Assessment of antibiosis potential of *Bacillus* sp. against the soil-borne fungal pathogen *Sclerotium rolfsii* Sacc. (*Athelia rolfsii* (Curzi) Tu & Kimbrough)

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**Abstract**

**Background:** This study aimed to investigate the rhizosphere bacterial isolates’ antagonistic property against the soil-borne fungal phytopathogen, *Sclerotium rolfsii* Sacc. (*Athelia rolfsii* (Curzi) Tu & Kimbrough). The chemical control of the disease caused by *S. rolfsii* is economically and environmentally unsustainable, and therefore, a bio-control agent in the form of rhizospheric bacteria is gaining importance.

**Main body:** Five rhizospheric *Bacillus* species viz. *B. subtilis* subsp. *Subtilis* str.168 (accession no. MH283878), *B. siamensis* strain PDA 10 (accession no. MH283879), *B. amylophilacogens* strain 1034 (accession no. MH283880), *B. velezensis* strain FZB42165 (accession no. MH283881), and *B. atrophaeus* strain NBRc 15539 (accession no. MH283882) were assessed for their antagonistic potential against *S. rolfsii* based on 3 different screening methods. Among these, 100% fungal growth inhibition by all 5 *Bacillus* spp. was observed in the novel ring method, whereas in the dual culture method, the maximum growth inhibition was (58%) exhibited by the strain NBRC 15539 of *B. atrophaeus*. The antagonistic activity showed by the modified dual culture method was also relatively high, and the highest activity (93.7%) was shown by the strain NBRC 15539 of *B. atrophaeus*. Besides, the *Bacillus* sp. was also evaluated for their plant growth-promoting attributes and other properties such as the production of siderophore, HCN, amylase, protease, lipase, and ammonia, including their assessment for chitinase and cellulase activity.

**Conclusion:** The study provided empirical evidence of *Bacillus* sp. antagonistic potential against *S. rolfsii* and should be of contributive value in developing a biocontrol agent for this highly important crop fungal pathogen.

**Keywords:** *Bacillus* species, Biocontrol, Antagonistic activity, *Sclerotium rolfsii*
the use of chemical fungicides is proven to be increasingly harmful to environmental as well as human health, besides their research and development being economically intensive. The development of resistance, i.e., the evolution of increasingly aggressive pathotypes, to the fungicidal molecules is another aspect which calls desisting their extensive use as control agents of fungal diseases of crop plants (Singh et al. 2016), including that caused by S. rolfsii. In this context, microbial role, particularly the bacterial antagonism, becomes essential as bacteria were selective, diverse, natural, and safe for environmental and human health (Iftikhar et al. 2020). Though their efficacy and efficiency against diverse fungal pathogens remain debated, biological control treatment of plant diseases utilizing antagonistic plant growth-promoting rhizobacteria (PGPR) gives a moderately effective, inexpensive, and environmentally secure alternative the conventional chemical pesticides. One added advantage of these microbial control agents is that besides their antagonistic effect against fungal pathogens, they often harbor plant growth-promoting activities (siderophore production, ammonia production, HCN production, etc.), making the agents of choice to the growers (Emmert and Handelsman 1999). Among these, the bacterial species belonging to Pseudomonas, Azospirillum, Klebsiella, Enterobacter, Alcaligenes, Arthrobacter, Burkholderia, Bacillus, and Serratia genera are harboring plant growth-promoting activities (PGPA) (Kour et al. 2019). The species belonging to Bacillus, Streptomyces, Pseudomonas, Burkholderia, and Agrobacterium are a few of the extensively studied ones as far as the PGPR activities are concerned. Among these, the Bacillus and Pseudomonas genera are particularly crucial for their antibiosis capability against major plant fungal pathogens (Karimi et al. 2012). Various Bacillus species have been extensively studied, and a variety of antimicrobial cyclic lipopeptides such as iturins, fengycins, and surfactants playing an essential role in the fungal antagonism have been reported to be synthesized by them (Nihorimbere et al. 2010). The production of various hydrolytic enzymes viz. amylase, cellulase, protease, lipase, and chitinase by the Bacillus strains is also a part of this antagonistic activity (Castillo et al. 2013), apart from the production of endospores and antibiotics (Jangir et al. 2019). Certain Bacillus species have also been reported to secrete volatile and non-volatile antimicrobial agents, which, in effect, inhibit the development of a variety of fungal infections (Athukorala et al. 2010). As part of the plant defense mutualism, Bacillus species secrete phenolic compounds, which are the secondary metabolites responsible for inhibiting mycelial growth (Gao et al. 2017). In the context of this background, the present study was focused on the isolation and screening of rice rhizospheric bacteria for potential antagonistic activity against S. rolfsii and their eventual evaluation for the synthesis of different antifungal metabolites, hydrolytic enzymes, and phenol producing capability.

