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Identification, physicochemical properties and molecular characterization of rhizospheric microflora of groundnut and antagonistic activity against *Sclerotium rolfsii*

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Stem rot caused by *Sclerotium rolfsii* Sacc. is a major nuisance in groundnut production, causingsubstantial yield losses in almost all groundnut-growing areas around the world. Biological control is regarded asa sustainable choice over the currently popular management strategy later has a negative impact on the environment. The present study evaluated the antagonistic effect of rhizosphere micro-flora of groundnut against stem rot of groundnut caused by*S. rolfsii*.A total of four bacterial isolates and 1 fungus isolated from groundnut rhizosphere soil wereevaluated for their antagonist activity against *S. rolfsii*under *in vitro*studies. Five isolates (four bacteria and one fungus)were chosen as prospective native 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. (*B. sphaericus, B. subtilis* and *B. paramycoides*) and fungal isolate as *Trichoderma asperellum* and N-fixing bacteria *Rhizobium*.The results of the study suggested that the groundnut rhizosphere micro-flora can be resource for biological control of groundnut stem rot pathogen *S. rolfsii*. Further research may enable the use of theisolated rhizosphere biocontrol agents.

Keywords: *Bacillus*, groundnut, rhizosphere, *Trichoderma*

INTRODUCTION

As a sustainable, efficient, and environmentally benign method of managing plant diseases, biological management is quickly gaining popularity. A comprehensive analysis of the biological control literature reveals a focus on soil-borne pathogens rather than foliar infections, with the latter receiving a more favourable response (Kumar and Thirumalaisamy 2016). In order to biologically manage a wide range of plant diseases, several bacterial and fungal agents are utilised. The most often used bacterial biocontrol agents are *Bacillus* and *Pseudomonas*, whereas *Trichoderma* is the most commonly used fungal biocontrol agent. Disease-suppressive soils must be highlighted when discussing biological control since they are the primary source of biocontrol agents.

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Plants are well protected against infection by soil-borne pathogens and the particular illness by soils that suppress diseasethat takes place in these soils is mostly microbiological in nature (Gomez Exposito *et al*. 2017). A hotspot for a variety of creatures, the rhizosphere is the small zone around and impacted by plant roots and is thought to be one of the most complex ecosystems on earth (Raaijmakers *et al*. 2009). Rhizospheric organisms are crucial in reprogramming the host plant's overall defence mechanism (Spence *et al*. 2014). The edible oilseed crop known as groundnut, sometimes known as "the king of oilseeds," is a member of the Fabaceae family and is widely used for oil production, domestic usage, and cooking.After China, India is the second-largest producer of groundnuts. Nevertheless, due to a number of biotic and abiotic restrictions, such as inadequate moisture, poor soil fertility, the occurrence of pests and diseases, the area under groundnut production

in India has decreased over time, from 8.30 million hectares during 1990–1991 to 6.02 million ha by 2020–2021. (INDIASTAT 2022). Stem rot (*Sclerotium rolfsii* Sacc.) and crown/collar rot (*Aspergillus niger*), two soil-borne diseases that afflict groundnuts, pose a hazard to effective groundnut production and can result in yield losses of up to 50%. (Joshi *et al*. 2020). Groundnut production is severely hampered by the stem rot disease, which is responsible for serious output losses in practically all groundnut-growing regions worldwide. *S. rolfsii*, a pervasive, polyphagous soil-borne pathogen that causes catastrophic plant diseases in several crop species, is the culprit. Due to *S. rolfsii* wide host range, prolific proliferation, and capacity to create chronic sclerotia, the disease causes significant economic losses (Cilliers *et al*. 2003). Stem rot management is challenging due to the pathogen's wide variety of hosts and ability to thrive in soil and plant tissues. One of the extensively used approaches to managing the condition is the use of chemicals. Nevertheless, it comes with a number of drawbacks, including the development of fungicide-resistant populations, disturbance of regional ecologies, higher labour costs, and risks to human health.As a result, it's crucial to create efficient and ecologically friendly disease management strategies, which is where biological control comes in. Researchers from all around the world have proven the potential of rhizosphere bacteria in regulating soil-borne plant diseases like *S. rolfsii* (Swaroopa and Madhuri, 2021). In order to effectively manage soil-borne diseases, beneficial microorganisms in the native rhizosphere must be studied for their antagonistic potential. *Bacillus, Pseudomonas,* and *Burkholderia* make up the majority of the groundnut rhizosphere's antagonistic bacterial community against *S. rolfsii* (Le *et al.*2018). According to Swaroopa and Madhuri (2021), *Bacillus* spp. isolated from the soil have the ability to stimulate plant development while also preventing *S. rolfsii* in groundnut from growing. Groundnut pathogens associated with pod rot, such as *S. rolfsii*, are successfully combated by native isolates of *Trichoderma* spp. and rhizosphere bacteria from groundnut rhizosphere soil (Ramanjaneyulu *et al.*2021).

