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IDENTIFICATION AND PATHOGENIC CHARACTERIZATION OF BACTERIA CAUSING RICE GRAIN DISCOLORATION IN PAKISTAN

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ABSTRACT

Rice grain morphology is affected by discoloration disease that responsible for causing huge yield loss every year and its threat increasing significantly. Disease scores and identification of pathogen provides the information to control this disease timely. Disease scoring was done at the maturity stage of the crop to classify the rice lines on the basis of disease severity. Different diseased leaf samples were collected in the year 2018 from different rice varieties grown in the research fields of Punjab University, Pakistan to identify the pathogen. From the collected diseased samples, the casual organisms *Bacillus cereus* from collected rice samples was isolated, purified and identified on the basis of various morphological characteristics and molecular analysis. For this purpose, The DNA was used as a template to amplify the 16S rRNA gene by means of primers 27F 5' (AGA GTT TGA TCM TGG CTC AG) 3 and 1492R 5' (TAC GGY TAC CTT GTT ACG ACT T) 3' (fD1 50-AGAGTTTGATCCTGGCTCAG-30 and rD1 50-AAGGAGGTGATCCAGCC-30) for isolation and identification of causal bacterial species from total genomic DNA. On morphological basis the pathogen *Bacillus cereus* was re-isolated and reconfirmed from the artificially inoculated rice plant leaves through Koch's postulates. To our information *Bacillus cereus was* reported first time in Pakistan and majorly causing grain discoloration disease in rice. For controlling and management of this disease, the current study will be very useful for research scientific and farmers community.

Keywords: Rice Grain discoloration, DNA, *Bacillus cereus*, Disease, Pathogen

INTRODUCTION

Rice (*Oryza sativa* L) is an important staple food and grown on large area every year (Zhu *et al*., 2017). About 90% of rice produced and consumed in Asia and China and remains largest producing countries among the World (Li *et al*., 2015; Zahra *et al*., 2018). Rice is affected by number of diseases i.e. grain rot, panicle blight, brown spot, blast, bacterial leaf blight and grain discoloration. A complexity of pathogen involved for causing these diseases. Among them discoloration of rice causes serious reduction in yield in Pakistan and deformation of grain shape and size (Ashfaq *et al*., 2017). The severity of disease may be minor to major by abrupt changes in environmental conditions (Wasim *et al.*, 2002). Rice grain discoloration caused by both fungus and bacteria is an emerging as well as a potent threat in Pakistan that effects texture and grain quality (Rajappan *et al*., 2001).

This disease has ultimate effects on breaking of rice grains, the grain quality, weight loss, exports, crop yield potential, post-harvest losses and badly effect the economy (Chelliah and Gunathilagaraj, 2013). Rice grain discoloration deteriorates the morphology (size and shape) of grain and lowers the yield of rice crop. It also affects the shelling, milling, drying and processing of the rice (Yu *et al.*, 2008).

Throughout growing season pathogens of rice present on the phyllo plane and in stored seeds during winter at room temperature (Arshad *et al*., 2009; Baite *et al*., 2019). Other sources of infection include weeds which present in the rice field, rice crop residues buried in the soil and improper soil cultivation. Plant diseases are controlled by eliminating sources of contamination. Grain discoloration reduces crop yield every year. The bacteria causing rice grain discoloration includes *Burkholderia glumae* (formerly *Pseudomonas glumae* and *Bacillus cereus.* Bacteria are also found in discolored seed and involved in (28-80%) for causing grain discoloration (Chhabra *et al.*, 2020).

The symptoms include black or brown spots on grain and panicle are lighter in weight. Blackish stripes appear on grains. Firstly, lesions appear on the lemma that is light, rusty, water soaked and then turned into brown. It frequently occurs at early flowering stage. On panicles, grains that are immature and lighter in weight are present. In this disease, bacterial ooze is not present. Unfilled grains are present in infected panicle and its threats increasing

year by year (Wu *et al.,* 2012). Utilization and screening of resistant varieties, disease scoring, isolation and identification of actual causal organism provides the information to control and timely management of this disease.

