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RESEARCH

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Antagonist activities of native rhizosphere micro-flora against groundnut stem rot pathogen, *Sclerotium rolfsii* Sacc.

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Abstract

Background: Stem rot caused by *Sclerotium rolfsii* Sacc. is a major nuisance in groundnut production, causing substantial yield losses in almost all groundnut-growing areas around the world. Biological control is regarded as a sustainable choice over the currently popular management strategy i.e., chemical control, as later has a negative influence on the environment. The present study evaluated the antagonistic effect of native rhizosphere micro-flora against groundnut stem rot pathogen *S. rolfsii*.

Results: A total of 111 bacterial isolates and 9 *Trichoderma* isolates isolated from groundnut rhizosphere soil were evaluated for their antagonist activity against *S. rolfsii* in vitro. Eight isolates (seven bacterial and one *Trichoderma*) were chosen as prospective biocontrol candidates based on the findings of the dual culture assay. Molecular characterization of these isolates by 16S rDNA and ITS rDNA sequencing confirmed the identity of bacterial isolates as *Bacillus* spp. (six *B. subtilis* and one *B. amyloliquefaciens*) and fungal isolate as *Trichoderma asperellum*. Also, the selected seven bacterial isolates recorded favourable results for antagonism-promoting biochemical traits.

Conclusion: The results of the current study suggested that the native groundnut rhizosphere micro-flora can be exploited for biological control of groundnut stem rot pathogen *S. rolfsii*. Further research may enable the use of the isolated rhizosphere biocontrol agents as single organisms or in a consortium for sustainable management of the groundnut stem rot pathogen.

Keywords: Groundnut stem rot, *Sclerotium rolfsii*, Biological control, Rhizosphere micro-flora, *Bacillus*, *Trichoderma*

Background

Biological control is fast gaining importance as a sustainable strategy for managing plant diseases which is effective and environment friendly. A critical review of the literature on biological control suggests skewed research towards soil-borne pathogens, as the response has been more positive in this area as compared to foliar pathogens (Kumar and Thirumalaisamy 2016). Numerous bacterial and fungal agents are used for the biological control of a variety of plant diseases. *Bacillus* and *Pseudomonas* are majorly exploited bacterial biocontrol agents, while *Trichoderma* is the widely exploited fungal biocontrol agent.

While talking about biological control, disease-suppressive soils have to be emphasized as it is the major source of biocontrol agents. Disease-suppressive soils offer effective protection to plants against infection by soil-borne pathogens and the specific disease suppression that occurs in these soils is mostly microbial in origin (Gómez Expósito et al. 2017). Rhizosphere, the narrow zone surrounding and influenced by plant roots, is a hotspot for a wide variety of organisms and is regarded as one of the exceedingly complex ecosystems on the earth (Raaijmakers et al. 2009). Rhizospheric organisms

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play a critical role in reprogramming the entire defence response of the host plant (Spence et al. 2014).

Groundnut [*Arachis hypogaea* L.], "the king of oilseeds", is a member of the family Fabaceae and is an edible oilseed crop that is extensively used for oil extraction, cooking, and domestic purposes. India is the second largest producer of groundnut after China. However, the area under groundnut cultivation in India has declined over the years from 8.30 million ha during 1990–1991 to 6.02 million ha by 2020–2021 owing to various biotic and abiotic constraints including low moisture, poor soil fertility, the incidence of pests, and diseases (INDIASTAT 2022). Among the soil-borne diseases affecting groundnut, stem rot (*Sclerotium rolfsii* Sacc.) and crown/collar rot (*Aspergillus niger*) are threats to successful groundnut cultivation and can cause yield losses up to 50% (Joshi et al. 2020).

Stem rot disease is a major constraint in groundnut production, causing severe yield losses in almost all groundnut-growing areas around the world. It is caused by *S. rolfsii*, an ubiquitous, polyphagous soil-borne pathogen that causes destructive plant diseases of different crop species. The wide host range of *S. rolfsii* due to its prolific growth and ability to produce persistent sclerotia contribute to the large economic losses associated with this disease (Cilliers et al. 2003).

Management of stem rot is difficult because the pathogen can survive in the soil and plant tissues and has a wide host range. The use of chemicals is one of the widely practiced methods to manage the disease. However, it has many disadvantages such as the emergence of fungicide-resistant populations, disruption of local ecosystems, increased cost and labour and threats to human health. Therefore, it is important to develop effective and environmentally friendly methods to control the disease and biological control comes to play here.

The potential of rhizosphere microorganisms in controlling soil-borne plant pathogens like *S. rolfsii* has been well-established by researchers around the world (Swaroopa and Madhuri 2021). Beneficial microbes present in native rhizosphere hold immense potential in managing soil-borne pathogens, so it is necessary to study their antagonist potential. The antagonistic bacterial population in the groundnut rhizosphere against *S. rolfsii* majorly belongs to *Bacillus, Pseudomonas* and *Burkholderia* (Le et al. 2018). Swaroopa and Madhuri (2021) reported that *Bacillus* spp. isolated from the soil are capable of promoting plant growth, while simultaneously inhibiting the growth of *S. rolfsii* in groundnut. Native isolates of *Trichoderma* spp. and rhizosphere bacteria from groundnut rhizosphere soil are effective against pod rot associated pathogens of groundnut including *S. rolfsii* (Ramanjineyulu et al. 2021).

Various other scientists have reported the ability of rhizosphere micro-flora in suppressing plant diseases caused majorly by soil-borne pathogens like *S. rolfsii* and promoting plant growth in a variety of crops including groundnut (Leona et al. 2020). So, the present investigation focussed on determining the antagonistic activity of native rhizosphere micro-flora against the groundnut stem rot pathogen *Sclerotium rolfsii* in vitro.

Methods

Test pathogen *Sclerotium rolfsii* Sacc.

The pathogen *S. rolfsii* was isolated from the stems of infected groundnut plants with white mycelial growth on the collar region on potato dextrose agar (PDA) medium by tissue segment method (Rangaswami and Mahadevan 1999). A pathogenicity test was conducted, and the isolate was molecularly confirmed by ITS rDNA sequencing as *Athelia rolfsii* (Fig. 1). Gene sequence of the test pathogen was submitted to NCBI GenBank as *Athelia rolfsii* isolate GNS1 under the accession number OL150603.1.

Collection of rhizosphere soil samples

A roving survey was conducted in groundnut-growing regions of Nagarkurnool district of Telangana, India, for the collection of rhizosphere soil samples. Rhizosphere soil samples adhering to the roots of groundnut plants were collected for the isolation of rhizosphere micro-flora. Collected rhizosphere soil samples were stored in an icebox for transportation to the laboratory.

Isolation of rhizosphere micro-flora

The serial dilution and plating method by Timonin (1940) was used for the isolation of microorganisms from groundnut rhizosphere soil. Briefly, 10 g of rhizosphere soil is serially diluted to obtain dilutions of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6}. Dilutions of 10^{-3} and 10^{-4} were used for isolation in potato dextrose agar medium (PDA), Martin's Rose Bengal agar medium (RBA) and actinomycetes isolation agar medium (AIA), while dilutions of 10^{-5} and 10^{-6} were used for isolation in Nutrient agar medium (NA) and King's B medium (KB). Hundred µl of respective dilutions were spread onto potato dextrose agar medium (PDA) and Martin's Rose Bengal agar medium (RBA) for isolation of fungus and Nutrient agar medium (NA), King's B medium (KB) and actinomycetes isolation agar medium (AIA) for isolation of bacteria present in rhizosphere soil. These media plates were incubated at 25 ± 2 °C for 3–4 days and 28 ± 2 °C for 24–48 h for isolation of fungus and bacteria, respectively. After the completion of incubation, subculturing was done to obtain pure cultures. Pure cultures of fungal isolates were obtained by the single spore and single hyphal tip method. While bacterial pure cultures were obtained by