MATERIALS AND METHODS

Test pathogen-Sclerotium rolfsii Sacc.

Pathogen *S. rolfsii* was isolated from the stems ofinfected groundnut plants with white mycelial growth onthe collar region on potato dextrose agar (PDA) mediumby tissue segment method (Rangaswami and Mahadevan1999). The isolate was molecularly confirmed by ITS rDNA sequencingas *Athelia rolfsii*. Gene sequence of the test pathogen was submitted to NCBI GenBank as *Athelia rolfsii* isolate P1SR under the accession number ON171368.

Collection of rhizosphere soil samples

Soil samples were collected from rhizosphere of groundnut plants grown in fields of BhadradriKothagudem district of Telangana State were uprooted carefully, shoot portion cut off and roots along with the rhizosphere soil aseptically in small plastic bags / bottles were brought to the laboratory and prior to their processing kept at 4°C.10g of soil samples was suspended in 90ml of sterilized distilled water and 10- 1 dilution was obtained. Serial dilutions were prepared by mixing 1ml of the suspension made into 9ml sterilized water blanks until the 10^{-5} dilution was obtained. From these dilutions 100µl was spread on sterilized petri plates contain solidifiedNutrient Agar. These plates were then incubated at 30! and were observed for 2-7 days. The total bacterial types were counted after 48 hrs of incubation.

Physicochemical analysis of rhizosphere soil samples

To validate the potency of the bacterial PGPR activity field experiment was conducted where various analyses were done to evaluate strength of the soil before and after field experiment. Analysis of physio-chemical properties such as P^H, Electrical conductivity (EC), available phosphorus, organic carbon and organic matter (OC/OM), available nitrogen of Groundnut field soil was analysed by standard method.

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

The stem rot pathogen was in vitro tested against the rhizosphere isolates using the dual culture method (Dennis and Webster, 1971). Primary testing for antagonistic effects on *S. rolfsii* in rhizosphere isolates. All bacterial isolates underwent initial screening to see whether they had any inhibitory effects on *S. rolfsii* growth. On a sterile agar plate, a five-mm mycelial disc of a five-day-old culture of *S. rolfsii* was positioned in the centre and separate bacterial isolates were streaked 1 cm from the plate's edge on the other four sides. Once full development had occurred in a control plate containing only *S. rolfsii*, observations were performed there. It is not regarded as hostile if *S. rolfsii* mycelium spreads across the rhizosphere solitary streak.In another instance, the isolate was chosen for secondary screening if it prevented the mycelial development of *S. rolfsii*, while secondary screening was also applied to all rhizosphere fungal isolates classified as *Trichoderma*.

Identification of Isolates

DNA was isolated from the culture. Its quality was evaluated on 1.0% Agarose Gel, a single band of high-molecular weight DNA has been observed.Fragment 16S rDNA gene was amplified by 27F and 1492R Primers. A single discrete PCR amplicon band of 1500bp was observed when resolved on Agarose gel.The PCR amplicon was purified to remove contaminants.Forward and Reverse DNA sequencing reaction of PCR amplicon was carried out with forward primer and reverse primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer.Consensus sequence of 16S rDNA gene was generated from forward and reverse sequence data using aligner software.The 16s rDNA gene sequence was used to carry out BLAST with the database of NCBI genebank database. Based on maximum identity score first ten sequences were selected and aligned using multiple alignments software programme Clustal W. Distance matrix was generated and the phylogenetic tree was constructed using MEGA 7 software.