Main text

Materials and methods

Source of Sclerotium rolfsii

The fungus S. rolfsii employed in the present study was sourced from the Plant Pathology Laboratory of ICAR Research Complex for Eastern Region, Research Centre, Plandu, Ranchi, India.

Isolation of rhizospheric bacteria

Different bacterial isolates were obtained from the rhizospheric soils (acidic soils, pH 5.5) of rice (Oryza sativa) raised in the experimental field of National Bureau of Plant Genetic Resources, Regional Station, Ranchi, Jharkhand, India, and were used in the screening of antagonistic potential against phytopathogenic sclerotial fungi S. rolfsii. One gram of soil was suspended in sterile distilled water and vortexed to form a suspension followed by serial dilution preparation. One hundred-microliter aliquot of dilution was plated in triplicate on nutrient agar (NA) medium (Hi-Media, Mumbai, India). The plates were incubated at 37 °C for 24 h. The cultures were purified on nutrient agar slants at 37 °C in a static incubator, and well-isolated colonies were maintained on nutrient agar. The isolates were then tested for their antagonistic activity against S. rolfsii.

Screening for potential biocontrol agents

All the isolated bacterial isolates were subjected to primary screening for prospective antagonistic activity against S. rolfsii on potato dextrose agar (PDA) medium (Hi-Media, Mumbai, India) by using the dual culture technique (Rangeshwaran and Prasad 2000). An agar disc (5-mm) was cut from an actively growing (96 h) S. rolfsii and placed on the surface of a fresh PDA medium on one side of the Petri plates. A loopful of Bacillus isolates was inoculated opposite to the fungal disc on each plate.

The novel ring method and modified dual culture technique were used for testing the antagonistic activity of effective antagonistic bacterial isolates against S. rolfsii as secondary and tertiary screening (Zhao et al. 2014). A 5-mm-diameter mycelial disc of the fungal pathogen was inoculated at the center of a 90-mm diameter Petri plate containing 20 ml of sterile PDA medium. The bacterial isolate was streaked in a plate at a distance of 2.5 cm from the center in a circular pattern and 3 cm from the center at four sides in secondary and tertiary screening, respectively. The plates inoculated with phytopathogen and without bacteria were used as control. These plates were then incubated in an inverted position at room temperature (30 °C) for 7 days. The radial growth of the
Biochemical and molecular characterization of bacterial strains

Based on the antagonistic test, the strains showing significant antagonistic activities were selected and identified by biochemical methods following Bergey’s manual of determinative bacteriology (Holt et al. 1994). It involved the following biochemical tests: Gram’s test, oxidase test, nitrate test, glucose fermentation test, Voges-Proskauer test, citrate utilization, starch hydrolysis, lipid hydrolysis, H₂S production, catalase production, gelatin liquefaction, and acid production test by utilization of fructose, glucose, glycerol, mannitol, maltose, and sucrose. The bacterial cultures were inoculated on the nutrient agar slants for catalase test, incubated for 24 h at 37 °C for 48 h. The plates were filled with Gram’s iodine after incubation, and then they were observed around the streaked bacterial cultures for a transparent zone. A bright zone with a yellowish, pinkish, and whitish coloration was observed in the dark blue medium, indicating the generation of siderophore. The isolates were screened for the production of hydrogen cyanide (HCN) by adopting the method of Lorck (1948). Nutrient agar (NA) medium (Hi-Media, Mumbai, India) amended with 4.4 g/l glycine (Hi-Media, Mumbai, India) was prepared to streak the cultures on it. A Whatman filter paper No. 1 dipped in a 0.5% picric acid solution (in 2% sodium carbonate) was attached to the lid or plate’s inner portion. The plates were sealed using parafilm and kept under incubation at 37 °C for seven consecutive days. It was observed that the paper turned yellow to brown, signifying HCN production by the isolates. The concentration of HCN was measured periodically. The degree of antagonism was calculated by estimating the pathogen’s radial growth with bacterial culture vis-à-vis control, and the percentage of inhibition was calculated by the following equation (Riungu et al. 2008):

\[ I = \frac{100(C-T)}{C} \]

where \( I \) = % inhibition of mycelial growth, \( C \) = radial growth of fungus in control plate (mm or cm), and \( T \) = radial growth of fungus on the plate inoculated with bacteria (mm or cm).