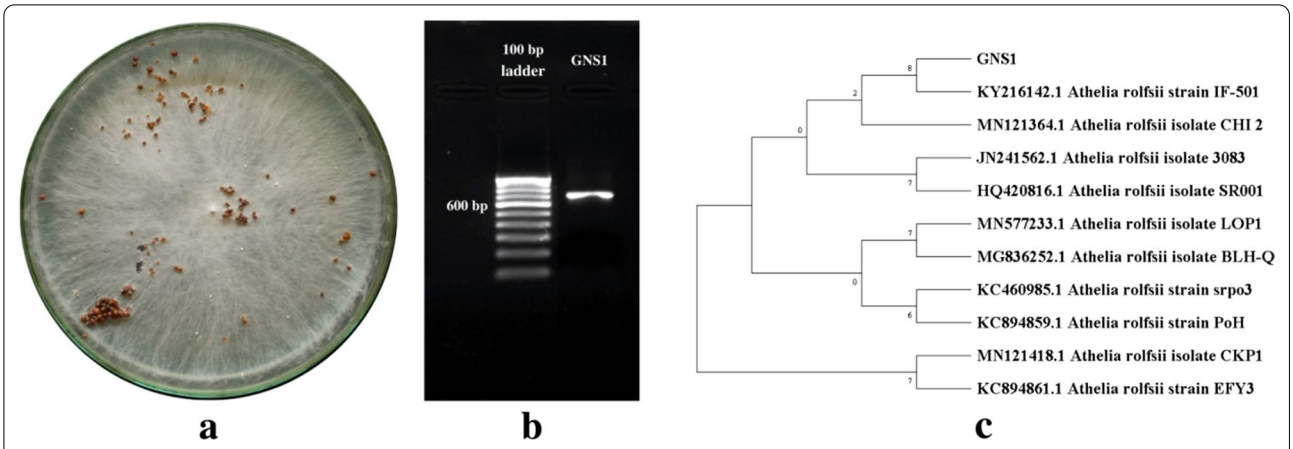

Fig. 1 Test pathogen *Sclerotium rolfsii* Sacc. **a** Pure culture, **b** Gel profile of PCR product of ITS rDNA gene sequence of test pathogen, **c** Molecular phylogenetic tree of partial ITS rDNA sequence of test pathogen

picking a single colony with a sterilized inoculation loop and streaking in fresh sterile media plates. The isolates were maintained by periodical subculturing.

Screening of antagonistic activity of rhizosphere isolates against *S. rolfsii* in vitro

The rhizosphere isolates were tested against stem rot pathogen in vitro by dual culture technique (Dennis and Webster 1971).

Primary screening of rhizosphere isolates for antagonism against *S. rolfsii*

All bacterial isolates were subjected to primary screening for assessing their inhibitory effect on the growth of *S. rolfsii*. Five-mm mycelial disc of 5-day-old culture of *S. rolfsii* was placed in the centre of a sterile agar plate and four distinct bacterial isolates streaked 1 cm away from the periphery on four sides without touching each other. A control plate with only *S. rolfsii* was also maintained and observations were made once complete growth has been reached in this plate. If the *S. rolfsii* mycelium grows over the streak of rhizosphere isolate it is considered non-antagonistic. In another case, if the mycelial growth of *S. rolfsii* was halted by the isolate, the isolate was selected for secondary screening, while all the rhizosphere fungal isolates identified as *Trichoderma* were considered in secondary screening.

Secondary screening of rhizosphere isolates for antagonism against *S. rolfsii*

Isolates showing inhibitory effects in primary screening were then tested for their antagonism against *S. rolfsii* in the dual culture. Loopful of 24-h-old pure cultures of test isolate was streaked 1 cm away from the periphery of PDA plates, and a 5-mm mycelial disc of 5-day-old culture of *S. rolfsii* was placed at the opposite end and incubated at 25 ± 2 °C. A control plate with only *S. rolfsii* was also maintained, while only the fungal rhizosphere isolates identified as *Trichoderma* through colony morphology and microscopy were studied for antagonism against *S. rolfsii*. Five-mm mycelial discs of a 5-day-old culture of *S. rolfsii* and the test fungal isolate were placed at opposite ends of the Petri dish 1 cm away from the periphery and incubated at 25 ± 2 °C. When full growth was achieved in the control plate, the mycelial growth of the pathogen was measured in each Petri dish separately and expressed in mm. Per cent inhibition of the mycelial growth of the pathogen by different test isolates was calculated using the formula given by Vincent (1947):

$$I = \frac{C - T}{T} \times 100$$

where I = Per cent inhibition of mycelial growth over control; C = Radial growth of the pathogen in control (mm); T = Radial growth of the pathogen in treatment (mm).

Morphological characterization of potential biocontrol isolates

Morphological and biochemical characterizations of the potential bacterial biocontrol isolates were done using standard methods outlined in Bergey's Manual of Determinative Bacteriology and Borkar (2017). Gram staining and biochemical characterization were performed on bacteria that had been cultured for 24 h. Potential *Trichoderma* isolates were inoculated in sterile potato dextrose agar (PDA) plates and incubated at 25 ± 2 °C for 7 days. The cultural characteristics of *Trichoderma* isolate on PDA plates were recorded. Further identification of *Trichoderma* isolate was done by examining the cultures

under the microscope for the characters of conidia and conidiophores (Barnett and Hunter 1972).

Molecular characterization of potential biocontrol isolates

Molecular characterization of the potential rhizosphere fungal isolate was done by ITS rDNA sequencing, while that of potential rhizosphere bacterial isolates was done by 16S rDNA sequencing. Genomic DNA of fungal and bacterial isolates was extracted by the methodologies proposed by Lee (1990) and Bazzicalupo and Fancelli (1997), respectively, with minor modifications. PCR amplification of 16S rDNA region of isolated bacterial DNA was carried out with 27F and 1492R primers while fungal DNA isolated was amplified at internal transcribed spacer region by ITS1F and ITS4 primers. A small volume (10 µl) reaction was carried out, followed by a large volume (50 µl) reaction. Base sequences of primers used, the composition of PCR mixture and cycling conditions and amplicon size are given in Table 1. PCR products were then sequenced, and sequence results obtained were analysed using BioEdit, MEGA11 and NCBI-BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi#). The top ten NCBI hits for each sequence were aligned using ClustalW, followed by Molecular Phylogenetic analysis by using the Maximum Likelihood method and Kimura 2-parameter model in MEGA11. Based on that, the closest homolog of each isolate from the NCBI GenBank database was identified.

Screening of potential bacterial isolates antagonism-promoting biochemical parameters

Selected potential antagonist bacterial isolates were studied for their antagonism-promoting biochemical parameters i.e., HCN, ammonia, siderophore, cellulase and pectinase productions.

HCN production

HCN production of test isolates was conducted as per the method of Castric and Castric (1983). The selected bacterial isolates were streaked on nutrient agar supplemented with 0.44% (w/v) glycine. A Whatman filter paper saturated with alkaline picric acid solution (2% sodium carbonate in

Table 1 Molecular characterization of potential biocontrol isolates

(a) Base sequences of 16S rDNA and ITS rDNA primers used

Primers	Primer ID	Sequence	Base pairs
16S rDNA	27F	5′-AGAGTTTGATCCTGGCTCAG-3′	20
	1492R	5′-TACGGYTACCTTGTTACGACTT-3′	22
ITS rDNA	ITS1F	5′-TCCGTAGGTGAACCTGCGG-3′	19
	ITS4	5′-TCCTCCGCTTATTGATATGC-3′	20

(b) PCR mixtures for 10 µl and 50 µl reaction volumes

Components	Quantity for one reaction	
	Total volume (10 µl)	Total volume (50 µl)
EmeraldAmp GT PCR Master Mix (2X premix)	5 µl	25 µl
Primers (2.5 pmol/µl)		
Forward	1 µl	5 µl
Reverse	1 µl	5 µl
Template DNA (100 ng/µl)	1 µl	5 µl
dH_2O	2 µl	10 µl
Total volume	10 µl	50 µl

(c) Cycling conditions and amplicon size for 16S rDNA and ITS rDNA amplification

Step	16S rDNA	ITS rDNA
Initial denaturation	96 °C for 4 min	94 °C for 5 min
35 cycles of		
Final denaturation	94 °C for 40 s	94 °C for 45 s
Primer annealing	57 °C for 1 min	55 °C for 45 s
Extension	72 °C for 80 s	72 °C for 1 min
End of cycle		
Final extension	72 °C for 10 min	72 °C for 5 min
Amplicon size	~1500 bp	~600 bp

0.5% picric acid) was placed on the upper lids of Petri plates and incubated at 30 ± 2 °C for 4 days. Plates are monitored for the development of red-brown shade from the yellow colour of filter paper, indicating HCN production.

Ammonia production

Estimation of ammonia production by test isolates was carried out by adding 0.5 ml Nessler's reagent to isolates grown in peptone water broth (5 g peptone and 10 g sodium chloride in 1 L water) at 30 ± 2 °C for 48 h. The development of slight yellow to brownish colour indicates the production of ammonia (Gupta and Pandey 2019).

