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Biocontrol Efficacy of *Trichoderma* and *Bacillus* Isolates against *Sclerotium rolfsii* under *in vitro* Conditions

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ABSTRACT

Background: *Sclerotium rolfsii* is a polyphagous pathogen which is uneconomical and difficult to control due to its broad host range and persistence due to its tough resting structures, *i.e*., sclerotia. Chemical control has been supplanted by biological phytopathogen control. Antibiotic microorganisms are regarded as one of the viable management techniques in an integrated approach for ecofriendly management.

Methods: Twenty three *Trichoderma* spp. and twenty one *Bacillus* species were isolated at Groundnut Pathology Laboratory, ICRISAT, Patancheru from rhizospheric soils of Telangana during *rabi* 2022-23 and 2023-24 and examined for antagonistic activity against *Sclerotium rolfsii* in an effort to design an effective biocontrol system for the management of *Sclerotium rolfsii* in groundnut.

Result: The highest antagonistic activity against *Sclerotium rolfsii* has been observed in *Trichoderma viride* and *Bacillus cereus*. In the dual culture assay, *Trichoderma viride* and *Bacillus cereus* inhibited *Sclerotium rolfsii* by up to 65.33 per cent and 58.22 per cent, respectively. In the metabolites assay, *Trichoderma viride* and *Bacillus cereus* inhibited *Sclerotium rolfsii* by 63.33 per cent and 55.40 per cent, respectively. Thus, *Trichoderma viride* and *Bacillus cereus* displayed promising biofungicidal capabilities against the stem rot pathogen.

Key words: Biofungicidal, Ecofriendly, Groundnut, *Sclerotium rolfsii*.

INTRODUCTION

Groundnut (*Arachis hypogea* L.) is an important oilseed crop grown in India, China, Nigeria, Senegal, Sudan, Burma and the United States (Reddy *et al*., 2003). Peanuts have high energy content (567 calories per 100 g) and are high in important nutrients, minerals, antioxidants and vitamins. They are high in mono-unsaturated fatty acids, primarily oleic acid. It aids in the reduction of LDL (bad cholesterol) and the increase of HDL (good cholesterol) levels within the blood (Balasubramanian *et al*., 2023).

Groundnut is grown to an extent of 29.59 mha worldwide, with an overall yield of 48.75 million tons (FAOSTAT, 2019). In India, the crop has been grown on 4.8 million hectares and produces 9.2 million tonnes (INDIASTAT, 2019). It is grown in Telangana state over an extent of 0.13 mha, with a yield of 0.30 mt and an average yield of 2364 kg ha^{-1} (Directorate of Economics and Statistics, 2019).

Pathogens that cause seed and seedling rots and stem rot diseases include *Aspergillus niger*, *Aspergillus flavus*, *Rhizoctonia bataticola* and *Sclerotium rolfsii*. *Sclerotium rolfsii* is one of the soil-borne fungal diseases that could threaten groundnut cultivation. Brown discoloration and decay can be seen in diseased tissues and white hyphae are widespread on the surfaces of affected areas. Affected plants gradually collapse and die. At the moment, no marketable groundnut cultivar is totally immune to the disease. Mayee and Datar (1988) reported yield decreases of up to 25% due to this illness as it approached maturity.

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Stem rot also known as Sclerotium blight, Sclerotium rot, southern blight, southern stem rot, Sclerotium wilt, root rot, pod rot and white mold caused by *Sclerotium rolfsii* Sacc is one of the major constraints in groundnut production as it severely affects the yield and quality of the produce (Babu and Deepika, 2022). Application of nitrogen reduced stem rot disease caused by *S. rolfsii*, however, this method is not recommended for groundnut cultivation being a

leguminous crop (Le *et al*., 2018). Bio-control has been shown to be a successful disease control technique, especially for soil-borne plant diseases. Several studies have found that *Trichoderma* spp. and *Bacillus* spp. are antagonistic to *S. rolfsii* (Kajalkumar and Chitreswar, 2000; Xu *et al*., 2020). It is crucial to discover isolates which are particularly effective against *S. rolfsii* in advance of a planned regional deployment. The current study aimed to find efficient isolates of *Trichoderma* spp. and *Bacillus* spp. antagonistic to *S. rolfsii* under *in vitro* conditions.

