

Chitinase-Mediated Suppression of Groundnut Stem Rot Pathogen (*Sclerotium rolfsii* Sacc.) by *Bacillus amyloliquefaciens* RRR16

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ABSTRACT

Groundnut (*Arachis hypogaea* L.) is one of the most important oilseed crops, but its production is severely affected by stem rot disease caused by *Sclerotium rolfsii*. The present study investigates the potential of a chitinase-producing *Bacillus amyloliquefaciens* strain GR16 for biological control of *S. rolfsii*. The isolate was obtained from rhizospheric soil of healthy groundnut plants and identified through morphological, biochemical, and molecular characterization. *B. amyloliquefaciens* RRR16 exhibited strong chitinolytic activity on colloidal chitin agar plate and significantly inhibited the mycelial growth of *S. rolfsii* in a dual culture assay with 92.86 % inhibition. The results indicate that *B. amyloliquefaciens* RRR16, through its chitinolytic mechanism, can serve as an efficient biocontrol agent for sustainable management of groundnut stem rot disease.

Keywords: *Sclerotium rolfsii*, *Bacillus amyloliquefaciens* RRR16, Chitinase, Biocontrol, Groundnut, and Stem rot

I. INTRODUCTION

Groundnut (*Arachis hypogaea* L.) is an economically important legume and oilseed crop extensively cultivated across tropical and subtropical regions.

However, its productivity and quality are severely constrained by several soil-borne fungal pathogens, among which *Sclerotium rolfsii* Sacc., the causal agent of stem rot, is the most destructive. *S. rolfsii* is a ubiquitous soil-borne pathogen prevalent in tropical

and subtropical agroecosystems worldwide (Elasmi *et al.*, 2015; Patra *et al.*, 2023). Stem rot caused by *S. rolfsii* can lead to substantial yield losses ranging from 50% to 80% in India under favourable environmental conditions (Mayee and Datar, 1988; Ambika *et al.*, 2023; Deepika *et al.*, 2025). The pathogen primarily infects the basal portion of the stem, resulting in sudden wilting, necrosis, and the formation of characteristic sclerotia on infected tissues.

In India, *S. rolfsii* is widespread across all major groundnut-growing regions, with particularly high incidence reported from Andhra Pradesh, Maharashtra, Gujarat, Madhya Pradesh, Karnataka, Odisha, and Tamil Nadu (Kumar *et al.*, 2013; Babu and Deepika, 2022). The disease incidence varies widely (5–86%) depending on climatic factors, soil type, and cropping systems. The pathogen survives adverse environmental conditions for extended periods through the formation of resilient sclerotial bodies in the soil (Vaniya *et al.*, 2022). The control of pathogens through conventional chemical means is difficult. Several fungicides can inhibit mycelial growth and sclerotial germination of *S. rolfsii* (Zmora *et al.*, 2008). The long-term use poses challenges related to environmental toxicity, non-target effects, and the emergence of resistant strains.

Commonly used fungicides such as Vitavax-Thiram, Vitavax-Captan, Thiram, Quintozene, Captan, Carbendazim, Benomyl, Oxycarboxin, and Triadimenol have shown varying degrees of efficacy against *S. rolfsii*. Among these, combinations such as Carboxin + Thiram and Quintozene formulations have demonstrated superior performance in suppressing sclerotial formation and inhibiting mycelial growth under laboratory and field conditions (Yaqub and Shahzad, 2006; Khan and Javaid, 2015; Chowdary *et al.*, 2024).

Traditionally, chemical fungicides have been used as a primary means of disease management. Although they provide quick and effective control, their continuous and indiscriminate use has led to environmental contamination, the development of resistant pathogen

strains, and harmful effects on non-target organisms, including humans (Cook & Baker, 1983; Patra *et al.*, 2024; Tyagi *et al.*, 2024). In contrast, biocontrol — the use of beneficial microorganisms such as *Bacillus*, *Trichoderma*, and *Pseudomonas spp.* — offers a sustainable and eco-friendly alternative for managing plant diseases (El-Saadony *et al.*, 2022; Nagrale *et al.*, 2023; Boro *et al.*, 2024). Biological control has emerged as a sustainable and eco-friendly alternative to chemical fungicides for managing soil-borne pathogens such as *Sclerotium rolfsii*. Among the diverse groups of beneficial microorganisms, *Bacillus spp.* has gained considerable attention due to their remarkable antagonistic potential, ability to form endospores, and adaptability to adverse environmental conditions (Miljaković *et al.*, 2020; Etesami *et al.*, 2023). These Gram-positive, spore-forming bacteria are known to colonize the rhizosphere effectively and employ multiple mechanisms to suppress phytopathogens through direct antagonism and indirect induction of plant defense responses (Sarangi *et al.*, 2023).

The present study focuses on isolating *Bacillus* spp. from the rhizospheric soil of groundnut and evaluating their antagonistic potential against *Sclerotium rolfsii* under *in vitro* conditions. To elucidate the possible biocontrol mechanism, the most promising *Bacillus* isolate was examined for *in vitro* chitinase activity.

