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MORPHOLOGICAL CHARACTERIZATION OF ENDOPHYTIC BACTERIAL STRAINS ISOLATED FROM DISCOLORED RICE GRAIN

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A B S T R A C T

Various bacterial species were isolated from different rice varieties: Kainat, Basmati-385, Super basmati, Basmati-386, KSK-133, Basmati-198, Basmati-2000x1053-2-2, Kasur, Stg 567989 and Basmati-2000x33797-1 collected from all agro ecological zones of Pakistan. When plated infected grain samples gave various bacterial colonies on Luria Bertani (L.B) agar medium. The isolates were identified on the basis of various morphological and biochemical features. Out of 22 isolates, five showed rod cell shape in microscope. Sixteen biochemical tests were conducted to characterize 22 isolates of bacteria. Gram stain demonstrated that three isolates were gram positive and rod shaped. All other isolates were gram negative. The presence of bacteria was also estimated in ten different varieties of rice. The highest presence of bacteria was observed in KSK-133, Kainat and Stg 567989. *Burkholderia* species and *Enterobacter* species have high frequency almost in all tested rice varieties. The overall objective of this study was to screen, classify and associate the bacterial species present on the basis of various morphological characteristics isolated from diverse rice genotype. The results demonstrated that collected and investigated rice varieties have a diverse range of bacterial species, some of which are considered as severe pathogens for plants.

Keywords: Rice grains, bacteria, isolation, identification, morphological and biochemical characterization.

INTRODUCTION

Rice (*Oryza sativa* L.) is one of the essential cereal grains and a staple food of more than 50% of the world's population. Crop productivity is based on numerous variables including climatic conditions, nutrients, biotic and abiotic factors (Luo *et al*., 1998). Pakistan rice grain yield/ unit area is very low in comparison with World developed countries and this low production is due the involvement of several environmental factors. Various diseases and drought stress are the main yield limiting factors of rice crop and its losses increasingly every year. Diseases causes' extensive damage to rice and severity of infection varies with different species and varieties (Asghar *et al*., 2007). Rice is affected by many micro-organisms i.e. viruses, fungi, bacteria, mycoplasma and nematodes that causes various diseases almost 76 (Hajano *et al.,* 2012; Sumangala *et al.*, 2009; Jabeen *et al*., 2012). A complex disease symptoms of rice discolored grains, blights, rots, blotch are due to the infection various microorganisms on the rice panicles. The goal of the present work was to isolate, purify and identify bacteria from different varieties of rice and their frequency distribution in all varieties. Rice grain discoloration is becoming a series threat to the Pakistan rice varieties and in Asia and its severity increases more and more with passage of time (Arshad *et al*., 2009; Phat *et al.,* 2005. Discoloration decreases the yield potential of rice crop along with other diseases, and account for the yield losses up to 6% (Savary *et al*., 2000). Rice grain discoloration affects the qualitative and quantitative traits (Sumangala *et al.,* 2009; Tariq *et al.,* 2012) and is ultimately responsible for yield reduction and other desirable traits. Losses due to brown spot infected grains have been recorded in the range of 16% to 43% (Datnoff *et al*., 1997). These diseases affect the grain quality, break of rice during milling, weight loss, exports, post-harvest losses, crop yield and ultimately badly affect the economy of

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Pakistan (Ghazanfar *et al.*, 2013). This problem can be solved by using resistant germplasm, chemicals, new agronomic practices, breeding techniques, mutations and new advanced molecular techniques. (Ribot, 2008). Usage of resistant varieties is the environmental friendly and economical method for the control of rice diseases; sometimes it completely fails due to occurrences of new pathogen races (Ghazanfar *et al*., 2009). The objectives of study were to (1) Isolation of endophytic bacterial strains from rice responsible for grain discoloration symptoms. (2) Categorization of bacterial species on the basis of their morphological traits.

MATERIALS AND METHODS

Collection of Rice Varieties: Various diseased rice grains were analyzed to study the frequency of various bacterial populations. Rice varieties with diseased grains were collected from Kala Shah Kaku (KSK), Lahore, Pakistan and USA as shown in Table 1.

Table 1. List of germplasm material used in the experiment.