The 16S rRNA gene is commonly used for successful identification of bacterial species and commonly present in all prokaryotes to classify the organisms at different taxonomic levels and identification of bacterial species (Sambo *et al.,* 2018). The accuracy of 16S rRNA sequencing strongly depends on the choice of the primer pairs obtained from in vitro culture species and this is most appropriate for identification of bacterial species (Kuczynski *et al*., 2012). Different16S universal primers are available for the identification of bacterial species. Herein, the *Bacillus Cereus* is responsible for causing grain discoloration of the rice crop and reported first time in Pakistan. The study will be very useful for scientists and farmers community for further evaluation of this disease.

MATERIALS AND METHODS

Rice disease sample collection and identification of pathogen

Different diseased rice samples were collected from the thirteen rice genotypes grown in University of the Punjab experimental field (Table 1). Seed morphology of selected rice varieties were shown in the Fig.1. Discolored panicles were collected from the field randomly from each genotype to determine the severity of disease on the basis discolored seeds (Fig. 2). Furthermore discolored seeds were placed on LBA media in petri plates for isolation of pathogen. The inoculated seeds cultures were kept at 25±2ºC in incubator for 7 days till the growth of bacteria appear and diseased seeds were sterilized with sodium hypochlorite solution (1.5%) for 1-2 minutes (Jan *et al.,* 2013). After this Luria Broth Agar (LBA) media was prepared in petri-plate to culture seed pathogen (Dhingra and Sinclair, 1995). The culture was transferred to the petri-plates having growth medium (LBA) to obtain fresh sample for further morphogenetic study of pathogen. On the other hand, Disease scoring was calculated by using the following formula:

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

Table 1. Plant material used in the experiment.

S.No	Plant name	Accession No	Taxon	Origin	Disease Reaction
1	IR-36	GSOR 301064 A11FA3 SD	*Oryzae sativa*	Philippines	Resistant
2	Jasmine-85	PI 595927	*Oryzae sativa*	China	Moderately Susceptible
3	Katy	PI 561735	*Oryzae sativa*	United States, Arkansas	Moderately resistant
4	Super Basmati	Approved variety	*Oryzae sativa*	Pakistan	Highly resistant
5	CB-42	1039	*Oryzae sativa*	Pakistan	Moderately Susceptible
6	NP-125	GSOR 310133 AO7FA3 SD	*Oryzae sativa*	USA	Susceptible
7	Sughdas	7620	*Oryzae sativa*	Pakistan	Resistant
8	IAGS-15	Advanced breeding line	*Oryzae sativa*	Pakistan	Highly resistant
9	Leah	GSOR 311045 A10FA3 SD	*Oryzae sativa*	United States, Louisiana	Susceptible
10	Zenith	Clor 7787 OR06 AR SD	*Oryzae sativa*	United States, Arkansas	Tolerant
11	IR-64	GSOR 311793 A10FA3 SD	*Oryzae sativa*	United States, Luzon	Resistant
12	CB-40	1038	*Oryzae sativa*	Pakistan	Tolerant
13	Sathi Basmati	GSOR 311134 AO7FA3 SD	*Oryzae sativa*	Pakistan, Punjab	Tolerant

Morphological features and other harmful effects of pathogen

Based on the morphology, 7 days old pure culture was carried out for characterization of isolated pathogen. Macroscopic characters of the bacterial colony were described i.e. dull gray to whitish and opaque colonies with rough matted surface, and have rod-shaped, endospore-forming aerobic Gram-positive bacteria are formed that are ubiquitous in nature (Fig. 3). The isolated pathogen was identified as 16S rRNA gene sequence and DNA was isolated as described by (Cheneby *et al*., 2004; Langille *et al*., 2013). After this the PCR product was completely sequenced and compared with the known sequences in the gene bank. On the basis of match resemblance our sequence 99% matches with *Bacillus cereus* (Altschul *et al*., 1997; Jan *et al*., 2019).