II. MATERIALS AND METHODS:

2.1 Soil Sampling and Isolation of Microbial Isolates:

Healthy Groundnut plants were collected from Kataneshwar (19°16'36.6"N 76°56'01.4"E) Parbhani district, Maharashtra, from the field and rhizosphere soil on their roots was gently collected with a soft brush after removing large pieces of soil from the roots. The soil was subsequently passed through a sieve with an aperture of 0.9 mm and stored at 4 °C. From the collected soil samples 1 gram was taken distinctly and dissolved in 9 ml of sterile normal saline

solution (0.85% w/v) and homogenized vigorously with a vortex mixer for 2 min. The sample solutions were heat-shocked at 80 °C for 10 min in a water bath to destroy non-spore formers and vegetative *Bacillus* cells. Then homogenized and heat-treated soil solutions were serially diluted in sterile normal saline to prepare 10^{-1} – 10^{-5} dilutions (Holt *et al.*, 1994; Cappuccino & Sherman, 2014). Then 100 µl volumes of serially diluted soil samples were taken and plated on nutrient agar medium and incubated aerobically at 37 °C for 24 hrs (Chilcott and Wigley, 1993; Alemu *et al.*, 2024).

Sclerotium rolfsii, isolated from a diseased groundnut stem in 2010 during the Doctoral research work and identified by cultural characteristics and Internal Transcribed Spacer sequence analysis was obtained from the Biokart Genomic Lab at 5M-612, 5th Main Road, OMBR Layout, Bengaluru, Karnataka 560043, Bengaluru - 560043, Karnataka, India.

2.2 Screening of Rhizospheric *Bacilli* for *In Vitro* Antifungal Activity:

The isolated *Bacillus* strains were screened for their antifungal activity against *Sclerotium rolfsii*, the causal agent of stem rot of groundnut, using the dual culture assay. The assay was performed on Potato Dextrose Agar (PDA) plates following the method described by Dennis and Webster (1971) and Raut *et al.*, (2023) with slight modifications.

After growth of *Sclerotium rolfsii* on Potato Dextrose Agar (PDA) for 5 days at 28 °C, a 5 mm diameter plug with mycelium was transferred to 1 cm away from the center of a 90 mm diameter fresh PDA plate. While in the opposite direction, 1 cm away from the center, a loopful of 24 – hour old culture of each rhizosphere soil *Bacilli* isolate was co-inoculated on the PDA 2 cm from *Sclerotium rolfsii*. Plates inoculated only with the pathogen served as controls. All plates were then incubated at 28 °C for 6 days. After incubation, the percentage of fungal growth inhibition was evaluated by measuring the radial growth of the pathogen in test

and control plates. The percent inhibition of mycelial growth (PIRG) was calculated using the formula:

$$\text{PIRG (\%)} = \frac{R1 - R2}{R1} \times 100$$

Where, R1 is radial growth by the pathogen in the opposite direction of the antagonist (a control value) and R2 is radial growth by the pathogen in the direction towards the antagonist (an inhibition value). (Skidmore & Dickinson, 1976).

2.3 Identification of Antagonistic *Bacillus spp.*

Total genomic DNA of efficient antagonistic *Bacillus spp.* was isolated using GeneElute Genomic DNA isolation kit (Sigma, USA) as per the manufacturer's instructions and used as template for PCR. Each reaction mixture contained approximately 10 ng of DNA; 2.5 mM MgCl₂; 1x PCR buffer (Bangalore Genei, Bangalore, India); 200 µM each dCTP, dGTP, dATP, and dTTP; 2 pmol of each, forward and reverse primer; and 1 U of Taq DNA polymerase (Bangalore Genei, Bangalore, India) in a final volume of 20 µl. 27F and 1492R primers were used to amplify almost entire 16S rRNA gene, as described previously (Rawlings 1995). The PCR was performed using the Eppendorf Gradient Mastercycler system with a cycle of 94 °C for 3 min; 32 cycles of 94 °C for 45 sec, 51 °C for 1 min and 72 °C for 1.30 min and final extension at 72 °C for 10 min, and the mixture was held at 4°C. The PCR product was precipitated using polyethylene glycol (PEG 6000, 8.5%) washed thrice using 70% ethanol and dissolved in Tris HCL (10mM, pH 8.0).

The ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Foster City, Calif.) was used for the sequencing of the PCR product. A combination of universal primers was chosen to sequence the nearly complete gene (Muyzer *et al.*, 1993; Rawlings, 1995). The sequencing reaction and template preparation were performed and purified in accordance with the directions of the manufacturer (Applied Biosystems). Samples were run

on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). The sequencing output was analyzed using the accompanying DNA Analyzer computer software (Applied Biosystems). The sequence was compared with EZTAXON for sequence identity.

