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# 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. amyloliquefaciens* 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*

## Background

*Sclerotium rolfsii* Sacc. (*Athelia rolfsii* (Curzi) Tu & Kimbrough) is a soil-borne plant pathogen, with a broad host range, causing seedling blight and stem rot of rice in tropical and subtropical regions, collar rot in chickpea and soybean, foot rot of ragi and damping off of tomato seedling, and stem rot diseases in groundnut (Zhong et al. 2016; Rangarani et al. 2017). It is a predominantly polyphagous, non-target, moisture-loving, and ubiquitous facultative parasitic basidiomycetes fungi that produce oxalic acid, poly-galacturonase, and cellulase as components of its pathogenesis (Chen et al. 2020). The characteristic growth feature of *S. rolfsii* in the infected plants is white fungal strands (mycelia or hyphae), which can be observed on the soil surrounding the plant at the base. The mycelia of *S. rolfsii* spread aggressively and remain active in the soil for longer durations of time in the form of sclerotia making the chemical or agronomic control viz. deep plowing, solarization, and crop rotation extremely difficult (Kumar et al. 2012). Not only this,

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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 Recourses, 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

pathogens was measured periodically. The degree of antagonism was calculated by estimating the pathogen's radial growth with bacterial culture vis-a-vis control, and the percentage of inhibition was calculated by the following equation (Riungu et al. 2008):

$$I = 100(C\text{-}T)/C$$

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).

### *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, $H_2S$ 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, and 3–4 drops of hydrogen peroxide were added following the growth of the isolates. The culture was observed for the presence or absence of the gas bubble. The bacterial culture was streaked on filter paper dipped in oxidase reagent during the oxidase test. After 15–30 s of streaking, the color change was observed. The deep blue color indicated a positive reaction, and the negative response was indicated by the presence of violet or purple. The starch agar media was prepared in the starch hydrolysis analysis followed by inoculation and incubation 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 region. However, confirmation of species was done using 16s rRNA sequencing (Eurofins, Bangalore, India). The DNA isolated from the bacteria was amplified with 16s rRNA specific primers 8F and 1492R. A single band of 1500 bp was observed on 1.2% agarose gel, which was then purified and was subjected to sequencing using Sanger's method followed by the polymerase chain reaction (PCR) amplification (94 °C for 4 min, 35 cycles of 95 °C for 1 min, 55 °C for 1 min, and 72 °C for 1.5 min, and a final extension at 72 °C for 10 min). To determine the similarity of the sequence obtained, multiple sequence alignment tool Clustal W was used. A phylogenetic tree was constructed using (MEGA) software version 6.0 (Tamura et al. 2013) using the neighbor-joining method with the bootstrap analysis based on 1000 replicates

### *Plant growth-promoting rhizobacteria and hydrolytic enzyme production*

Qualitative estimation of siderophore by chrome-azurol-S (CAS) plate assay was done as per Shrivastava and Kumar (2011). The discernment of siderophore generation was carried out by the CAS plate assay method. The bacterium was spot inoculated at the center of the plate and for 72 h at 37 °C. 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. Bacterial isolates were tested for ammonia production, as described by Cappuccino and Sherman (1992) and Awad et al. (2019). The isolates were grown in peptone water at 37 °C for 3 days. At the end of the incubation period, 0.5 ml of Nessler's reagent (Hi-Media, Mumbai, India) was added to each tube. The appearance of faint yellow to dark brown color after the addition of Nessler's reagent indicated ammonia. All five bacterial isolates were subsequently biochemically characterized by the test for the production of amylase (Ahmed et al. 2020), cellulase production (Cattelan et al. 1999), protease production (Awad et al. 2013), lipase production (Kumar et al. 2012), and chitinase activity (Atalla et al. 2020).

