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Molecular diagnosis of red rot of sugarcane in Pakistan and *in vitro* control of isolated pathogen using rhizobacteria

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Abstract: Sugarcane (*Saccharum officinarum* L.) is a key cash crop, and its production is influenced by a variety of phytopathogens in different parts of the world. During consecutive field surveys, sugarcane stalks with red rot symptoms were observed in three provinces of Pakistan (Punjab, Sindh, and Khyber Pakhtunkhwa). Cane samples with visible symptoms were plated on potato dextrose agar (PDA) media. Morphological and microscopic observations identified this pathogen as *Colletotrichum falcatum.* Comparative molecular study of amplified sequences of β-tubulin (Bt) and actin (ACT) genes showed 100% similarity and ITS sequence showed 99% similarity with *C. falcatum.* For *in vitro* control of red rot, eight bacterial strains from the rhizosphere of sugarcane were isolated. These strains showed variable growth inhibition of *C. falcatum* in dual culture method. Among all tested strains, *Bacillus amyloliquefaciens* and *B. altitudinis* exhibited best antifungal activities. Based on these results, tested bacterial strains (*B. amyloliquefaciens* and *B. altitudinis*) can be recommended as effective biocontrol agents to manage red rot disease of sugarcane.

Keywords: phytopathogens; *Colletotrichum falcatum*; Actin; *Bacillus amyloliquefaciens*; *Bacillus altitudinis*

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Sugarcane is the biggest source of commercial sugar, and it accounts for more than two-third of worldwide sugar production (Menossi et al. 2008). It is grown on more than 26.3 million hectares across the world and according to recent statistics, 1.9 billion tons of sugarcane are produced, annually (FAO 2018). The sugarcane cultivating countries of the world are situated between 36°N and 31°S latitudes on the equator, spanning tropical and subtropical zones. Brazil is the world's biggest producer of sugarcane, accounting for 40% of global output, followed by India, China, Thailand, Pakistan, Mexico, Philippines, United States of America, Australia, and Argentina (FAO 2018). Sugarcane is the second-largest cash crop of Pakistan, with 0.966 million hectares under cultivation and generating approximately 3.6% of the country's GDP (Federal Bureau of Statistics, Government of Pakistan 2005). In the last 50 years, the sugarcane area has escalated by 310%, productivity has increased by 566%, cane yield has climbed to 200%, and sugar recovery has enhanced from 7.50% to 8.70% (Qureshi & Afghan 2005).

Several biotic and abiotic factors influence sugarcane production in different parts of the world (Malathi et al. 2011). About 200 different diseases of sugarcane have been reported to be caused by viruses, fungi, bacteria, phytoplasmas and nematodes. Diseases caused by viruses are the main threat to worldwide sugarcane production, causing massive outbreaks and losses. Mosaic is a viral disease that affects sugarcane, maize, sorghum, and other members of the *Poaceae* family. Red rot, smut, sett rot, and wilt are the most widespread fungal diseases that cause considerable damage to sugarcane yield in the field. Bacterial diseases such as red stripe and leaf scald also drastically affect sugarcane yield (Gonçalves et al. 2012).

Among all reported diseases of sugarcane, the fungal disease, classified as red rot of sugarcane is one of the most destructive diseases and is called the "cancer" of sugarcane. This disease has a substantial impact on sugarcane yield and quality. *Colletotrichum falcatum* Went [Perfect state: *Glomerella tucumanensis* (Speg.)] (von Arx & Müller 1954), the causal organism of red rot disease is very diverse and often destroys resistant varieties of sugarcane (Ruchika & Sushma 2015). In most sugarcane-producing areas, red rot is the most significant obstacle to cane yield. The inoculum of the pathogen stays in the soil and is transmitted by sugarcane root borer and irrigation water, resulting in primary and secondary infections. Initially, this disease spreads through the soil and later it is transmitted through injury caused by poor cultural practices including borer galleries formed in the stem to extract the cane contents, as well as via a variety of certain other vectors. Red rot has a terrible impact on sugarcane production in countries such as Pakistan, Thailand, Australia, Fiji, Bangladesh, and United States of America. Overall, this disease has been reported in more than 77 countries (Putra & Damayanti 2012). Reddish spots on the leaf sheaths, elongated red lesions on the midribs, and often tiny black dots on the leaf blades are visible indications of red rot (Olahan et al. 2020)