Determination of total phenolic contents (TPC) in bacterial extracts

The concentrations of phenolics in bacterial extracts were assessed using the spectrophotometric method using folin-ciocalteu reagent (Hi-Media, Mumbai, India) (Singleton et al. 1999), and TPC was expressed as μg/ml. The samples were subjected to homogenization and subsequently centrifugation at 15000 rpm for 10 min. One ml of supernatant was taken, and one milliliter of ethyl acetate was added to it, and the content was mixed thoroughly. The mixture was subjected to 12 h of incubation. The upper layer was collected and allowed to evaporate by placing it in the hot air oven at 50 °C. After completing the oven’s drying process, 0.5 ml ethanol was added, followed by 5 ml of sterilized distilled water, and the
solution was mixed thoroughly. The phenolic estimation was conducted by taking a different concentration of samples and adding 2 ml of distilled water and 0.5 ml of the folin-ciocalteu reagent to each of the samples and mixed thoroughly. Three minutes of incubation at room temperature (30 °C) was carried out, after which 2 ml of 20% of Na₂CO₃ (Hi-Media, Mumbai, India) was added to each of the test samples. The solution was incubated at room temperature (30 °C) for 1 h. Subsequently, the absorbance of the reaction mixture was recorded at 650 nm.

**Results and discussion**

**Isolation and screening of the bacterial strains for antagonistic activity against S. rolfsii**

The total number of different bacterial isolates obtained in the present study was 120, and these were coded as CBK1001 to CBK17010. The primary, secondary, and tertiary screening based on the dual culture method, novel ring method, and modified dual culture method was conducted with co-culturing of the five bacterial isolates with the fungus along with S. rolfsii cultured without any bacterial isolate as control (Fig. 1).

In primary screening, 14 isolates (CBK2007, CBK3005, CBK5001, CBK6004, CBK9002, CBK10002, CBK11002, CBK12002, CBK13008, CBK13010, CBK14008, CBK15006, CBK16008 and CBK17001) were found to have antifungal activity against S. rolfsii by dual culture method (Table 1). Five isolates (CBK2007, CBK3005, CBK5001, CBK6004, and CBK17001) among these, which showed > 25% antifungal activity against S. rolfsii, were selected for further screenings. It was observed that the fungi covered 50% area of the 90-mm-diameter Petri plates within 48 h in the control plate. It was observed that the fungi covered 50% area of the 90-mm-diameter Petri plates within 48 h in the control plate. In the present study, the antagonistic potential estimated for all the bacterial isolates by the different methods, i.e., the dual culture method or modified dual culture method or novel ring method, were different. This finding has a practical value in that the bacterial isolates with the maximum average antagonistic activity across the three methods should ideally constitute candidates of choice for employing them as a biocontrol agent. CBK17001 was the isolate that showed maximum antifungal activity against S. rolfsii in all three methods, followed by CBK2007, CBK3005, CBK6004, and CBK5001. A very high level of antifungal activity, i.e., 100% inhibition of fungal growth in novel ring method observed in the present study is in agreement with earlier reports of Reyes-Ramírez et al. (2004), who reported complete inhibition of the hyphal growth of S. rolfsii by B. thuringiensis and Gholami et al. (2014) who reported the antagonistic activity of endophytic bacteria B. subtilis subsp. Subtilis (69.42%) and B. subtilis subsp. spizizenii (68.88%) against S. rolfsii.

**Characterization and identification of bacterial isolates**

All five isolates were characterized comprehensively by different biochemical tests by following Bergey’s microbial identification manual, which revealed that all isolates belonged to the Bacillus genus (Table 3).

However, for species identification, the sequencing of 16S rDNA (Eurofins, Bangalore, India) was undertaken. As 16S rDNA gene sequence give precise gathering of creature even at subspecies level, it is considered as an incredible asset for the fast-distinguishing proof of bacterial species (Clarridge 2004). Many researchers have reported the advantage of 16 s analysis over the other modern rapid identification methods such as MALDI Biotype analysis (Harba et al. 2020). Based on the phylogenetic analysis of the 16S rDNA sequence obtained from the strain CBK 2007, CBK 3005, CBK 5001, CBK 6004, and CBK17001, the sequence showed 99% similarity with B. subtilis subsp. Subtilis str.168 (accession no. MH283878), B. siamensis strain PDA 10 (accession no. MH283879), B. amyloliquefaciens strain 1034 (accession no. MH283880), B. velezensis strain FZB42165 (accession no. MH283881), and B. atrophaeus strain NBRC 15539 (accession no. MH283882) respectively. The BLAST algorithm with the NCBI Gene Bank database was used for the similarity search of the resulting consensus sequence. The sequences were selected based on maximum identity score and aligned using the multiple alignment software ClustalW. A phylogenetic tree was constructed using MEGA 6 software (Fig. 2). The 16S rRNA sequences CBK2007, CBK3005, CBK5001,
Fig. 1 (See legend on next page.)
CBK6004, and CBK17001 were submitted to DDBJ/EMBL/GenBank under accession numbers MH283878, MH283879, MH283880, MH283881, and MH283882 respectively.

