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Identifying and characterizing the main causal pathogen responsible for rice grain discoloration in Pakistan

Muhammad Ashfaq[1,*], Abdul Rasheed[1], Muhammad Ali[1], Muhammad Sajjad[2], Bilal Rasool[5], Shabnum Shaheen[3], Muhammad Shafiq[1], Muhammad Arshad Javed[1], Alia Anwar[1], Muhammad Saleem Haider[1], and Urooj Mubashar[4]

[1]Faculty of Agricultural Sciences, University of the Punjab, Quaid-e-Azam Campus, Lahore, Pakistan; [2]Department of Biosciences, COMSATS University Islamabad (CUI), Park Road, 45550, Islamabad, Pakistan;[3]Department of Botany, Lahore College for Women University, Pakistan;[4]Government Teachers Education Training Academy Ghakkhar Mandi, Gujranwala, Pakistan;[5]Department of Zoology, Faculty of Life Sciences, Government College University, Faisalabad, Pakistan

*Corresponding author's e-mails: ashfaq.iags@pu.edu.pk; ashfaq_qs@yahoo.com

Grain discoloration affects grain quality, quantity, and yield of rice crop worldwide. Herein, morphological, molecular, and pathogenic characterizations of the pathogen causing rice grain discoloration were performed. A set of 20 rice genotypes were scored for disease infestation in field and scanned with 12 unlinked polymorphic SSR primer pairs for genetic diversity assay. Pathogens from the discolored rice panicles were isolated to investigate the disease-causing pathogen on DNA sequencing basis. The universal primer pairs were used for DNA sequencing of the isolated pathogen. Furthermore, the new pathogen was reconfirmed morphologically by using Koch's postulates. The bacterial species *Bacillus licheniformis* was identified as a major pathogen causing grain discoloration disease in rice. The genotypes found to be resistant to rice grain discoloration include Super-basmati, Rondo, Gulfmont, KSK-133 and L-203 which can be used as potential genetic resources for breeding rice grain discoloration resistant cultivars. The study provided information to the researchers and farmers for managing and controlling rice grain disease by the adoption of *Bacillus licheniformis.* The study is equally beneficial both for researchers and scientific community to start up a new research program by utilization of this study.
Keywords: Rice, disease, *Bacillus licheniformis*, grain discoloration, characters and resistant.

INTRODUCTION

Rice is an important export commodity and major food crop in Pakistan. Rice crop feeds more than half of the world population and provides various supplements for the daily requirements (Fageria 2007; Seck *et al*., 2012). Various rice diseases that affect grain quality, quantity and reduce grain yield of the crop. Grain discoloration is becoming a serious threat to the rice crop in Pakistan and its occurrence is increasing every year (Phat *et al*., 2005; Ashfaq *et al*., 2017). This disease is caused by various types of pathogens i.e., fungal, bacterial, and viral or may be due to the complexity of pathogens along with environmental factors (Modarresi *et al*., 2015).

Rice with its small genome size (400Mb) and high genetic polymorphism is used as genetic model for other cereals (Wing *et al*., 2018). Due to genetic diversity in germplasm including land races, wild species, obsolete cultivars, modern cultivars, cultivated forms of wild species, approved varieties, advanced homozygous breeding material and mutants etc. genetic resources resistant to new disease like grain discoloration can be identified (Ashfaq *et al*., 2017).

Rice pathogens associated with discolored grains i.e., *Bipolaris oryzae*, *Fusarium* spp, *Burkholderia glumae*, *Erwinia* spp, and *Burkholderia gladioli* that affect seed health, seed quality, germination ability, weight loss, seed morphology of rice (Cottyn *et al*., 2009; Bigirimana *et al*., 2015; Zarbafi *et al*., 2019; Akter *et al*., 2019). Such types of pathogens are responsible for causing other diseases of rice ultimately restricting the yield potential of the crop (Amini *et al*., 2015; Dirchwolf *et al*., 2018; Abebrese *et al*., 2019). These pathogens are found to be associated with 28-65% of discolored seed and causing a huge loss in yield (Uma and Wesely 2013; Ashfaq *et al*., 2017). Selection of grain discoloration resistant rice with desired traits, disease scoring and disease management provides the information for the

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selection of disease free rice and evolution of new rice varieties through hybridization and interspecific crosses (Dirchwolf *et al*., 2018; Abebrese *et al*., 2019; Pampana *et al*., 2019).