2.4 Scanning Electron Microscopy (SEM) Analysis of Antifungal Interaction:

The ultrastructural interactions between *Bacillus* RRR16 and *Sclerotium rolfii* were examined using Scanning Electron Microscopy (SEM) to visualize the morphological alterations on the fungal hyphae caused by bacterial antagonism. The study was conducted following the method described by Ziedan *et al.*, (2008) and modified as per Kumar *et al.*, (2013). For SEM observation, small agar blocks (8 mm) were aseptically excised from the interaction zone of the dual culture plate after five days of incubation. Samples were gently washed with 0.1 M phosphate buffer (pH 7.2) and fixed in 4 % glutaraldehyde for 12 hours at 4 °C. The fixed samples were then washed three times with phosphate buffer and post-fixed in 2 % osmium tetroxide for 4 hours. After fixation, samples were dehydrated through a graded ethanol series (30%, 50%, 70%, 90%, and 100%), air-dried, and mounted on aluminum stubs using double-sided carbon tape. The specimens were then sputter-coated with gold and examined under a scanning electron microscope at an accelerating voltage of 15 kV. The occurrence of morphological changes in the hyphae of *Sclerotium rolfii* was recorded. Fungal discs without antagonists served as the controls.

2.5 Assay of Chitinase Enzyme:

2.5.1 Preparation of Colloidal Chitin:

To assess chitinase enzyme production, rhizospheric isolates were examined for chitinase activity. Colloidal chitin was prepared following the method described by Hsu and Lockwood (1975). In this method 4 g of crab shell chitin was dissolved in 40 ml concentrated HCl by stirring for 30-50 min. The chitin was precipitated as a colloidal suspension by adding it

slowly to 200 ml of cold water at 5-10°C. The suspension was collected by filtration with a coarse filter paper and then washed by suspending it in about 500 ml of tap water and re-filtered. The washing was repeated until the pH of the suspension was about 3.5. The pH of the suspension was adjusted to 7.0 by the addition of 5 N NaOH and the colloidal suspension was washed several times with distilled water for desalting. After desalting, the suspension was centrifuged at 8000 rpm for 10 min. The colloidal chitin obtained had a soft, pasty consistency with 90–95% moisture and was autoclaved and stored at 4°C until further use.

2.5.2 Detection of Chitinase Enzyme Production:

The chitinase production of efficient rhizospheric isolates was determined by using Colloidal Chitin Agar (Hsu & Lockwood, 1975) amended with 0.4 % colloidal chitin. The Colloidal Chitin Agar consists of 6 g Na₂HPO₄, 3 g KH₂PO₄, 0.5 g MgSO₄, 1g NH₄Cl, 0.5 g NaCl, 0.05 g yeast extract, and 4 g colloidal chitin dissolved in 1000 ml distilled water. The pH of the medium was adjusted 7.0 and 20 g agar was added as a solidifying agent. The medium was sterilized at 121°C for 20 min and plates were prepared. The chitin agar plate was spot inoculated with bacterial suspension and incubated at room temperature for 4 days. The plates were observed for the development of zone of a chitin hydrolysis around the spot inoculated culture was considered positive for chitinase production.

III.RESULTS:

3.1 Isolation of *Bacillus* spp. from Groundnut Rhizosphere:

A total of 102 *Bacillus* isolates were isolated from the rhizosphere soil of groundnut plants. The rhizospheric soil sample yielded a diverse set of *Bacillus*-like colonies after heat treatment and plating on nutrient agar, consistent with the spore-forming nature of this genus. Colonies showing typical *Bacillus* morphology, such as irregular, opaque, dry to mucoid colonies with Gram-positive rods and endospores, were purified and

maintained as putative *Bacillus spp.* for subsequent screening (Sarvani & Reddy, 2013; Akash *et al.*, 2022). These isolates were tentatively named as *Bacillus spp.* RRR1 to *Bacillus spp.* RRR102.

3.2 Screening Soil *Bacilli* for *In Vitro* Antifungal Activity:

All *Bacillus* isolates underwent initial *in vitro* screening utilizing the dual-culture assay against *Sclerotium rolfsii* (Table 1.0).

Table 1.0: Primary Screening of *Bacillus spp* as effective Microbiological Control Agents against *Sclerotium rolfsii* Sacc. under *In Vitro* conditions.

