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# Bioprospecting Indigenous *Bacillus* spp. for Antagonistic Potential against *Sclerotium rolfsii* Causing Groundnut Stem Rot

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***Authors' contributions***

*This work was carried out in collaboration between both authors. Author TD conceptualized the study, conducted the experiments, collected and analysed the data, performed the investigations and prepared the original manuscript. Author PB supervised the research, provided guidance on the experimental design & methodology and critically reviewed and edited the manuscript. Both authors read and approved the final manuscript.*

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## Abstract

Groundnut (*Arachis hypogaea* L.) stem rot, caused by *Sclerotium rolfsii*, is an economically important soil-borne disease that causes considerable yield losses. The present study aimed to isolate, characterise and evaluate the antagonistic potential of soil-derived *Bacillus* spp. against *S. rolfsii*. Fifteen bacterial isolates were obtained from groundnut rhizosphere and forest soils collected from different regions of Tamil Nadu, India. The isolates were characterised on the basis of cultural, morphological and biochemical characteristics. All isolates exhibited Gram-positive, rod-shaped morphology and positive reactions for catalase, oxidase, starch hydrolysis and citrate utilisation. Their antagonistic potential was evaluated using the dual culture technique. Among the fifteen isolates, BaLDP exhibited the highest inhibition of mycelial growth (66.64%), with the minimum pathogen growth of 30.02 mm, followed by BaMPY (63.84%) and BaPDR (60.43%). Based on their superior antagonistic activity, five promising isolates were

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selected for molecular identification through 16S rRNA gene sequencing. BLASTn analysis revealed that BaLDP, BaPRG and BaPDR showed the highest similarity to *Bacillus subtilis*, while BaVPD and BaMPY were closely related to *B. velezensis* and *B. mycoides*, respectively. Phylogenetic analysis further supported their molecular identification. The obtained sequences were deposited in the NCBI GenBank database. Scanning electron microscopy of the most effective isolate, BaLDP, revealed elongated rod-shaped cells with rounded ends and a relatively smooth surface architecture. Overall, the findings highlight the antagonistic potential of indigenous *Bacillus* isolates, particularly BaLDP, as promising candidates for the biological management of groundnut stem rot.

*Keywords: Bacillus spp.; groundnut; rhizosphere soil; biochemical characterisation; biocontrol.*