Screening of antagonistic activity of rhizosphere isolates against S. rolfsiiin vitro

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

Primers	Primer ID	Sequence	Base pairs
16S rDNA	F27	5'-AGAGTTTGATCCTGGCTCAG-3'	20
	R1492	5'-TACGGYTACCTTGTTACGACTT-3'	22
ITS rDNA	ITS1F	5'-TCCGTAGGTGAACCTGCGG-3'	19
	ITS4	5'-TCCTCCGCTTATTGATATGC -3'	20

(b) PCR mixtures for 10 ìl and 50 ìl 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	1µl	5 µl
Reverse	1 µl	5 µl
Template DNA (100 ng/µl)	1 µl	5 µl
dH2O	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

Secondary screening of rhizosphere isolates for antagonism against S. rolfsii

In the dual culture, isolates that shown inhibitory effects in the first screening were examined for their antagonistic effects on *S. rolfsii*. A 5 mm mycelial disc of a five day old culture of *S. rolfsii* was put at one end of the PDA plate, and a loopful of pure cultures of the test isolate that were 24 hours old was streaked 1 cm away from the plate's edge. The plate was then incubated at 25 2 °C. Just the fungal rhizosphere isolates identified as Trichoderma by colony morphology and microscopy were investigated for antagonistic activity against *S. rolfsii*, while a control plate containing only *S. rolfsii* was also kept. When the control plate had fully developed, the pathogen's mycelial development was assessed in each Petri dish independently and expressed as Per cent inhibition of the mycelialgrowth of the pathogen by different test isolates was calculatedusing the formula given by Vincent (1947).

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

Table 1: Physicochemical properties of collected rhizosphere soil sample of groundnut

Village Details	Location	P^H	Electrical conductivity (dS m^{-1})	Available			Organic carbon (%)
				Nitrogen (kg ha^{-1})	Phosphorus (kg ha^{-1})	Potassium (kg ha^{-1})	
Asupaka, Aswaraopeta, Bhadradri Kothagudem, Telangana.	Lat. 17.3655802 Long. 81.1153104	6.23 ± 0.12	0.268 ± 0.008	189.23 ± 3.24	32.18 ± 1.34	486.37 ± 3.45	0.62 ± 0.05

Table 2: Details of accession numbers obtained from NCBI GenBank.

Isolate ID	Submitted as	NCBI Genbank accession number
P1SR	*Athelia rolfsii* P1SR	ON171368
S1BC1	*Trichoderma asperellum* S1BC1	OR789507
S2BC2	*Bacillus spharicus* S2BC2	OR789549
S3BC3	*Bacillus paramycoides* S3BC3	OR789558
S5BC4	*Bacillus pumilus* S5BC4	OR740656
S4NB1	*Rhizobiumfredii* S4NB1	CP071454

Table 3 : Efficacy of isolated bio control agents against *Sclerotium rolfsii* under *in vitro* conditions

Treatment	Mycelial growth (cm)	Mycelial inhibition %
Trichoderma asperellum	4.16	53.70
Bacillus spharicus	4.63	48.33
Bacillus paramycoides	4.46	50.37
Bacillus subtilis	3.43	61.70
Rhizobium	5.13	42.96

Table 4 : Cultural and morphological characteristics of isolated bio control agents of groundnut isolated on PDA, NA and YEMA.

Sample	Media	Colony Shape	Margin	Elevation	Size	Texture	Pigmentation	Shape	Gram staining
S1BC1	PDA	Rings	Entire	Flat	Moderate	Smooth	Whitish Green	Voval	Fungus
S2BC2	NA	Circular	Entire	Raised	Small	Butyrous	Creamy white	Cocos	+
S3BC3	NA	Circular	Entire	Raised	Medium	Smooth	Creamy white	Cocos	+
S4NB1	YEMA	Circular	Wavy	Raised	Moderate	Smooth	Pinkish white	Rod	-
S5BC4	NA	Circular	Wavy	Flat	Moderate	Moist	Cream	Rod	+

