG-668, CSSG-676, NSG-311, CPSG-3481, NSG-59, CPSG-2923, CSSG-212, CPSG-104, HoSG-1257, CPSG-25, CSSG-239, CPSG-2713	0-10	Resistant	16
SPF-238, CP77-400, SPF-213, GT-11	11-30	Moderately resistant	4
HoSG-315, CPSG-437, NSG-49, CPSG-2453, CP-NIA-82-223	31-50	Moderately susceptible	5
CSSG-2402, US-114	51-70	Susceptible	2
-----	71 and above	Highly susceptible	0

were reported by Christopher and Edgerton (1930), Klement (1968) and Dange and Payak (1973). Table 6

shows that out of 27 varieties, 16 were found resistant, four moderately resistant, five moderately susceptible and two susceptible. There was no highly susceptible clone. Iqbal et al. (1995) reported that the performance of sugarcane clones by artificial inoculation with the red stripe pathogen varies from natural infection under field conditions.

Conclusions

Morphological appearance and biochemical characterizations identified the bacteria as *A. avenae* subsp. *avenae*. *In vitro* screening for the efficacy of various antibiotics to inhibit the growth of *A. avenae* subsp. *avenae* on YDC media showed that ampicillin and vancomycin were most effective. Artificial inoculation on sugarcane against red stripe disease was observed. Observations were made up to six weeks for disease development. Out of 27 varieties, 16 were found resistant, four moderately resistant, five moderately susceptible and two susceptible

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