Bacillus licheniformis is well known plant growth promoting rhizobacteria (PGPR) and biocontrol agent (Fira *et al*., 2018; Saxena *et al*., 2020). However, inconsistent performance of PGPR and biocontrol agents in the field conditions is evident as a major hindrance for their successful commercialization. There is growing evidence that horizontal gene transfer (HGT) is a potent evolutionary force in prokaryotes that can transfer genes between different species in the rhizosphere and endosphere of plants. This may result in the loss of PGP traits from beneficial microbes and *vice versa* (Emamalipour *et al*., 2020; Tiwari *et al*., 2020).

Shrestha *et al*. (2016) reported *B. licheniformis* as one of the strains associated with rice grains. They reported that among the species of Bacillus included in the cluster analysis, *B. anthracis*, *B. cereus*, *B. pumilus*, and *B. licheniformis* were grouped as separate clades from the antagonistic rice associated bacteria (RAB), indicating that the RABs were distantly related to these species. Although exactly mechanism of HGT has not been explored yet but it would be an interesting aspect to be studied. Furthermore, traits associated with each type strains are different due to variation in their genetic makeup. So, it is also possible that the strain of *B. licheniformis* associated with rice grains is specific in their pathogenicity towards rice. *Bacillus licheniformis* is gram positive and endospore-forming bacteria that lives in plant and soil (Veith *et al*., 2004)

Grain discoloration and panicle blight disease appeared at the time of panicle emergence and crop maturity stage that affected the rice grains, quality, size, shape, weight, yield, and other qualitative and quantitative traits. Some environmental factors are also responsible for causing discolored grains i.e., rains, dew drops, humidity, seed injuries, insect vectors and high wind pressure. Various pathogens i.e., fungi, bacteria, viruses, and some insect vectors also cause this severe disease (Uematsu *et al*., 1976; Cottyn, 2001). The leading rice growing countries are badly affected by these pathogens that causing a huge yield loss due to hollow panicles, discolored grains and grains weight loss ((Hajano *et al*., 2012; Jabeen *et al*., 2012; Tariq *et al*., 2012; Ghazanfar *et al*., 2013).

Use of various modern breeding, identification, mutation techniques and management strategies will be very useful for controlling such types of pathogens. Here in this study, a diverse set of 20 rice genotypes was scored for rice grain discoloration. The pathogens from infected panicles were isolated and characterized to identify the major rice grain discoloration causing pathogen that was *Bacillus licheniformis.*

MATERIALS AND METHODS

***Plant material and sample collection*:** Twenty rice germplasm lines were studied based on various desired traits under Randomized Complete Block Design (RCBD) with three replications (Table 1). Different disease grain discoloration samples of rice panicles were collected randomly from paddy fields across different locations i.e.,

Table 1. Rice (*Oryza sativa*) genotypes used in the experiment

Sr.	Variety Name	Taxon	Accession No	Origin	Disease reaction
1.	CB-38	Oryza sativa	4439	Pakistan	Susceptible
2.	WW8/2290	Oryza sativa	GSOR 310494	Netherlands	Susceptible
3.	Bas-515	Oryza sativa	Approved variety	Pakistan	Susceptible
4.	Zhen Shan 97B	Oryza sativa	PI 474580	China	Susceptible
5.	IR-64	Oryza sativa	GSOR 311793	Philippines, Luzon	Susceptible
6.	IR-6	Oryza sativa	Approved variety	Philippines, IRRI	Susceptible
7.	CDR 448	Oryza sativa	PI 615009	China	Susceptible
8.	Bas-370	Oryza sativa	Approved variety	Pakistan	Susceptible
9.	Super basmati	Oryza sativa	Approved variety	Pakistan	Resistant
10.	Shaheen Bas	Oryza sativa	Approved variety	Pakistan	Susceptible
11.	Chaoyang No. 1	Oryza sativa	PI 615219	China	Susceptible
12.	Basmati-Pak	Oryza sativa	Approved variety	Pakistan	Susceptible
13.	Dhankasarwala	Oryza sativa	0120	Pakistan	Susceptible
14.	Rondo	Oryza sativa	PI 657830	United States, Arkansas	Resistant
15.	Bas-198	Oryza sativa	Approved variety	Pakistan	Susceptible
16.	Jasmine 85	Oryza sativa	PI 595927	China	Susceptible
17.	Gulfmont	Oryza sativa	PI 502967	United States, Texas	Resistant
18.	KSK-133	Oryza sativa	Approved variety	Pakistan	Resistant
19.	L-203	Oryza sativa	PI 547249	United States, California	Resistant
20.	CB-36	Oryza sativa	Breeding line	Pakistan	Susceptible