PDA- Potato Dextrose Agar; NA-Nutrient Agar; YEMA – Yeast Extract Mannitol Agar. Appearance- All samples smooth; All samples Optically opaque

Table 5 : Closest homolog of sequences of test pathogen and potential biocontrol isolates in the NCBI nucleotide sequence database

Isolate ID	Description	Max. score	Total score	Percent query cover	Percent identity	Acc. length	Acc. number
P1SR	*Atheliarolfsii* isolate P1SR internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence.	1358	1358	100	100	735	ON171368.1
S1BC1	*Trichoderma asperellum* isolate S1BC1 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence	1018	1018	100	100	551	OR789507.1
S2BC2	*Bacillus paramycoides*strain MCCC 1A04098 16S ribosomal RNA, partial sequence	2736	2736	99	99.87	1509	NR157734.1
S3BC3	*Lysinibacillussphaericus* strain DSM 28 16S ribosomal RNA, partial sequence	2501	2501	98	96.68	1515	NR042073.1
S4NB1	*Rhizobium fredii* NGR 234 plasmid pNGR234a, complete sequence	65.8	1674	98	100	5361	CP071454.1
S5BC4	*Bacilluspumilus*strain2.16SribosomalRNA gene, partial sequence	518	2329	93	100	1415	MK027246.1

RESULTS

Collection and physicochemical properties of collected rhizosphere soil sample

The surveyed in the Asupaka village of Aswaraopetamandal of BhadradriKothagudem district, Telangana State. Groundnut rhizosphere soil samples were collected from sites of groundnut growing soils. 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 1.

Table 4 shows the result of P^H (6.23); EC (0268); Available Nitrogen (189.23); Available Phosphorus (32.18); Available Potassium (486.37) and Organic carbon (0.62%).

Isolation and identification of rhizosphere microflora

Rhizosphere micro-flora was isolated by serial dilution method on different media *viz*., Nutrient agar medium (NA), potato dextrose agar medium (PDA) and Yeast Extract Mannitol agar medium (YEMA) from the collected rhizosphere soil samples. A total of 4 bacterial isolates were obtained from NA and YEMA and 1 fungal isolate was obtained from PDA and respectively. All the bacterial and fungal isolates were maintained by periodical subculturing for use in further experiments.

All the bacterial and fungal isolates were identified by molecular characterization and BLAST technique. P1SR-*Sclerotium rolfsii*, S1BC1-*Trichoderma asperellum*, S2BC2-*Bacillus spharicus*, S3BC3-*Bacillus paramycoides*, S5BC4-*Bacillus pumilus* and S4NB1-*Rhizobium fredii*. Results are presented in Table 2.

Secondary screening of rhizosphere isolates for antagonism against S. rolfsii

A total of 4 bacterial isolates (3 from NA, 1 from YEMA) were selected through primary screening and 1 native *Trichoderma* isolates isolated from rhizosphere soil were tested for their antagonistic

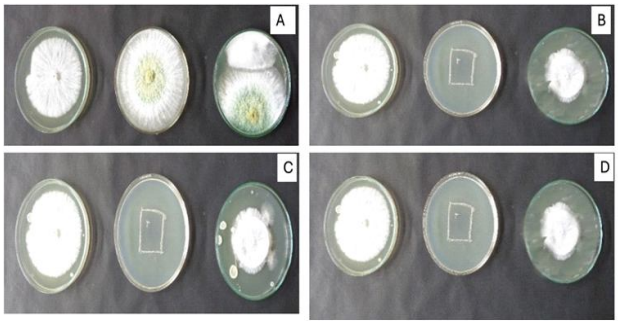

Fig.1. Antifungal tests of isolated microorganisms against *Atheliarolfsii* in vitro. a- *Trichoderma asperellum;* b- *Bacillus spharicus;* c-*Bacillus paramycoides;* d- *Bacillus subtilis.*

