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Research Article

MOLECULAR CHARACTERIZATION AND ANTAGONISTIC ACTIVITY OF *BACILLUS* STRAINS AGAINST *SCLEROTIUM ROLFSII*

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ABSTRACT

The devastating soil-borne pathogen *Sclerotium rolfsii* has become increasingly significant worldwide, affecting several crops. However, biological disease management, particularly using antagonistic microbes, offers an environmentally friendly alternative to chemical control. *Bacillus* species, in particular, have demonstrated strong antagonistic potential against various fungal pathogens. In this study, the morpho-microscopic and biochemical characterization of *Bacillus* isolates obtained from rhizosphere soil was conducted. Furthermore, their antagonistic activities were assessed *in vitro* using dual culture and well diffusion techniques against *S. rolfsii*. During primary screening, four isolates viz. S3C9, S1C9, S2C9, and S6C8 strongly suppressed fungal mycelial growth, with inhibition zones measuring 18.3, 14.7, 14.3, and 14.7 mm, respectively. Three isolates (S2C9, S3C9, and S4C9) were further evaluated for inhibitory properties using cell-free supernatant through the well diffusion method, yielding inhibition zones of 6.3, 4.7, and 4.7 mm, respectively. Moreover, molecular analysis identified two isolates, S1C9 and S3C9, as strains of *Bacillus subtilis*, with GenBank accession codes OP962345 and OQ180051, respectively. This study provides evidence that the local soil of Kot Diji, Sindh Province, Pakistan, harbors beneficial microbes with significant antifungal activity, which could be utilized for future biocontrol strategies against phytopathogenic fungi.

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INTRODUCTION

Various pathogens, including viruses, bacteria, fungi, and nematodes, pose significant threats to crop productivity by causing severe plant diseases, leading to substantial yield losses (Etesami et al., 2023). Among these, fungal pathogens are particularly destructive, reducing global crop yields by approximately 15% annually (Doty et al., 2022).

Sclerotium rolfsii is a versatile soilborne saprotroph that functions as a facultative parasite, infecting over 500 plant species, including tomato, chili, sunflower, cucumber, eggplant, soybean, maize, groundnut, beans, and watermelon (Veena et al., 2025). It causes various plant diseases, such as collar rot, Sclerotium wilt, stem rot, charcoal rot, seedling blight, damping-off, foot rot,

stem blight, and root rot (Veena et al., 2025).

In particular, *S. rolfsii* is responsible for southern blight in chilies and stem rot in groundnuts (Iquebal et al., 2017; Javaid et al., 2020). It also causes collar rot in cotton, sugarcane, tomato, and potato (Billah et al., 2017). Recent reports indicate that *S. rolfsii* induces stem rot in peanuts, potentially reducing crop yields by 10% to 40% (Dodia et al., 2019).

Plant pathogen control can be achieved through the extensive application of fungicides; however, their excessive use may have adverse effects on the environment and human health (Keinath and DuBose, 2017; Iqbal and Mukhtar, 2020a). On the other hand, non-chemical methods, including biocontrol agents, have been widely used for disease management in crops (Sharf et al., 2021; Saeed et al., 2023; Aziz et al., 2024). Biocontrol agents are often cost-effective and environmentally safe compared to synthetic chemical treatments (Sunil and Thara, 2022; Mukhtar et al., 2013; Iqbal and Mukhtar, 2020b).

Recently, plant growth-promoting rhizobacteria (PGPR) have gained significant attention in modern agricultural systems due to their ability to enhance plant growth and soil health (Mohamed et al., 2019; Shahzaman et al., 2015). PGPR employ distinct mechanisms of action, including antibiosis, hyperparasitism, competition, and induced systemic resistance (Verma et al., 2019). Several bacterial genera, such as *Azospirillum, Alcaligenes, Arthrobacter, Burkholderia, Bacillus, Enterobacter, Klebsiella, Pseudomonas*, and *Serratia*, have been reported to exhibit plant growth-promoting activities (Kour et al., 2019). Among these, *Bacillus, Burkholderia, Pseudomonas*, and *Streptomyces* are well-documented for their roles in PGPR activities. Notably, *Bacillus* and *Pseudomonas* have been recognized as key biocontrol agents due to their strong antibiosis activity against various fungal pathogens (Karimi et al., 2012; Guenoun et al., 2019; Hadri et al., 2019).

In particular, *Bacillus* species play a crucial role in antimicrobial activity. Cyclic lipopeptides, such as fengycins, iturins, and surfactins, are essential antimicrobial compounds that contribute to fungal antagonism (Nihorimbere et al., 2010). Moreover, antagonistic *Bacillus* strains produce various hydrolytic enzymes, including amylase, cellulase, protease, lipase, and chitinase, which enhance their biocontrol potential (Shekhar et al., 2006). Some *Bacillus* species can also produce volatile and non-volatile antimicrobial compounds that are highly effective against fungal pathogens (Athukorala et al., 2010).