Genomic analysis of isolated pathogen

Universal primers were used to study the specific 16S rRNA gene region of isolated pathogen (Alwahshi *et al*., 2019). To ensure the good quality of DNA the simple PCR was conducted along the entire length of amplicon Crous *et al*. (2006). The samples were taken from established bacterial colonies for the isolation pathogen DNA (Gams *et al*., 2007). By using CTAB method the complete DNA of the pathogen was isolated (Wilson, 2001) and measured by Nano drop to check its quality and quantity. This DNA was used as template to amplify 16S rRNA gene in thermal cycler PCR (Weisburg, 1991) by using universal primer pairs 27F 5' (AGA GTT TGA TCM TGG CTC AG) 3 and 1492R 5' (TAC GGY TAC CTT GTT ACG ACT T) 3' and 785F 5' (GGA TTA GAT ACC CTG GTA) 3', 907R 5' (CCG TCA ATT CMT TTR AGT TT) 3' (White *et al.,* 1990; Bellemain *et al.,* 2010). The 16S rRNA gene was sequenced by Sanger's method through Macrogen (South Korea).

Pathogenicity test

Koch's postulates were used for confirmation of pathogenicity of isolated pathogen and tested on healthy plants. Plant leaves were taken from 1- month- old rice plants grown in earthen pots filled with clay loamy soil 35 cm in diameter. Sterile distilled was used for the preparation of spore suspension from freshly prepared isolate on LBA media. Control plants were injected with sterilized water and endospores were (1×10^5 spores/mL) injected using sterile syringe (0.5 mL/leaf) for inoculation of plants. Under controlled condition i.e. 30 °C, 80% humidity and 12 hours of photoperiod all the inoculated plants were kept in greenhouse for observation. Different necrotic spots (8 to 12 mm) i.e. oval shaped of blackish brown color were appeared after two weeks of inoculation. On morphological basis pathogen further reconfirmed with Koch's postulates.

RESULTS

Bacillus cereus the pathogen clearly confirmed the pathogenicity on basis of symptoms and morphology.

Morphological Traits

Bacterial colonies effuse, whitish cream, velvety, brownish white, reaching 3 to 4 cm in diameter with reverse whitish cream color. The growth characteristics recorded including presence or absence of precipitation zone, if applicable, and colony morphology after overnight incubation at 30°C. With desired information the pathogen was clearly identified as *Bacillus cereus*.

16S rRNA Gene Region Analysis

Universal primer pairs (forward and reverse) 27F 5' (AGA GTT TGA TCM TGG CTC AG) 3 and 1492R 5' (TAC GGY TAC CTT GTT ACG ACT T) 3' and 785F 5' (GGA TTA GAT ACC CTG GTA) 3', 907R 5' (CCG TCA ATT CMT TTR AGT TT) 3' were used to amplify the 16S gene region of genome of bacteria and approximately 1535bp DNA band sizes was perceived on 2 % agarose gel (Fig. 5). Gene sequence was BLAST the nucleotides sequence of 16S gene region using NCBI and the European Bioinformatics Institute (EBI) bioinformatics websites deposited in Gene bank.

DNA sequencing and 16S universal primers have been used to analyze the phylogenetic relationship of various bacterial species. For the identification of pathogenic bacteria PCR technology is the easiest, quickest and authentic approach. Such types of techniques are very useful for genomic fingerprinting, classification and identification of various species that rely on 16S universal primers. The genus bacillus is most famous and prevalent among bacterial genera.