There are three primary approaches to suppress disease: (I) usage of a resistant cultivar, (II) fungicide treatment (III) biological control via microorganisms that are hostile to one another (Patel et al. 2019). Nowadays, the need for organic fertilizers is steadily increasing (Campbell & Campbell 1989). The control of phytopathogens through biological means involves a variety of methods that impede the survival of the pathogen. The use of rhizobacteria as biocontrol agents is thought to be a long-term solution for disease management (Srivastava et al. 2016). Several plant-associated rhizobacteria from various genera, such as *Bacillus* have gained importance in recent years due to their capability to control diseases caused by soil-borne pathogens in many crops (Cazorla et al. 2007; Romero et al. 2007). Several rhizobacteria have also been found to manage and suppress various fungal diseases of sugarcane. The objectives of this study were to evaluate the incidence of red rot, isolate red rot pathogen, and control its spread by using different rhizobacteria.

MATERIAL AND METHODS

Field survey. To see the status of sugarcane red rot, extensive field surveys were conducted in major sugarcane-producing areas of three provinces (Punjab, Sindh, and Khyber Pakhtunkhwa) of Pakistan (Table 1). Random sampling of diseased samples was done from different farmer and sugar mill fields. Following previous studies, Z scheme was followed for plant sampling (Teng 1983). Ten plant samples were collected from each randomly selected site/field. Google Earth was used to label the sample locations (Figure 1). Collected plant

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Table 1. List of districts surveyed across provinces Punjab, Sindh, and Khyber Pakhtunkhwa (KPK)

S. No.	KPK	Punjab	Sindh
1	Swabi	Faisalabad	Sanghar
2	Charsadda	Lodhran	Badin
3	Mardan	Layyah	Shaheed Benazirabad
4	Nowshera	Multan	Mirpur Khas
5	Peshawar	Jhang	Tando Allah Yar
6	–	Muzaffargarh	Naushahro Feroze
7	–	Rahimyar Khan	Tando Muhammad Khan
8	–	Toba Tek Singh	Sukkur
9	–	Bahawalpur	Ghotki
10	–	–	Larkana
11	–	–	Shikarpur
12	–	–	Khairpur
13	–	–	Hyderabad
14	–	–	Kashmore

samples were placed in iceboxes and transported to the lab, for further analysis.

Incidence of disease. Based on symptomatic data, the incidence of red rot disease was estimated. Following previous protocol (Nayyar et al. 2017), symptoms were observed as red rot lesions along the stalk length, nodal transgression, and white spots. Here, the incidence referred to the number of new disease occurrences, which may be stated as a risk or as an incidence rate (Noordzij et al. 2010). Using the below formula, the incidence of disease was determined:

$$\text{Disease incidence \%} = \frac{\text{number of diseased plants}}{\text{total plants observed}} \times 100 \quad (1)$$

Isolation of disease-causing fungus. Diseased samples were observed in the field and infected cane samples were collected and sliced open with a sterilized blade to assess the typical red rot symptoms. Reddish tissues extending throughout the stem and transverse white spots were seen in the cross-section of infected canes. For the isolation of disease-causing pathogen, a previously described protocol was followed (Hossain et al. 2021). The diseased cane tissues were chopped into 2–3 mm small pieces and surface-sterilized with 5% Clorox for 30–60 seconds. The infected pieces of tissue were rinsed with distilled water, wiped on blotting paper, and placed on potato dextrose agar (PDA) slants at 28 ± 2 °C. Later, the fungus was isolated from a single spore on Petri plates containing PDA.

Morphological and microscopic identification of isolated pathogen. After 10 days of incubation, colony characteristics, colour, topography, and margin shape were recorded (Sangdit et al. 2014). Under a light microscope, the conidia length and width were measured. Mycelial growth and morphology were observed at 40× magnification.