Bacillus species may secrete phenolic compounds (secondary metabolites) that inhibit mycelial growth (Gao et al., 2017). *Bacillus subtilis* is widely used in agriculture due to its environmental safety, industrial applicability, and biocontrol activity (Wang et al., 2020). It exhibits key biocontrol traits, including plant growth promotion, root colonization, activation of induced systemic resistance, and pathogen suppression (Hashem et al., 2019).

Studies have also demonstrated the role of *B. subtilis* in biofilm formation on plant root surfaces and its effectiveness in controlling plant diseases (Gao et al., 2013; Al-Ali et al., 2018). The genus *Bacillus* plays a crucial role in managing peanut stem rot and bean root rot caused by *S. rolfsii* (Gholami et al., 2014; Chen et al., 2020). Among various isolates, *B. subtilis* strain B4 has exhibited the strongest antagonistic effect against *S. rolfsii*, significantly inhibiting fungal mycelium compared to isolates B7, B8, and B10 (Ahmad et al., 2019). Several studies have evaluated the antagonistic activity of *B. subtilis* strains against *S. rolfsii* (Kumari et al., 2021). Jia et al. (2023) reported that *Bacillus* strains CB13 and CB19 significantly suppressed fungal mycelial growth. A recent study found that *Bacillus* spp. inhibited *A. rolfsii* growth by 80% *in vitro* (Wang et al., 2024).

These findings prompted us to isolate and evaluate *Bacillus* strains from local soil in the Kot Diji region for their antagonistic activity against *S. rolfsii*. The primary objectives of this study were to isolate and identify rhizospheric bacteria with strong antifungal properties and to molecularly characterize the most effective isolates. Our findings provide a foundation for the potential use of rhizospheric *Bacillus* spp. as an eco-friendly alternative to chemical treatments for managing fungal diseases in local crops in Sindh, Pakistan.

MATERIALS AND METHODS

Collection of soil samples

Rhizosphere soil samples were collected from cotton and sugarcane crops grown in Taluka Kot Diji, district Khairpur Mir, Sindh, Pakistan. The samples were properly labeled and transferred to an ice bag for transportation to the Postgraduate Research Laboratory at the Institute of Microbiology, Shah Abdul Latif University (SALU), Khairpur. They were then stored at 4°C for further use in the isolation of bacterial strains.

Isolation and cultural conditions of rhizosphere bacteria

For bacterial isolation from the collected soil samples,

the tenfold serial dilution method was used (Sabir et al., 2013). One gram of each sample was transferred into a 10 ml tube containing sterile distilled water, thoroughly mixed, and left at room temperature for 30 min to allow soil particles to settle. Subsequently, 1 ml was taken from the initial tube and transferred to a second tube labeled 1/100, and this process was continued up to a dilution of 1/10,000, with each tube shaken well. From the last three dilution tubes, 0.1 ml (aliquots) was taken and inoculated for bacterial isolation.

For isolation, the Luria Bertani agar (LB agar) medium was prepared following the method described by Hägg et al. (2004). The medium contained the following components (gL^{-1}): yeast extract (5.0), tryptone (10.0), agar (10.0), and NaCl (5.0). The inoculated media were then incubated at 37°C for 24 h. After incubation, cultural characteristics such as colony size, color, margin, elevation, texture (roughness or smoothness) were observed (Todorova and Kozhuharova, 2010). The samples were then subcultured on nutrient agar medium, and bacterial colonies exhibiting smooth, elevated, dry, and creamy-white characteristics were selected for further research.

Pure *Bacillus* species were preserved at -20°C in nutrient broth containing 17% (v/v) glycerol for long-term storage, while for short-term preservation, strains were maintained at 4°C on nutrient agar slants.

Morphological and biochemical characterization of the selected isolates

Morphological and microscopic characteristics such as Gram staining, motility, capsule formation, and endospore tests were performed following method described by Cheesbrough (1981). Biochemical characterization included catalase, nitrate reduction, urease production, oxidase, indole production, Voges-Proskauer, Methyl Red, citrate utilization, and sugar fermentation tests for glucose, lactose, galactose, maltose, and mannitol.

Catalase test

The catalase test was performed using a sterile wire loop. A pure bacterial culture was transferred to the surface of a clean glass slide, followed by the addition of three to four drops of 3% H_2O_2. The appearance of bubbling within 20-30 sec indicated a positive result.

Nitrate reduction test

Each bacterial isolate was aseptically inoculated into nitrate broth and incubated at 37°C for 24 h. Following incubation, 0.5 ml of solution B (alpha-naphthylamine) and 0.5 ml of solution A (sulfanilic acid) were added to the culture, as described by Cheesbrough (1981), to determine nitrate reduction.