Cao et al. (2005) confirmed that the HCN, ammonia, and siderophore were part of the Bacillus sp. antifungal properties. According to Afsharmanesh et al. (2010), fungal growth is mainly inhibited by HCN production and siderophore production. Such antifungal traits have been reported in many strains of B. subtilis (Kumar et al. 2020). Some reports are available that have confirmed the presence of these antifungal traits (HCN, ammonia, and siderophore) in B. amyloliquefaciens strains; B. velezensis strain (Chen et al. 2019), and B. atrophaeus strain (Gholami et al. 2014). However, the present study is the first to report an antifungal activity of these five strains (B. subtilis subsp. Subtilis str.168, B. siamensis strain PDA 10, B. amyloliquefaciens strain 1034, B. velezensis strain FZB42165, B. atrophaeus strain NBRC 15539) with this antifungal trait. Volatile compounds such as ammonia and hydrogen cyanide are produced by several rhizobacteria and are reported to play an essential role in biocontrol (Junaid et al. 2013).

**Table 1** Antagonistic activity of different isolates against Sclerotium rolfsii

| Isolates | % Inhibition |
|----------|-------------|
| CBK2007  | 41.85 ± 1.09|
| CBK3005  | 36.67 ± 1.11|
| CBK5001  | 29.63 ± 1.06|
| CBK6004  | 36.3 ± 1.22 |
| CBK9002  | 23.43 ± 2.01|
| CBK10002 | 24.32 ± 2.13|
| CBK11002 | 20.89 ± 1.28|
| CBK12002 | 24.54 ± 1.51|
| CBK13010 | 19.18 ± 2.12|
| CBK13008 | 23.43 ± 1.07|
| CBK14008 | 15.78 ± 2.01|
| CBK15006 | 18.54 ± 1.45|
| CBK16008 | 24.76 ± 1.26|
| CBK17001 | 58.45 ± 1.28|

**Hydrolytic enzyme production**

All five bacterial isolates showed positive results for amylase, protease, and lipase production. B. siamensis strain PDA 10 and B. atrophaeus strain NBRC 15539 also showed a positive chitinase activity. Cellulase activity was not observed in any of the 5 isolates (Table 4).

Enzymatic dissolution of cell walls leading to loss of fungal protoplasm is one of the main antagonistic mechanisms involved in biocontrol agents’ activity. The results were consistent when compared to Castillo et al. (2013) indicating that the Bacillus species were well-known biological control agents that can inhibit soil-borne phytopathogens S. rolfsii by direct antagonism or by secreting several cell wall degrading enzymes. The studies were also consistent with a report from Prapagdee et al. (2008), reporting that chitinase enzymes were responsible for the degradation of the cell wall of S. rolfsii. Therefore, it can be concluded for the present study that the antagonistic behavior of B. siamensis strain PDA 10 and B. atrophaeus strain NBRC 15539 should have their root in the chitinase activity.

**Estimation of total phenol**

The total phenolic content of the bacterial extracts (B. subtilis subsp. Subtilis str.168, B. siamensis strain PDA 10, B. amyloliquefaciens strain 1034, B. velezensis strain FZB42165, and B. atrophaeus strain NBRC 15539) alone (TPC1) and of their interaction with S. rolfsii (TPC2) (B. subtilis subsp. Subtilis str.168) S. rolfsii, B. siamensis strain PDA 10 S. rolfsii, B. amyloliquefaciens strain 1034 S. rolfsii, B. velezensis strain FZB42165 S. rolfsii, and B. atrophaeus strain NBRC 15539 S. rolfsii) showed that the amount of phenol increases when the sample increases (Fig. 3). Both TPC1 and TPC2 were different at all the tested concentrations (20, 40, 60, 80, and 100 μl/ml) when compared to each other. In TPC1, the maximum amount of phenol was produced by B. atrophaeus strain NBRC 15539 (45.34 μg/ml), followed by B. siamensis strain PDA 10 (36.97 μg/ml), B. subtilis subsp. Subtilis str.168 B. amyloliquefaciens strain 1034 (22.23 μg/ml), and B. velezensis strain FZB42165 (22.23 μg/ml). In the case of TPC2, maximum amount of phenol was produced by B. atrophaeus strain NBRC 15539 (50.39 μg/ml) after that B. siamensis strain PDA 10 (40.99 μg/ml) followed by B. subtilis subsp. Subtilis str.168 (32.17 μg/ml), B. velezensis strain FZB42165...
and B. amyloliquefaciens strain 1034 (26.26 μg/ml). In the comparison between TPC1 and TPC2, TPC2 had a greater value than TPC1 at all concentrations. The obtained results were consistent with Patel and Saraf (2017). It was reported that the level of total phenol activity increased after pathogen inoculation that showed enhancement of plant defense mechanism by T. asperellum MSST against Fusarium oxysporum sp. lycopersici in tomato. The present results were also consistent with the study of Singh et al. (2003), who observed that two Pseudomonas strains induced more phenol acids in treated than in non-treated and control plants of chickpea (Cicer arietinum) in the presence of culture filtrate of S. rolfsii.