Sr. No	Name of Rice Varieties	Accession No.	Source	Collection site
01	Kainat	NA	Subcontinent	Gujranwala
02	Basmati-385	Approved Variety	KSK, Pakistan	Sheikhupura
03	Super basmati	Approved Variety	KSK, Pakistan	Gujranwala
04	Basmati- 386	Approved Variety	KSK, Pakistan	Gujranwala, Sialkot
05	KSK-133	Approved Variety	KSK, Pakistan	Hafizabad, Sheikhupura
06	Basmati-198	Approved Variety	KSK, Pakistan	Lahore
07	Basmati-2000 x 1053-2-2	F_4	KSK, Pakistan	PU, IAGS
08	Kasur	NA	Punjab, Pakistan	Hafizabad
09	Stg 567989	GSOR 311705	USA, Arkansas	PU, IAGS
10	Basmati-2000 x 33797-1	F_4	KSK, Pakistan	PU, IAGS

Media Preparation: Luria Bertani agar (LBA) medium (Difco and high media) was prepared by using the following mixture Agar: 15.0 g; Trypton: 10.0 g; NaCl: 5.0 g and Yeast extract: 5.0 g in distilled water. pH was adjusted 7.4 before the addition of Agar. At 15 lb. pressure for 15 minutes media was sterilized by autoclaving and then used for bacterial isolation (Ali and Naseem, 2011, 2012).

Sterilization of Discolored Rice Grains: Rice grains with symptoms of disease were washed in running tap water and classified by surface appearance in order to exclude samples that showed superficial damage. Surface sterilization was done by stepwise washing in 70% ethanol for 5 min, sodium hypochlorite solution for 5 min, and 70% ethanol for 30 second, followed by three rinses in sterile distilled water (Araujo, 2002).

Isolation of bacterial strains: Various parts of discolored rice grains were homogenized in 5 ml of sterile saline solution with sterilized spreader and serial dilutions (1ml of 10^5) (Ali and Naseem, 2011). On the other hand, sterilized grains were cut into 2 to 3 mm long pieces and placed on LBA medium (g/L) plates incubated at 37°C for 24 hours to allow endophytic bacterial growth from rice grains. Streaking of isolates was done to obtain individual colonies on agar plates (Beishir, 1991). Pure bacterial cultures were stored in 20% sterile glycerol at -20 °C until further analysis.

Identification of bacterial strains: Bacterial species were identified on the basis of various phenotypic characteristics, e.g., cell shape, colony color, morphology, growth rate and motility etc. The purified colonies were characterized using gram staining, biochemical tests and consulting literature (Holt *et al*., 2000; Koneman *et al*., 1997; Benson, 1996). Bacteria were identified by providing the results of all above mentioned biochemical tests to Microgen Identification System software. The relative frequency (%) of bacterial species was calculated as: (bacterial species colonies/ total bacterial colonies) × 100 (Mukhtar *et al*., 2010).

RESULTS

Bacterial species isolated on the L.B agar medium from different rice varieties were used in this study collected from KSK, Lahore, Pakistan and USA. Different isolates were purified with dilution of distilled water and plating on LBA medium for further studies of various morphological traits of bacterial colonies. Single colony of each plate was touched with a flamed bacteriological loop with diverse cultural characteristics and then streaked on LBA medium for incubation at 37°C for 24 hr. After this each culture plate was stored in micro-centrifuge tubes at -20°C in 20% glycerol for later studies in future prospectus and identification of bacterial species. The results were described in described in table 2 to 4.Frequency distribution of bacterial species as shown in Table 4 and Figure 1 and 2 was different within varieties and location. The frequency of bacteria from KSK-133, Basmati-385, Kainat and Stg 567989 was higher than that of Basmati-86, Basmati-198 and Kasur. It was found that *Burkholderia pseudomallai, Burkholderia glumae, Enterobacter asburiae* and *Acidovorax facilis* have high frequency almost in all tested varieties (Figure 1 and 2).

Table 2. Bacterial Species Isolated from Rice Grains on Basis of Morphology.