Oxidase test

The oxidase test was performed using filter paper placed at the center of an empty Petri plate. Four to five drops of freshly prepared oxidase reagent were added to the filter paper. A bacterial smear was prepared on the filter paper using a sterile wooden stick. The appearance of a blue-purple color within 20 sec indicated a positive result, whereas the absence of a color change within the same time frame indicated a negative result.

Urease production

The urease test was performed in normal saline. A 0.30 ml aliquot of a packed, milky suspension of the isolated bacteria was prepared, sealed in a tube, and incubated for up to five hours at 36°C. The results were recorded after the addition of a urease tablet. A yellow/orange color indicated a negative (−ve) test, while a red/purple color indicated a positive (+ve) test.

Indole production

The indole test was conducted using tryptophan (an amino acid) as a substrate to assess the ability of the isolate to produce indole. The test bacteria were inoculated in tryptone broth and incubated for 24 h at 37°C. To interpret the results, 0.4 mL of Kovac's reagent was added to 2 ml of the inoculated broth. A prominent pink ring in the top layer indicated a positive (**+**ve) indole test.

Methyl red (MR) test

The Methyl red test was conducted aseptically. Bacterial isolates were inoculated into Clark's broth and incubated at 37°C for 24 h. A 2 ml aliquot of the inoculated culture was taken, and 0.5 ml of methyl red indicator was added and mixed. A red color indicated a positive (**+**ve) test.

Voges-Proskauer (VP) test

Bacterial isolates were inoculated in Clark's broth and incubated for 24 h at 37°C. A 2 ml aliquot of the inoculated culture was transferred to a tube, followed by the addition of 0.7 ml of Barritt's reagent A (5% α-naphthol) and Barritt's reagent B (40% potassium hydroxide). The mixture was blended and left at room temperature for 15 min to observe results. The development of a pink color indicated a positive (+ve) test, while the absence of color change indicated a negative (−ve) test.

Citrate utilization test

The Citrate utilization test was conducted following the method described by Monica (1991). Bacterial isolates were inoculated onto Simmons' citrate agar slants and

incubated at 37°C for 24 h. If no color change was observed, the incubation was extended for another 24 h. A color change indicated a positive (+ve) test, whereas the absence of a color change indicated a negative (−ve) test.

Sugar fermentation

Using a sterile wire loop, pure isolated bacterial colonies were inoculated into 0.6% solutions of various labeled sugars (glucose, galactose, lactose, maltose, and mannitol). Each sugar solution was inoculated into test tubes, with uninoculated sugar solutions serving as negative controls. The tubes were incubated for 24 h at 37°C. A color change from red to yellow indicated a positive (+ve) fermentation result, while the absence of a color change indicated a negative (−ve) result.

Source of plant fungal pathogen (*Sclerotium rolfsii*)

The fungal plant pathogen *Sclerotium rolfsii* used in this study was obtained from the Botany Department, SALU, Khairpur. The fungal strain was cultured on potato dextrose agar (PDA).

Primary antagonistic screening by bacterial cultures

The antagonistic efficacy of isolated bacterial strains was evaluated *in vitro* using the dual culture plate assay (Saha et al., 2012). Specifically, the inhibition test was conducted using a 5 mm mycelial mat of the fungal culture, which was placed at one corner of a Petri plate containing PDA medium. Each bacterial isolate was streaked in a single line (2 to 3 cm) on the PDA medium at the opposite corner of the plate. The plates were then incubated at 28°C, and fungal mycelial inhibition by the bacterial isolates was observed. Mycelial inhibition was measured in millimeters (mm) and compared with the control after five days of incubation. Each experiment was conducted in triplicate. Plates without bacterial inoculation served as controls.

Secondary antagonistic screening by cell-free culture filtrate

All 31 *Bacillus* isolates were inoculated into conical flasks containing 100 ml of sterile nutrient broth and incubated in a shaker incubator at 150 rpm and 37°C for 24 h to obtain a cell-free supernatant. The cultures were then centrifuged at 4,000 rpm for 15 min, and the supernatants were stored at 4°C. The secondary antagonistic potential of the cell-free culture filtrates was assessed using the agar well diffusion method described by Tan et al. (2013). PDA plates were prepared, and wells (~3 cm apart) were created using a sterile cork borer. A 5 mm disc of *S. rolfsii* was placed at the center of each plate, and 50 µl of the cell-free supernatant was added to each well. Uninoculated PDA plates served as negative controls, while PDA plates inoculated with the test fungal strain served as positive controls. The plates were incubated at 28°C for five days, after which the inhibition zones were measured in millimeters. Each experiment was performed in triplicate.

Molecular characterization of the isolated bacteria

Molecular characterization was performed for two bacterial cultures, S1C9 and S3C9, based on their strong antagonistic activity against the test fungal cultures. These two isolates were selected and sent for PCR amplification and sequencing at Macrogen Inc., Seoul, Korea, using the universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3'). Phylogenetic analysis and tree construction were conducted following the methods described by Saand et al. (2015).