District Gujranwala Punjab, District Lahore, and experimental field the Institute of Agricultural Sciences, University of the Punjab Lahore, Pakistan. All the samples were collected at panicle and grain filling stage. The discolored panicles were cut with scissor and placed in plastic bags, all the samples properly labeled with date, name, location and stored in envelopes for further study in the laboratory on The Luria Bertani Agar (LBA) culture media. Randomly single panicle of each variety was selected and counted the infected seeds based on disease incidence/symptoms of grain discoloration of rice. Disease severity/incidence was determined using the scale described by (Malavolta *et al*., 2007), Where 0= with no symptom; 1= small spots on the rice glumes; 2= occurrence of 25% spots on the grain surface; 3= occurrence of 26 to 50% spots on grain surface; 4= spots exceed more than 50% on grain surface. The seed panicles with no disease symptoms considered to be resistant and other found to be susceptible having disease symptoms. Disease scoring/grain discoloration (GD) percentage was calculated by using the following formula (Bodalkar *et al*., 2014).

$$\text{Disease scoring} = \frac{\text{No. of infected seeds}}{\text{Total number of seeds}} \times 100$$

***Seed traits measurement and classification*:** Seed traits i.e., seed length, seed width; seed thickness and seed length width ratio of all genotypes were measured with the help of a Vernier caliper and thousand seed weight was measured with weighing balance. For size and shape measurement fifteen random seeds of each genotype were selected to investigate the long and short grain of the varieties that helped in their proper utilization in the research program. Seed size of each genotype was defined in its longest dimension and classified in the following order as very long (>7.50 mm), long (6.61-7.50 mm), medium (5.51-6.60 mm) and short (< 5.50 mm). Seed length width ratio provides the information regarding shape of the seed and ranges from slender (3.0 or>3.0), medium (2.1-3.0), bold (2) and round (1 or < 1) (Ashfaq *et al*., 2012) and measured with the help of the following formula.

$$\text{Seed length width ratio} = \frac{\text{Seed Length (mm)}}{\text{Seed Width (mm)}}$$

***DNA extraction and PCR analysis*:** Rice leaf samples were collected at the seedling stage of every genotype and DNA was extracted by using (CTAB) method Cetyl Trimethyl Ammonium Bromide (Muray and Thompson, 1980). DNA samples of all the genotypes were checked based on their qualitative and quantitative parameters using Nano Drop (ND 1000) spectrophotometer, Sigma chemical company USA. The 40 ng DNA of each genotype was used in the experiment. All samples were found good quality and quantity of DNA and very suitable for PCR analysis.12 SSR primer pairs were used for PCR of all the genotypes by using (Paunad, 1996). Furthermore, PCR samples were scanned on a gel documentation system to see genetic differences in morphological based data.