Extraction of genomic DNA. Following a standard protocol, the fungal DNA was extracted (Lin et al. 2009). For this purpose, the fungal mycelia of each strain were picked carefully, crushed, and homogenized in 500 µL of extraction buffer (1 M KCl, 100 mM Tris-HCl, 10 mM EDTA). Af-

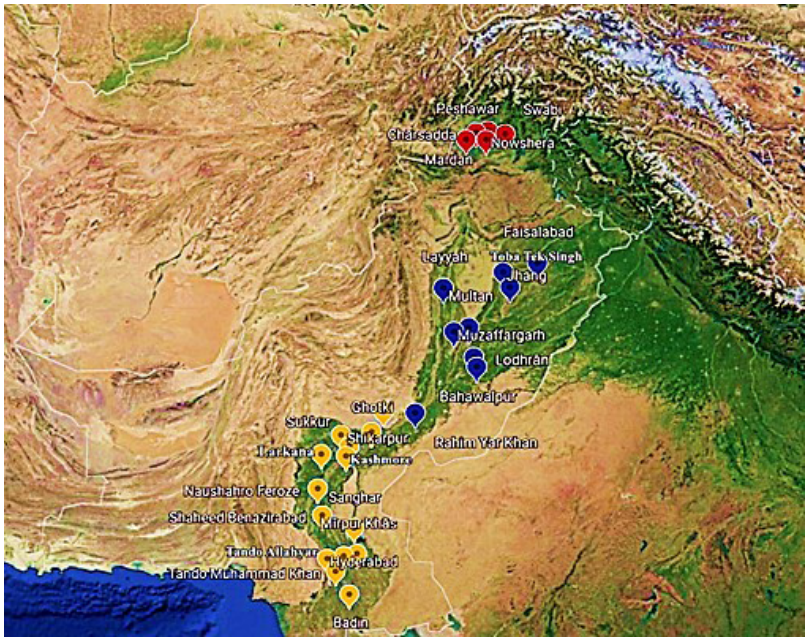

Figure 1. Diseased sample collection from different regions of Punjab province (blue labels), Sindh province (yellow labels) and Khyber Pakhtunkhwa province (red labels)
Map was created using Google earth computer program

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ter disintegration, the culture was centrifuged at 10 000 rpm for 7 minutes. In the supernatant, 300 µL of isopropanol was added and centrifuged for 10 min at 12 000 rpm. The supernatant was removed, and the pallet was washed with 70% ethanol. The tubes were air-dried, and the DNA pellet was suspended in nuclease-free water. The purity of DNA was checked, and its concentration was adjusted at 20 ng/µL.

PCR amplification and phylogenetic analysis. For the identification of isolated pathogen, purified DNA was used as template to amplify ribosomal RNA gene (White et al. 1990) and two protein coding genes viz. β-tubulin (Bt) (Wang et al. 2014) and actin (ACT) (Carbone & Kohn 1999), using specific gene primers ITS-1 (CTTGGTCATTTAGAGGAAGTAA), ITS-4 (TCCTCCGCTTATTGATATGC), Bt-1 (AACATGCGTGAGATTGTAAGT), Bt-2 (ACCCTCAGTGTAGTGACCCTTGGC), and ACT-F (ATGTGCAAGGCCGGTTTCGC), ACT-R (TACGAGTCCTTCTGGCCCAT), respectively. For this purpose, PCR reactions were performed in 10 µL volume using 5 µL Hot start master mix, 1 µL DNA template, 0.5 µL (10 pmol) of each forward and reverse primer, and 3 µL of nuclease-free water. Initial denaturation was performed at 95 °C for 2 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 56–58 °C for 30 s, extension at 72 °C for 45 s, and final extension at 72 °C for 10 minutes. The amplified PCR products were observed using a UV transilluminator after staining with ethidium bromide in 1.2% agarose gel. The sequencing of the PCR product of one random strain (S2S1) was performed (Macrogen sequencing services, South Korea). The obtained sequences were aligned using BLAST of NCBI database (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to validate the genera to which the isolated sequence belonged. Mega-11 software was used to investigate the phylogenetic and evolutionary relationships of the obtained sequence.