Statistical analysis

Data were analyzed using Statistics software (Version 8.1) and Microsoft Excel (2010). Analysis of variance (ANOVA) was performed, and Duncan's multiple range test (DMRT) was used to determine significant differences at $p < 0.05$.

RESULTS

Isolation, morpho-microscopic, and biochemical characterization of bacterial isolates

A total of 31 bacterial isolates were preliminarily identified based on their morphological characteristics, including colony morphology and microscopic features. The cultural characteristics of bacterial isolates from four samples are illustrated in Figure 1, showing creamy, off-white, yellow, and white colonies with a dry-centered appearance.

Further, various morpho-microscopic, biochemical, and sugar fermentation tests were conducted to comprehensively characterize the bacterial isolates. All isolates were Gram-positive, motile (flagellated), and spore-forming (Table 1). The biochemical characterization revealed that all isolates tested positive for catalase (C), oxidase (O), methyl red (MR), and nitrate reduction (NR), except for isolate S5C9, which tested negative for catalase. However, most isolates tested negative for indole production (IP), Voges-Proskauer (VP), and urease (U) tests, with the exception of isolate S2C8, which tested positive for the VP test (Table 1).

Additionally, most isolates exhibited a positive response to carbohydrate fermentation. All 31 isolates

demonstrated 100% positive results for mannitol (M) and glucose (GL). Except for two isolates, all tested positive for maltose (MA). However, the galactose (GA) test showed predominantly negative results, with 29 isolates testing negative and only two testing positive. Interestingly, lactose (L) fermentation results were variable, with 12 isolates testing positive and 19 testing negative (Table 1).

Screening of bacterial isolates for antifungal activity

Initially, all 31 bacterial isolates were evaluated for their antagonistic activity against *S. rolfsii* using the streak plate method. The primary screening revealed that four isolates, S3C9, S1C9, S2C9, and S6C8, exhibited strong antagonistic activity against *S. rolfsii*. Among these, the S3C9 isolate showed the highest inhibition zone of fungal mycelial growth, measuring 18.3 mm in diameter, followed by S1C9, S2C9, and S6C8, with inhibition zones of 14.7 mm, 14.3 mm, and 14.7 mm, respectively (Figure 2a). In contrast, the remaining isolates exhibited smaller inhibition zones, ranging from 4.0 mm to 8.7 mm (Figure 2a).

Table 1. Details of the identified isolates.

Isolate	Morpho-microscopy	Biochemical Characteristics										Sugar Fermentation				
	Shape	SS	FS	GS	O	C	MR	IP	VP	U	NR	GA	M	GL	MA	L
S1A8	Bacilli	+	+	+	+	+	+	-	-	-	+	-	+	+	-	+
S1A9	Bacilli	+	+	+	+	+	+	-	-	-	+	-	+	+	+	-
S1B8	Bacilli	+	+	+	+	+	+	-	-	-	+	-	+	+	+	-
S1B9	Bacilli	+	+	+	+	+	+	-	-	-	+	-	+	+	+	+
S1C8	Bacilli	+	+	+	+	+	+	-	-	-	+	-	+	+	+	-
S1C9	Bacilli	+	+	+	+	+	+	-	-	-	+	-	+	+	+	-
S2A8	Bacilli	+	+	+	+	+	+	-	-	-	+	-	+	+	+	-
S2B8	Bacilli	+	+	+	+	+	+	-	-	-	+	-	+	+	+	+
S2C8	Bacilli	+	+	+	+	+	+	-	+	-	+	+	+	+	+	+
S2C9	Bacilli	+	+	+	+	+	+	-	-	-	+	-	+	+	+	+
S3A8	Bacilli	+	+	+	+	+	+	-	-	-	+	-	+	+	+	-
S3A9	Bacilli	+	+	+	+	+	+	-	-	-	+	-	+	+	+	+
S3B8	Bacilli	+	+	+	+	+	+	-	-	-	+	-	+	+	+	-
S3B9	Bacilli	+	+	+	+	+	+	-	-	-	+	-	+	+	+	-
S3C8	Bacilli	+	+	+	+	+	+	-	-	-	+	-	+	+	+	-
S3C9	Bacilli	+	+	+	+	+	+	-	-	-	+	-	+	+	+	-
S4A8	Bacilli	+	+	+	+	+	+	-	-	-	+	-	+	+	+	-
S4A9	Bacilli	+	+	+	+	+	+	-	-	-	+	-	+	+	+	+
S4B8	Bacilli	+	+	+	+	+	+	-	-	-	+	-	+	+	+	-
S4B9	Bacilli	+	+	+	+	+	+	-	-	-	+	-	+	+	+	-
S4C8	Bacilli	+	+	+	+	+	+	-	-	-	+	-	+	+	+	-
S4C9	Bacilli	+	+	+	+	+	+	-	-	-	+	-	+	+	+	-
S5A8	Bacilli	+	+	+	+	+	+	-	-	-	+	-	+	+	+	+
S5A9	Bacilli	+	+	+	+	+	+	-	-	-	+	-	+	+	-	+
S5B8	Bacilli	+	+	+	+	+	+	-	-	-	+	-	+	+	+	+
S5B9	Bacilli	+	+	+	+	+	+	-	-	-	+	-	+	+	+	-
S5C8	Bacilli	+	+	+	+	+	+	-	-	-	+	-	+	+	+	-
S5C9	Bacilli	+	+	+	+	-	+	-	-	-	+	+	+	+	+	-
S6A8	Bacilli	+	+	+	+	+	+	-	-	-	+	-	+	+	+	+
S6B8	Bacilli	+	+	+	+	+	+	-	-	-	+	-	+	+	+	-
S6C8	Bacilli	+	+	+	+	+	+	-	-	-	+	+	+	+	+	-