According to BLAST analysis bacterial isolates were 99 % identical to nucleotide sequence showed (Fig. 4) with other cultures of *Bacillus cereus* in GenBank (Accession No. NR_115714.1). The sequence of our isolate indicated that the causing organism of grain discoloration of rice in Pakistan is *Bacillus cereus.*

Koch`s Postulates Confirmation

On the basis of morphological characters the plants showed similar symptoms after two weeks with those diseased plants that were observed primarily in the research field. By using Koch's postulates the pathogen was isolated and reconfirmed morphologically as *Bacillus cereus*.

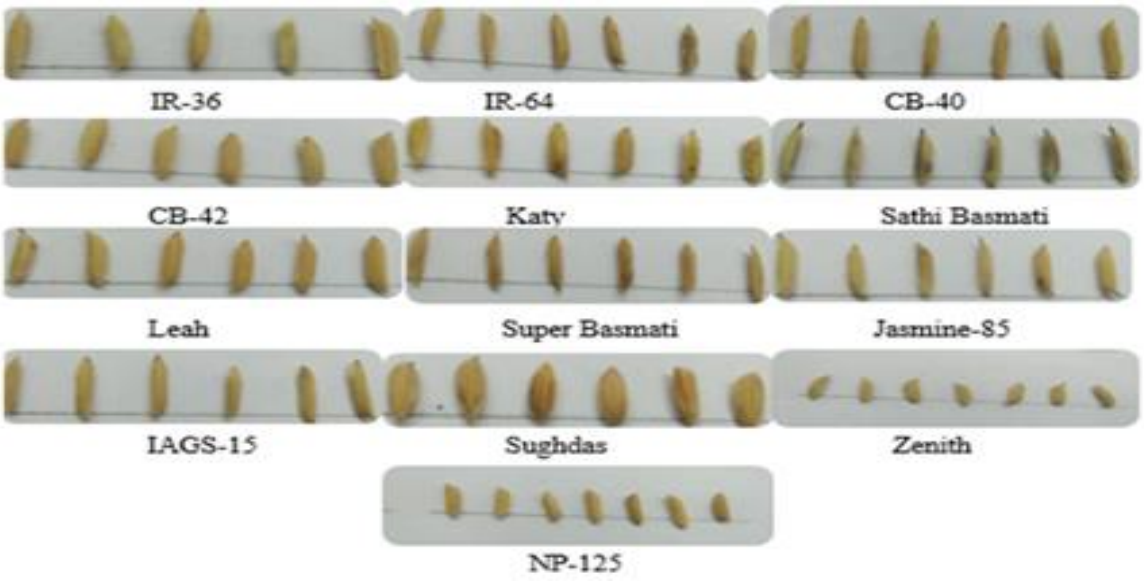

Fig.1 Morphology of different rice varieties.