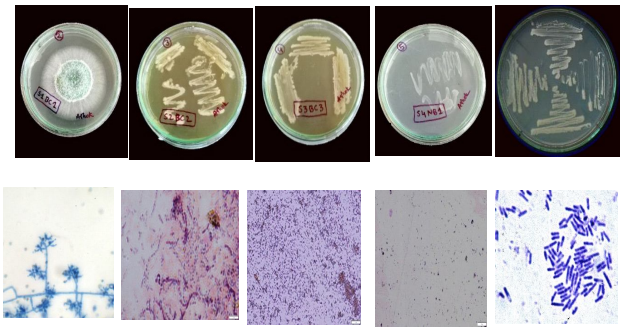

Fig.2: Morphological (Top row- Lto R) and microscopic (Bottom row- L to R) images of isolated biocontrol agents.***i -*** *Trichoderma asperellum* (S1BC1)**;** ***ii -*** *Bacillus spharicus* (S2BC2)***; iii -*** *Bacillus paramycoides* (S3BC3)***; iv –*** *Rhizobium fredii* (S4NB1)***; v -*** *Bacillus pumilus* (S5BC4)*.*

Fig. 3 : Gel profile of PCR amplicon of fungal biocontrol isolate (S1BC1) and test pathogen (P1SR) obtained using ITS primers (ITS1F and ITS4)

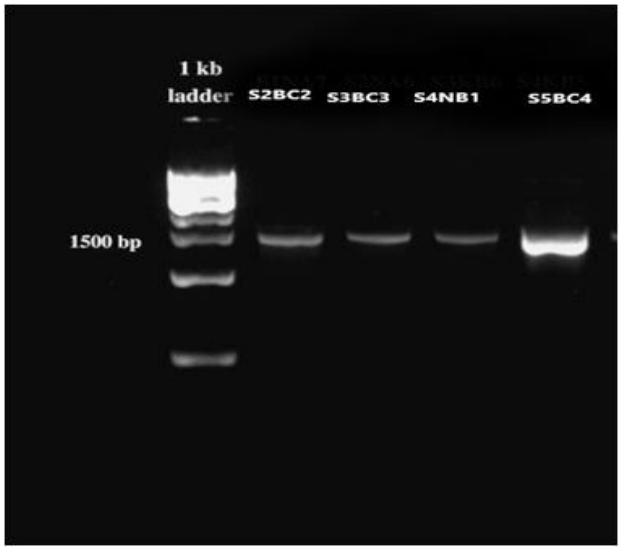

Fig. 4: Gel profile of PCR amplicon of bacterial biocontrol isolates (S2BC2, S3BC3, S4NB1 and S5BC4) obtained using 16S rDNA primers (27F and 1492R)

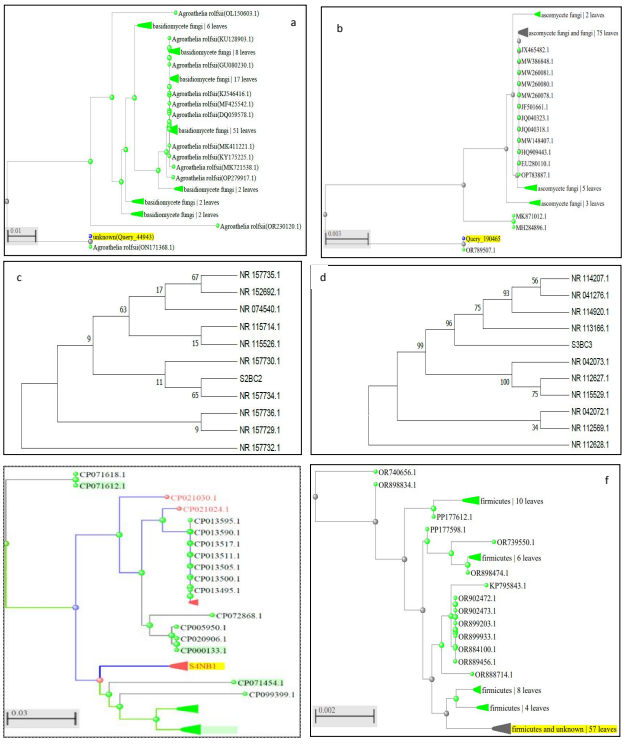

Fig. 5 : Molecular phylogenetic tree of isolated biocontrol agents.. a-P1SR; b- S1BC1; c-S2BC2; d-S3BC3; e-S4BC5; f-S5NB1.

activity against *S. rolfsii* by dual culture technique. Observations were taken on the day when the radial growth of *S. rolfsii* in the control plate was full. Among the 4 bacterial isolates tested, isolate S5BC4, S3BC3 and S2BC2 recorded maximum inhibition of 61.70, 50.37 and 48.33 % over control, respectively. Significantly minimum inhibition of 11.11% over control was recorded by isolate S4NB1. (Table 3; Fig 1). Further, the native isolate

of S1BC1- *Trichoderma asperellum* was tested and, recorded the maximum inhibition of 53.70 % over control.