Tentative Name of <i>Bacillus spp.</i>	Inhibition of <i>S. rolfsii</i> (%)	Tentative Name of <i>Bacillus spp.</i>	Inhibition of <i>S. rolfsii</i> (%)	Tentative Name of <i>Bacillus spp.</i>	Inhibition of <i>S. rolfsii</i> (%)
<i>Bacillus spp.</i> RRR1	1	<i>Bacillus spp.</i> RRR35	2	<i>Bacillus spp.</i> RRR69	1
<i>Bacillus spp.</i> RRR2	2	<i>Bacillus spp.</i> RRR36	1	<i>Bacillus spp.</i> RRR70	2
<i>Bacillus spp.</i> RRR3	2	<i>Bacillus spp.</i> RRR37	1	<i>Bacillus spp.</i> RRR71	2
<i>Bacillus spp.</i> RRR4	1	<i>Bacillus spp.</i> RRR38	1	<i>Bacillus spp.</i> RRR72	2
<i>Bacillus spp.</i> RRR5	1	<i>Bacillus spp.</i> RRR39	1	<i>Bacillus spp.</i> RRR73	2
<i>Bacillus spp.</i> RRR6	2	<i>Bacillus spp.</i> RRR40	1	<i>Bacillus spp.</i> RRR74	2
<i>Bacillus spp.</i> RRR7	1	<i>Bacillus spp.</i> RRR41	1	<i>Bacillus spp.</i> RRR75	2
<i>Bacillus spp.</i> RRR8	1	<i>Bacillus spp.</i> RRR42	2	<i>Bacillus spp.</i> RRR76	2
<i>Bacillus spp.</i> RRR 9	1	<i>Bacillus spp.</i> RRR43	1	<i>Bacillus spp.</i> RRR77	1
<i>Bacillus spp.</i> RRR10	1	<i>Bacillus spp.</i> RRR44	2	<i>Bacillus spp.</i> RRR78	1
<i>Bacillus spp.</i> RRR11	2	<i>Bacillus spp.</i> RRR45	1	<i>Bacillus spp.</i> RRR79	1
<i>Bacillus spp.</i> RRR12	2	<i>Bacillus spp.</i> RRR46	1	<i>Bacillus spp.</i> RRR80	1
<i>Bacillus spp.</i> RRR13	2	<i>Bacillus spp.</i> RRR47	1	<i>Bacillus spp.</i> RRR81	2
<i>Bacillus spp.</i> RRR14	2	<i>Bacillus spp.</i> RRR48	1	<i>Bacillus spp.</i> RRR82	1
<i>Bacillus spp.</i> RRR15	3	<i>Bacillus spp.</i> RRR49	1	<i>Bacillus spp.</i> RRR83	1
<i>Bacillus spp.</i>	4	<i>Bacillus spp.</i>	2	<i>Bacillus spp.</i>	1

Tentative Name of <i>Bacillus spp.</i>	Inhibition of <i>S. rolfsii</i> (%)	Tentative Name of <i>Bacillus spp.</i>	Inhibition of <i>S. rolfsii</i> (%)	Tentative Name of <i>Bacillus spp.</i>	Inhibition of <i>S. rolfsii</i> (%)
<i>RRR16</i>		<i>RRR50</i>		<i>RRR84</i>	
<i>Bacillus spp.</i> <i>RRR17</i>	1	<i>Bacillus spp.</i> <i>RRR51</i>	1	<i>Bacillus spp.</i> <i>RRR85</i>	2
<i>Bacillus spp.</i> <i>RRR18</i>	2	<i>Bacillus spp.</i> <i>RRR52</i>	1	<i>Bacillus spp.</i> <i>RRR86</i>	2
<i>Bacillus spp.</i> <i>RRR19</i>	2	<i>Bacillus spp.</i> <i>RRR53</i>	3	<i>Bacillus spp.</i> <i>RRR87</i>	2
<i>Bacillus spp.</i> <i>RRR20</i>	2	<i>Bacillus spp.</i> <i>RRR54</i>	1	<i>Bacillus spp.</i> <i>RRR88</i>	1
<i>Bacillus spp.</i> <i>RRR 21</i>	1	<i>Bacillus spp.</i> <i>RRR55</i>	1	<i>Bacillus spp.</i> <i>RRR89</i>	2
<i>Bacillus spp.</i> <i>RRR22</i>	1	<i>Bacillus spp.</i> <i>RRR56</i>	1	<i>Bacillus spp.</i> <i>RRR90</i>	2
<i>Bacillus spp.</i> <i>RRR23</i>	1	<i>Bacillus spp.</i> <i>RRR57</i>	2	<i>Bacillus spp.</i> <i>RRR91</i>	2
<i>Bacillus spp.</i> <i>RRR24</i>	1	<i>Bacillus spp.</i> <i>RRR58</i>	1	<i>Bacillus spp.</i> <i>RRR92</i>	2
<i>Bacillus spp.</i> <i>RRR25</i>	1	<i>Bacillus spp.</i> <i>RRR59</i>	1	<i>Bacillus spp.</i> <i>RRR93</i>	2
<i>Bacillus spp.</i> <i>RRR26</i>	2	<i>Bacillus spp.</i> <i>RRR60</i>	2	<i>Bacillus spp.</i> <i>RRR94</i>	1
<i>Bacillus spp.</i> <i>RRR27</i>	2	<i>Bacillus spp.</i> <i>RRR61</i>	2	<i>Bacillus spp.</i> <i>RRR95</i>	1
<i>Bacillus spp.</i> <i>RRR28</i>	2	<i>Bacillus spp.</i> <i>RRR62</i>	1	<i>Bacillus spp.</i> <i>RRR96</i>	2
<i>Bacillus spp.</i> <i>RRR29</i>	3	<i>Bacillus spp.</i> <i>RRR63</i>	2	<i>Bacillus spp.</i> <i>RRR97</i>	2
<i>Bacillus spp.</i> <i>RRR30</i>	3	<i>Bacillus spp.</i> <i>RRR64</i>	2	<i>Bacillus spp.</i> <i>RRR98</i>	1
<i>Bacillus spp.</i> <i>RRR31</i>	3	<i>Bacillus spp.</i> <i>RRR65</i>	2	<i>Bacillus spp.</i> <i>RRR99</i>	1
<i>Bacillus spp.</i> <i>RRR32</i>	2	<i>Bacillus spp.</i> <i>RRR66</i>	2	<i>Bacillus spp.</i> <i>RRR100</i>	1
<i>Bacillus spp.</i> <i>RRR33</i>	1	<i>Bacillus spp.</i> <i>RRR67</i>	2	<i>Bacillus spp.</i> <i>RRR101</i>	0
<i>Bacillus spp.</i> <i>RRR34</i>	1	<i>Bacillus spp.</i> <i>RRR68</i>	1	<i>Bacillus spp.</i> <i>RRR102</i>	2

Each number is mean of three replicates. 0 – none, 1= inhibition zone 1 – 25 %, 2= inhibition zone 26 – 50 %, 3= inhibition zone 51 – 75 %, 4= inhibition zone 76 – 100 %.