Strain No.	Morphological characters									
	Colony color	Colony texture	Reverse color	Odour	Cell Shape	Gram Stain	Capsule Stain	Motility test	Growth on 5% NaCl	Growth on 5% Glucose
01	Off white	Slimy	Yellow	+	Cocci	-	-	-	-	+
02	Off white	Slimy	Yellow	+	Cocci	-	-	-	+	+
03	Off white	Slimy	Yellow	+	Cocci	-	-	-	-	+
04	White	Slimy	White	-	Cocci	-	-	+	+	+
05	White	Slimy	White	-	Cocci	-	-	-	+	+
06	White	Slimy	White	-	Cocci	-	-	-	+	+
07	White	Rough slimy	White	-	Cocci	-	-	+	-	+
08	White	Rough slimy	White	-	Cocci	-	-	+	+	+
09	Dusty white	Dry crust	White	+	Rods	+	-	+	+	+
10	Dusty white	Dry crust	White	+	Rods	+	-	-	-	-
11	Dusty white	Dry crust	White	+	Rods	+	-	+	+	-
12	Off white	Slimy	Light yellow	-	Rods	-	-	-	+	-
13	Off white	Slimy	Light yellow	-	Rods	-	-	-	+	-
14	Off white	Slimy	White	-	Cocci	-	-	+	-	-
15	Bright yellow	Slimy	Yellow	-	Cocci	-	-	+	+	+
16	Bright yellow	Slimy	Yellow	-	Cocci	-	-	-	+	+
17	Bright yellow	Slimy	Yellow	-	Cocci	-	-	+	+	+-
18	Dirt white	Slimy	White	-	Cocci	-	-	-	-	+
19	Dirty white	Slimy	White	-	Cocci	-	-	+	+	-
20	White	Slimy	White	-	Cocci	-	-	-	+	+
21	White	Slimy	White	-	Cocci	-	-	+	+	-
22	White	Slimy	White	-	Cocci	-	-	+	+	-

Table 3. Bacterial species Isolated from Rice Grains on the Basis of Biochemical Tests.

Strain No	Biochemical Tests																Identified Bacterial Species
	Lysine Test	Glucose Test	Raffinose Test	Arabinose Test	Nitrate Reductase Test	Oxidase Test	Urease Test	Methyl Red Test	Citrate Utilization Test	Hydrogen Sulfide Production Test	Catalase Test	Indole Test	Rhamnose Test	Mannitol Test	Xylose Test	Sorbitol Test	
01	-	-	-	-	+	-	-	-	-	-	-	-	+	-	-	+	*Enterobacter asburiae*
02	+	-	-	-	-	-	-	+	-	-	-	-	-	-	+	-	*Enterobacter* sp.
03	-	+	-	-	-	+	-	-	-	-	+	-	-	-	-	-	*E. cloacae*
04	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	+	*Acidovorax* sp.
05	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	*A. temperans*
06	-	-	-	-	+	-	-	+	-	-	+	-	-	-	-	+	*A. facilis*
07	-	+	-	-	-	-	-	-	-	-	-	-	-	-	+	-	*Citrobacter* sp.
08	-	-	-	-	+	-	+	-	+	-	-	-	-	-	-	-	*C diversus*
09	+	-	-	-	-	-	-	-	+	-	+	+	-	+	-	+	*Kurthia* sp.
10	-	-	-	-	+	-	+	-	-	+	-	+	-	-	+	-	*K. sibirica*
11	-	-	-	+	-	+	+	+	+	+	-	-	-	-	-	+	*K. zopfii*
12	-	+	-	-	+	-	-	-	-	+	+	-	-	+	+	-	*Acinetobacter* sp.
13	-	-	-	-	-	+	+	+	-	-	-	-	-	-	-	-	*A. junii*
14	-	-	-	-	+	-	-	-	+	-	-	-	-	+	-	+	*Kluyvera* sp.
15	-	-	-	-	-	-	-	+	-	+	-	+	+	-	+	-	*Xanthobacter* sp.
16	-	-	+	-	-	+	+	-	-	-	-	-	+	-	-	+	*X. agilis*
17	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	*X. flavus*
18	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	*Aureobacterium* sp.
19	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	*A. liquefaciens*
20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	*Burkholderia* sp.
21	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	*B. glumae*
22	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	*B. pseudomallei*

Table 4. Number of colonies of bacteria from various varieties of rice grains.