***Isolation and identification of pathogen*:** Rice discolored samples were collected from the experiment field at the maturity stage of the crop. The Luria Bertani Agar (LBA) culture media was prepared for growth, isolation, and identification of pathogen on the basis of morphology and genetics. DNA was also extracted from LBA culture media of discolored rice samples to investigate the pathogen using Genomic DNA Purification kit (Sigma chemicals, USA). The quality and quantity of the extracted DNA was assessed using Nano Drop ND-1000 spectrophotometer (Sigma chemical company USA). The DNA concentration of the tested specie was adjusted to 40 ng/ul for the PCR analysis. For this purpose universal primer pairs were used i.e., fD2: 5′-AGA GTT TGA TCA TGG CTC AG-3′ and rP1: 5′-ACG GTT ACC TTG TTA CGA CTT-3′ and 27F 5' (AGA GTT TGA TCM TGG CTC AG) 3 and 1492R 5' (TAC GGY TAC CTT GTT ACG ACT T) 3' for DNA sequencing of the isolated pathogen. For this purpose, Koch's Postulates and two universal primer pairs were used for the identification of pathogens that mainly responsible for causing of grain discoloration disease in the rice crop. The pathogen was further reconfirmed through pathogenicity test by the preparation and application of inoculum at panicle emergence stage in glass house to identify the actual causal organisms.

***Pathogenicity test*:** For confirmation Koch's postulates were proved for pathogenicity of pathogen. The healthy plants were selected for the inoculation of tested pathogen. For this purpose, 90 days old healthy plants were shifted into glasshouse at flowering stage in earthen pots (25 cm diameter, with sandy soil and organic matter) for inoculation. Single isolate was prepared on LBA and spores' suspension was prepared in sterile distilled water. Bacterial spore suspension was adjusted to 1 x 10^5 spores/mL by using Scanning Electron Microscopy and used to spray on the outer surface of the panicle at the stage of emergence (0.5 mL/panicle). On the other hand, control plants were sprayed with sterilized water. Inoculated plants were kept in glasshouse at 30 °C, under 12 hours of photoperiod and 75% humidity. About 21 days of post inoculation, black or brown spots/lesions (6 to 10 mm) were observed on the lemma, palea and glumes of the grains. The pathogen was isolated and reconfirmed morphologically and based on molecular analysis which fulfilled the Koch's postulates.

***Statistical analysis*:** The data recorded on different morphological traits were analyzed by using SAS version 9.2 (SAS Institute, 2008) for all the genotypes included in this study. Variance and correlation analysis of the diverse traits was done by using (Steel *et al*., 1997). Genetic diversity studies of the entire set of genotypes were analyzed by the application of Power Marker (Liu & Muse, 2005).

Table 2. Seed morphological analysis of various traits of rice genotypes

Source of variation	D.F	SL	SW	ST	L/W	TGW
Genotypes	19	2.196**	0.806**	1.422**	0.0430**	0.2010**
Replications	2	0.003	0.668	0.023	0.0001	0.0016
Error	38	0.040	0.470	0.020	0.0041	0.1330

Level of significance * Indicates $p < 0.05$ and ** indicates $p< 0.01$. SL: Seed length, SW: Seed width, ST: Seed thickness, L/W: Length/width ratio and TGW: 1000-grain weight.

RESULTS

***Analysis of variance (ANOVA) and Correlation analysis*:** To test level of variation among 20 selected rice genotypes for the studied traits analysis of variance (ANOVA) was performed. The ANOVA revealed that the traits under study had highly significant ($p< 0.01$) variances among the twenty genotypes (Table 2). Prevalence of significant variance for all studied traits implicates the usefulness of the selected set of genotypes for genetics analysis and scoring for rice grain discoloration infestation.

To determine the pattern of genetic association of the studied traits correlation analysis was performed. The correlation analysis revealed negative significant correlations between SL and SW; SL and ST; SW and L/W. Positive significant correlations were observed between SL and L/W; SL and TGW; SW and ST; TGW and ST. Non-significant correlation was observed between TGW and SW (Table 3). The positive correlation between TGW and SL can be used for improving grains yield and grain length in the hybrids/pure line varieties which will be developed using the selected germplasm.