During the secondary screening, six *Bacillus spp.*—namely RRR16, RRR15, RRR29, RRR30, RRR31, and RRR53—which showed strong antagonistic properties in the primary screening, were selected for further testing against *Sclerotium rolfii* using the dual culture method. Among the isolates, *Bacillus spp.* RRR16 exhibited the highest antagonistic activity, producing a maximum mycelial growth inhibition of 92.30 %, (Table 2.0) followed by isolates *Bacillus spp.* RRR15 *Bacillus spp.* RRR29, *Bacillus spp.* RRR30, *Bacillus spp.* RRR31 and *Bacillus spp.* RRR53 with inhibition values of 53.21%, 64.58, 63.72, 69.03 and 71.26%, respectively. The antagonist *Bacillus spp.* RRR16 produced a distinct inhibition zone and restricted radial growth of the pathogen, indicating strong antibiosis and competitive rhizosphere colonization potential, was selected for further studies. (Photo plate 1.0).

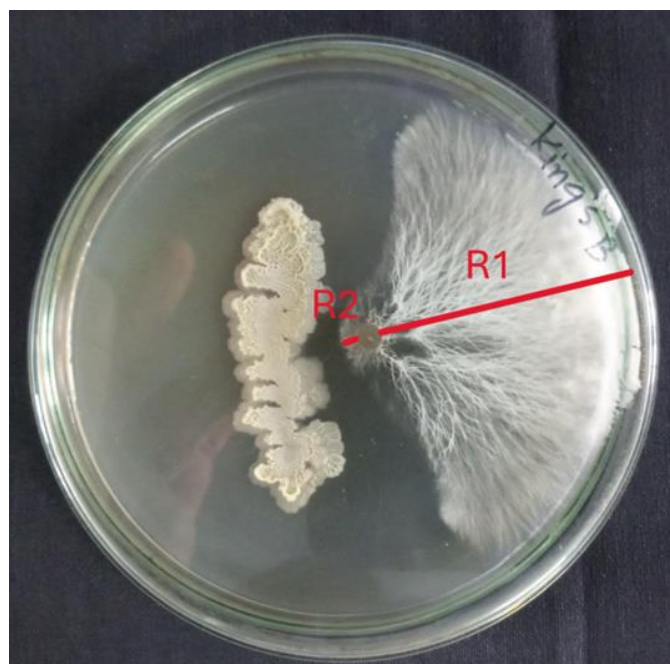


Photo Plate 1.0: *In Vitro* secondary screening of effective *Bacillus spp.* RRR16 against *Sclerotium rolfii* using the dual culture method

Table 2.0: *In Vitro* secondary screening of effective microbiological control agent, *Bacillus spp.*, selected during primary screening against *Sclerotium rolfii* Sacc.

Tentative Name of <i>Bacillus spp.</i>	Radial growth by the pathogen in the opposite direction of the antagonist (a control value) R1 (mm)	R2 is radial growth by the pathogen in the direction towards the antagonist (an inhibition value) R2(mm)	Percent Inhibition (%) of <i>Sclerotium rolfii</i>
<i>Bacillus spp.</i> RRR16	37.33±1.53	2.67±0.85	92.86±1.54

Each value is mean of three replicates with their standard deviation.

3.3 Identification of Antagonistic *Bacillus spp.*:

Bacillus spp. RRR16 was cultured on the NA plate for 48 h. The colony surface was dry, wrinkled, opaque, and white, with irregular or nearly circular edges. The bacteria were characterized as rod shaped, Gram positive and spore [Photo plate 2.0]. The 16S rRNA sequence lengths of *Bacillus spp.* RRR16 were 1232 bp. The sequences had been deposited in GenBank of National Center for Biotechnology Information (NCBI), U.S. with the accession number [MN749517](#). Blast comparison results showed that RRR16 was 99.91% homologous to *Bacillus amyloliquefaciens* strain MPA 1034. A phylogenetic tree, constructed

based on 16S rRNA sequences, showed a close genetic relationship between *Bacillus spp.* RRR16 and *Bacillus amyloliquefaciens* strain MPA 1034 [Photo plate 3.0]. According to morphological, physiological, and biochemical characteristics and 16S rRNA sequence analysis, RRR16 was identified as *Bacillus amyloliquefaciens* RRR16.