MATERIALS AND METHODS

Isolation of *Trichoderma* and *Bacillus* species from peanut rhizosphere soil

Trichoderma and *Bacillus* species have been isolated at Groundnut Pathology Laboratory, ICRISAT, Patancheru from the rhizosphere soils collected in diverse groundnut growing locations throughout Telangana during *rabi* 2022-23 and 2023-24 (Table 1). The plants were carefully pulled while their root systems remained intact and the soil clinging over the roots was collected. Ten grams of this rhizosphere soil was placed in a 250-milliliter Erlenmeyer flask with 100 mL distilled water. One milliliter of the final dilution of 10^{-3} was put into a sterile Petri dish with Trichoderma specific medium to isolate fungal antagonists Trichoderma. Similarly, one ml of an aliquot from the final dilutions 10^{-5} and 10^{-6} was put into a sterile Petri dish with Bacillus specific medium and plates were incubated for 24 hours at 27°C. Isolates of *Bacillus* were purified on Nutrient agar medium using the streak plate method (Rangaswami, 1993), whereas *Trichoderma* isolates were grown on Potato dextrose agar. From groundnut rhizosphere soil, twenty three isolates of *Trichoderma* and twenty one isolates of *Bacillus* have been isolated. Light microscopy was used to identify the morphology of the cultures and pure cultures were preserved at 4°C on appropriate agar slants.

Dual culture assay of *Trichoderma* isolates against *Sclerotium rolfsii*

The dual culture experiment (Dennis and Webster, 1971a) was used to evaluate the capacity of twenty three Trichoderma isolates to suppress the radial growth of a virulent *S. rolfsii* strain. On Petri dishes with solidified Potato dextrose agar, six-millimeter mycelial discs of rapidly growing Trichoderma isolates and the pathogen were positioned roughly 5 cm apart. The plates were then incubated at 27°C for 5 days with an appropriate control. The efficiency of Trichoderma isolates were assessed by computing the percent inhibition of pathogen radial growth by the formula (Kamaruzzaman *et al*., 2021).

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

Where,

I= Inhibition as a percentage of control.

C= *S. rolfsii* radial development in control plates.

T= *S. rolfsii* radial development in the presence of Trichoderma isolates.

Metabolite assay of *Trichoderma* spp.

The metabolite test method was utilized to assess the influence of possible *Trichoderma* isolates' volatile metabolites on the pathogen. The lid was substituted with the bottom dish of a different potato dextrose agar plate that had been centrally placed with a 6 mm *S. rolfsii* mycelial disc. Both plates were secured with tape together and incubated for 5 days at 27°C (Dennis and Webster, 1971b). As control, two bottom lids containing *S. rolfsii* discs were used. Using the provided formula, the percentage inhibition over control was calculated.

Dual culture assay of *Bacillus* isolates against *S. rolfsii*

The dual culture technique was used to evaluate all twenty one Bacillus isolates against a virulent isolate of *S. rolfsii* (Vidhyasekaran and Muthamilan, 1999). Their efficacy was measured by their capacity to block the pathogen's radial growth. *Bacillus* isolates were streaked independently on one side of the pathogen on a Petri dish with PDA medium and a virulent *S. rolfsii* isolate's 6 mm mycelial disc was placed on the opposite side. The plates were incubated at 27°C for 5 days with an appropriate control. Using the above

Table 1: Geographical origin of isolates of *Trichoderma* and *Bacillus* collected from major groundnut growing areas of Telangana.

Geographical origin		
State	District	Village
Telangana	Warangal	Kadarigudem
Telangana	Warangal	Ramojikummarigudem
Telangana	Warangal	Kammapally
Telangana	Warangal	Nashkal
Telangana	Warangal	Ellanda
Telangana	Warangal	Madannapet
Telangana	Warangal	Chandraypally
Telangana	Warangal	Dasaripally
Telangana	Warangal	Muchimpla
Telangana	Warangal	Lenkalapalli
Telangana	Warangal	Rangapur
Telangana	Wanaparthy	Palem
Telangana	Wanaparthy	Apparaala
Telangana	Wanaparthy	Polikapadu
Telangana	Wanaparthy	Yedutla
Telangana	Wanaparthy	Buddaram
Telangana	Nagarkurnool	Zamisthapur
Telangana	Nagarkurnool	Bopally
Telangana	Nagarkurnool	Pedduru
Telangana	Nagarkurnool	Gattunallykuduru
Telangana	Nagarkurnool	Gattuthummen
Telangana	Nagarkurnool	Jinkunta
Telangana	Nagarkurnool	Godal

formula, the efficacy of several *Bacillus* isolates was estimated as a per cent suppression of pathogen radial growth over control.

Metabolite assay of *Bacillus* spp.