### *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 $Na_2CO_3$ (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 (CBK 2007, CBK 3005, CBK 5001, CBK 6004, 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 and covering the complete area within 72 h. By the dual culture method (Table 2), the isolate CBK17001 (58.45 ± 1.28%) showed maximum inhibition followed by CBK2007 (41.85 ± 1.09%), CBK3005 (36.67 ± 1.11%), CBK6004 (36.3 ± 1.22%), and CBK5001 (29.63 ± 1.06%). The secondary screenings of the isolated bacterial strains were conducted by a novel ring method (Fig. 1) in which 100% inhibition of *S. rolfsii* by all the five bacterial isolates was observed. Table 2 (modified dual culture method) showed that CBK17001 (93.7 ± 1.29%) increase in inhibition than other bacterial isolates, whereas CBK2007 (73.22 ± 2.23%) and CBK3005 (72.78 ± 1.29%) were showing almost the same result followed by CBK6004 (43.33 ± 1.26%) and CBK 5001 (31.11 ± 1.28%). All the isolates exhibited a difference in their percentage of inhibition of mycelial growth of *S. rolfsii* after 72 h when compared to each other.

It was observed that the antagonistic effect of bacterial isolates in the novel ring method was maximum compared to the other two methods employed in the present study. A similar finding was reported by Fakoya et al. (2009) that the antagonistic activity against *S. rolfsii* was better in the novel ring method by providing sufficient screen relative to the dual culture method, which was reported to be less effective.