Table 3. Association of rice traits at the time of maturity

Traits	SL	SW	ST	L/W	TGW
SL	1.000				
SW	-0.211**	1.000			
ST	-0.105**	0.222**	1.000		
L/W	0.632**	-0.332**	-0.24**	1.000	
TGW	0.079**	0.031^{NS}	0.049*	-0.135**	1.000

SSR analysis: Selected SSR primers were used for the estimation of molecular diversity. All polymorphic primers were used. Different genetic characters i.e., total number of alleles, percentage of polymorphism and polymorphic information content (PIC) etc. were calculated. The polymorphic SSRs produced 3-6 alleles with an average unit of 4.16. The PIC values ranged from 0.264 to 0.733 with average mean of 0.521 (Table 4). These polymorphic and multi-allelic SSR markers can be used for marker-trait associations (MTAs), marker assisted selection (MAS) in segregation populations raised from crosses of the selected germplasm.

Disease scoring and identification of causal organism: Rice grain discoloration is an emerging threat of rice crop in Pakistan and its threat increasing very fast rate in every year. It often occurred at early flowering and maturity stage of rice grains. Black or brown spots/lesions were appeared on the lemma, palea and glumes of the grains. At the time of harvest of the crop some immature and lighter grains were observed due to this disease. No bacterial ooze was observed in the discolored grains. Fifteen random samples of susceptible rice varieties were selected for the isolation of the pathogen. The surface sterilized sample was cut into small portions for bacterial isolation and these samples placed on LBA (Luria Bertani Agar) media plates under aseptic conditions. The inoculated plates were incubated at 30 ºC for 24hours to count the single colonies for estimation of bacterial cells in the suspension. Bacterial isolates were re-cultured to obtain pure bacterial isolates. Ten isolates were recovered from the fifteen samples of discolored rice. Eight isolates were selected based on purity of culture for identification of the pathogen. The pure culture was prepared of all the isolates for PCR study (Fig. 3). Based on morphological and molecular characters the major pathogen was identified as *Bacillus licheniformis* (Accession No. LT860212) in all the isolates. The results were shown in the Fig. 1 and Fig. 2.

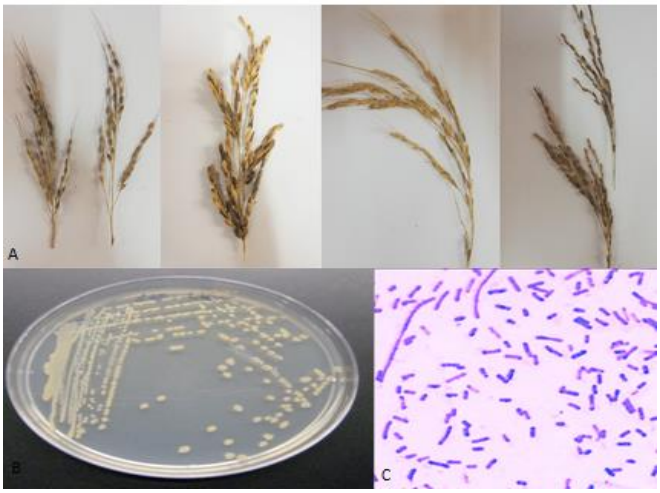

Figure 1. A: Rice grain discoloration symptoms at panicle stage, B: Bacterial growth of *Bacillus licheniformis* on petri plates, C: Gram staining of isolated bacterial species under compound microscope

Table 4. Alleles, and PIC values calculated for a set of 20 diverse rice genotypes.

SSR marker	Sequence	Chromosomes location	Product Size(bp)	Total no. of alleles	No. of polymorphic alleles	% polymorphism	PIC
RM529	F: CCCTCCCTTCTGTAAGCTCC R:GAAGAACAATGGGGTTCTGG	1	145	4	4	100	0.364
RM208	F:TCTGCAAGCCTTGTCTGATG R:TAAGTCGATCATTGTGTGGACC	2	190	4	3	75	0.643
RM250	F: GGTTCAAACCAAGCTGATCA R: GATGAAGGCCTTCCACGCAG	2	140	3	3	100	0.344
RM251	F: GAATGGCAATGGCGCTAG R: ATGCGGTTCAAGATTCGATC	3	230	4	3	75	0.630
RM267	F:TGCAGACATAGAGAAGGAAGTG R:AGCAACAGCACAACTTGATG	5	166	4	4	100	0.286
RM204	F:GTGACTGACTTGGTCATAGGG R:GCTAGCCATGCTCTCGTACC	6	190	5	5	100	0.660
RM248	F: TCCTTGTGAAATCTGGTCCC R: GTAGCCTAGCATGGTGCATG	7	122	5	4	75	0.733
RM52	F: CTACTCGCGCGTGGAGTT R: TGTCTTACTGGTGAAGCTGG	8	272	4	4	100	0.469
RM215	F:CAAAATGGAGCAGCAAGAGC R:TGAGCACCTCCTTCTCTGTAG	9	173	4	3	75	0.370
RM258	F: TGCTGTATGTAGCTCGCACC R: TGGCCTTTAAAGCTGTCGC	10	230	4	3	75	0.350
RM254	F: AGCCCCGAATAAATCCACCT R: CTGGAGGAGCATTTGGTAGC	11	180	6	6	100	0.570
RM277	F: CGGTCAAATCATCACCTGAC R:CAAGGCTTGCAAGGGAAG	12	150	3	3	100	0.349
	Means			4.16	3.75	89.58	0.521