The purpose of this study was to see how volatile metabolites produced by potential *Bacillus* spp. isolates affected S. *rolfsii*. In the center of the PDA plates, a Bacillus isolate was streaked and the cover was substituted by the bottom dish of other potato dextrose agar plate infected with a 6 mm *S. rolfsii* mycelial disc. The two plates were secured with tape together and incubated for 5 days at 27°C (Dennis and Webster, 1971b). Both bottom lids with *S. rolfsii* discs served as control. In both control and *Bacillus*-inoculated plates, pathogen radial growth was recorded. Using the previously mentioned formula, the per cent inhibition over control was computed.

Molecular characterization of isolates of *Trichoderma*

The genomic DNA was extracted from the Trichoderma isolates mycelium using the CTAB method with slight modifications (Murray and Thompson, 1980). Isolated DNA samples were run on 0.8% agarose gel containing ethidium bromide for 45 min at 60 V in 1X TAE buffer to check the quality and quantity of DNA. PCR-based molecular characterization was carried out by amplifying the rDNA-ITS region of all 23 Trichoderma isolates using universal fungal primers, *viz*., ITS1 (5′-TCC GTA GGT GAA CCT GCG G-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (White *et al*., 1990). For this, a 2X PCR Taq Master mix (Applied Biological Materials, Richmond, Canada) containing dNTPs, DNA polymerase, buffer and MgCl2, primers (Integrated DNA Technologies, Coralville, US) and nuclease-free water were used. The PCR thermal cycler (Bio-Rad, California, US) reaction contained 50 ng genomic DNA, 1X PCR Taq Master mix and 0.25 mM of each primer in a 25 ml reaction volume. The PCR program was as follows: 1 cycle at 94°C for 5 min, 35 cycles at 94°C for 1 min followed by a cycle at 55°C for 1 min and another at 72°C for 2 min and 1 cycle at 72°C for 5 min and then the products were held at 4°C. The PCR products were analyzed on 1% agarose gel electrophoresis for 45 min at 60 V in 1X TAE buffer (40 mM Tris base, 20 mM Acetic acid and 1 mM EDTA), stained with ethidium bromide and photographed by using Gel Documentation System (Bio-Rad, California, US). PCR amplicons were purified using NucleoSpin Gel and PCR clean-up kit (Macherey-Nagel, Düren, Germany) and sequenced at eurofins Genomics facility.

Molecular characterization of isolates of *Bacillus*

The genomic DNA of 21 bacterial isolates was extracted from the cultures growing on nutrient agar for 18 h using QIAGEN DNA bacteria kit (QIAGEN, Germany). The extracted genomic DNA was detected by gel electrophoresis and then stored at -20°C for further use. The gene encoding 16S rRNA was amplified by PCR using universal primers 27F (5-AGAGTTTGATCCTGGC TCAG-3) and 1492R (5-GGTTACCTTGTTA CGACTT-3). The PCR reaction mixture was made in a total volume of 50 µL containing 25 µL of master mix (Promega, USA), 2 µL of template DNA, 2 µL each of forward and reverse primers and 19 µL of nuclease-free water. The PCR amplification conditions were as follows: an initial denaturation step of 96°C for 3 min followed by 27 cycles of 96°C for 30 s, annealing of 56°C for 25 s and elongation at 72°C for 15 s and final extension step at 72°C for 10 min (Miyoshi *et al*., 2005). The PCR products were photographed by using Gel Documentation System (Bio-Rad, California, US). PCR amplicons were purified using NucleoSpin Gel and PCR clean-up kit (Macherey-Nagel, Düren, Germany) and sequenced at eurofins Genomics facility.

RESULTS AND DISCUSSION

***Trichoderma* spp. dual culture assay**

Five putative *Trichoderma* spp. isolates were examined in a dual culture experiment against a virulent isolate of *S.rolfsii* (SrPWp). The isolate T2 considerably decreased pathogen radial growth (65.33%) when compared to the control. T1 (60.22) and T3 (60.88) isolates were revealed to be the best antagonists with equivalent activity against the pathogen. The pathogen was shown to be considerably less effective against T4 (55.77) and T5 (58.00) isolates (Table 2; Plate 1).

Paramasivan (2006) reported that *T. viride* and *T. harzianum* were particularly effective in inhibiting the radial growth of *S. rolfsii* in dual culture. Srinivasulu *et al.* (2005) and Kotasthane *et al*. (2014) also observed in vitro reduction in *S. rolfsii* radial mycelial growth.

Metabolite assay of *Trichoderma* spp.