Phenolic acids are formed in response to pathogens’ ingress, and their appearance is considered part of an active defense response. Gao et al. (2017) reported that plant pathogenic fungi’s mycelial growth could be inhibited by the phenol (4-chloro-3-methyl) synthesized by B. velezensis. The obtained results are confirmatory to Ray et al. (2020), reporting that the phenolic acids can inhibit the growth and development of S. rolfsii. It is interesting to report that the biocontrol potential of isolate B. atrophaeus strain NBRC 15539 was higher due to a high

| Incubation time (hour) | Percentage of inhibition (%) (dual culture method) | Percentage of inhibition (%) (modified dual culture method) |
|------------------------|----------------------------------------------------|----------------------------------------------------------|
|                        | CBK2007    | CBK3005    | CBK5001    | CBK6004    | CBK17001 | CBK2007 | CBK3005 | CBK5001 | CBK6004 | CBK17001 |
| 24                     | 14.58 ± 3.61 | 14.58 ± 3.61 | 10.41 ± 3.61 | 20.83 ± 3.61 | 10.67 ± 1.54 | 18.67 ± 2.31 | 23.33 ± 5.77 | 18.67 ± 2.31 | 43.33 ± 5.77 |
| 48                     | 11.33 ± 2.46 | 7.22 ± 1.43 | 9.02 ± 3.01 | 12.15 ± 1.42 | 14.6 ± 2.85 | 7.29 ± 1.93 | 69.65 ± 0.72 | 61.76 ± 1.44 | 61.34 ± 0.72 | 92.93 ± 0.72 |
| 72                     | 41.85 ± 0.64 | 36.67 ± 1.11 | 29.63 ± 1.28 | 36.3 ± 1.28 | 58.44 ± 1.35 | 73.25 ± 0.13 | 72.96 ± 0.64 | 31.11 ± 2.22 | 43.33 ± 1.11 | 93.7 ± 0.61 |

Table 3 Biochemical characteristics of strain CBK2007, CBK3005, CBK5001, CBK6004, and CBK17001

| Test                     | CBK 2007 | CBK 3005 | CBK 5001 | CBK 6004 | CBK 17001 |
|--------------------------|----------|----------|----------|----------|-----------|
| Growth on MacConkey      | −ve      | +ve      | −ve      | −ve      | −ve       |
| Indole                   | −ve      | −ve      | −ve      | −ve      | −ve       |
| Nitrate test             | −ve      | −ve      | −ve      | −ve      | −ve       |
| Voges-Proskauer test     | +ve      | +ve      | +ve      | −ve      | +ve       |
| Citrate utilization      | +ve      | −ve      | −ve      | +ve      | −ve       |
| Starch hydrolysis        | +ve      | +ve      | +ve      | +ve      | +ve       |
| Casein hydrolysis        | +ve      | +ve      | +ve      | +ve      | +ve       |
| Lipid hydrolysis         | +ve      | +ve      | +ve      | +ve      | +ve       |
| H₂S production           | −ve      | −ve      | −ve      | −ve      | −ve       |
| Catalase                 | +ve      | +ve      | +ve      | −ve      | +ve       |
| Oxidase                  | +ve      | −ve      | +ve      | +ve      | +ve       |
| Oxidation/fermentation   | O        | O        | O        | O        | O         |
| Gelatin liquefaction     | −        | −        | −        | −        | −         |
| Acid production from D-glucose | +          | +          | +          | +          | +          |
| D-ribose                | +        | +        | +        | +        | +          |
| Galactose                | +        | +        | +        | +        | −          |
| Fructose                 | +        | +        | +        | +        | +          |
| Maltose                  | +        | +        | +        | +        | +          |
| Mannitol                 | +        | +        | +        | +        | −          |
| Sucrose                  | +        | +        | +        | +        | +          |
| Glycerol                 | +        | +        | +        | +        | +          |
Fig. 2 a-e Phylogenetic tree of CBK2007, CBK3005, CBK5001, CBK6004, and CBK17001 respectively as constructed using MEGA 6 software by the neighbor-joining method indicating the phylogenetic relationship of the strain to closely related sequences from the Gene Bank database. Bootstrap values (expressed as percentages of 1000 replications) greater than 50% are shown at the branch points. Scale bar represents 0.005 substitutions per nucleotide position

Table 4 Plant growth-promoting activity and hydrolytic enzyme production by the isolated strains

| Different metabolites       | CBK2007 | CBK3005 | CBK5001 | CBK6004 | CBK17001 |
|----------------------------|---------|---------|---------|---------|----------|
| Amylase activity           | +       | +       | +       | +       | +        |
| Cellulase activity         | −       | −       | −       | −       | −        |
| Protease activity          | +       | +       | +       | +       | +        |
| Chitinase activity         | −       | −       | −       | −       | −        |
| Lipase activity            | +       | +       | +       | +       | +        |
| Siderophore production     | +       | ++      | ++      | −       | −        |
| HCN production             | +       | +       | −       | −       | −        |
| Ammonia production         | +       | +++     | +++     | ++      | ++       |

*+++* stronger production, *++* moderate production, *+* low production, *−* no production
amount of total phenolic acid. The results are consistent with those of Kumar et al. (2012), where they reported phenolic acid’s role in the biocontrol potential of Trichoderma harzianum. Therefore, it can also be concluded that bacterial extracts’ phenol content could be responsible for the antifungal activity against the phytopathogen, though knowing the exact role of phenol in antifungal activity is the subject matter of further investigation.