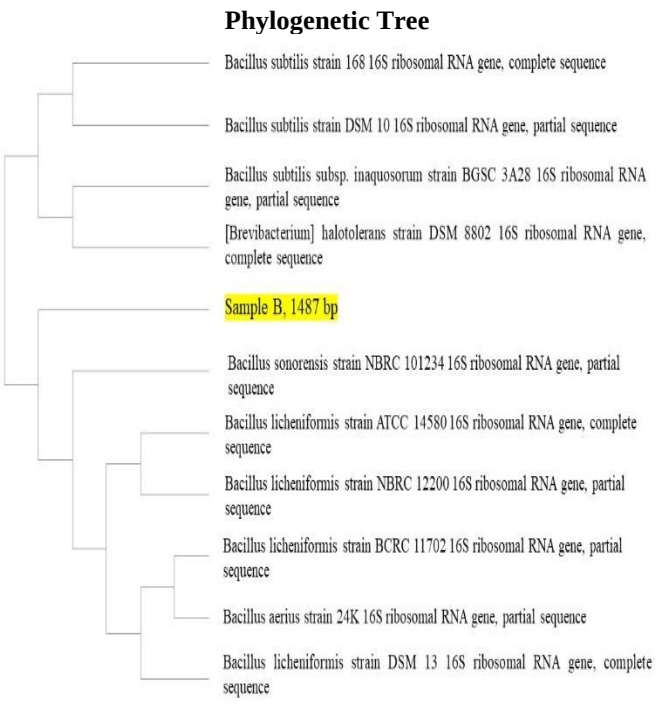

Figure 2. Phylogenetic tree of 1487 bp gene showing similarity with *Bacillus licheniformis* which confirms the identified specie is *Bacillus licheniformis* (NCBI).

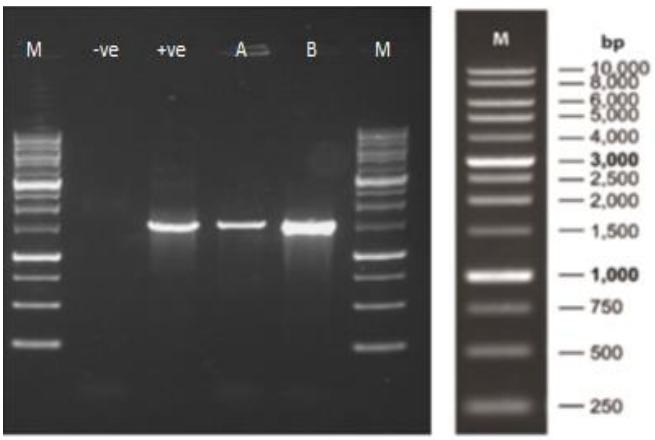

Figure 3. Gel picture showing Amplified Internal Transcribed Spacer (ITS) region and one compact band using polymerase chain reaction (PCR)

DISCUSSION

Discoloration in rice is very destructive disease in rice that causes a very huge loss in yield and its severity increasing and increasing every year. In the present study different sets of rice lines were studied on the basis of various morphological and pathogenic characteristics. The evaluation of diverse rice lines and identification of pathogens can be utilized in

breeding program for further screening and selection of resistant rice against grain discoloration (Xie *et al*., 2003; Rao *et al*., 2018).