Five putative *Trichoderma* spp. isolates were compared to a virulent isolate of *S. rolfsii* (SrPWp) in a metabolite experiment. When compared to the control, isolate T2 had the highest percent of pathogen radial growth inhibition (63.33%). Furthermore, pathogen radial mycelial growth

Table 2: Antagonistic efficacy of potential isolates of *Trichoderma* spp. against *Sclerotium rolfsii.*

Isolates	Per cent inhibition of radial growth over control	
	Dual culture assay	Metabolite assay
T1	60.22 (50.89)	58.20 (49.72)
T2	65.33 (53.92)	63.63 (52.91)
T3	60.88 (51.31)	58.22 (49.77)
T4	55.77 (48.31)	48.36 (44.05)
T5	58.00 (49.60)	54.40 (46.26)
CD (0.01)	3.21	4.71
S.Em.±	1.01	1.49
CV (%)	3.47	5.38

was suppressed much less (48.36%) by isolate T4 than by the control (Table 2; Plate 2).

The findings are comparable with those of Fravel (1988) and Kotasthane *et al*. (2014), who explored the influence of volatile metabolites from *Trichoderma* spp. on *S. rolfsii*, *T. harzianum*, according to Fravel (1988), creates alkyl pyrones that suppress *S. cepivorum*. Similarly, Kotasthane *et al*. (2014) reported that *Trichoderma viride* isolates had the strongest antagonistic activity against *Scelrotium rolfsii* and *Rhizoctonia solani*, two soil-borne plant pathogens.

Dual culture assay of *Bacillus* isolates against *S. rolfsii*

A dual culture study of 5 putative *Bacillus* isolates against *S. rolfsii* demonstrated that *Bacillus* isolates were similarly effective as *Trichoderma* spp. B5 was determined to be the most efficient against the test pathogen among the five possible isolates tested, with a significantly greater reduction in radial development (58.22 per cent) than the control, followed by B4 (56.32). Furthermore, isolate B3 performed the worst, with a 50.44 per cent reduction in radial growth above control, comparable to isolates B2 (53.16) and B1 (55.32). (Plate 3; Table 3).

The results are comparable with the findings reported by Solanki *et al*. (2012), who reported that *Bacillus* spp. strain MB101 suppressed the radial growth of *R. solani*. The *Bacillus subtilis* strain (EU07) had the largest growth rate *in vitro* decrease in *Fusarium oxysporum* f. sp. *radicis-lycopersici*, the causative organism of tomato fusarium wilt, according to Baysal *et al*. (2008).

Metabolites assay of *Bacillus* spp

A similar pattern was found when 5 putative *Bacillus*. isolates were tested in a metabolic assay for their efficiency against *S. rolfsii*. The strain B5 was shown to be the most successful in reducing pathogen radial development by 55.40% more than the control. Isolates B1 (50.42), B2 (49.33) and B3 (49.11) were the subsequent best effective isolates, all of which were comparable. In addition, the B4 isolate performed the worst, with a 48.44 reduction in pathogen radial development (Table 3; Plate 4).

Knox *et al*. (2000) reported that two strains of *B. subtilis* suppressed many plant pathogenic fungi on agar plates

Table 3: Antagonistic efficacy of potential isolates of *Bacillus* spp. against *Sclerotium rolfsii.*

Isolates	Per cent inhibition of radial growth over control	
	Dual culture assay	Metabolite assay
B1	55.32 (48.05)	50.42 (45.24)
B2	53.16 (46.81)	49.33 (44.61)
B3	50.44 (45.25)	49.11 (44.49)
B4	56.32 (48.63)	48.44 (44.10)
B5	58.22 (49.73)	55.40 (48.10)
CD (0.01)	1.69	3.44
S.Em.±	0.53	1.09
CV (%)	1.95	4.18

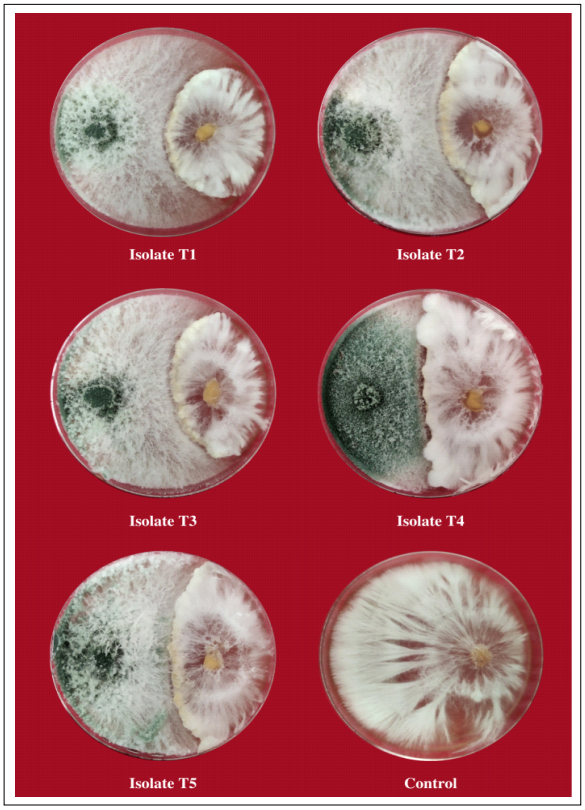

Plate 1: Dual culture assay of five potential isolates of *Trichoderma* spp. against virulent isolate of *Sclerotium rolfsii.*