The Bacillus spp.-specific interaction with S. rolfsii is a very little-explored aspect of the microbial antagonism. The exact mechanism and molecular interactions leading to this phenomenon could be highly encouraging and useful for biocontrol of this economically highly important pathogen, though in the present study, we could find that every Bacillus strain had its characteristic antagonistic ability against the pathogen S. rolfsii, and it can be better utilized individually and in combination to produce a biofertilizer cum biocontrol agent for growth enhancement as well as disease suppression in the rice crop among others. Many of the secondary metabolites extracted from these strains should substitute certain fungicides and inorganic fertilizers, making the agricultural production systems more sustainable from both the environment and economic point of view.

**Conclusion**

The present study demonstrated the antifungal properties of Bacillus spp. isolated from rhizospheric soil of healthy rice crop plants against the pathogenic fungi S. rolfsii. All the five strains (CBK 2007 (B. subtilis subsp. Subtilis str.168), CBK 3005 (B. siamensis strain PDA 10), CBK 5001 (B. amyloliquefaciens strain 1034), CBK6004 (B. velezensis strain FZB42165), CBK 17001 (B. atrophaeus strain. NBRC 15539)) were found to exhibit antagonistic effects against fungal pathogen. Among all the five Bacillus spp., B. atrophaeus strain NBRC 15539 (accession no. MH283882) showed maximum inhibition of S. rolfsii by all three methods. Evidences suggest that the bacterial isolates identified in the present study can be used as an effective biocontrol agent solely or in combination for control of diseases caused by S. rolfsii. These strains can substitute certain chemical fungicides and inorganic fertilizers, making the agricultural production systems economically and environmentally sustainable.

**Abbreviations**

HCN: Hydrogen cyanide; PGPR: Plant growth-promoting rhizobacteria; PGPA: Plant growth-promoting activities; PDA: Potato dextrose agar; MEGA: Molecular evolutionary genetics analysis; CAS: Chrome-azurol-S; TPC: Total phenolic contents

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Authors' contributions
First author PK carried out the experiment, analyzed the data, interpreted the data, and contributed to the writing of the manuscript along with SC and SKB while SC and SKB also conceptualized the study. All the authors have read and approved the manuscript.