Siderophore production

A qualitative assay of siderophore production activity of test isolates was tested on Chrome Azurol S (CAS) agar medium (Schwyn and Neilands 1987). The test bacterial isolates were spot-inoculated on CAS agar plates and incubated at 30 ± 2 °C for 3–4 days. The formation of a yellow to orange coloured zone around the colony indicates siderophore production. Based on the clearing halo zone length, solubilization index (SI) and solubilization efficiency (SE) were calculated for each isolate using the following formulas:

$$\text{Solubilization index (SI)} = \frac{C+Z}{C}$$

$$\text{Solubilization efficiency (SE)} = \frac{Z}{C} \times 100$$

where Z = Solubilization zone (mm); C = Colony diameter (mm).

Chrome Azurol S reagent

Solution 1	
Chrome azurol S	0.0605 g
Distilled water	50 ml
Solution 2	
Ferric chloride	0.0027 g
10 mM Hydrochloric acid	10 ml
Solution 3	
HDTMA	0.0729 g
Distilled water	40 ml

Solution (1) was mixed with 9 ml of solution (2) this was mixed with solution (3). The solution should now be in blue. Autoclave and store in a plastic container.

CAS agar plates: King's B medium was prepared separately and autoclaved after which 99 ml of Chrome Azurol S reagent (prepared as above) was added to 750 ml of medium aseptically which gave it a greenish-blue colour. The medium was then poured into plates and allowed to solidify.

Cellulase production

Cellulose production activity was screened on carboxymethylcellulose (CMC) agar medium [sodium nitrate-2 g; potassium dihydrogen phosphate-1 g; magnesium sulphate-0.5 g; potassium chloride-0.5 g; carboxymethylcellulose-2 g; peptone-0.2 g; agar-20 g; distilled water-1000 ml] according to the method of Hankin and Anagnostakis (1977) as modified by Kasana et al. (2008). Test isolates were spot-inoculated on CMC agar medium plates and incubated at 30 ± 2 °C for 3–4 days. After incubation, plates were flooded with Gram's iodine (2.0 g KI and 1.0 g iodine in 300 ml distilled water) for 3 to 5 min. Positive cellulase production activity was indicated by the formation of a zone of clearance around the colony. Based on the clearing halo zone length, solubilization index (SI) and solubilization efficiency (SE) were calculated for each isolate.

Pectinase production

Screening for pectinase production activity by isolates was studied on pectinase screening agar medium (PSAM) [disodium hydrogen phosphate-6 g; potassium dihydrogen phosphate-3 g; sodium chloride-5 g; ammonium chloride-2 g; magnesium sulphate-0.1 g; pectin-10 g; agar-20 g; distilled water-1000 ml; final pH-4.5 ± 0.5] (Oumer and Abate 2018). Test isolates were spot inoculated on PSAM agar plates and incubated at 30 ± 2 °C for 2 days. After incubation, plates were flooded with Gram's iodine (2.0 g KI and 1.0 g iodine in 300 ml distilled water) for 3 to 5 min. Positive pectinase production activity was indicated by the formation of a zone of clearance around the colony. Based on the clearing halo zone length, solubilization index (SI) and solubilization efficiency (SE) were calculated for each isolate.

Statistical analysis

The data of the experiments were subjected to one-way analysis of variance (ANOVA), and means were separated ($p \leq 0.05$) by Fisher's least significant difference (LSD) using statistical analysis software GRAPES 1.0.0, Kerala Agricultural University, Kerala, India (Gopinath et al. 2021).

Results

Collection of rhizosphere soil samples

The survey covered 10 villages in 6 mandals of Nagarkurnool district viz., Bijinapalle, Tadoor, Kalwakurthy, Nagarkurnool, Peddakothapally and Kollapur. Groundnut rhizosphere soil samples were collected from 12 different sites in these six mandals. The soil pH, electrical conductivity, available nitrogen, available phosphorous, available potassium and organic carbon of survey soil samples were analysed and are presented in Table 2.

Table 2 Physicochemical properties of groundnut rhizosphere soils collected from different locations of Nagarkurnool district

Sample ID	Collected from		Field location		pH	Electrical conductivity (dS m^{-1})	Available nitrogen (kg ha^{-1})	Available phosphorous (kg ha^{-1})	Available potassium (kg ha^{-1})	Organic carbon (%)
	Mandal	Village	Latitude (°N)	Longitude (°E)						
S1	Bijinapalle	Palem	16.51448	78.250468	6.11 ± 0.17	0.354 ± 0.005	175.56 ± 2.55	39.33 ± 0.29	527.25 ± 2.83	0.58 ± 0.01
S2		Palem	16.51645513	78.23946526	6.09 ± 0.14	0.455 ± 0.002	128.31 ± 0.73	42.67 ± 0.76	502.88 ± 3.90	0.55 ± 0.01
S3	Tadoor	Polmur	16.55449166	78.41018216	6.62 ± 0.22	0.382 ± 0.002	114.24 ± 1.92	28.50 ± 2.50	280.88 ± 2.60	0.29 ± 0.01
S4	Kalwakurthy	Tharnikal	16.63257867	78.47112144	6.65 ± 0.07	0.369 ± 0.006	109.83 ± 3.69	35.83 ± 0.76	297.38 ± 4.55	0.22 ± 0.01
S5	Nagarkurnool	Vanapatla	16.44346687	78.34063245	7.42 ± 0.07	0.380 ± 0.005	184.38 ± 0.96	38.67 ± 0.58	307.50 ± 0.65	0.47 ± 0.01
S6		Vanapatla	16.442247	78.341947	7.57 ± 0.03	0.434 ± 0.003	190.47 ± 2.91	48.50 ± 0.87	535.13 ± 5.20	0.52 ± 0.02
S7		Deshitkyal	16.44037965	78.34143955	7.09 ± 0.11	0.371 ± 0.002	116.97 ± 2.98	28.17 ± 0.29	267.75 ± 2.98	0.41 ± 0.01
S8	Peddakothapally	Adirala	16.363835	78.359953	7.46 ± 0.14	0.341 ± 0.003	136.92 ± 1.31	34.50 ± 0.50	392.25 ± 1.30	0.47 ± 0.01
S9		Chandrakal	16.352544	78.359924	6.91 ± 0.03	0.342 ± 0.002	137.13 ± 5.36	41.33 ± 0.76	357.38 ± 8.52	0.48 ± 0.01
S10		Chennapuraopally	16.192217	78.322912	7.02 ± 0.14	0.336 ± 0.002	102.27 ± 1.92	26.67 ± 0.29	352.88 ± 7.32	0.32 ± 0.01
S11	Kollapur	Ankiraopally	16.167291	78.315946	7.40 ± 0.11	0.425 ± 0.003	141.12 ± 3.51	36.33 ± 0.29	441.38 ± 2.34	0.25 ± 0.01
S12		Choutabetla	16.12870577	78.30240042	7.11 ± 0.09	0.408 ± 0.002	104.37 ± 1.59	44.50 ± 0.50	329.63 ± 1.95	0.29 ± 0.01

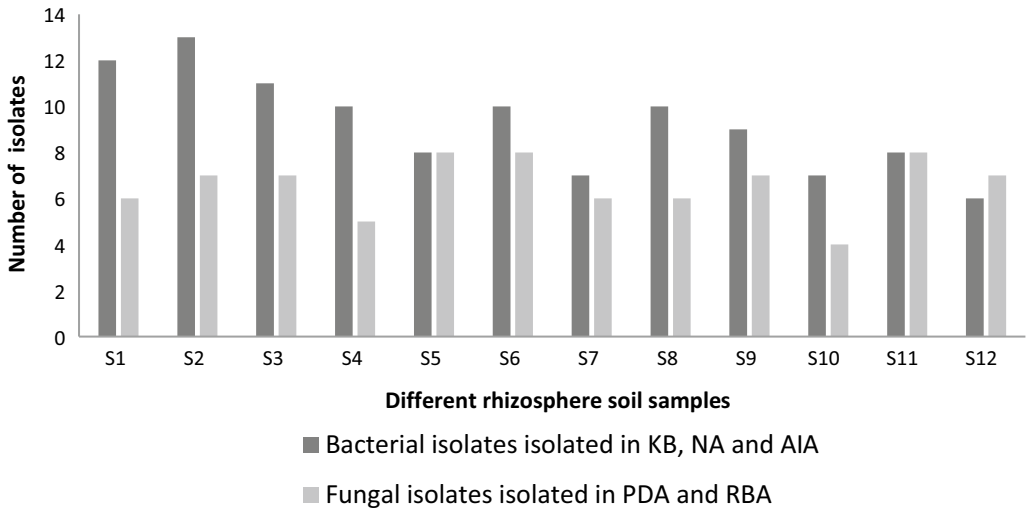

Fig. 2 Number of isolates isolated from collected groundnut rhizosphere soil samples

Isolation of rhizosphere micro-flora

Rhizosphere micro-flora was isolated by serial dilution and plating method on five different media viz., King's B medium (KB), nutrient agar medium (NA), actinomycetes isolation agar medium (AIA), potato dextrose agar medium (PDA) and Martin's Rose Bengal agar medium (RBA) from the collected 12 rhizosphere soil samples. A total of 111 bacterial isolates were obtained from KB, NA and AIA, and 79 fungal isolates were obtained from PDA and RBA, respectively (Fig. 2). All the bacterial and fungal isolates were maintained by periodical subculturing for use in further experiments.