Abbreviations used in the table: SS (Spore staining), FS (Flagella staining), GS (Gram staining), O (Oxidase), C (Catalase), MR (Methyl Red), IP (Indole production), VP (Voges-Proskauer), U (Urease), NR (Nitrate reduction), GA (Galactose), M (Mannitol), GL (Glucose), MA (Maltose), and L (Lactose).

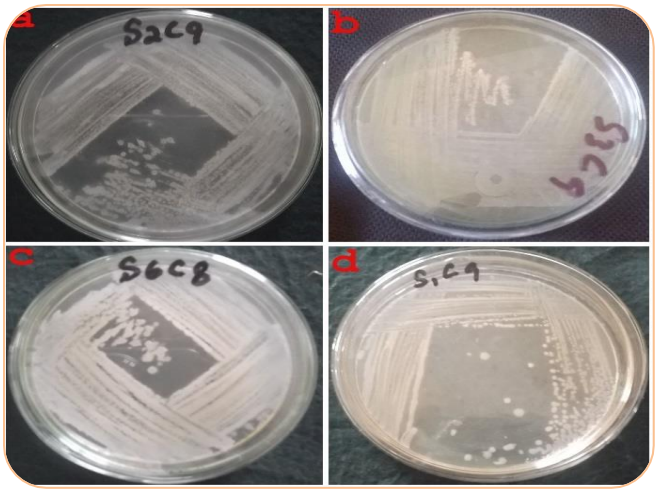

Figure 1. Cultural characteristics of the identified isolates. a, b, c, and d represent the respective isolates.

Furthermore, a dual culture assay was conducted, comparing bacterial isolates grown with *S. rolfsii* and without *S. rolfsii* as a control (Figure 3). The results demonstrated a reduction in fungal mycelial growth in the presence of bacterial isolates, particularly S1C9 and S3C9, compared to the control at two days post-inoculation (2 dpi) (Figure 3).

For secondary screening, all bacterial isolates were cultured in liquid nutrient broth medium. The cell-free culture supernatant from each isolate was subsequently collected and tested for antifungal activity using the agar well diffusion method. The results of the secondary screening against *S. rolfsii* are presented in Figure 2b. In this assay, the findings differed from the primary screening. Only three isolates, S2C9, S3C9, and S4C9, demonstrated intermediate antifungal activity, producing inhibition zones of 6.3 mm, 4.7 mm, and 4.7 mm, respectively. In contrast, isolates S1A9, SIB8, and S5B9 exhibited minimal antagonistic activity.

Molecular identification and characterization of bacterial isolates

For molecular identification, the two most effective bacterial isolates that exhibited significant antagonistic activity against *S. rolfsii* were selected for 16S rRNA sequencing (Macrogen Inc., Seoul, Korea). The obtained 16S rRNA sequences were used as queries in a BLAST search to identify homologous sequences in the National Center for Biotechnology Information (NCBI) database. Sequence similarities were determined based on the maximum identity score, and the phylogenetic relationships of the retrieved sequences with the respective isolates were analyzed using the multiple alignment program ClustalW in MEGA 5 software.

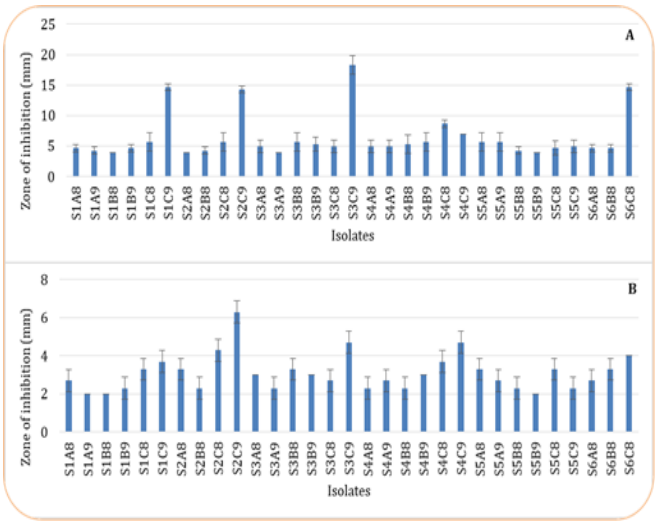

Figure 2. Antagonistic activity of isolates against *S. rolfsii*. (A) and (B) show the inhibition zones for primary and secondary screening, respectively. Different lowercase letters indicate significant differences in inhibition zones among isolates, as determined by ANOVA followed by DMRT in a triplicate experiment at $p < 0.05$.