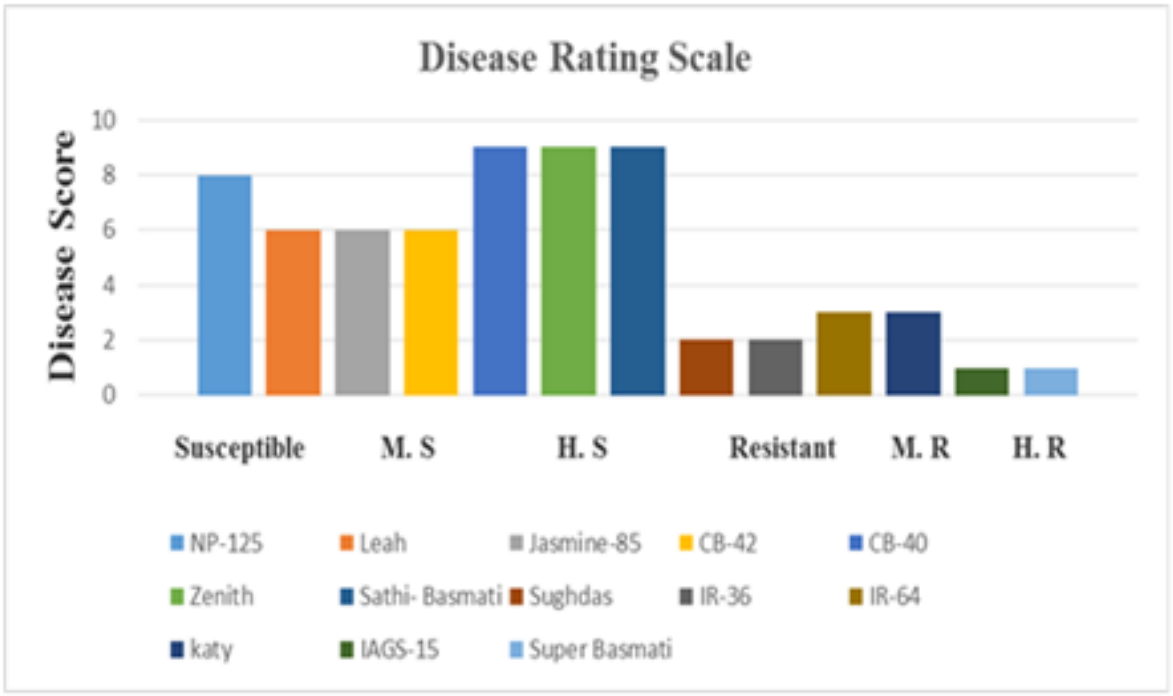

Fig.2. Disease rating scale of selected rice samples analyzed. Graph legend: M.S.= Moderately Susceptible, H.S.= Highly Susceptible, M.R. Moderately Resistant, H.R.= Highly Resistant.

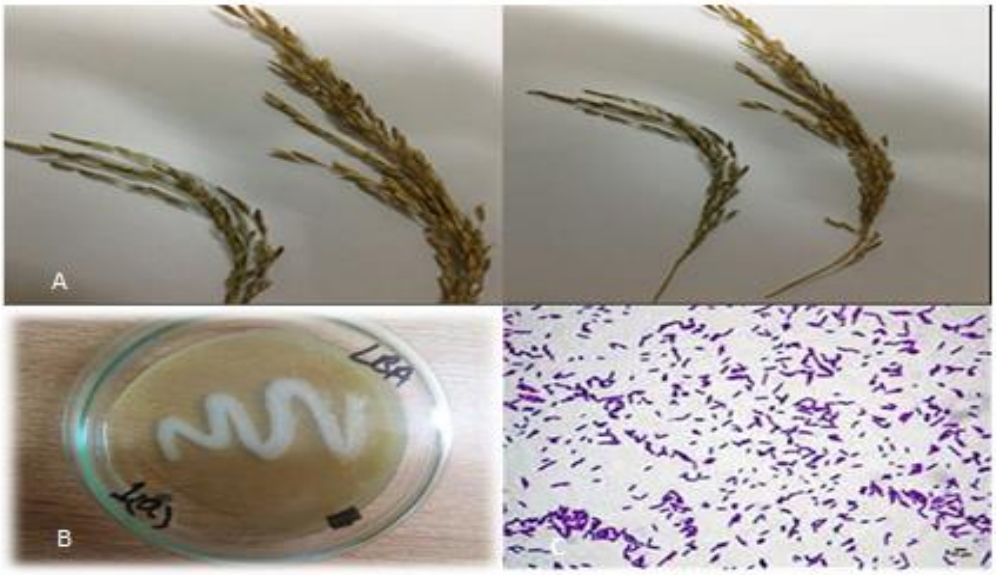

Fig.3. Rice grain discolouration symptoms at panicle stage, B: Bacterial growth of *Bacillus cereus* in petriplates, C: Grain staining of isolated bacterial species under compound microscope.