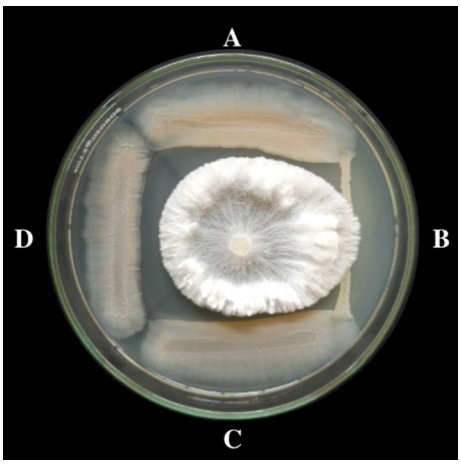

Fig. 3 Primary screening of rhizosphere bacterial isolates for antagonism against *Sclerotium rolfsii*; here A, C, and D are antagonistic while B is non-antagonistic

Primary screening of rhizosphere isolates for antagonism against *S. rolfsii*

All 111 bacterial isolates obtained were considered for primary screening for antagonism against *Sclerotium rolfsii* (Fig. 3). Among these, 33 isolates (12 from KB, 15 from NA and 6 from AIA) showed antagonism against *S. rolfsii*, which were then evaluated for antagonism against *S. rolfsii* in dual culture. Among the 79 fungal isolates, 9 isolates were identified as *Trichoderma* and these were selected for testing in dual culture for antagonism against *S. rolfsii*.

Secondary screening of rhizosphere isolates for antagonism against *S. rolfsii*

A total of 33 bacterial isolates (12 from KB, 15 from NA, and 6 from AIA) was selected through primary screening and 9 native *Trichoderma* isolates isolated from rhizosphere soil were tested for their antagonistic activity against *S. rolfsii* by dual culture technique (Figs. 4, 5). Observations were taken on the day when the radial growth of *S. rolfsii* in the control plate was full. Among the 33 bacterial isolates tested, isolate S3KB6, S9KB4 and S1NA7 recorded maximum inhibition of 62.82, 61.70 and 61.11% over control, respectively, followed by isolates S2NA6 (58.52%), S10KB2 (57.25%), S4KB5 (55.39%) and S8KB2 (54.64%). Significantly minimum inhibition of 11.11% over control was recorded by isolate S11A1, which is on par with the values recorded by isolates S6A1 (11.48%) and S3KB2 (13.01%). The zone of inhibition was maximum in isolate S3KB6 (37.33 mm), followed by S1NA7 (27.67 mm). However, isolates S3KB2, S6A1 and S11A1 did not record any zone of inhibition (Table 3; Fig. 4).

Table 3 Antagonistic activity of rhizosphere bacterial isolates on radial growth of *Sclerotium rolfsii* by dual culture technique

Isolate	Mycelial growth (mm)	Per cent mycelial inhibition	Zone of inhibition (mm)
S1KB2	66.00^{de}	26.39^{qr}	7.67^{p}
S1NA3	63.67^{fg}	29.26^{op}	4.67^{r}
S1NA4	68.33^{c}	24.07^{r}	1.33^{u}
S1NA5	44.67^{n}	50.37^{fg}	20.67^{f}
S1NA7	35.00^{s}	61.11^{ab}	27.67^{b}
S2KB4	46.33^{mn}	48.33^{gh}	10.67^{l}
S2NA1	61.67^{g}	31.48^{no}	8.33^{op}
S2NA2	51.33^{j}	42.96^{jk}	9.33^{mn}
S2NA3	67.67^{cd}	24.81^{r}	3.67^{s}
S2NA6	37.33^{r}	58.52^{bc}	19.00^{gh}
S3KB2	78.00^{b}	13.01^{s}	0^{v}
S3KB6	33.33^{s}	62.82^{a}	37.33^{a}
S4A1	64.00^{ef}	28.89^{opq}	1.33^{u}
S4KB2	55.33^{i}	38.28^{l}	19.33^{g}
S4KB4	44.67^{n}	50.18^{g}	8.33^{op}
S4KB5	40.00^{pq}	55.39^{de}	19.67^{g}
S6A1	79.67^{b}	11.48^{s}	0^{v}
S6NA1	52.00^{j}	42.22^{k}	15.33^{j}
S6NA5	42.33^{o}	52.96^{ef}	10.67^{l}
S7NA3	57.67^{h}	35.93^{lm}	6.33^{q}
S8A1	48.33^{lm}	46.30^{hi}	22.33^{e}
S8A2	59.33^{h}	34.07^{mn}	8.67^{no}
S8KB2	40.67^{op}	54.64^{e}	25.00^{cd}
S8NA1	41.67^{op}	53.70^{e}	18.33^{hi}
S9KB1	49.00^{kl}	45.35^{ij}	11.67^{k}
S9KB2	41.67^{op}	53.53^{e}	17.67^{i}
S9KB4	34.33^{s}	61.70^{a}	24.67^{d}
S9NA1	52.00^{j}	42.22^{k}	2.67^{t}
S9NA2	50.67^{jk}	43.70^{ijk}	9.67^{m}
S9NA3	66.67^{cd}	25.93^{r}	7.67^{p}
S10KB2	38.33^{qr}	57.25^{cd}	25.67^{c}
S11A1	80.00^{b}	11.11^{s}	0^{v}
S11A2	66.00^{de}	26.67^{pqr}	2.33^{t}
Control	90.00^{a}	0^{t}	0^{v}

Values presented are mean of three replications; Values in columns with the same letters after them indicate insignificant differences at the 5% significance level

Further, among the nine native isolates of *Trichoderma* tested, isolate Tricho5 recorded maximum inhibition of 70.37% over control, followed by isolates Tricho9 (63.33%) and Tricho6 (62.59%). Significantly minimum inhibition of 52.59% over control was recorded by isolates Tricho1 and Tricho7 (Table 4; Fig. 5).

Morphological characterization of potential biocontrol isolates

Eight rhizosphere isolates were selected as potential biocontrol isolates against *S. rolfsii* based on secondary screening. These include seven bacterial isolates (S1NA7, S2NA6, S3KB6, S4KB5, S8KB2, S9KB4 and S10KB2) and one *Trichoderma* isolate (Tricho5). The morphological