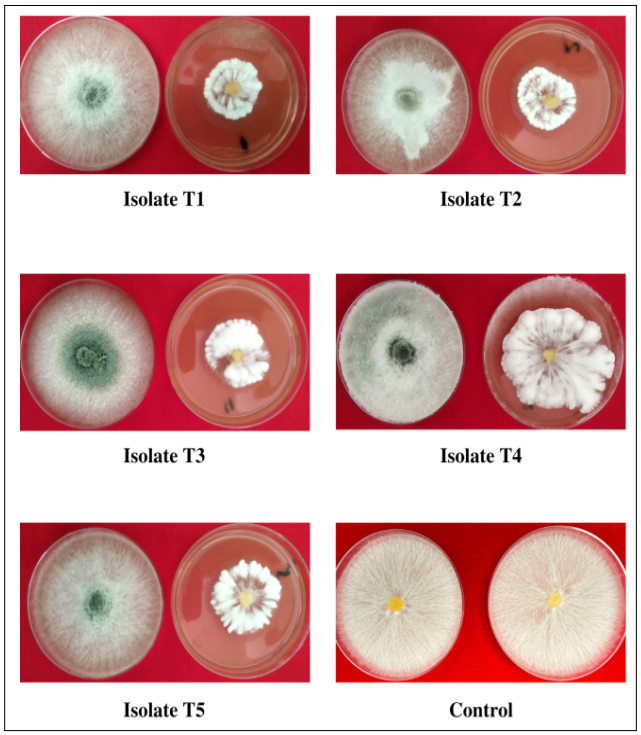

Plate 2: Metabolite assay of five potential isolates of *Trichoderma* spp. against virulent isolate of *Sclerotium rolfsii.*

and they hypothesized that this was due to antifungal volatile substances (AFS) produced by them. Likewise, Ashok *et al*. (2014) discovered that *Bacillus subtilis* produces a bioactive chemical that inhibits the growth of *S. rolfsii*. Giorgio *et al*. (2015) discovered that eight Bacillus strains prevented the growth of *Sclerotinia sclerotiorum* by creating volatile organic compounds (VOCs). Furthermore, Li *et al*. (2015) discovered that a Bacillus strain produced VOCs that suppressed *Fusarium solani* mycelial development *in vitro* substantially.

Molecular characterization of potent isolates of *Trichoderma* and *Bacillus*

To test twenty three Trichoderma and twenty one Bacillus isolates biocontrol ability, isolates were pre-screened against SrPWp, the virulent isolate of *S. rolfsii*. Fungal antagonist isolates demonstrated varying levels of biocontrol efficacy against virulent *S. rolfsii* isolate. Furthermore, the five most promising antagonistic fungal isolates were chosen for further study and serially labeled from T1 to T5. Table 4 contains information about these isolates. ITS-rDNA amplification, sequencing and phylogeny were used to identify these promising antagonistic fungal isolates. The ITS-rDNA amplification was performed using primers ITS 1 and ITS 4, yielding a 600 bp amplicon (Fig 1). The NCBI databases was nucleotide blasted with the quality forward as well as reverse sequence data of 5 isolates' amplified fragments and all isolates were confirmed as *Trichoderma* spp. MEGA7 software was used to create the phylogenetic tree.

In the phytogenic tree, isolates T1, T2 were clustered with *Trichoderma viride*, isolate T3, isolate T5 with *Trichoderma harzianum* and isolate T4 with *Trichoderma hamatum* reference strains (Fig 2).

Antagonistic fungi, particularly *Trichoderma* and *Gliocladium* spp., have been employed more widely than bacteria (Ganesan, 2004; Ganesan and Sekar, 2004a). *Sclerotium rolfsii* was controlled with *Trichoderma harzianum* by Ganesan, (2004). Muthamilan and Jeyarajan (1996) discovered that mixing *T. harzianum*, *Rhizobium* and carbendazim improved groundnut root rot management. Similarly, Ekundayo *et al*. (2016) discovered *Trichoderma viride* to be efficient in lowering the incidence of southern blight of tomato in pot culture tests. Five of the most promising *Trichoderma* spp. isolates were chosen and evaluated for biocontrol against a virulent *S. rolfsii* isolate.