Isolation of bacterial strains. Soil from the rhizosphere of healthy sugarcane plants growing near symptomatic plants was obtained. Bacteria were isolated from soil samples on Luria–Bertani (LB) agar using the serial dilution technique. Serial dilutions ranging from 10^{-1} to 10^{-6} were obtained by dissolving 1 g of strongly adherent soil with roots in 9 mL saline. On LB agar plates, 100 µL of each dilution was poured and incubated at 28 °C. Distinct colonies of bacteria that grew on LB agar after 24–72 h of incubation were selected and subcultured on new LB agar plates to isolate pure colonies of bacteria.

Gram staining. The gram staining method was used to differentiate gram-positive and gram-negative bacteria (O'Toole 2016). In the first step, the initial staining of the slide was performed with crystal violet dye. To avoid colour removal, iodine was used to create a crystal violet-iodine complex. Using acetone, the dye was removed and safranine was applied to the smear for 2 minutes. Finally, the slide was examined under a light microscope at 40× magnification.

Antagonism assay of bacteria. Antagonistic activity of all bacterial strains was tested against the isolated fungal pathogen using dual culture technique (Anith et al. 2003). The three diagonals of the PDA Petri plate were streaked with bacterial isolates. In the center, a plug of fungal mycelia was placed, equidistant from the bacterial isolate. PDA plate inoculated only with fungus was considered as control. The antagonistic activity of each bacterial strain was tested by measuring the growth inhibition and growth rate of the fungus after 72–120 h of incubation at 28 ± 2 °C. Using the below formulas, the percentage inhibition was computed:

$$\text{Replicate wise inhibition \%} = \frac{C - T}{T} \times 100 \quad (2)$$

$$\text{Strain wise inhibition \%} = C - \frac{T1 + T2}{2} / C \times 100 \quad (3)$$

where: C – the colony diameter of fungus in control; T – the colony diameter of fungal pathogen; $T1$ and $T2$ – the first and second replicates of the fungal pathogen.

Based on the antagonistic potential of eight tested bacterial strains, the two best strains were used for molecular characterization and rDNA sequence-based identification.

Molecular identification and phylogenetic analysis of bacterial isolates. The GeneJET Genomic DNA extraction Kit (catalog number K0721) was used to extract total genomic DNA. Multiskan TM GO Microplate Spectrophotometer was used to evaluate the purity of DNA at wavelengths A260/A280. The concentration of the purified DNA was adjusted between 20 ng/µL. PCR amplification of the 16S-rDNA regions of two best-performing bacterial isolates was performed using bacterial universal forward B16SF

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(AGAGTTTGATCCTGGTCAGAACGAACGCT) and reverse B16SR (TACGGCTACCTTGTTACGACTTCACCCC) primers. PCR mixture (10 µL) was comprised of 5 µL Hotstart master mix, 1 µL DNA template, 0.5 µL (10 pmol) of each forward and reverse primer, and 3 µL nuclease-free water. PCR was performed with an initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 30 s, and final extension at 72 °C for 10 minutes. The amplified PCR products was sequenced (Macrogen sequencing services in Korea). To authenticate the genera to which the isolates belong, the sequences were subjected to BLAST analysis on the NCBI database (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The obtained sequences were deposited in the GenBank.

RESULTS

Incidence of disease. Overall, the incidence of sugarcane red rot was recorded in the range of 10.0 to 45.6%, across all surveyed districts of Punjab, Sindh, and Khyber Pakhtunkhwa (KPK) (Figure 2). The maximum incidence of red rot was found in Rahimyar Khan (45.6%), followed by Sukkur (29.0%) and Ghotki (27.5%). The districts of Badin and Hyderabad had the lowest red rot incidence (10.0%).

Morphological and microscopic identification of the isolated pathogen. Isolated pathogen developed whitish grey (light type), dark grey (dark type), usually flat, raised fluffy, and some less fluffy colonies (Figures 3A, 3B). Under the light microscope at 40× magnification, all conidia appear falcate-shaped while the mycelia of all strains are septate (Figure 3C). Except one isolate with irregular margins, all isolates had smooth margins (Table 2).

PCR amplification and phylogenetic analysis. PCR reaction successfully amplified desired genes of isolated pathogen (Figure 3D). Amplified sequences of Bt and ACT genes showed 100% similarity with *Colletotrichum falcatum,* GenBank accession numbers MW001869.1 and MK867398.1, respectively. The obtained ITS sequence showed 99% similarity with IR-06 isolate of *C. falcatum* (AB242422.2) (Figure 4).