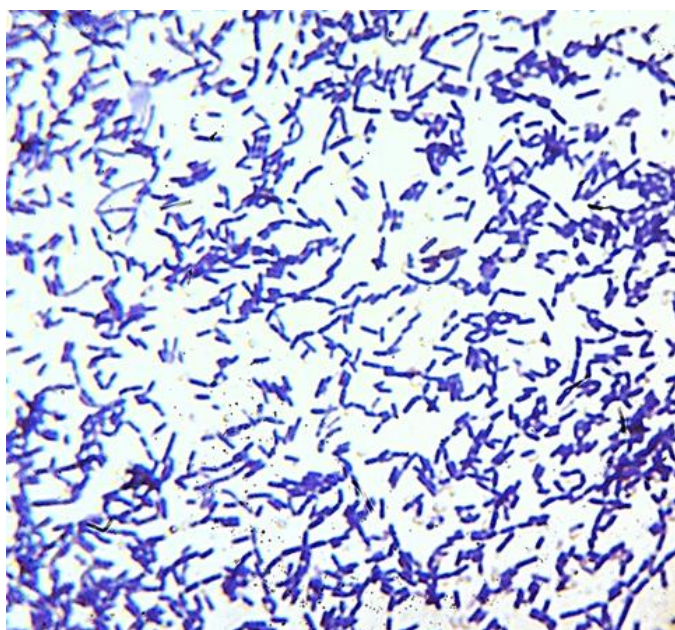


Photo plate 2.0: Gram's staining of *Bacillus amyloliquefaciens* RRR16

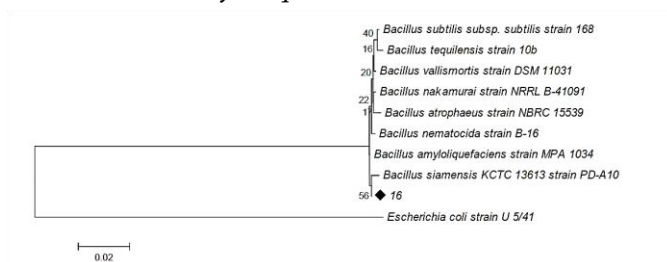


Figure 1.0: Neighbour-joining 16S rRNA gene phylogenetic tree of *Bacillus amyloliquefaciens* RRR16

3.4 Scanning Electron Microscopy (SEM) Analysis of Antifungal Interaction:

Scanning Electron Microscopy (SEM) analysis demonstrated that *Bacillus amyloliquefaciens* RRR16 exhibited a pronounced antibiosis effect, leading to complete inhibition of sclerotial production by *Sclerotium rolfsii* both in proximity to and at a distance from the zone of inhibition. Notable morphological abnormalities were observed in the hyphae of *S. rolfsii* as a result of exposure to *B. amyloliquefaciens* RRR16. Hyphae located near the inhibition zone displayed a loss of structural integrity as well as subsequent destruction and colonization of pathogen hyphae by *B. amyloliquefaciens* RRR16 was detected (Photo Plate 3.0 (a)). In contrast, control plates maintained intact and structurally normal *S. rolfsii* hyphae (Photo Plate 3.0 (b)).

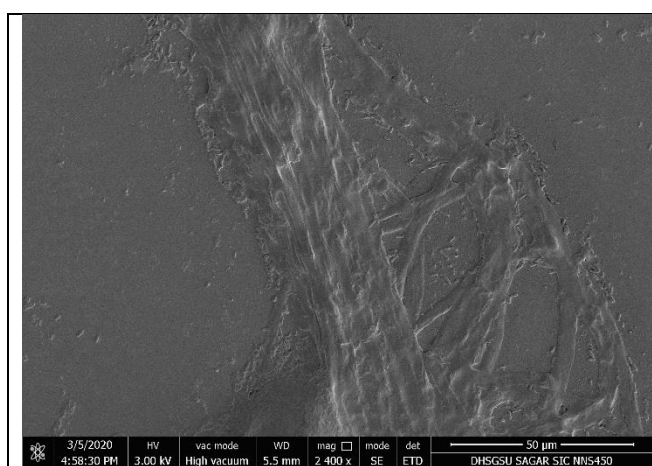


Photo Plate 3.0 (a): inhibition zone displayed a loss of structural integrity and subsequent destruction of *S. rolfsii*

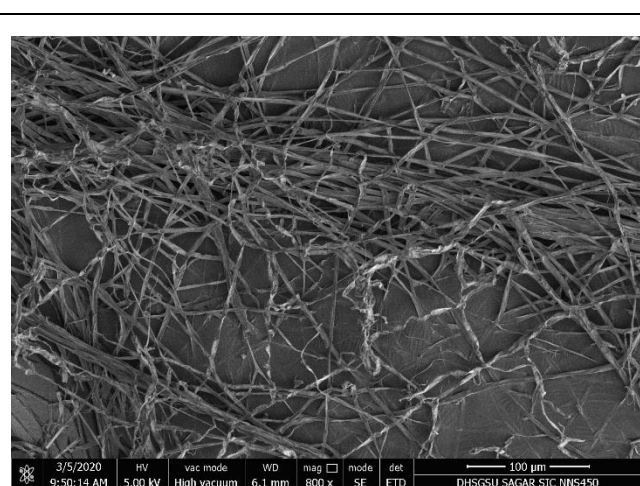


Photo Plate 3.0 (b): control plates maintained intact and structurally normal *S. rolfsii* hyphae

Photo Plate 3.0: Scanning Electron Microscopy of Inhibition Zone between *S. rolfsii* and *Bacillus amyloliquefaciens* RRR16

3.5 Assay of Chitinase Enzyme

The chitinolytic activity of the rhizobacterial isolate *Bacillus amyloliquefaciens* RRR16 was quantitatively evaluated through a chitin agar plate assay. The isolate exhibited a distinct zone of clearance measuring 14 mm in diameter surrounding

the bacterial colony (Photo Plate 4.0), indicative of extracellular chitinase secretion. The formation of this hydrolytic zone confirms the organism's potential to produce chitin-degrading enzymes responsible for the breakdown of chitin (Table 3.0).