In the preliminary screening, the isolates of bacterial antagonists demonstrated various levels of biocontrol features against the virulent isolate of *S. rolfsii*. Furthermore, the five most promising bacterial antagonist isolates were chosen for future testing and were labeled serially from B1 to B5. Table 5 contains details regarding the bacterial isolates. Using 16S rDNA amplification, sequencing and phylogeny, the researchers identified five potential antagonistic bacterial isolates. The 16S rDNA amplification was performed with 24 F and 1492 R primers, yielding

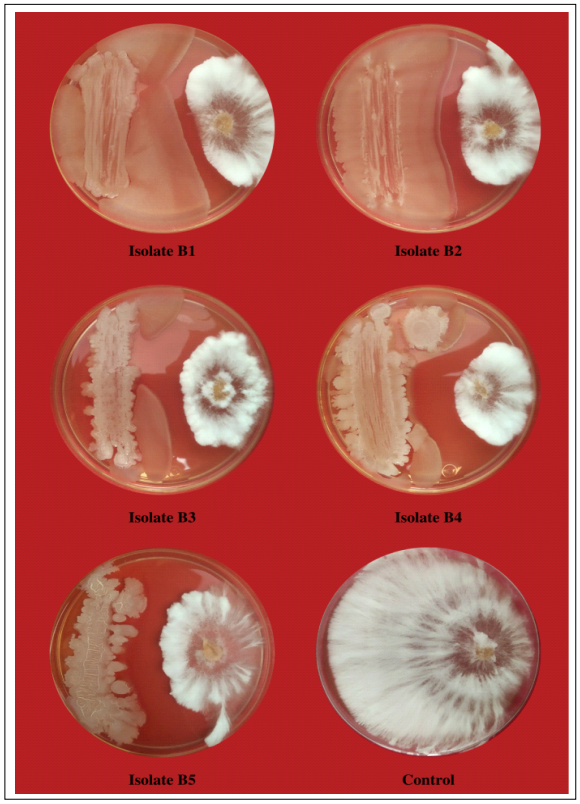

Plate 3: Dual culture assay of five potential isolates of *Bacillus* spp. against virulent isolate of *Sclerotium rolfsii.*

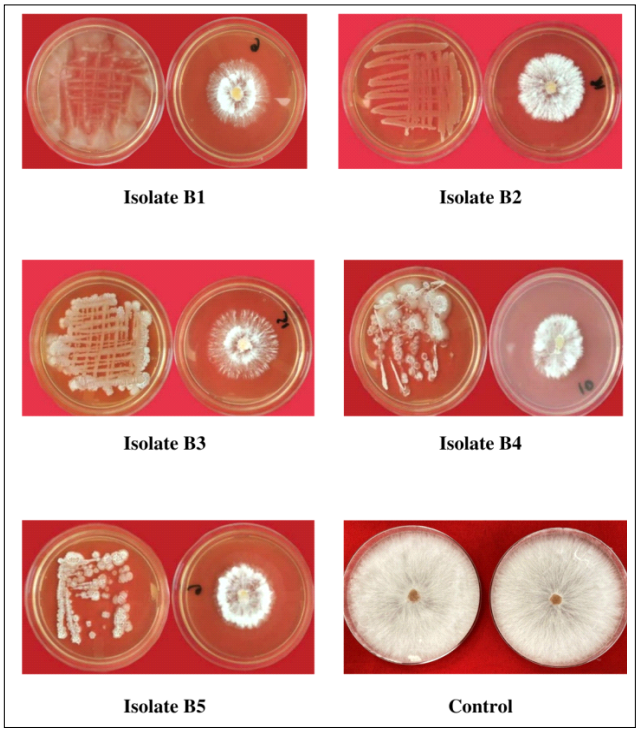

Plate 4: Metabolite assay of five potential isolates of *Bacillus* spp. against virulent isolate of *Sclerotium rolfsii.*

Table 4: List of potential isolates of *Trichoderma* spp. isolated from groundnut rhizosphere soils.

Soil sample location	*Trichoderma*isolate	18S rDNA sequence identification
Ellanda (Warangal)	*Trichoderma* spp. (T1)	*Trichoderma viride* (MZ836869.1)
Nashkal (Warangal)	*Trichoderma* spp. (T2)	*Trichoderma viride* (MZ836868.1)
Rangapur (Warangal)	*Trichoderma* spp. (T3)	*Trichoderma harzianum* (MZ836872.1)
Buddaram (Wanaparthy)	*Trichoderma* spp. (T4)	*Trichoderma hamatum* (MZ836871.1)
Pedduru (Nagarkurnool)	*Trichoderma* spp. (T5)	*Trichoderma harzianum* (MZ836870.1)