Gram staining. Under the light microscope, gram-negative bacteria had a pale to dark red appearance while gram-positive bacteria showed dark purple appearance (Figure 5). Among eight tested bacterial strains, only one was gram-positive (Table 3).

Antagonism assay of bacterial strains. In this study, bacterial isolates exhibited variable growth inhibition of *C. falcatum* (Figures 6A–6D). The bacterial strain ZAB3 demonstrated the highest level of inhibition (76.7%), followed by ZAB1 (65.1%), FS-9 (62.5%), and GS14 (60%) (Table 3).

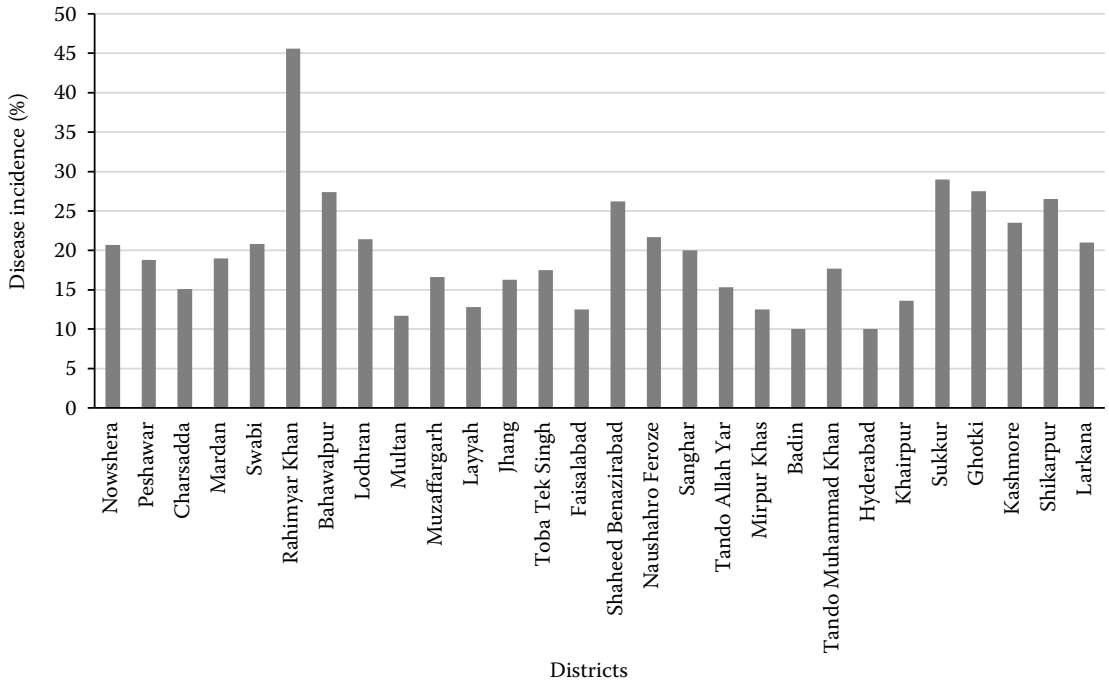

Figure 2. Disease incidence of sugarcane red rot in Pakistan

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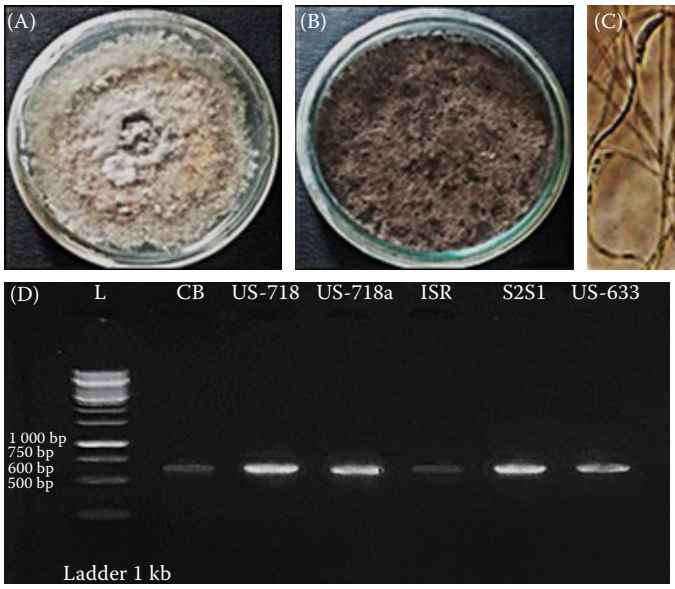