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,

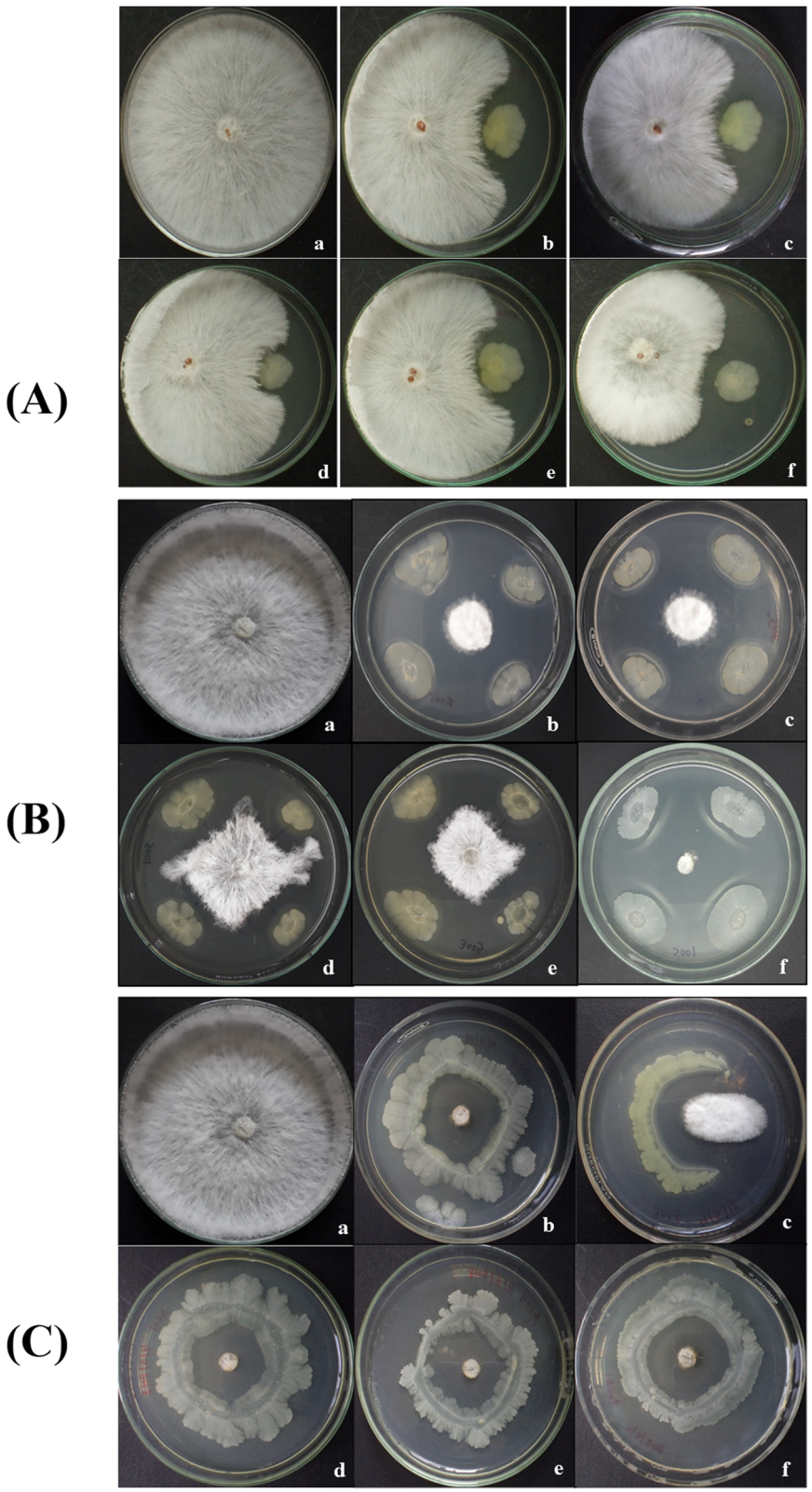


**Fig. 1** (See legend on next page.)

(See figure on previous page.)
**Fig. 1** In vitro interaction between bacterial isolates and *S. rolfsii* on PDA (potato dextrose agar) media plate at third day after incubation at 28 °C. **a** Dual culture method (bacterial isolates were inoculated with the distance 3 cm from the colony of *Sclerotium rolfsii*, **b** modified dual culture method (bacterial isolates were inoculated at 4 sites on PDA plate with equal distance, 3 cm apart from the colony of *S. rolfsii*), and **c** novel ring method (bacterial isolates were inoculated in circular way in PDA plates with the radius 2.5 cm away from the colony of *S. rolfsii*). **a** Five-millimeter agar plug of *S. rolfsii* on the center of PDA plate, **b** CBK2007, **c** CBK3005, **d** CBK5001, **e** CBK6004, and **f** CBK17001

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 (27.1 μg/ml), *Bacillus 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

**Table 2** Antifungal activity of bacterial isolates (CBK2007, CBK3005, CBK5001, CBK6004, and CBK17001) by dual culture method and modified dual culture method against *Sclerotium rolfsii* (*S. rolfsii*) (in percentage (%)) (results are expressed as means of three experiments and values are expressed as mean ± SD)

| 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 | 8.33 ± 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 | 72.29 ± 1.93 | 69.65 ± 0.72 | 61.76 ± 1.44 | 61.34 ± 1.25 | 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 |

(28.17 μg/ml), 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

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

| Test | CBK 2007 | CBK 3005 | CBK 5001 | CBK 6004 | CBK 17001 |
|---|---|---|---|---|---|
| Biochemical characterization | | | | | |
| 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 |
| $H_2S$ 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 | + | + | + | + | + |
| Ribose | + | + | + | + | + |
| Galactose | + | + | + | + | - |
| Fructose | + | + | + | + | + |
| Maltose | + | + | + | + | + |
| Mannitol | + | + | + | + | - |
| Sucrose | + | + | + | + | + |
| Glycerol | + | + | + | + | + |