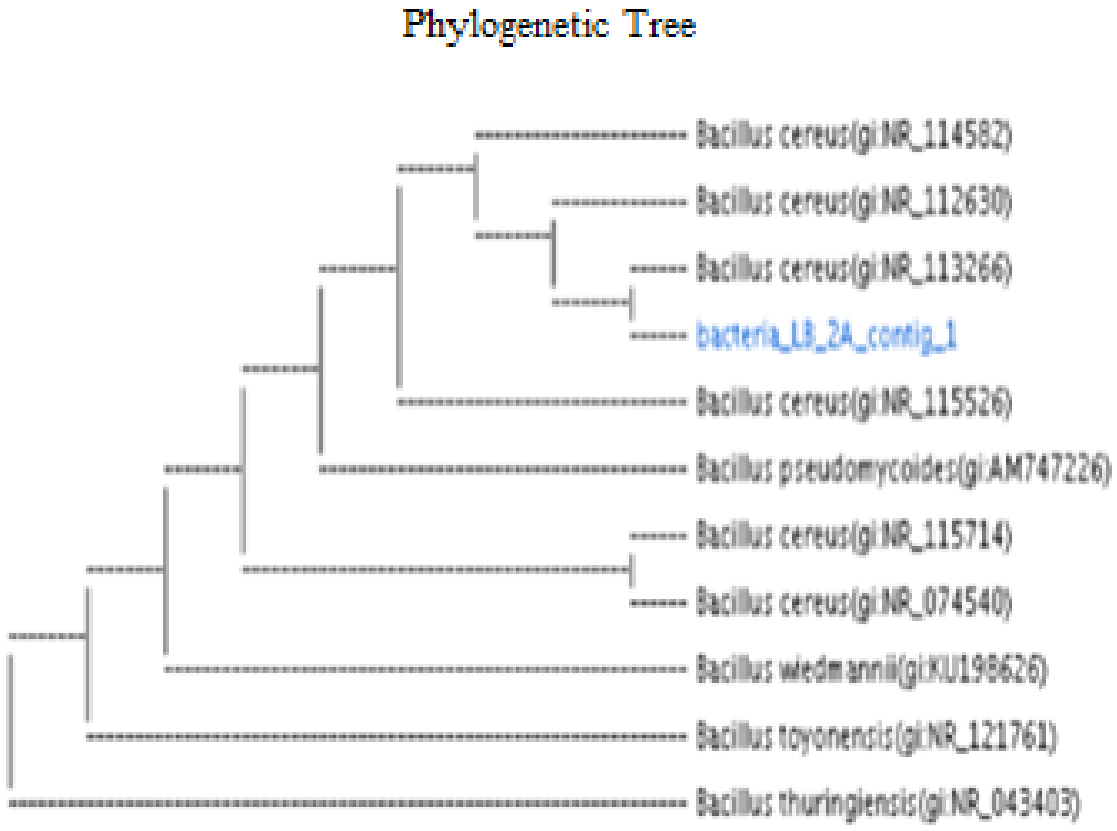

Fig.4. Phylogenetic tree of 1535 bp gene showing similarity with *Bacillus cereus* which confirms the identified species is *Bacillus cereus* (NCB1).

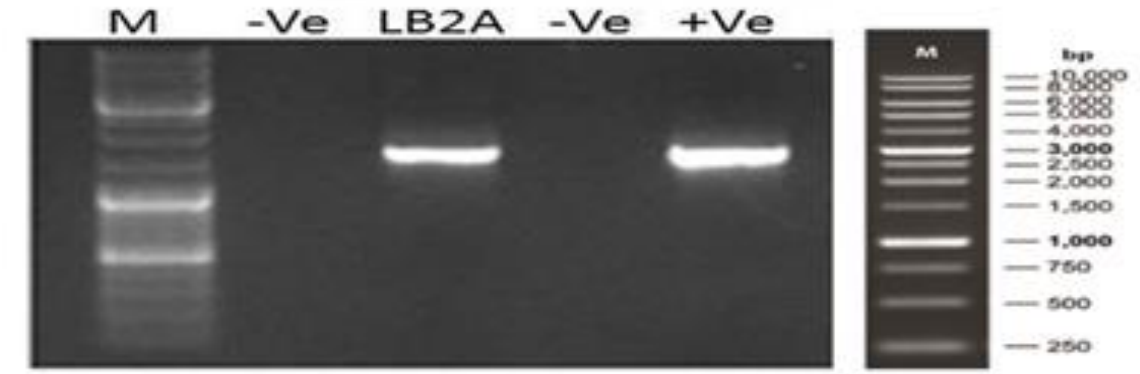

Fig.5. Gel picture showing one compact band of Amplified Internal Transcribed Spacer (ITS) region using polymerase chain reaction (PCR).