Figure 3. Morphological, microscopic and molecular identification of isolated fungus
(A and B) Growth pattern of fungus on PDA media (light and dark colonies of isolated fungus), (C) conidia and mycelia of fungus under light microscope at 40× magnification and (D) PCR amplicons of ITS-1F/ITS-4 region of isolated pathogen

Table 2. Colonies characteristics of isolated pathogen

Strain code	Colony colour	Topography	Margin
CB	greyish white	flat	smooth
US-718	dark grey	less fluffy	smooth
US-718a	dark grey	less fluffy	smooth
ISR	grey	flat	smooth
S2S1	whitish grey	raised fluffy	irregular
US-633	greyish white	flat	smooth

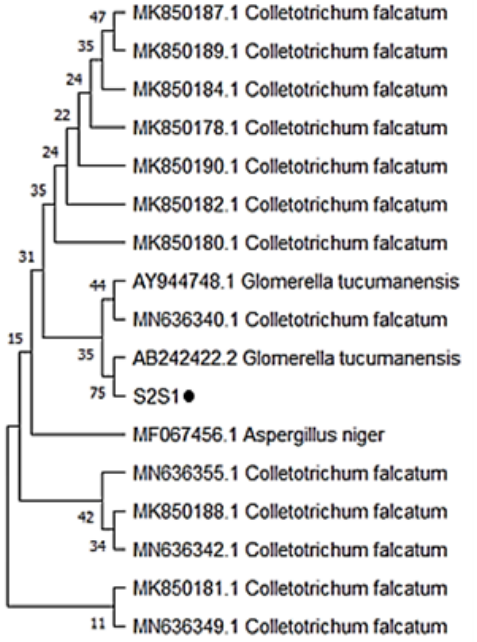

Figure 4. At 1 000 bootstrap value, the Neighbor-Joining approach was used to infer the evolutionary relationship of ITS sequence of isolated pathogen (S2S1) with 15 related sequences; *Aspergillus niger* was used as outgroup taxon

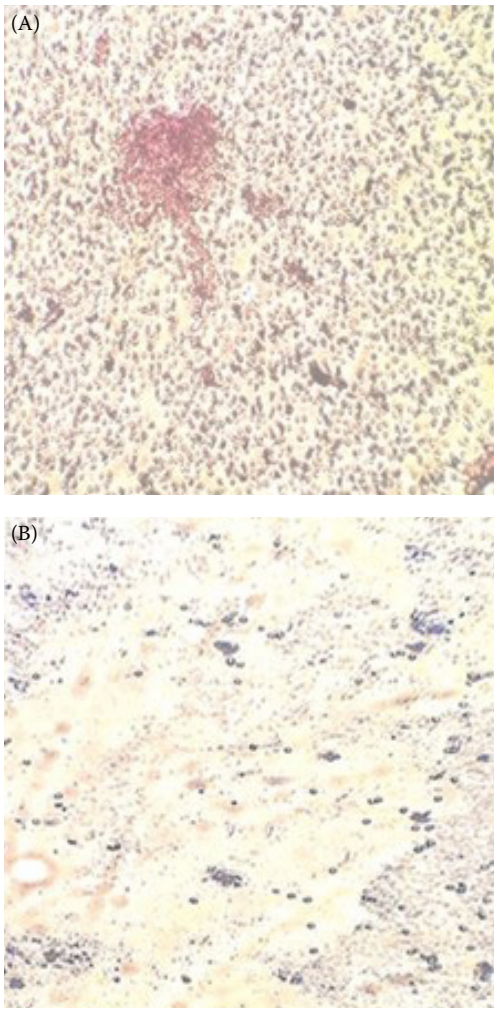

Figure 5. Microscopic identification of bacterial strains
(A) Gram-negative bacteria and (B) Gram-positive bacteria