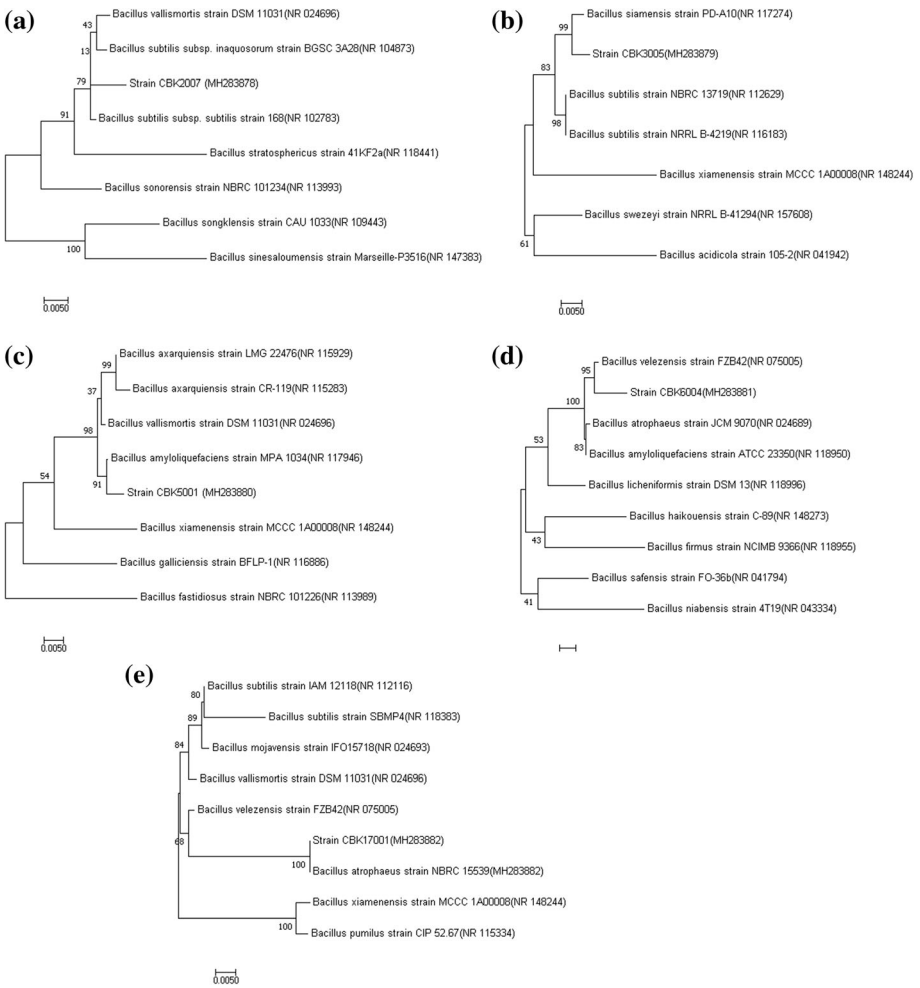


**Fig. 2 a**–**e** Phylogenetic tree of CBK2007, CBK3005, CBK5001, CBK6004, and CBK17001 respectively as constructed using MEGA 6 software by the neighbor-joining method indicating the phylogenetic relationship of the strain to closely related sequences from the Gene Bank database. Bootstrap values (expressed 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

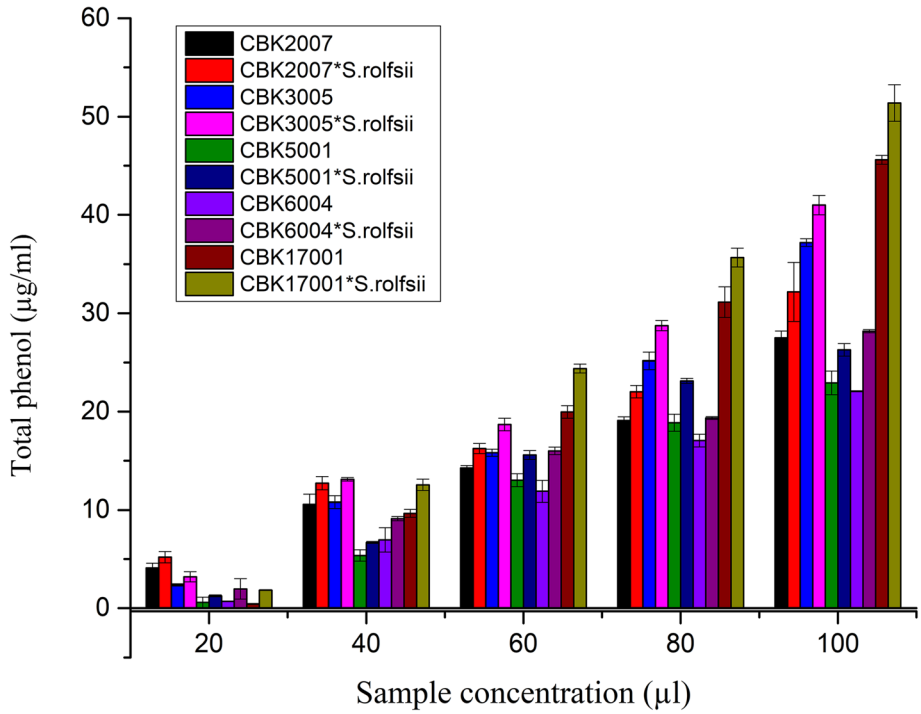


**Fig. 3** Total phenol in the culture filtrate of bacterial isolates alone and of their interaction with *Sclerotium rolfsii* (values are expressed as mean ± SD ($n$ = 3))

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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