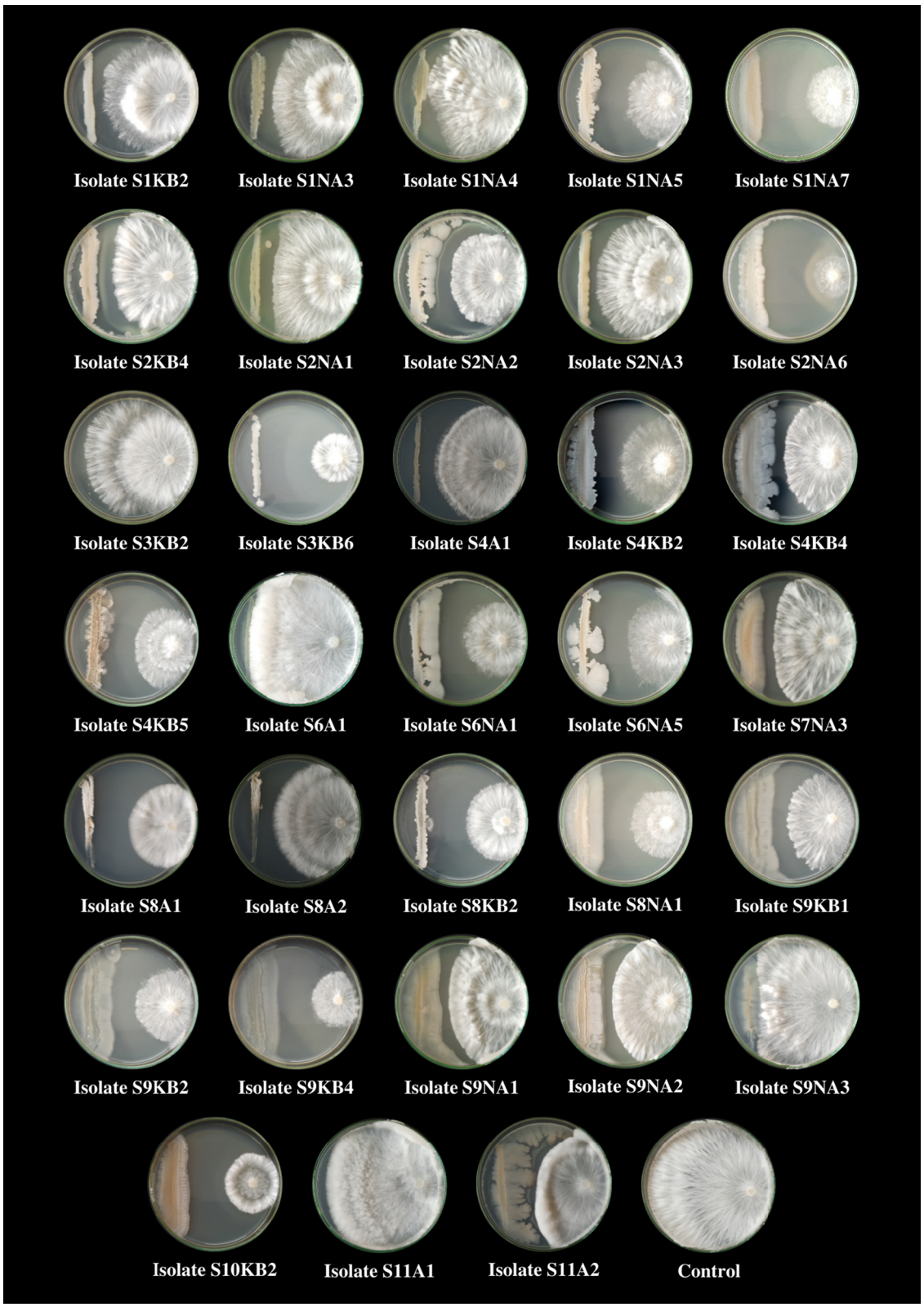

Fig. 4 Antagonistic activity of bacterial isolates from groundnut rhizosphere against *Sclerotium rolfsii* in vitro

Table 4 Antagonistic activity of *Trichoderma* isolates from groundnut rhizosphere on radial growth of *Sclerotium rolfsii* by dual culture technique

Isolate	Mycelial growth (mm)	Per cent mycelial inhibition
Tricho1	42.67[b]	52.59[e]
Tricho2	39.67[d]	55.93[c]
Tricho3	41.33[bc]	54.07[de]
Tricho4	40.33[cd]	55.19[cd]
Tricho5	26.67[f]	70.37[a]
Tricho6	33.67[e]	62.59[b]
Tricho7	42.67[b]	52.59[e]
Tricho8	39.33[d]	56.30[c]
Tricho9	33.00[e]	63.33[b]
Control	90.00[a]	0[f]

Values presented are mean of three replications; Values in columns with the same letters after them indicate insignificant differences at the 5% significance level

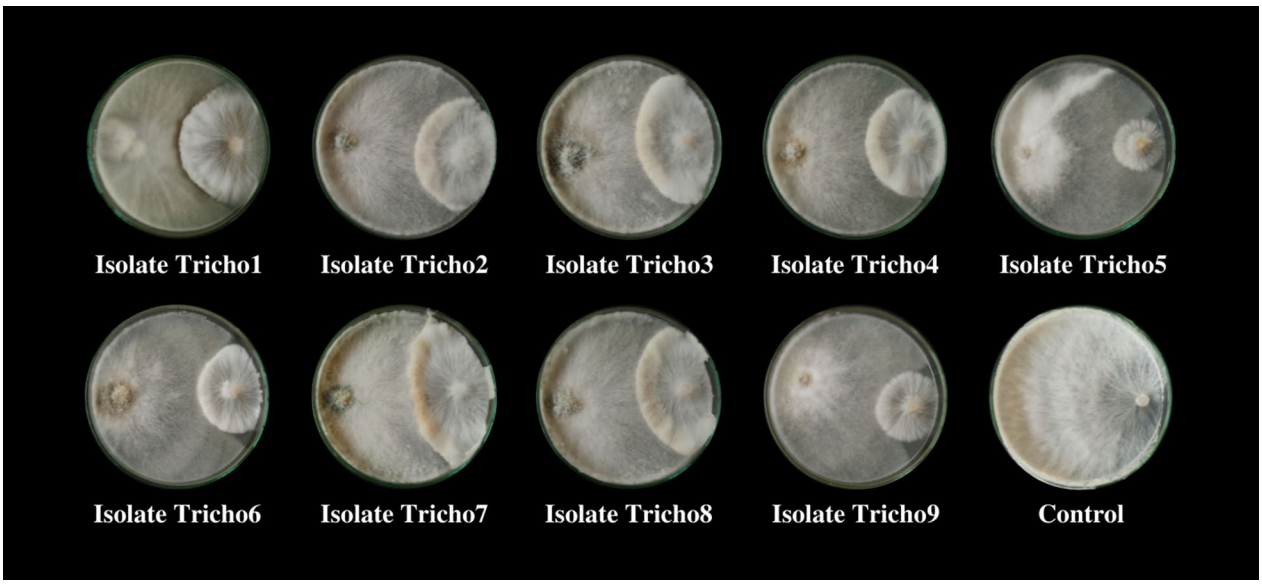

Fig. 5 Antagonistic activity of *Trichoderma* isolates from groundnut rhizosphere against *Sclerotium rolfsii* in vitro

and biochemical characterization of the bacterial isolates was done. Also, cultural and morphological characteristics of the *Trichoderma* isolate (Tricho5) were recorded. The results are presented in Table 5.

Molecular characterization of potential biocontrol isolates

Molecular characterization of eight selected biocontrol isolates viz., seven bacterial isolates (S1NA7, S2NA6, S3KB6, S4KB5, S8KB2, S9KB4 and S10KB2) and one *Trichoderma* isolate (Tricho5) were done by ITS rDNA sequencing (*Trichoderma* isolate) and 16S rDNA gene sequencing (bacterial biocontrol isolates). The fungal biocontrol isolate Tricho5 showed 99.82 per cent identity to *Trichoderma asperellum* (MH013955.1). Bacterial biocontrol isolates S1NA7, S2NA6, S3KB6, S4KB5, S8KB2 and S10KB2 showed highest per cent identity to *Bacillus subtilis* sequences, GU125629.1 (100%), MH160718.1 (99.86%), MT111002.1 (99.83%), GU125629.1 (99.72%), KC438378.1

Table 5 Morphological characteristics of selected potential biocontrol isolates

Potential rhizosphere bacterial isolates										
Isolate ID	**Colony morphology**	**Indole production test**	**Methyl red test**	**Voges proskaeur's test**	**Citrate utilization**	**Oxidase test**	**Catalase test**	**KOH test**	**Gram staining**	**Shape**
S1NA7	Cream coloured moderate smooth circular colonies with wavy margin	−	−	+	+	−	+	−	+	Rod
S2NA6	White coloured small veined irregular colonies with wavy margin	−	−	+	+	−	+	−	+	Rod
S3KB6	Greyish white coloured large rough irregular colonies with wavy margin	−	−	+	+	−	+	−	+	Rod
S4KB5	Buff coloured large rough irregular colonies with wavy margin	−	−	+	+	−	+	−	+	Rod
S8KB2	Cream coloured veined moderate irregular colonies with wavy margin	−	−	+	+	−	+	−	+	Rod
S9KB4	White coloured small wrinkled irregular colonies with entire margin	−	−	−	−	+	+	−	+	Rod
S10KB2	Cream coloured moderate veined irregular colonies with wavy margin	−	−	−	−	−	+	−	+	Rod

Potential rhizosphere *Trichoderma* isolate		
Isolate ID	**Cultural characteristics**	**Microscopic characteristic**
Tricho5	White coloured culture turns to green later based on conidia colour	Globose conidia produced on bottle shaped conidiophores

(99.72%) and GU125629.1 (99.71%) respectively (Figs. 6, 7). The bacterial biocontrol isolate S9KB4 showed 99.72 per cent identity to *Bacillus amyloliquefaciens* (AB983212.1). Partial gene sequences of the potential biocontrol agents were submitted to NCBI GenBank and cultures are submitted to the National Agriculturally Important Microbial Culture Collection (NAIMCC), NBAIM, Mau, India and accession numbers were obtained (Table 6).