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Table 3. Results of gram staining and mycelial growth inhibition of bacterial isolates

Strain code	Gram staining	Mycelial growth inhibition (%)
GS-14	Gram negative	60
FS-11	Gram negative	48
FS-15	Gram positive	42.8
ZAB1	Gram negative	65.1
FS-30	Gram negative	11
FS-12	Gram negative	18.1
ZAB3	Gram negative	76.7
FS-9	Gram negative	62.5

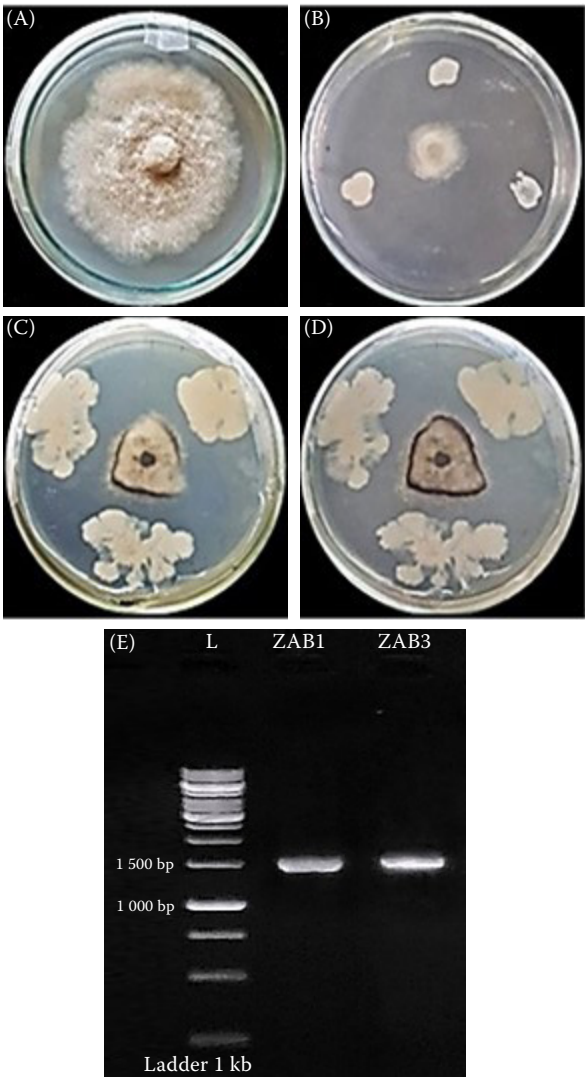

Figure 6. Growth inhibition and molecular identification of bacterial isolates

(A) In the absence of any bacterial inoculation, fungal growth was normal, (B) significant growth inhibition of fungal mycelia was observed after three days, (C) five days, (D) seven days of dual culture, (E) PCR amplicons of 16SrDNA region of bacterial isolates

Molecular identification and phylogenetic analysis of bacterial isolates. PCR successfully amplified the desired fragment (1 500 bp) in both isolates (Figure 6E). BLAST of isolated strains showed similarity with two different species of *Bacillus.* ZAB1 showed 99% similarity to *B. amyloliquefaciens* (KKU1) (GenBank accession number MH114079.1) while ZAB3 was 99% similar to *B. altitudinis* (T86) (GenBank accession number KC764971.1) (Figure 7). The sequences of strains