DISCUSSION

Rice grain discoloration affected the grain quality, quantity and texture which ultimately reduce the yield of the crop. Changes in panicle color, discoloration of rice seeds, spikes sterility, lemma palea and glumes discoloration are the symptoms of this disease. The panicles are infected with the presence of pathogenic cells in the sheath of the rice crop that causes the discoloration in rice. Different pathogens i.e. B. glumae and B. gladioli that can grow and survive in the leaf sheaths without showing any signs of disease (Bigirimana *et al*., 2015; Zarbahfi and Ham, 2019). At panicle emergence stage, which is the most sensitive stage for growing of pathogens and most important part for invasion of pathogen is lemma, palea and glumes. The pathogen invaded into intercellular spaces of grains and causing the disease infection. Grain discoloration is an emerging threat in the rice world, which causes a huge loss in yield every year. This disease is spreading very rapidly in every year. To control this disease we should improve the isolation and identification of real causal organisms (Eyre *et al*., 2019; Chhabra *et al*., 2020).

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 (Fig 1). 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. On morphological basis of various characteristics of the new pathogen *Bacillus cereus* was identified in almost all samples and all the selected isolates on the basis of purity were 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 rice varieties, after three weeks similar symptoms were observed like samples collected in 2017. These were further isolated and identified as *Bacillus Cereus.* The pathogen was further identified with DNA molecular markers (Joshi *et al*., 2006; Links *et al*., 2014).

Identification was done by 16S rDNA sequence analysis. DNA was extracted followed by amplification and sequencing of the ITS gene region by using universal primers (Joshi *et al*., 2006; Langille *et al*., 2013). The resulting sequence was deposited in GenBank (Accession No. NR_115714.1) and BLAST analysis revealed that the isolates were 99 % identical with other cultures of *Bacillus* species in GenBank (for instance Accession Nos. NR_115526, NR_113266 and 115714) (Fig. 4 and Fig. 5). On the basis of pathogenicity test and DNA sequencing test the pathogen was identified as *Bacillus cereus* (Cottyn *et al*., 2009), which causes grain discoloration of coarse and fine varieties of rice and ultimately affected the global rice yield (Yan *et al*., 2010; Chhabra *et al.*, 2020).

To spray inoculating panicles of coarse and fine varieties of rice were completed at grain filling stage with cell suspensions containing 1 x 10^8 spores/mL of single fresh strain at 25 to 29°C by using Koch's postulates. To validate the results plants were inoculated with each fresh strain and controls were sprayed with water. After inoculation all the plant panicles showed similar symptoms that observed in the field. From symptomatic panicles yellow pigmented bacteria were re-isolated and confirmed by pathogenicity test. These results indicates that the pathogen is *Bacillus cereus* (Cottyn *et al*., 2009), which causes grain discoloration of coarse and fine varieties of rice and ultimately affected the global rice yield. To our information, *Bacillus Cereus* reported first time in Pakistan that

caused grain discoloration in rice. The disease cycle on rice crop and different management strategies in the regions are being further studied to control this disease for the improvement of rice yield.

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CONCLUSION

Our isolate is completely similar morphologically symptoms similarity in Koch's postulate confirmed that the *Bacillus Cereus* is causing organism of grain discoloration of rice in Pakistan. This first report on grain discoloration in rice will be used to control and manage the disease in Pakistan.

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