S. No.	Diseased Rice Grains sample Collection	Rice Varieties	Total No. of Bacteria	Bacterial species	No. of colonies	Culture media (g/L)
1	Gujranwala, KSK, Pakistan	Kainat	10	*Acidovorax*sp.	03	Luria Bertani Agar(NA)
				Enterobacter sp.	02	=
				Acidovorax temperans	02	=
				Kurthia zopfii	03	=
2	Sheikhupura, KSK, Pakistan	Basmati-385	08	*Burkholderia*sp.	03	=
				Acidovorax temperans	02	=
				Enterobacter asburiae	02	=
				Aureobacterium sp.	01	=
3	Gujranwala, KSK, Pakistan	Super basmati	07	*Burkholderiapseudomallei*	02	=
				Acidovorax facilis	03	=
				Citrobacter sp.	01	=
				Aureobacterium liquefaciens	01	=
4	Gujranwala, Sialkot KSK, Pakistan	Basmati 86	05	*Acinetobacter junii*	02	=
				Burkholderiaglumae	02	=
				Aureobacterium sp.	01	=
5	Hafizabad, Sheikhupura, KSK, Pakistan	KSK-133	11	*Kurthia sibirica*	04	=
				Acidovorax facilis	03	=
				Xanthobacter agilis	02	=
				Burkholderiaglumae	02	=
6	Lahore, Pakistan	Basmati-198	05	*Kluyvera* sp.	01	=
				Enterobacter asburiae	02	=
				Aureobacterium liquefaciens	01	=
				Burkholderiaglumae	01	=
7	IAGS, P.U, Lahore	Basmati-2000x1053-2-2	07	*Burkholderiapseudomallei*	02	=
				Citrobacter diversus	02	=
				Enterobacter sp.	03	=
8	Hafizabad, Sheikhupura, KSK, Pakistan	Kasur	05	*Acidovorax facilis*	01	=
				Acinetobacter junii	01	=
				Burkholderiaglumae	01	=
				Xanthobacter flavus	02	=

Continue…

9	IAGS, P.U, Lahore	Stg 567989	09	*Burkholderia glumae*	01	=
				Xanthobacter agilis	04	=
				Enterobacter cloacae	02	=
				Kurthia sp.	02	=
10	IAGS, P.U, Lahore	Basmati-2000x33797-1	07	*Acinetobacter* sp.	01	=
				Enterobacter asburiae	03	=
				Xanthobacter sp.	02	=
				Burkholderia glumae	01	=
				Total	77	