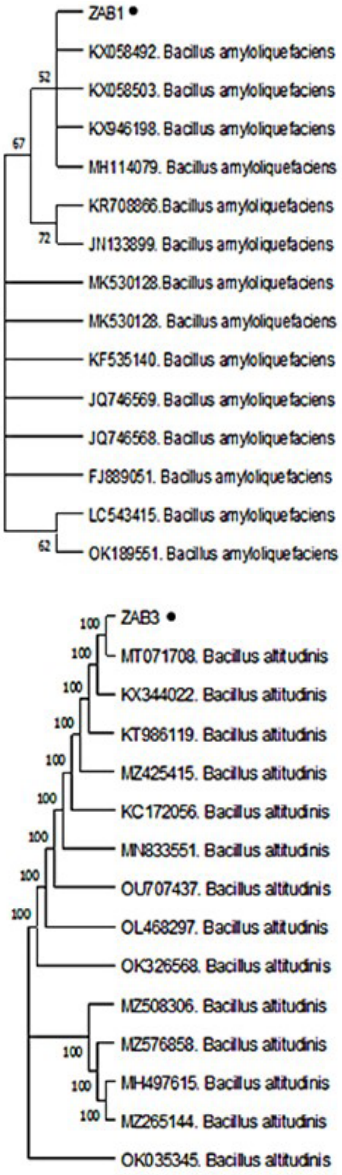

Figure 7. The Neighbor-Joining approach was used at 1 000 bootstrap value to infer the evolutionary relationship of the sequence of two isolated bacterial strains with related sequences

Escherichia coli was used as outgroup taxon. Isolated strains of current study have been highlighted using bullets (•)

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ZAB1 and ZAB3 were deposited in GenBank with accession numbers OL721873 and OL7218-73, respectively.

DISCUSSION

Red rot is referred as one of the most devastating diseases of sugarcane. In this study, field surveys described the status of sugarcane red rot in Pakistan. The isolated pathogen was identified as *Colletotrichum falcatum.* This pathogen has been reported to infect the leaf, stalk, roots, and buds of the sugarcane plant. The disease severity is increased when the pathogen attacks the sugarcane stem. Infected sugarcane spreads the disease from one place to another (Malathi et al. 2011). A survey of prominent sugarcane-producing areas in Punjab, Sindh, and KPK suggested moderate incidence of sugarcane red rot. In all three provinces, disease severity was not evenly distributed throughout the fields. This is because many resistant cultivars are planted in commercial fields to reduce the disease.

C. falcatum isolates displayed variable morphology. Concerning pathogenicity, variation in *C. falcatum* has been characterized culturally as light and dark isolates at the beginning of the 20th century (Abbott 1935). The colour of colonies ranged from whitish grey to dark grey. Most isolates had a flat, less fluffy topography, and just a couple are raised fluffy. Previous studies have also described variable morphological and cultural features of *C. falcatum* (Prema et al. 2013). Although *C. falcatum* isolates are classified based on the morphological and colony features, these methods are neither accurate nor reliable because of several environmental conditions that influence fungal colony and morphological characteristics. To further validate the results, the molecular basis of *C. falcatum* was studied. The molecular approach is known for allowing quick identification of isolates as well as demonstrating the relationships between fungal species (Shahnazi et al. 2013).

Several strategies have been formulated to manage red rot disease, but none of them have been successful. One of the main causes is that the fungus evolves into new races (Kumar et al. 2011). Microorganisms with rhizosphere colonization and biocontrol potential might play a major role in crop protection against soil-borne plant diseases (El-Hassan & Gowen 2006). Biological management using rhizobacteria is a cost-effective and simple way to manage soil-borne diseases (Whipps 2001). Nowadays, several *Bacillus* species have attracted much importance for their biological management of many fungal diseases. *B. amyloliquefaciens* has the potential to be an effective biocontrol agent against *F. oxysporum* f. sp. *spinaciae* (Zhao et al. 2014). *B. subtilis*, a soil bacterium isolated from the Chili rhizosphere, displayed excellent antagonistic activity against *C. gloeosporioide* (Ashwini & Srividya 2014). *B. velezensis* isolated from endogenous root tissue of olive trees exhibited the best antifungal activity under *in vitro* conditions against *Verticillium dahliae* with a 92% inhibition rate (Azabou et al. 2020). The current research was effective in identifying the two most promising bacteria (*B. amyloliquefaciens* and *B. altitudinis*) from the rhizosphere of sugarcane. The *in vitro* results, together with the bacteria's molecular identification by sequencing the 16S rRNA gene, revealed that the chosen rhizobacteria are taxonomically distinct and can suppress the spread of sugarcane red rot sugarcane.

CONCLUSION

Results of this study revealed that *B. amyloliquefaciens* and *B. altitudinis* have the potential to control the growth of *C. falcatum*. These two bacterial strains can be considered effective biocontrol agents to manage the red rot disease of sugarcane.

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