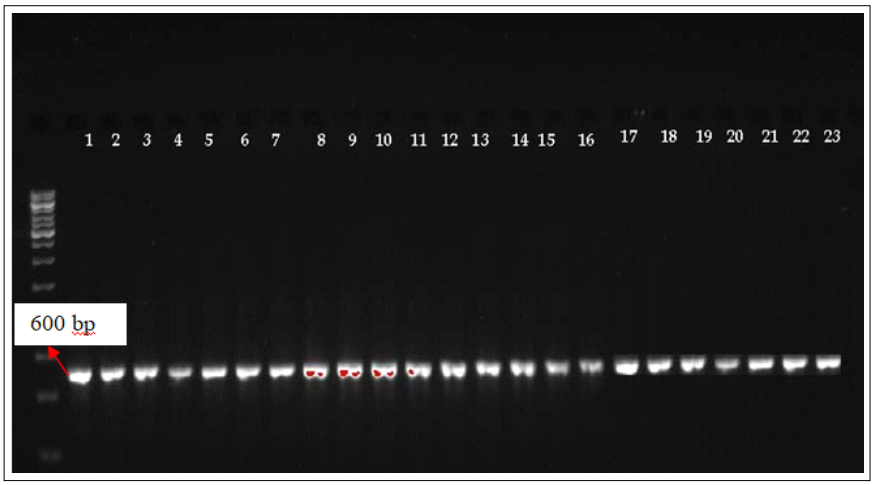

Fig 1: ITS-rDNA region amplified product of twenty three isolates of *Trichoderma* spp.

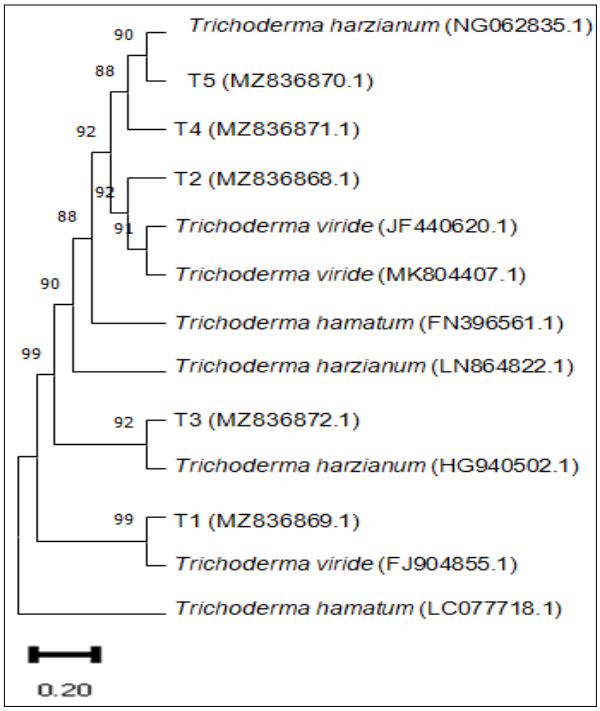

Fig 2: Phylogeny of 18S rDNA sequences of five potential isolates of *Trichoderma* spp. using Neighbor-joining tree.

amplicons of 1500 bp (Fig 3). Five isolates' forward and reverse sequence data were nucleotide blasted in the NCBI data base and identified as *Bacillus* spp. For phylogenetic analysis, the sequences were matched to reference *Bacillus* spp. sequences from the NCBI database. The phylogenetic tree was created using the MEGA7 software.

Isolates B1, B3 and B4 have been linked with *Bacillus velezensis* in the phylogenetic tree, whereas isolates B2 and B5 were grouped together with *Bacillus tequilensis* and *Bacillus cereus* reference strains, respectively (Fig 4).

Several *Bacillus* spp. strains have been identified as bacterial antagonists capable of suppressing soil-borne plant diseases and improving plant growth (Zhao *et al*., 2014; Shrestha *et al*., 2016). Suslow and Schroth (1982) observed that *Bacillus* spp. efficiently inhibited *S. rolfsii* infection in groundnut, chickpea and beans. Because of their rapid rhizosphere growth, *Bacillus* spp. may have an advantage over fungal antagonists in controlling sclerotial fungus.

Furthermore, Singh and Dwivedi (1987) reported that *Bacillus subtilis, Bacillus licheniformis, Pseudomonas aerginosa* and *Streptomyces diastaticus* strains considerably reduced *S. rolfsii*-caused barley foot rot. Similarly, Abeysinghe (2009) discovered that *B. subtilis* has an enhanced ability to reduce *S. rolfsii* incidence in chilli

Table 5: List of potential isolates of *Bacillus* spp. isolated from groundnut rhizosphere soils.