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References
Afsharmanesh H, Ahmadzadeh M, Javan-Nik bah M, Behboudi K (2013) Characterization of the antagonistic activity of a new indigenous strain of Pseudomonas fluorescens isolated from onion rhizosphere. J Plant Pathol 92:187–194
Ahmed NE, El Sharny AR, Awad HM (2020) Optimization and immobilization of amylase produced by Aspergillus terreus using pomegranate peel waste. Bull Natl Res Cent 44:1–12. https://doi.org/10.1186/s44269-020-00363-3
Atalla SM, Gamal NGE, Awad HM (2020) Chitinase of marine Penicillium chrysogenum MH745129: isolation, identification, production, and characterization as controller for citrus fruits postharvest pathogens. Jordan J Biol Sci 13:19–28
Athukorala SN, Fernando WD, Rashid KY, De Kievit T (2010) The role of volatile and non-volatile antibiotics produced by Pseudomonas chlororaphis strain PA23 in its root colonization and control of Sclerotinia sclerotiorum. Biocontrol Sci Technol 20:875–890. https://doi.org/10.1080/09583157.2010.484484
Awad HM, El Deen AMN, Mostafa ESE, Hassabo AA (2019) Biochemical studies and biological activities of L-glutaminase from rhizosphere soil Streptomyces rochei SAH2, CW1MSG: Egypt Pharm J 18:27. https://doi.org/10.4103/epjp.epj_32_18
Awad HM, Mostafa ESE, Saad MM, Selim MH, Hassan HM (2013) Partial purification and characterization of extracellular protease from a halophilic and thermotolerant strain Streptomyces pseudogriseolus NRC-15. Indian J Biochem Biophys 50:305–311
Cao L, Qu H, You J, Tan H, Zhou S (2005) Isolation and characterization of endophytic streptomycese antagonists of Fusarium wilt pathogen from surface-sterilized banana roots. FEMS Microbiol Lett 247:147–152. https://doi.org/10.1016/j.femsle.2005.05.006
Cappuccino JC, Sherman N (1992) Microbiology: A laboratory manual. 3rd edn. Benjamin Cummings, New York, pp 125–179. https://doi.org/10.2307/1380615
Castillo HF, Reyes CF, Morales GG, Herrera RR, Aguilar C (2013) Biological control of root pathogens by plant growth promoting Bacillus spp. Weed and pest control-conventional and new challenges.79–103. https://doi.org/10.5772/54229
Cattelan AJ, Hartel PG, Fuhrmann JJ (1999) Screening for plant growth-promoting rhizobacteria to promote early soybean growth. Soil Sci Soc Am J 63:1670–1680. https://doi.org/10.2136/ussa19996361670x
Chen L, Shi H, Heng J, Wang D, Bian K (2019) Antimicrobial, plant growth-promoting and genomic properties of the peanut endophyte Bacillus velezensis LDO2. Microbiol Res 218:41–48. https://doi.org/10.1016/j.micres.2018.10.002
Chen L, Wu YD, Chong XY, Qin QH, Wang DX, Bian K (2020) Seed-borne endophytic Bacillus velezensis LHS81 mediate the biocontrol of peanut stem rot caused by Sclerotium rolfsii. J Appl Microbiol 128:803–813. https://doi.org/10.1111/jam.14508
Clarridge JE (2004) 3rd. Impact of 16S rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious diseases. Clin Microbiol Rev 17:940–862. https://doi.org/10.1128/cmr.17.4.840-862.2004
Emmett EA, Handselman J (1999) Biocontrol of plant disease: a (Gram)+ positive perspective. FEMS Microbiol Lett 171:1–9. https://doi.org/10.1111/j.1574-6968.1999.tb13405.x
Fakoya S, Fakoya A, Adetuyi FC, Akinyosoye FA (2009) Antifungal activities of microbial isolates from sweet cassava (Manihot patnato, Grantz) Starch. Int J Appl Environ Sci 4:151–160
Gao Z, Zhang B, Liu H, Han J, Zhang Y (2017) Identification of endophytic Bacillus velezensis ZSY-1 strain and antifungal activity of its volatile compounds against Alternaria solani and Botrytis cinerea. Bioline 105:27–39. https://doi.org/10.1016/j.biocontrol.2016.11.007
Gholami M, Khakvar R, Niknam G (2014) Introduction of some new endophytic bacteria from Bacillus and Streptomyces genera as successful biocontrol agents against Sclerotum rolfsi. Arch Phytopathol Pflanzenschutz 7–122–130. https://doi.org/10.1007/s0335408.2013.805043
Harba M, Jawhar M, Arabi MIE (2020) In vitro antagonistic activity of diverse Bacillus species against Fusarium culmorum and F. solani pathogens. Open Agric 14. https://doi.org/10.2174/1747831502014010157
Holt JC, Krieg NR, Sneath PHA, Staley JT, Williams ST (1994) Bergey's manual of determinative bacteriology. Williams and Wilkins, Baltimore
Iftikhar Y, Saad A, Shakeel Q, Ahmad Z, Haq ZU (2020) Biological antagonism: a safe and sustainable way to manage plant diseases. In: Plant Disease Management Strategies for Sustainable Agriculture through Traditional and Modern Approaches, Springer, Cham, pp 83–109. https://doi.org/10.1007/978-3-030-35955-3_5
Jangir M, Pathak R, Sharma A, Sharma S, Sharma S (2019) Volatiles as strong markers for antifungal activity against Fusarium oxysporum f. sp. lycopersici. Indian Phytopathol 72:681–687. https://doi.org/10.5897/1429260-14290723
Jnaind JM, Dar NA, Bhat TA, Bhat AH, Bhat MA (2013) Commercial biocontrol agents and their mechanism of action in the management of plant pathogens. Int J Modern Plant Ani Sci 1:39–57
Karin M, Amini J, Harighi B, Bahmannejad B (2012) Evaluation of biocontrol potential of Pseudomonas and Bacillus spp. against fusarium wilt of chickepea. Aust J Crop Sci 6:695
Kour D, Rana KL, Yadav N, Yadav AN, Kumar A, Meena VS, Singh B, Chauhan VS, Dhalwals HS, Saxena AK (2019) Rhizospheric microorganisms: biodegradation, mechanisms of plant growth promotion, and biotechnological applications for sustainable agriculture. In: Plant Growth Promoting Rhizobacteria for Agricultural Sustainability, Springer, Singapore, pp 19–65. https://doi.org/10.1007/978-1-839-1753-8_3
Kumar DP, Anupama PD, Singh RK, Thennosu R, Nagasathya A, Thajuddin N, Paneerselvam A (2012) Evaluation of extracellular lytic enzymes from indigenous Bacillus isolates. J Microbiol Biotechnol 2:129–137
Kumar SN, Siji JV, Ramya R, Namibsan B, Mohandas C (2020) Improvement of antimicrobial activity of compounds produced by Bacillus spp. associated with a Rhodotillium sp. (entomopathogenic nematode) by changing carbon and nitrogen sources in fermentation media. J Microbiol Biotechnol Food Sci 9:1424–1438
Lorck H (1948) Production of hydrocyanic acid by bacteria. Physiol Plant 1:142–146. https://doi.org/10.1111/j.1399-3049.1948tb0718x
Nihorimbere V, Ongena M, Cavoy H, Brostaux Y, Kakana P, Jourdan E, Thonart P (2010) Beneficial effects of Bacillus subtilis on field-grown tomato in Burundi: reduction of local Fusarium disease and growth promotion. Afr J Microbiol Res 4:1135–1142. https://doi.org/10.5897/AJMR.0900123
Patel S, Saraf M (2017) Biocontrol efficacy of Trichoderma asperellum MSA5 against tomato wilting by Fusarium oxysporum f. sp. lycopersici. Arch Phytopathol Pflanzenschutz 50:228–238. https://doi.org/10.1080/03235408.2017.1287236
Prapagdee B, Kuekulvong C, Mongkolsuk S (2008) Antifungal potential of extracellular metabolites produced by Streptomyces hygroscopicus against phytopathogenic fungi. Int J Biol Sci 4:338. https://doi.org/10.7150/ijbs.4.338
Rangasami A, Rajan CRD, Harathimal BN, Bhaskar B, Santhiya V (2017) Evaluation of fungicides and herbicides on Sclerotium rolfsii, incitant of stem rot diseases in groundnut (Arachis hypogeal). Int J Pure App Biosci 5:92–97. https://doi.org/10.21878/2320-7513.3040
Rangeshwaran R, Prasad RD (2000) Biological control of Sclerotium rolfs rot of sunflower. Indian Phytopathol 53:444–449
Ray S, Swapnil P, Singh S, Sarma BK, Singh HB (2020) Endophytic Alcaligenes faecalis mediated redesigning of host defense itinerary against Sclerotium rolfsii through induction of phenolics and antioxidant enzymes. Biol Control 150:104355. https://doi.org/10.1016/j.biocontrol.2020.104355