All the genotypes studied showed significant differences along with entire set of traitsat the level of 1% and 5% significance. It was observed that the strong association between genotypes and a particular trait that provides the information for the screening and selection of rice lines. A strong association is very helpful in selection and identification process for further to start up a new breeding research. Various molecular traits or diversity indices were studied to evaluate the genetic diversity amongst the rice lines used in the current study i.e., total number of alleles (4.16), polymorphic alleles (3.75), percentage of polymorphism (89.58) and PIC values 0.521 etc. (Table 4).

Rice discoloration is mainly affected by complexity of pathogens and major role for its infection is fungi and bacteria. This disease can be a major threat to rice crop production in coming years along with facing global warming affects (Ham *et al*., 2011).It was found that the disease appeared at the panicle emergence stage, booting stage and maturity stage severely affecting the yield potential of rice crops (Hikichi *et al*., 1993; Hikichi *et al*., 1998). On the other hand, various environmental factors also involved for affecting the rice grains and lowering the yield potential of the crop (Bala and Pannu, 2017; Rohit and Layanya, 2019).

In 2017, fifteen seed panicle samples of discolored rice lines were collected at the maturity stage for the isolation of pathogen. Pathogens were identified based on morphology and molecular analysis (Fig 1, Fig 2). Identification of bacterial species was done on the basis of various morphological features colony (color, shape, size, texture, margins and odor etc.) and cell microscopic characters i.e., shape, color, cell wall, contents, arrangement, gram staining, spore staining etc. Based on morphological characteristics the pathogen *Bacillus licheniformis* was identified in almost all samples and all the selected isolates on the basis of purity it was facultative anaerobic Gram-positive rods that measured 1.4 to 2.3 × 0.4 to 0.6 µm and had three to six peritrichous flagella. Colonies on LBA were yellow and raised with smooth margins (Cottyn *et al*., 2001). Inoculum was prepared for further testing the isolated pathogen on rice plant. In 2018, the inoculum was applied at the panicle emergence stage on coarse and fine twenty rice varieties, after three weeks similar symptoms were observed like samples collected in 2017. These were further isolated and identified as *Bacillus licheniformis.* The pathogen was further identified with DNA molecular markers.

Identification was done by 16S rDNA sequence analysis. DNA was extracted followed by amplification and sequencing of the ITS gene region by using universal primer pairs (Tsoktouridis *et al*., 2014). The resulting sequence was deposited in Gene Bank (Accession No.LT860212) and BLAST analysis revealed that the isolates were 100 % identical with other cultures of *Bacillus* species in Gene Bank (for instance Accession Nos. CP021677.1, CP021669.1, KY886137.1, CP020352.1, KY595454.1, KY063593.1and KX641746.1) (Fig. 2 and Fig. 3). On the basis of pathogenicity test and DNA sequencing test the pathogen was identified as *Bacillus licheniformis* (Cottyn *et al*., 2009), causing grain discoloration of coarse and fine varieties of rice and ultimately affected the global rice yield.

***Conclusion*:** By this study, a new pathogen (*Bacillus licheniformis)* was identified as a major rice grain discoloration causing agent in both coarse and fine rice genotypes in Pakistan. The finding of the study is very useful for researchers and farmer's community for timely control of the disease. The disease cycle on rice crop and different management strategies such as proper selection of genetic resources and precise identification of pathogen in the regions are being further studied to control this disease for the improvement of rice yield. The genotypes Super basmati, Rondo, Gulfmont, KSK-133 and L-203 could be used as potential genetic resources for breeding rice grain discoloration resistant cultivars.

***Conflict of interest*:** The authors have no conflicts of interest

***Authors Contributions Statement*:** M.A. and A.R. conceived the idea of the study. M.A. and M.S. helped in organizing the experimental material for further recording the data. B.R. and S.S. designed the experiment. M.S., M.A.J., A.A., M.S.H. and U.M. helped in data analysis, supported in write up and review the manuscript**.**

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