Table 3.0: Chitinase activity shown by *Bacillus amyloliquefaciens* RRR16

Rhizospheric isolates	Total zone of hydrolysis including colony (mm)	Diameter of colony (mm)	Diameter of Zone of chitin hydrolysis (mm)
<i>Bacillus amyloliquefaciens</i> RRR16	28±1.0	12±1.0	14±1.0

Each value is mean of three replicates with their standard deviation.

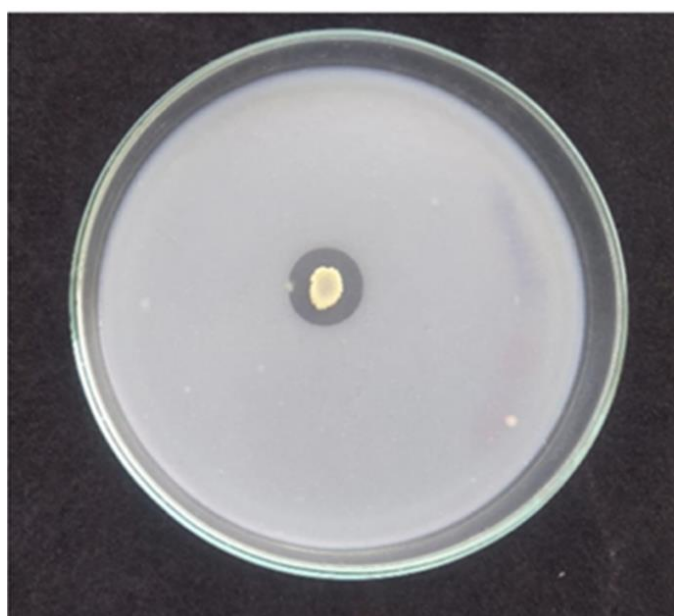


Photo Plate 4.0: Chitinase activity shown by *Bacillus amyloliquefaciens* RRR16 on chitin agar plate

IV. DISCUSSION:

The present investigation highlights the potential of groundnut rhizosphere-associated *Bacillus spp.* as effective biocontrol agents against *Sclerotium rolfsii*, a destructive soil-borne pathogen responsible for stem rot disease. The successful isolation of a large population of *Bacillus spp.* (102 isolates) from the groundnut rhizosphere confirms that this niche serves as a rich reservoir of spore-forming, antagonistic bacteria. Heat treatment followed by nutrient agar

plating selectively enriched endospore-forming bacteria, which is a well-established approach for isolating *Bacillus spp.* from soil environments. The observed colony characteristics—irregular, opaque, dry to mucoid colonies with Gram-positive, rod-shaped, endospore-forming cells—agree with earlier reports describing rhizospheric *Bacillus* populations (Sarvani & Reddy, 2013; Akash *et al.*, 2022). The diversity among isolates (RRR1–RRR102) suggests strong adaptation of *Bacillus spp.* to the groundnut rhizosphere, likely driven by root exudates and selective microbial interactions.

Primary screening revealed that although the majority of isolates exhibited low to moderate antagonistic activity, a small proportion demonstrated substantial inhibition of *S. rolfsii*. This observation is consistent with the general understanding that only a limited number of rhizobacterial *Bacillus* isolates possess strong antifungal potential, often due to specific genetic and metabolic traits such as antibiotic production, lytic enzymes, or superior competitive ability (Kumar *et al.*, 2011; Mardanov *et al.*, 2016). Primary dual-culture screening identified promising antagonists like RRR16, RRR15, RRR29, RRR30, RRR31, and RRR53, with inhibition scores up to 4 (76–100%), while most isolates showed modest activity (1–2), indicating natural variability in

biocontrol potential against *Sclerotium rolfsii* (Kumar *et al.*, 2012; Chang *et al.*, 2025).

During secondary co-culture screening, *Bacillus* spp. RRR16 exhibited the highest *in vitro* inhibition of *Sclerotium rolfsii*, achieving an inhibition rate of 92.86%. The identification of isolate RRR16 as *Bacillus amyloliquefaciens* based on morphological traits and 16S rDNA sequence analysis, showed the high sequence homology (99.91%) with *B. amyloliquefaciens* strain MPA 1034 and its clear clustering in the phylogenetic tree confirm its taxonomic placement. The strain was deposited at GenBank of National Center for Biotechnology Information (NCBI), U.S. with the accession number [MN749517](#).