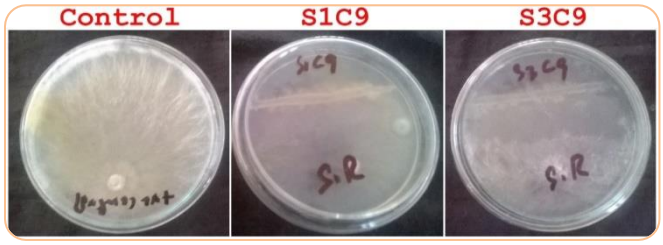

Figure 3. Inhibition zones of dual-culture isolates against *S. rolfsii*.

A phylogenetic tree constructed using fifteen sequences retrieved from the BLAST search for the S1C9 isolate demonstrated its similarity to *Bacillus* species. The phylogenetic analysis revealed that the S1C9 isolate clustered closely with *B. subtilis* strain A29 (16S rRNA partial sequence, accession no. OQ165119.1) and an uncultured bacterium clone (16S rRNA partial sequence, accession no. MH458858.1). Based on these findings, the S1C9 isolate was identified as *B. subtilis* (Figure 4). The sequence of S1C9 was submitted to NCBI under GenBank accession no. OP962345.

Similarly, fifteen sequences retrieved from the BLAST search for the S3C9 isolate indicated its similarity to *Bacillus* species. The phylogenetic analysis showed that the S3C9 isolate clustered closely with *B. subtilis* strain LSRBMoFPIKRGCFTR17 (16S rRNA partial sequence, accession no. MN882713.1) (Figure 5). Therefore, the S3C9 isolate was also identified as *B. subtilis*. The sequence of the S3C9 isolate was deposited in NCBI, and the corresponding accession number (OQ180051) was obtained.

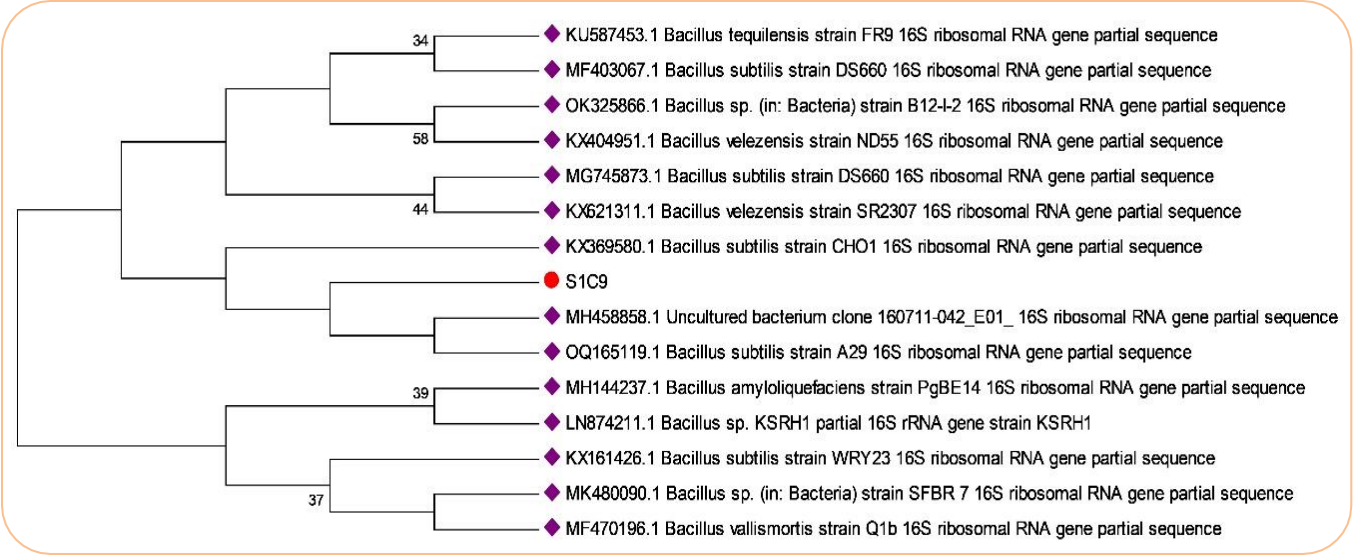

Figure 4. Phylogenetic tree of S1C9. The phylogenetic tree was constructed using the methods described by Saand et al. (2015).