Screening of potential bacterial isolates antagonism-promoting biochemical parameters

Selected seven potential bacterial isolates were evaluated for antagonism antagonism-promoting biochemical parameters (Table 7; Fig. 8). All the tested bacterial isolates registered negative results for HCN production, except for *B. subtilis* isolates S4KB5 and S8KB2, which recorded slightly positive reactions. Ammonia production for all of the examined bacterial isolates was moderately positive. All the isolates except *B. subtilis* isolate S10KB2 recorded positive reactions for siderophore production with *B. subtilis* isolate S4KB5 recording the highest values for solubilization efficiency ($306.67 \pm 11.55\%$) and solubilization index (4.07 ± 0.115). While all the tested isolates recorded positive results for cellulase and pectinase production with *B. amyloliquefaciens* isolate S9KB4 recording the highest values for both (Table 7).

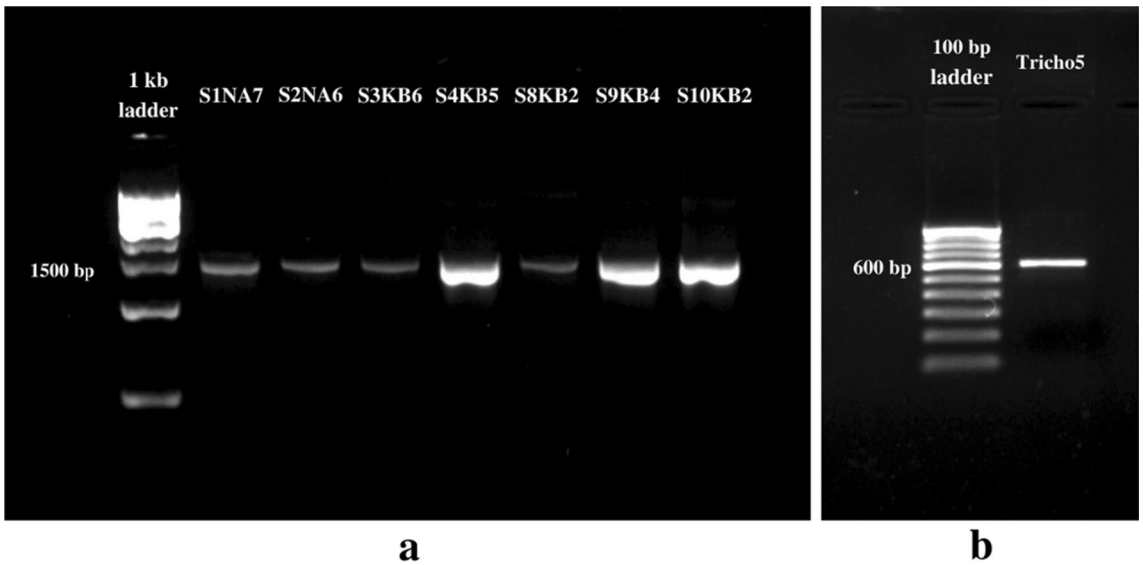

Fig. 6 Gel profile of PCR products of partial **a** 16S rDNA gene sequences of selected potential bacterial isolates (S1NA7, S2NA6, S3KB6, S4KB5, S8KB2, S9KB4 and S10KB2), **b** ITS rDNA gene sequences of selected potential *Trichoderma* isolate (Tricho5)

Table 6 Details of accession numbers of potential biocontrol isolates obtained from NCBI GenBank and NAIMCC

Isolate ID	Submitted as	NCBI GenBank accession number	NAIMCC accession number
S1NA7	*Bacillus subtilis* isolate S1NA7	OK655678.1	NAIMCC-B-03057
S2NA6	*B. subtilis* isolate S2NA6	OK655682.1	NAIMCC-B-03055
S3KB6	*B. subtilis* isolate S3KB6	OK655683.1	NAIMCC-B-03056
S4KB5	*B. subtilis* isolate S4KB5	OK655727.1	NAIMCC-B-03058
S8KB2	*B. subtilis* isolate S8KB2	OK655743.1	NAIMCC-B-03059
S9KB4	*Bacillus amyloliquefaciens* isolate S9KB4	OK655745.1	NAIMCC-B-03053
S10KB2	*B. subtilis* isolate S10KB2	OK655744.1	NAIMCC-B-03054
Tricho5	*Trichoderma asperellum* isolate Tricho5	OK655746.1	NAIMCC-F-04250

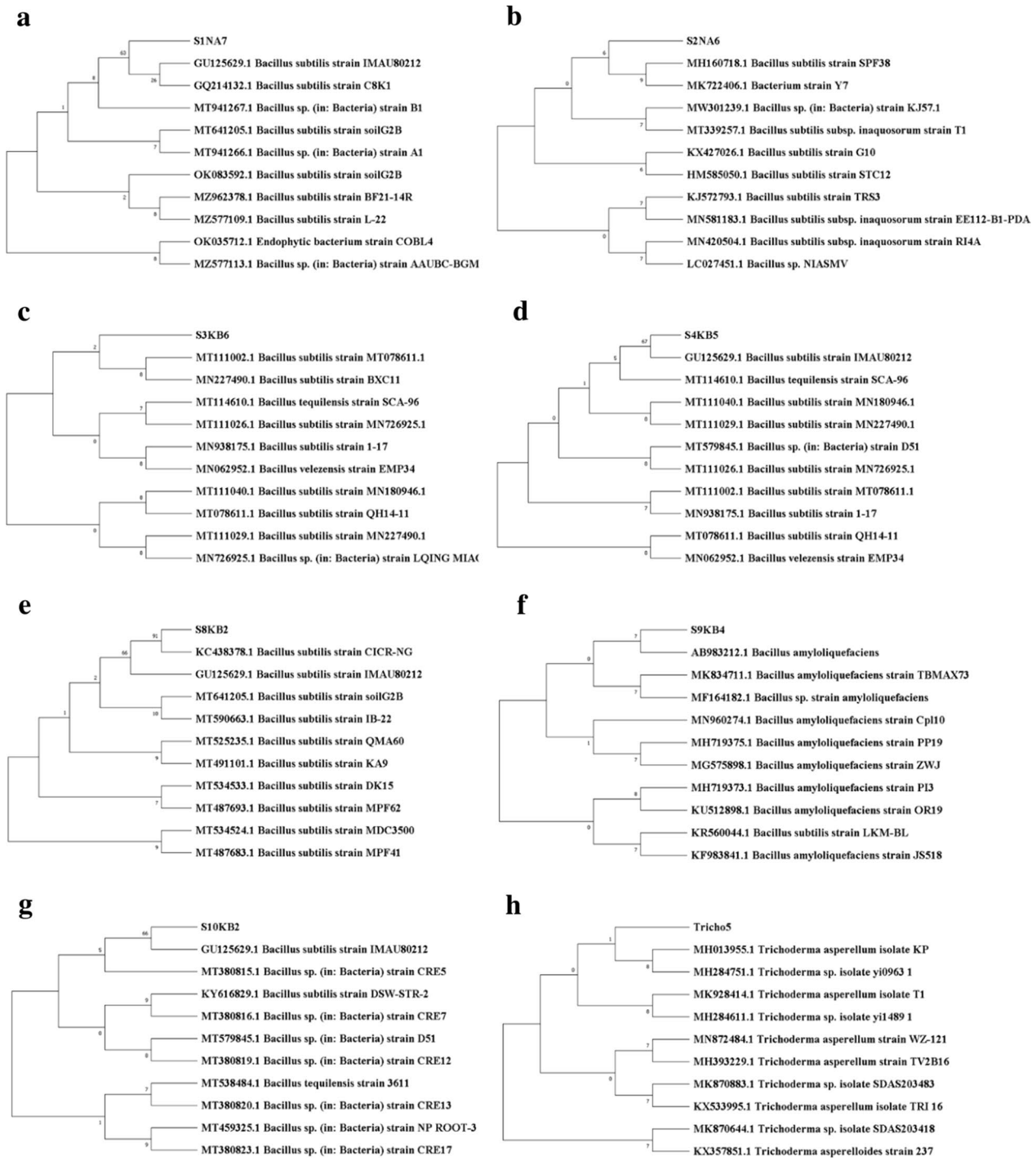

Fig. 7 Molecular phylogenetic trees of partial **a** 16SrDNA gene sequences of selected potential bacterial isolates (S1NA7, S2NA6, S3KB6, S4KB5, S8KB2, S9KB4 and S10KB2), **b** ITS rDNA gene sequence of selected potential *Trichoderma* isolate (Tricho5)

Table 7 Screening of potential bacterial biocontrol isolates for antagonism-promoting biochemical traits in vitro