Akash *et al.* (2022) utilized twelve groundnut rhizosphere soil samples to isolate native rhizospheric microflora, resulting in the recovery of 111 bacterial and 79 fungal isolates. These isolates were subsequently screened for antagonistic activity against *Sclerotium rolfsii*. During primary screening, 33 out of the 111 bacterial isolates exhibited antagonistic behavior toward *S. rolfsii* and were further evaluated using the dual culture technique. All selected isolates significantly suppressed the radial growth of the pathogen, with isolates S3KB6, S9KB4, and S1NA7 showing the highest levels of inhibition at 62.82%, 61.70%, and 61.11%, respectively, compared to the control. These findings corroborate the results of Safni and Antastia (2018), who reported substantial antagonistic activity of rhizobacterial species against *S. rolfsii*, with inhibition levels reaching up to 60%. Similar inhibitory effects of soil-derived *Bacillus* spp. against *S. rolfsii* in groundnut were reported by Swaroopa and Madhuri (2021). Furthermore, Ramanjineyulu *et al.*, (2021) also documented significant *in vitro* suppression of *S. rolfsii* radial growth by rhizosphere-associated bacterial isolates, thereby supporting these observations.

However, in comparison with these earlier reports, the results obtained in the present study demonstrate a comparatively higher and more pronounced

antagonistic potential of *Bacillus amyloliquefaciens* RRR16 (92.86 %) against *S. rolfsii*.

SEM analysis provided direct visual evidence of the antagonistic interaction between *B. amyloliquefaciens* RRR16 and *S. rolfsii*. The severe deformation, collapse, and destruction of fungal hyphae near the inhibition zone indicate a strong lytic and inhibitory effect exerted by the bacterial isolate. The absence of sclerotial formation in treated plates is particularly significant, as sclerotia serve as the primary survival and infection structures of *S. rolfsii*. The observed colonization of fungal hyphae by RRR16 further suggests mycoparasitic or contact-mediated antagonism, which, together with antibiosis, enhances the overall biocontrol potential. In contrast, the intact and normal hyphal structures in the control clearly demonstrate that the morphological damage was specifically induced by the bacterial antagonist. Santhosh *et al.*, (2024) similarly reported that the isolate *Bacillus amyloliquefaciens* SS_CR10, obtained as an endophytic bacterial strain from chilli (*Capsicum annum* L.), exhibited notable effects on *A. rolfsii* mycelia. Specifically, *Bacillus amyloliquefaciens* SS_CR10 induced marked deformation and cell death in the mycelia, as demonstrated by live/dead staining assessed via fluorescence microscopy and confirmed through scanning electron microscopy (SEM).

Chitinase activity exhibited by *B. amyloliquefaciens* RRR16 provides a plausible mechanistic explanation for the observed antifungal effects. Chitin is a major structural component of fungal cell walls, and the production of chitinase enables rhizobacteria to degrade fungal hyphae, leading to growth inhibition and cell lysis. The substantial zone of chitin hydrolysis (14 mm) recorded in this study indicates strong chitinolytic activity, which likely contributed to hyphal deformation and suppression of sclerotial formation observed under SEM. Similar associations between chitinase production and effective suppression of *S. rolfsii* have been documented for *Bacillus* spp., reinforcing the role of lytic enzymes as key biocontrol determinants. The enzymatic

breakdown of cell wall, resulting in the loss of fungal protoplasm, constitutes a primary antagonistic mechanism underlying the efficacy of biocontrol agents. These findings align with those reported by Castillo *et al.*, (2013), which recognized *Bacillus species* as established biological control agents capable of suppressing the soil-borne phytopathogen *S. rolfsii* through direct antagonism or secretion of various cell wall-degrading enzymes. Furthermore, the results corroborate the observations of Prapagdee *et al.*, (2008), who identified chitinase enzymes as responsible for the degradation of *S. rolfsii* cell walls. Overall, the present study demonstrates that the groundnut rhizosphere harbors highly effective antagonistic *Bacillus spp.*, with *Bacillus amyloliquefaciens* RRR16 emerging as a particularly potent biocontrol agent against *S. rolfsii*.

V. CONCLUSION:

The present study demonstrates that the groundnut rhizosphere is a rich source of antagonistic *Bacillus spp.* with significant potential for the biological control of *Sclerotium rolfsii*, the causal agent of stem rot in groundnut. A total of 102 *Bacillus* isolates were successfully isolated using selective heat treatment and morphological characterization, indicating the predominance and adaptability of spore-forming bacteria in the rhizosphere. Systematic *in vitro* screening identified a limited number of highly effective antagonists, among which *Bacillus amyloliquefaciens* RRR16 exhibited the strongest antifungal activity, achieving more than 90% inhibition of pathogen mycelial growth.

Molecular identification based on 16S rDNA sequencing confirmed the isolate as *B. amyloliquefaciens* RRR16, a species well known for its biocontrol and plant growth-promoting attributes. Scanning electron microscopy provided direct evidence of severe hyphal deformation, loss of structural integrity, and complete suppression of sclerotial formation in *S. rolfsii* when exposed to

RRR16, indicating a strong antibiosis and hyphal-destructive mechanism. The pronounced chitinase activity exhibited by this isolate further supports the involvement of lytic enzymes in pathogen suppression.

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