Cultural and morphological characteristics of isolated biocontrol agents

Cultural and morphological characters of isolated biocontrol agents were identified in the stereo binocular microscope and the characters were shown different patterns, characters were shown in the Table 4 and Fig. 2**.**

Molecular characterization of potential biocontrol isolates

PCR products of test pathogen and isolated biocontrol isolates were purified and sequenced at Eurofins Genomics Laboratory, Bengaluru, India and the data were processed using Bio Edit and MEGA11 software. The consensus sequences generated using Bio Edit software were used to perform NCBI-BLAST against the NCBI GenBank database (https://blast.ncbi .nlm.nih.gov/Blast.cgi#). The top ten NCBI hits for each sequence were aligned using Clustal W followed by that distance matrix and the phylogenetic tree was created using MEGA11. Based on the phylogenetic tree and the pair-wise distance matrix, the closest homolog of each isolate from the NCBI GenBank database was identified (Table **5**). Briefly, the test pathogen isolate P1SR showed 100 per cent identity to *Atheliarolfsii* (ON171368.1) and fungal biocontrol isolate S1BC1 showed 100 per cent identity to *Trichoderma asperellum* (OR789507.1). Bacterial biocontrol isolates S2BC2 showed 99.87 per cent identity to *Bacillus paramycoides* (NR157734.1). The bacterial biocontrol isolate S3BC3 showed 96.68 per cent identity to *Bacillus spharicus* (NR042073.1). Bacterial isolate S5BC4 showed the 100 per cent identity to *Bacillus pumilus*, (MK027246.1). Bacterial isolate S4NB1 shows highest per cent identity to *Rhizobium fredii* (CP071454.1). All the sequences were submitted to NCBI GenBank and the accession numbers were obtained (Table 2; Figs. 3-5).

All the bacterial and fungal isolates were identified by molecular characterization and BLAST technique and submitted to NCBI Genbank and obtained the Accession numbers. P1SR-*Sclerotium rolfsii* (ON171368); S1BC1-*Trichoderma asperellum* (OR789507); S2BC2-*Bacillus spharicus* (OR789549); S3BC3-*Bacillus paramycoides* (OR789558); S5BC4-*Bacilluspumilus* (OR740656) and S4NB1-*Rhizobium fredii* (CP071454).

DISCUSSION

Groundnut rhizosphere soil samples were used to isolate the native rhizosphere micro-flora, yielding a total of 4 bacterial and 1 fungal isolate. Then, the isolates were tested for antagonistic activity against *S. rolfsii*. In primary screening, 4 of the 4 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 S5BC4, S3BC3, S2BC2 and S4NB1 recording maximum inhibition of 61.70, 50.37, 48.33 and 42.96 % 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). One isolate identified as Trichoderma spp. from the fungal isolates were tested for antagonistic activity against *S. rolfsii* in dual culture assay. Tested isolate showed significant inhibition of radial growth of *S. rolfsii* and recorded a maximum inhibition of 53.70% 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). Molecular characterization of selected biocontrol isolates by 16S rDNA and ITS rDNA sequencing confirmed the identity of bacterial isolates as *Bacillus* spp. (*B. subtilis, B. spharicus* and *B. paramycoides*) and fungal isolate as *Trichoderma* sp. (*Trichoderma asperellum*). 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 4 bacterial isolates and *Trichoderma* isolates from groundnut rhizosphere soil in controlling *S. rolfsii* under *in vitro* conditions. Out of these, the identities of 4 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.

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DECLARATIONS

Conflicts of interest: The authors declares that there is no conflict of interests.

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