Isolate ID	HCN production	Ammonia production	Siderophore production		Cellulase production		Pectinase production	
			SE (%)	SI	SE (%)	SI	SE (%)	SI
Bacillus subtilis isolate S1NA7	−	++	207.33 ± 7.15	3.07 ± 0.071	495.24 ± 8.25	5.95 ± 0.082	373.33 ± 11.55	4.73 ± 0.115
B. subtilis isolate S2NA6	−	++	151.27 ± 5.35	2.51 ± 0.054	410.30 ± 16.8	5.10 ± 0.168	473.33 ± 23.09	5.73 ± 0.231
B. subtilis isolate S3KB6	−	++	232.32 ± 4.63	3.32 ± 0.046	462.04 ± 11.23	5.62 ± 0.112	189.68 ± 9.01	2.90 ± 0.090
B. subtilis isolate S4KB5	+	++	306.67 ± 11.55	4.07 ± 0.115	389.63 ± 10.02	4.90 ± 0.100	386.67 ± 11.55	4.87 ± 0.115
B. subtilis isolate S8KB2	+	++	252.19 ± 5.83	3.52 ± 0.058	275.09 ± 3.17	3.75 ± 0.032	217.04 ± 5.13	3.17 ± 0.051
Bacillus amyloliquefaciens isolate S9KB4	−	++	138.79 ± 2.10	2.39 ± 0.021	577.78 ± 19.25	6.78 ± 0.192	555.56 ± 19.25	6.56 ± 0.192
B. subtilis isolate S10KB2	−	++	−	−	385.93 ± 5.13	4.86 ± 0.051	310.12 ± 20.85	4.10 ± 0.209

Values expressed are mean of replications ± standard deviation

SE, solubilization efficiency; SI, solubilization index

−: negative; +: slightly positive; ++: moderately positive; +++: highly positive; ++++: extremely positive

Discussion

Twelve different groundnut rhizosphere soil samples were used to isolate the native rhizosphere micro-flora, yielding a total of 111 bacterial and 79 fungal isolates. Then, the isolates were tested for antagonistic activity against *S. rolfsii*. In primary screening, 33 of the 111 bacterial isolates showed antagonistic behaviour towards *S. rolfsii,* and they were further evaluated in dual culture. All the isolates significantly inhibited radial growth of *S. rolfsii* with isolates S3KB6, S9KB4 and S1NA7 recording maximum inhibition of 62.82, 61.70 and 61.11% over control respectively. Results are in agreement with the findings of Safni and Antastia (2018) who reported that rhizobacterial species showed significant antagonistic activity against *S. rolfsii* with inhibition up to 60%. Swaroopa and Madhuri (2021) found that *Bacillus* spp. isolated from the soil inhibited the growth of *S. rolfsii* in groundnut. The in vitro inhibition of radial growth of *S. rolfsii* by rhizosphere isolates was also reported by Ramanjineyulu et al. (2021). Nine isolates identified as *Trichoderma* spp. from the 79 rhizosphere fungal isolates were tested for antagonistic activity against *S. rolfsii* in dual culture assay. All the isolates showed significant inhibition of radial growth of *S. rolfsii* and isolate Tricho5 recorded a maximum inhibition of 70.37% over control. Results obtained are in conformity with Karthikeyan et al. (2006), who reported inhibition of *S. rolfsii* radial growth of mycelium in dual culture by *Trichoderma* isolates ranging between 39.93 and 69.40% with isolate Tv1 of *T. viride* recording highest inhibition over control. Likewise, Hirpara et al. (2017) tested 11 *Trichoderma* isolates against *S. rolfsii*. *T. virens* NBAII Tvs12 exhibited maximum growth inhibition of *S. rolfsii* (87.91%), followed by *T. koningii* MTCC 796 (67.03%), *T. viride* NBAII Tv23 (63.74%) and *T. harzianum* NBAII Th1 (60.44%). The in vitro inhibition of radial mycelial growth of *S. rolfsii*by *Trichoderma* was also reported by Pacheco et al. (2016). Eight isolates (seven bacterial and one *Trichoderma*) were selected as potential biocontrol ones. Molecular characterization of selected biocontrol isolates by 16S rDNA and ITS rDNA sequencing confirmed the identity of bacterial isolates as *Bacillus* spp. (*B. subtilis* and *B. amyloliquefaciens*) and fungal isolate as *Trichoderma* sp. (*Trichoderma asperellum*). Further, the selected bacterial isolates recorded favourable results for antagonism-promoting biochemical parameters i.e., HCN production, ammonia production, siderophore production, cellulase production and pectinase production, which is comparable to the findings of Syed et al. (2020). The cultures of the potential biocontrol were deposited to NAIMCC, NBAIM, Mau, India. The use of these isolates in the biological control of *S. rolfsii* may be made possible with further study, thus offering a sustainable solution for the management of groundnut stem rot disease.

Conclusion

Results of the present study proved the effectiveness of 33 bacterial isolates and 9 *Trichoderma* isolates from groundnut rhizosphere soil in controlling *S. rolfsii* under in vitro conditions. Of these, the identities of seven bacterial isolates and one *Trichoderma* isolate, which recorded significantly high inhibition of radial growth of *S. rolfsii* were morphologically and molecularly confirmed. Further research may enable the use of the isolated rhizosphere biocontrol agents as single organisms or in a consortium for sustainable management of the groundnut stem rot pathogen.

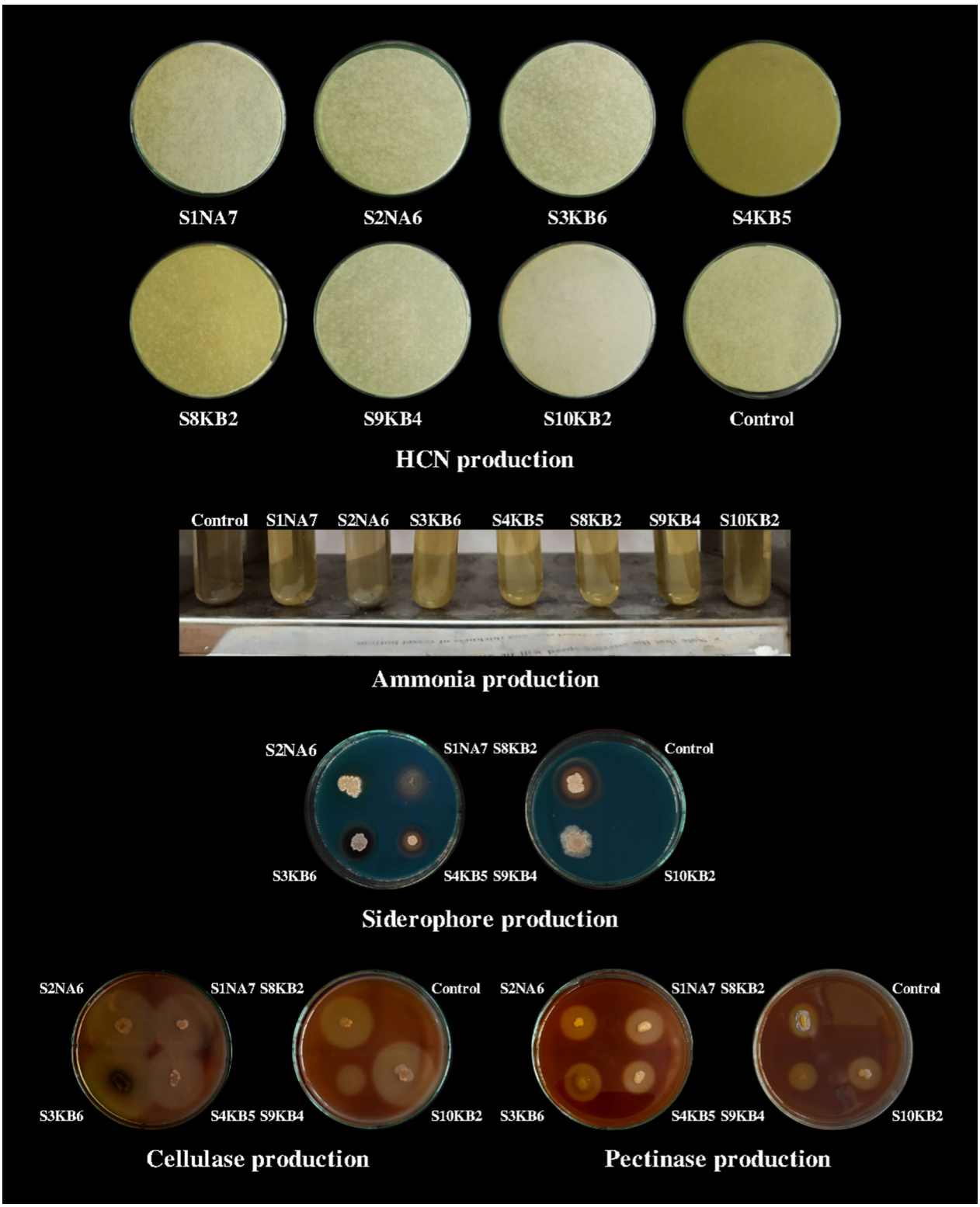

Fig. 8 Screening of selected potential bacterial isolates for antagonism-promoting biochemical traits

Abbreviations
KB: King's B medium; NA: Nutrient agar medium; AIA: Actinomycetes isolation agar medium; PDA: Potato dextrose agar medium; RBA: Martin's Rose Bengal agar medium; ITS: Internal Transcribed Spacer; PCR: Polymerase chain reaction; ANOVA: Analysis of variance; NBAIM: National Bureau of Agriculturally Important Microorganisms.