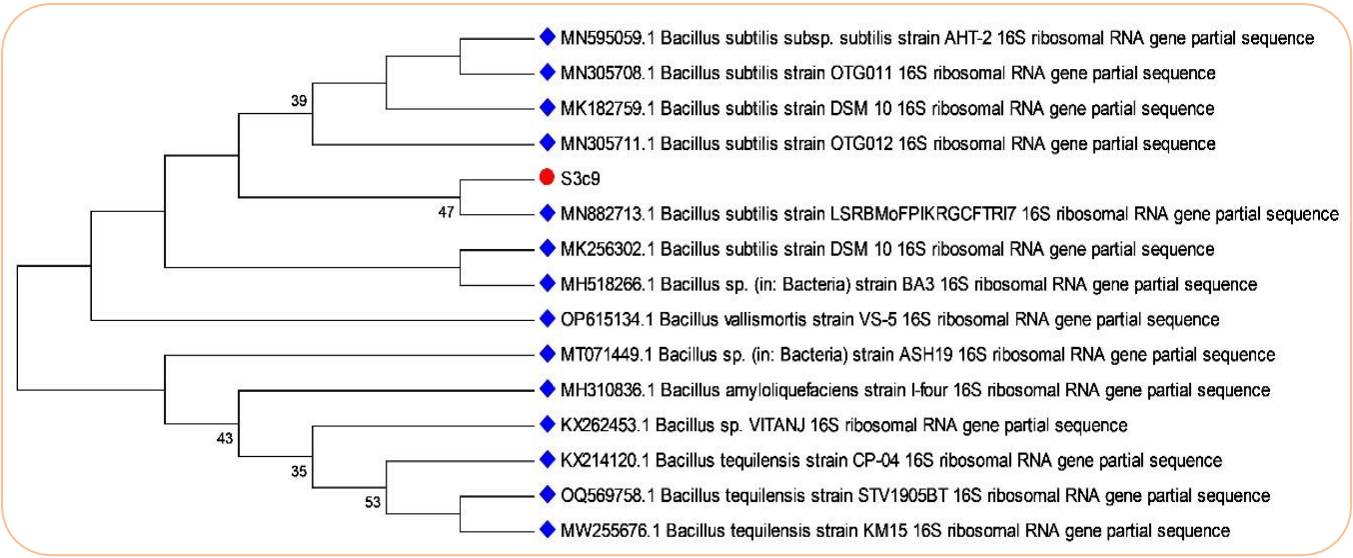

Figure 5. Phylogenetic tree of S3C9. The phylogenetic tree was constructed using the methods described by Saand et al. (2015).

DISCUSSION

PGPR have been reported as beneficial bacteria capable of either promoting plant growth by colonizing roots or exhibiting antagonistic properties, making them effective biocontrol agents against plant diseases (Almoneafy et al., 2014). Among PGPR, *Bacillus* spp. has been extensively studied for its plant growth-promoting activities and antagonistic effects against fungal pathogens (Kour et al., 2019; Kumari et al., 2021). Several studies have also provided evidence supporting the antagonistic potential of *Bacillus* spp. against *S. rolfsii* (Kumari et al., 2021; Li et al., 2023).

Previously, six *Bacillus* isolates were identified as Gram-positive, catalase-positive, mannitol-positive, glucose-positive, and VP-negative (Sethi and Mukherjee, 2018). Similar findings were observed in this study (Table 1). Furthermore, our results showed that these six *Bacillus* isolates exhibited strong inhibition of *S. rolfsii* in *in vitro* assays, consistent with the findings of Sethi and Mukherjee (2018). Another study demonstrated that *Bacillus* spp. isolated from Moroccan soils inhibited 90% of the *S. rolfsii* mycelial zone (Bidima et al., 2021). Moreover, *B. subtilis* isolates have been tested in *in vitro* assays against *Rhizoctonia solani*, where their antagonistic activity significantly reduced fungal mycelial development (Krishnan et al., 2024).

Molecular, physiological, and biochemical analyses have previously identified *Bacillus velezensis* (Jia et al., 2023). In their study, two *Bacillus* strains, CB13 and CB19, exhibited strong antagonistic activity, significantly inhibiting *S. rolfsii* mycelial growth (Jia et al., 2023). More recently, Wang et al. (2024) reported that *Bacillus* spp. could suppress *Agroathelia rolfsii* growth by up to 80% in *in vitro* assays. In our study, some *Bacillus* isolates significantly inhibited the growth of *S. rolfsii* (Figure 2).

Comparative analysis revealed that four isolates viz. S3C9, S1C9, S2C9, and S6C8 exhibited the largest inhibition zones in the primary screening test (Figure 2a). In the secondary test, three isolates, S2C9, S3C9, and S4C9, showed the highest inhibition zones (Figure 2b). Notably, two isolates, S2C9 and S3C9, consistently reduced fungal growth in both primary and secondary tests (Figure 2a, b).