Reyes-Ramírez A, Escudero-Abarca B, Aguilar-Uscanga G, Hayward-Jones PM, Barboza-Corona JE (2004) Antifungal activity of Bacillus thuringiensis chitinase and its potential for the biocntrol of phytopathogenic fungi in soybean seeds. J Food Sci 69:131–134. https://doi.org/10.1111/j.1365-2621.2004.tb10721.x

Riungu GM, Muthomi JW, Narla RD, Wagacha JM, Gathumbi JK (2008) Management of Fusarium head blight of wheat and deoxynivalenol accumulation using antagonistic microorganisms. Plant Pathol J 7:13–19. https://doi.org/10.3923/ppj.2008.13.19

Shrivastava UP, Kumar A (2011) Biochemical characterization of siderophore producing plant growth promoting rhizobacteria of rice rhizosphere. Nep J Integr Sci 1:31–37

Singh R, Maunya S, Upadhyay RS (2016) The improvement of competitive saprophytic capabilities of Trichoderma species through the use of chemical mutagens. Braz J Microbiol 47:10–17. https://doi.org/10.1016/j.bjm.2015.11.003

Singh UP, Sarma BK, Singh DP (2003) Effect of plant growth-promoting rhizobacteria and culture filtrate of Sclerotium rolfsii on phenolic and salicylic acid contents in chickpea (Cicer arietinum). Curr Microbiol 46:0131–0140. https://doi.org/10.1007/s00284-002-3834-2

Singleton AH, Oukaci R, Goodwin JG (1999) Processes and catalysts for conducting fischer-tropsch synthesis in a slurry bubble column reactor. Energy Int Corp 5:939, 350. U.S. Patent. https://doi.org/10.1016/s0140-6701(00)94202-4

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013) MEGA6: molecular evolutionary genetics analysis version 6.0. Mol Biol Evol 30:2725–2729. https://doi.org/10.1093/molbev/mst197

Zhao Y, Selvaraj JN, Xing F, Zhou L, Wang Y, Song H, Tan X, Sun L, Sangare L, Folly YME, Liu Y (2014) Antagonistic action of Bacillus subtilis strain SG6 on Fusarium graminearum. PLoS One 9. https://doi.org/10.1371/journal.pone.0092486

Zhong J, Chen D, Zhu HJ, Gao BD, Zhou Q (2016) Hypovirulence of Sclerotium rolfsii caused by associated RNA mycovirus. Front Microbiol 7:1798. https://doi.org/10.3389/fmicb.2016.01798

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