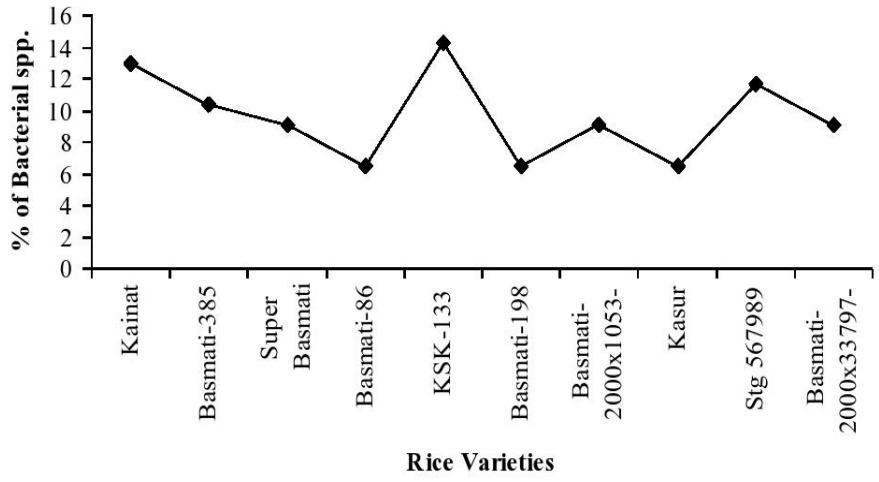

Figure 1. Percentage of Bacterial spp. isolated from each Rice varieties

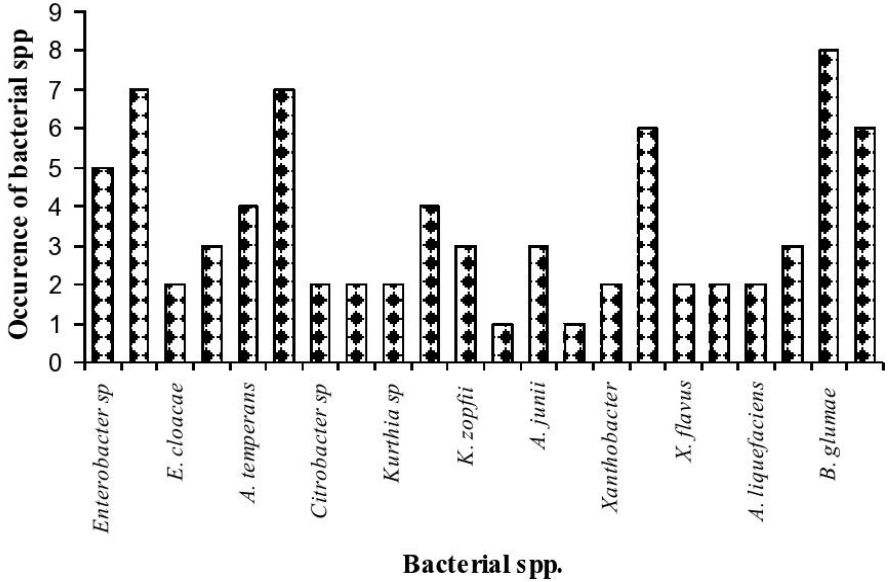

Figure 2. Occurrence of Bacterial spp. in each Rice samples

DISCUSSION

All isolated bacterial species were identified by indicating of their biochemical as well as macroscopic characters and further classified using Bergey's Manual of Determinative Bacteriology (9thEdition) (Akhtar etal., 2013).Colony morphology gave an indication of variation among bacterial strains. Purification of bacterial species was done due to uniqueness or differences with other in colony morphology (Ali et al., 2013). The identified bacterial species that was isolated from rice grains included: *Enterobacter* sp., *E. asburiae, E. cloacae, Acidovorax*sp., *A. temperans, A. facilis, Citrobacter*sp., *C diversus, Kurthia*sp., *K. sibirica, K. zopfii , Acinetobacter* sp., *A. junii, Kluyvera*sp., *Xanthobacter*sp., *X. agilis, X. flavus, Aureobacterium* sp., *A. liquefaciens, Burkholderia* sp., *B. glumae* and *B. pseudomallei*. Of the 22 identified bacterial species three were gram positive and nineteen gram negative. The cell shapes of five bacterial species were bacilli (rod) and the other were cocci.

Interestingly, Gram positive and Gram negative isolates were equally distributed between all rice varieties. An earlier study reported a predominance of Gram negative bacteria in the tissues of rice grains (Ashfaq *et al*., 2013). However, Akhtar *et al*. (2000) reported an equal presence of gram negative and gram positive bacteria in micro-fauna of Lahore soil. Pathogens associated with discolored rice grain disease have been reported before (Khan *et al*., 2000; Javed *et al*., 2002). Rice yield reduction is caused by many rice diseases and worldwide it is estimated to be about 14-18% (Mew *et al*., 2004; Mew and Gonzale, 2002). Although grain discoloration disease has been minor disease in Pakistan but it is becoming a serious problem and have heavy yield losses ranging from 50 to 90%, if no proper attention is given to this disease, it may become a potential threat for the rice crop (Arshad *et al*., 2009). In Tamil Nadu India yield losses up to 39% were reported (Shanmugam *et al*., 2006). Rice grain discoloration is also a major limiting factor in rice yield (Rajpan *et al*., 2001). Rice molecular markers also play a very important role for screening, selection and identifying the new resistant rice lines against diseases and other biotic and abiotic stresses (Choudhary *et al*., 2013; Pinta *et al*., 2013).

CONCLUSION

The diversity and characterization of different bacterial species isolated from diverse rice germplasm lines were classified. The study may be utilized for the screening of various rice germplasm lines on the basis of various isolated bacterial species and classification of the genotypes with respect to their various kinds of pathogen. This study may also equally helpful for the scientist and farmers community for the classification (on the basis of resistant, tolerant and susceptibility of rice genotypes against various pathogens and also provides the information for further investigation in to new insight in scientific field.

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