On the contrary, one *Bacillus* isolate (ZMR6) exhibited 33% inhibition against *S. rolfsii* in an *in vitro* analysis (Sharf et al., 2021). Zou et al. (2023) examined the endophytic bacterium *B. subtilis* JY-7-2L, isolated from *Aconitum carmichaelii*, and its potential for biocontrol and plant growth promotion. The strain effectively reduced southern blight in both *in vitro* and field conditions.

In the present study, the antagonistic activity of *B. subtilis* (Bs-BKN) against *S. rolfsii* was evaluated under *in vitro* conditions. The results demonstrated that this bioagent exhibited lower inhibitory effects compared to other bacterial strains (Meena et al., 2024). Our findings revealed that four isolates showed higher inhibition in primary biocontrol screening against *S. rolfsii*, while three isolates exhibited significantly greater inhibition than others in secondary screening against fungal pathogens. The suppression of mycelial growth by *Bacillus* strains is likely due to the production of antimicrobial substances, including antibiotics and other bioactive compounds (Singh et al., 2008). Previously, *Bacillus* strains have demonstrated significant disease control against necrotrophic fungal species such as *Solani* spp., *S. rolfsii*, and *S. oryzae* (Sethi and Mukherjee, 2018). *Bacillus* isolates have been reported from various field crops, including groundnuts, beans, chilies, and rice (Iquebal et al., 2017; Sethi and Mukherjee, 2018; Javaid et al., 2020; Li et al., 2023). Similarly, PGPR species have been isolated from sugarcane rhizospheres (Santos and Rigobelo, 2021). In Pakistan (Punjab Province), Zain et al. (2019) reported PGPR species isolated from cotton and sugarcane rhizospheres and examined their antagonistic effects on *Fusarium* spp.

The present study cultivated PGPR isolates from the rhizospheres of cotton and sugarcane crops grown in a different ecological zone of the country. These findings underscore the significance of rhizospheres in supporting beneficial microbial communities in the same crops across different regions, potentially playing a crucial role in managing fungal pathogens.

Previously, *B. subtilis* strain JY-7-2L was evaluated as a potential biocontrol agent against *S. rolfsii*, and its molecular characterization was performed using multi-locus sequence analysis (Zou et al., 2023). Phylogenetic analysis of *Bacillus* isolates revealed their clustering into two major groups, logically identifying *B. subtilis* strains based on 16S rRNA sequencing (Sagar et al., 2024). Furthermore, the molecular identification of *Bacillus* isolates through 16S rRNA gene sequencing has provided insights into their phylogenetic relationships at the bacterial species level (Sagar et al., 2024).

Moreover, our previous study demonstrated that *Streptomyces thermolilacinus* (isolated from the soil of Kot Diji, Sindh, Pakistan) exhibited significant antagonistic activity against six multidrug-resistant (MDR) bacterial strains (Talpur et al., 2020). This study also highlights the potential of rhizosphere soil from the same region, where bacterial isolates demonstrated a significant reduction in fungal mycelial growth under *in vitro* conditions.

Importantly, this study contributes to the molecular characterization of *Bacillus* isolates, marking one of the first scientific investigations in this area, as few or no studies have been conducted on the rhizosphere soils of crops cultivated in Kot Diji, Sindh, Pakistan. However, this study is limited to identifying *B. subtilis* strains based solely on 16S rRNA sequencing. Nonetheless, the findings emphasize the richness and significance of the rhizosphere soil in the Khairpur District of Sindh Province.

Bacillus species are well-known PGPR and have been widely used as biocontrol agents against various plant fungal pathogens, playing a crucial role in sustainable agriculture (Khan et al., 2022). Due to constraints in time and resources, this study did not extend to field-based *in vivo* analyses to evaluate the antagonistic potential of *Bacillus* species against fungal pathogens. However, it underscores the importance of PGPR applications for eco-friendly and sustainable agriculture. Consequently, our study lays the groundwork for future research on effective biocontrol strategies through promising *in vivo* approaches.

CONCLUSION

The thirty-one bacterial isolates were characterized based on morpho-microscopic and biochemical analyses. Their antagonistic activity was evaluated against *Sclerotium rolfsii*. Among them, four isolates, S3C9, S1C9, S2C9, and S6C8, exhibited strong antagonistic activity against the fungus in an *in vitr*o primary screening test. In the secondary screening, three isolates (S2C9, S3C9, and S4C9) significantly reduced fungal growth. Molecular characterization identified two isolates (S1C9 and S3C9) as *Bacillus subtilis* strains. These strains hold potential as effective biocontrol agents against fungal pathogens and may contribute to maintaining a healthy rhizosphere. Their antifungal properties, as demonstrated in field trials, support their use in sustainable agriculture.

AUTHORS' CONTRIBUTIONS

SAW and MAS designed the study; AAU, NAK, and SFH performed the experiments; QAM, SG, and GHS conducted bioinformatics analysis; NAK supervised the work; SAW and MAS wrote the manuscript; AAU and NAK proofread the paper.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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