Soil sample location	*Bacillus* isolate	Gram stain	Cell shape	16S rRNA sequence identification
Dasaripally (Warangal)	*Bacillus* spp. (B1)	+	Rod	*Bacillus velezensis* (MZ831872.1)
Kadarigudem (Warangal)	*Bacillus* spp. (B2)	+	Rod	*Bacillus tequilensis* (MZ831870.1)
Rangapur (Warangal)	*Bacillus* spp. (B3)	+	Rod	*Bacillus velezensis* (MZ831871.1)
Pedduru (Nagarkurnool)	*Bacillus* spp. (B4)	+	Rod	*Bacillus velezensis* (MZ831869.1)
Jinkunta (Nagarkurnool)	*Bacillus* spp. (B5)	+	Rod	*Bacillus cereus* (MZ843638.1)

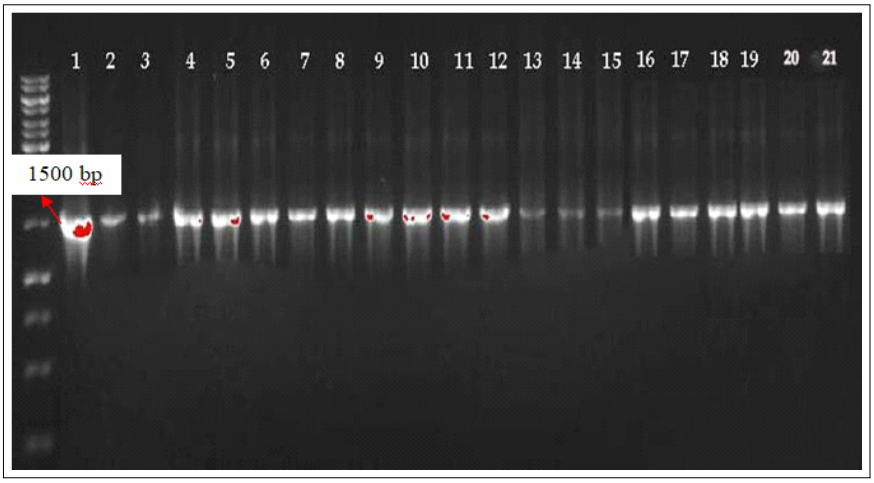

Fig 3: 16S-rRNA region amplified product of twenty one isolates of *Bacillus* spp.

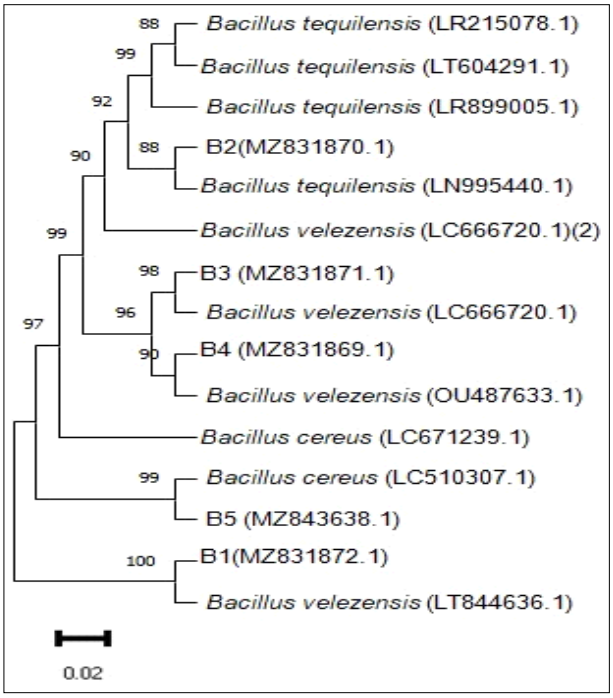

Fig 4: Phylogeny of 16S rDNA sequences of five potential isolates of *Bacillus* spp. using Neighbor-joining tree.

via seed and root bacterization, leading to a greater number of bacteria at the collar region of chilli plants and protecting of the pathogen's most vulnerable area, resulting in greater protection.

The five most promising *Bacillus* spp. isolates from the previous investigation were chosen and tested for biocontrol characteristics against an aggressive isolate of *S. rolfsii.*

CONCLUSION

Antibiotic producing microorganisms have proven to be one of the viable management techniques in a holistic strategy due to their eco-friendliness. The fungal bioagent *Trichoderma viride* and the bacterial bioagent *Bacillus cereus* exhibited the best antagonistic action against *Sclerotium rolfsii* in both the dual culture and metabolite experiments.

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Conflict of interest

The authors declare that they have no conflict of interest.

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