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Author contributions
AUA, VR, GUD, SNCVLP and ST were involved in the conceptualization of research work and designing of experiments. AUA carried out the experiments and recorded data. AUA, VR and SNCVLP were involved in the statistical analysis and interpretation of data. AUA wrote the first manuscript. VR revised the manuscript. All authors read and approved the final manuscript.

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Declarations

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Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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References

Barnett HL, Hunter BB (1972) Illustrated genera of imperfect fungi, 3rd edn. Burgess Publishing Company, Minnesota, p 241

Bazzicalupo M, Fancelli S (1997) DNA extraction from bacterial cultures. In: Micheli MR, Bova R (eds) Fingerprinting methods based on arbitrarily primed PCR. Springer, Berlin, pp 41–45. https://doi.org/10.1007/978-3-642-60441-6_7

Borkar SG (2017) Biochemical tests used in identification of bacteria. In: Borkar SG (ed) Laboratory techniques in plant bacteriology, 1st edn. CRC Press, Boca Raton, pp 93–109. https://doi.org/10.1201/9781315206882

Castric KF, Castric PA (1983) Method for rapid detection of cyanogenic bacteria. Appl Environ Microbiol 45(2):701–702. https://doi.org/10.1128/aem.45.2.701-702.1983

Cilliers AJ, Pretorius ZA, Van Wyk PS (2003) Integrated control of *Sclerotium rolfsii* on groundnut in South Africa. J Phytopathol 151(5):249–258. https://doi.org/10.1046/j.1439-0434.2003.00715.x

Dennis CJ, Webster J (1971) Antagonistic properties of species-groups of Trichoderma: I. Production of non-volatile antibiotics. Trans Br Mycol Soc 57(1):25–39. https://doi.org/10.1016/S0007-1536(71)80077-3

Gómez Expósito R, de Bruijn I, Postma J, Raaijmakers JM (2017) Current insights into the role of rhizosphere bacteria in disease suppressive soils. Front Microbiol 8(2529):1–12. https://doi.org/10.3389/fmicb.2017.02529

Gopinath PP, Parsad R, Joseph B, Adarsh VS (2021) grapesAgri1: collection of shiny apps for data analysis in agriculture. J Open Source Softw 6(63):3437. https://doi.org/10.21105/joss.03437

Gupta S, Pandey S (2019) ACC deaminase producing bacteria with multifarious plant growth promoting traits alleviates salinity stress in French bean (*Phaseolus vulgaris*) plants. Front Microbiol 10:1506. https://doi.org/10.3389/fmicb.2019.01506

Hankin L, Anagnostakis SL (1977) Solid media containing carboxymethylcellulose to detect Cx cellulase activity of micro-organisms. Microbiology 98(1):109–115. https://doi.org/10.1099/00221287-98-1-109

Hirpara DG, Gajera HP, Hirpara HZ, Golakiya BA (2017) Antipathy of *Trichoderma* against *Sclerotium rolfsii* Sacc.: evaluation of cell wall-degrading enzymatic activities and molecular diversity analysis of antagonists. J Mol Microbiol Biotechnol 27(1):22–28. https://doi.org/10.1159/000452997

INDIASTAT (2022) Season-wise area, production and productivity of groundnut in India (1949–1950 to 2021–2022 - 3rd advance estimates). Retrieved from https://www.indiastat.com/table/agriculture/season-wise-area-production-productivity-groundnut/17354#

Joshi E, Sasode DS, Singh N, Chouhan N (2020) Diseases of groundnut and their control measures. Biotica Res Today 2(5):232–237

Karthikeyan V, Sankaralingam A, Nakkeeran S (2006) Biological control of groundnut stem rot caused by *Sclerotium rolfsii* (Sacc.). Arch Phytopathol Plant Prot 39(3):239–246. https://doi.org/10.1080/03235400500094688

Kasana RC, Salwan R, Dhar H, Dutt S, Gulati A (2008) A rapid and easy method for the detection of microbial cellulases on agar plates using Gram's iodine. Curr Microbiol 57(5):503–507. https://doi.org/10.1007/s00284-008-9276-8

Kumar V, Thirumalaisamy PP (2016) Diseases of groundnut. In: Dubey SC, Agarwal R, Patro TSSK, Sharma P (eds) Disease of field crops and their management. Today and Tomorrow's Printers and Publishers, New Delhi, pp 445–494

Le CN, Thai TH, Tran DH, Nguyen TL, La TTH, Nguyen XV (2018) Genetic diversity of groundnut rhizosphere antagonistic bacteria and biological control of groundnut wilted diseases in central Vietnam. Legume Res Int J 42(3):405–410. https://doi.org/10.18805/LR-427

Lee SB (1990) Isolation of DNA from fungal mycelia and single spores. In: PCR protocols, a guide to methods and applications, pp 282–287

Leona G, Sudhakar R, Uma Devi G, Uma Maheswari T (2020) Management of stem rot of groundnut caused by *Sclerotium rolfsii* Sacc with actinomycetes. Int J Curr Microbiol Appl Sci 9(12):3587–3601. https://doi.org/10.20546/ijcmas.2020.912.427

Oumer OJ, Abate D (2018) Screening and molecular identification of pectinase producing microbes from coffee pulp. BioMed Res Int 2018:2961767. https://doi.org/10.1155/2018/2961767

Pacheco KR, Viscardi BSM, de Vasconcelos TMM, Moreira GAM, do Vale HMM, Blum LEB (2016) Efficacy of *Trichoderma asperellum*, *T. harzianum*, *T. longibrachiatum* and *T. reesei* against *Sclerotium rolfsii*. Biosci J 32(2):412–421

Raaijmakers JM, Paulitz TC, Steinberg C, Alabouvette C, Moënne-Loccoz Y (2009) The rhizosphere: a playground and battlefield for soilborne pathogens and beneficial microorganisms. Plant Soil 321(1):341–361. https://doi.org/10.1007/s11104-008-9568-6

Ramanjineyulu P, Viswanath K, Nagamani P, Kumar NK (2021) Evaluation of rhizospheric antagonistic microorganisms and fungicides against pod rot associated pathogens of groundnut (*Arachis hypogaea* L.). Pharma Innov J 10(5):374–379

Rangaswami G, Mahadevan A (1999) An agar block technique for isolating soil micro organisms with special reference to pythiaceous fungi. Sci Cult 24:85

Safni I, Antastia W (2018) *In vitro* antagonism of five rhizobacterial species against *Atheliarolfsii* collar rot disease in soybean. Open Agric 3(1):264–272. https://doi.org/10.1515/opag-2018-0028

Schwyn B, Neilands JB (1987) Universal chemical assay for the detection and determination of siderophores. Anal Biochem 160(1):47–56. https://doi.org/10.1016/0003-2697(87)90612-9

Spence C, Alff E, Johnson C, Ramos C, Donofrio N, Sundaresan V, Bais H (2014) Natural rice rhizospheric microbes suppress rice blast infections. BMC Plant Biol 14(1):1–17. https://doi.org/10.1186/1471-2229-14-130

Swaroopa ZM, Madhuri RJ (2021) Bio-control activity of plant growth promoting rhizobacteria on *Sclerotium rolfsii*. Plant Arch 21(1):379–383. https://doi.org/10.51470/PLANTARCHIVES.2021.v21.no1.052

Syed S, Tollamadugu NP, Lian B (2020) *Aspergillus* and *Fusarium* control in the early stages of *Arachis hypogaea* (groundnut crop) by plant growth-promoting rhizobacteria (PGPR) consortium. Microbiol Res 240:126562. https://doi.org/10.1016/j.micres.2020.126562
Timonin MI (1940) The interaction of higher plants and soil micro-organisms: I. Microbial population of rhizosphere of seedlings of certain cultivated plants. Can J Res 18(7):307–317. https://doi.org/10.1139/cjr40c-031
Vincent JM (1947) Distortion of fungal hyphae in the presence of certain inhibitors. Nature 159:850–852. https://doi.org/10.1038/159850b0

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