## 1. Introduction

In India, groundnut (*Arachis hypogaea* L.) is cultivated extensively across diverse agroclimatic regions, except in high-altitude areas. The major groundnut-producing states include Gujarat, Andhra Pradesh, Tamil Nadu, Karnataka, Maharashtra, Rajasthan, Madhya Pradesh, Odisha and Uttar Pradesh. India ranks second globally in terms of both the area under groundnut cultivation and total production, accounting for approximately 5 million hectares and 8 million tonnes, respectively (Jegadeeswari *et al.*, 2025). It is an economically significant oilseed crop that is highly susceptible to various soil-borne pathogens, which adversely affect crop productivity. Among these, stem rot is one of the major diseases, posing a serious threat to groundnut production and causing substantial yield losses. Bacterial endophytes isolated from groundnut nodules have demonstrated *In vitro* inhibition of *Sclerotium rolfsii*, supporting the screening of indigenous bacteria for stem rot management (Deva *et al.*, 2024). The management of soil-borne diseases requires sustainable approaches that exploit the potential of beneficial soil microorganisms. Soil is one of the most biologically diverse ecosystems, containing a vast array of microorganisms that play essential roles in nutrient cycling, organic matter decomposition and maintenance of soil fertility. Soil microbial communities strongly influence plant growth and productivity through their interactions with plant roots and the surrounding rhizosphere environment. Beneficial soil microorganisms, particularly plant growth-promoting rhizobacteria (PGPR), enhance plant health by improving nutrient uptake, stimulating plant growth and suppressing plant pathogens (Aloo *et al.*, 2022; Jiao *et al.*, 2021). Among these, bacteria belonging to the genus *Bacillus* are widely distributed and have gained considerable attention for their agricultural importance. *Bacillus* species are Gram-positive, endospore-forming bacteria capable of surviving diverse environmental conditions due to their ability to produce resistant spores. Several species function as PGPR and enhance plant productivity through mechanisms such as phytohormone production, nutrient solubilisation and synthesis of antimicrobial compounds (Dobrzyński *et al.*, 2022; Lee *et al.*, 2024). In addition to plant growth promotion, many *Bacillus* species exhibit strong antagonistic activity against plant pathogens through the production of lipopeptides, antibiotics and hydrolytic enzymes. These metabolites suppress pathogens and enhance plant resistance to diseases, making *Bacillus* an important component of biological control strategies in sustainable agriculture (Patel *et al.*, 2023). Recent work on groundnut has shown that volatile metabolites of *Bacillus subtilis* can inhibit mycelial growth and sclerotial germination of *S. rolfsii*, while formulations containing *Bacillus licheniformis* have shown potential for reducing stem rot under greenhouse conditions (Cheng *et al.*, 2025; Jeevanantham *et al.*, 2026). Isolation and characterisation of beneficial microorganisms from diverse soil ecosystems is therefore an important step in identifying novel microbial strains with potential agricultural applications. Soil environments such as forest soils and agricultural fields often harbour diverse microbial communities that differ in their ecological functions and metabolic capabilities. Exploring microorganisms from different soil ecosystems increases the likelihood of identifying novel isolates with beneficial traits such as plant growth promotion and pathogen suppression (Devi *et al.*, 2023). Variation in *In vitro* antagonism against *S. rolfsii* has also been reported among *Bacillus* isolates from mangrove rhizosphere soils in a study of tomato collar rot, illustrating the value of screening isolates from distinct environments (Anbu & Sanjeevkumar, 2026). Morphological characterisation remains a fundamental preliminary step in microbial identification. Observations of colony morphology, pigmentation, growth patterns and microscopic characteristics provide useful information for distinguishing bacterial genera prior to advanced analyses. In addition, biochemical characterisation plays a crucial role in the identification and differentiation of bacterial isolates. Biochemical assays such as catalase and oxidase tests, starch hydrolysis, nitrate reduction and other enzymatic activities are commonly used to evaluate metabolic and physiological characteristics. These tests aid in confirming bacterial identity and understanding functional capabilities and are widely applied in microbial taxonomy (Franco-Duarte *et al.*, 2019). However, conventional phenotypic methods are often insufficient for differentiating closely related species, particularly within the genus *Bacillus*, owing to their considerable morphological and physiological similarity. Therefore, molecular characterisation has emerged as a reliable and precise approach for bacterial identification and taxonomic resolution. Among molecular techniques, 16S rRNA gene sequencing is widely regarded as a standard method, as it contains conserved and variable regions that enable accurate phylogenetic analysis. Amplification of the ~1500 bp 16S rRNA gene followed by sequencing and comparison with databases such as NCBI using BLAST allows reliable identification at the genus and species level (Golnari *et al.*, 2024; Sagar *et al.*, 2024). Nevertheless, 16S rRNA gene sequences alone may not adequately distinguish closely related species within the *Bacillus subtilis* and *Bacillus cereus* groups, and species assignments based on this marker should therefore be interpreted with caution (Bisaschi *et al.*, 2025). Furthermore, phylogenetic analysis based on sequence homology provides insights into evolutionary relationships among isolates (Noor *et al.*, 2024). Thus, integrating molecular techniques with morphological and biochemical characterisation enhances the accuracy of bacterial identification and provides a comprehensive understanding of microbial diversity and function. Therefore, the isolation, characterisation and evaluation of beneficial bacteria for their antagonistic potential represent essential steps in exploring microbial resources for sustainable agriculture. Despite the documented antagonistic potential of beneficial bacteria, the relative inhibitory activity and phenotypic diversity of indigenous *Bacillus* isolates from groundnut rhizosphere and forest soils in Tamil Nadu warrant further investigation. Integrating preliminary morphological and biochemical characterisation with molecular identification of selected antagonists can help prioritise isolates for subsequent evaluation as potential biocontrol agents against groundnut stem rot.

With this background, the present study was undertaken to isolate and characterise *Bacillus* spp. from forest and agricultural soils using an integrated approach, followed by the evaluation of their antagonistic activity against *Sclerotium rolfsii*, the causal organism of groundnut stem rot.

## 2. Materials and Methods

### 2.1 Collection of Soil Samples

Soil samples were collected from agricultural rhizosphere soils of groundnut (*Arachis hypogaea* L.) and forest soils from different regions of Tamil Nadu, India. Rhizosphere soil samples were collected from groundnut fields located in Coimbatore, Erode, Perambalur, Villupuram, Kallakurichi, Cuddalore and Tiruvannamalai districts. Healthy plants were carefully uprooted and the soil adhering to the root surface was considered rhizosphere soil and was collected using sterile spatulas following standard rhizosphere sampling procedures (Abdullahi *et al.*, 2022). Forest soil samples were collected from relatively undisturbed forest regions located in Nilgiris, Coimbatore, Tiruvannamalai, Tirupathur, Theni and Madurai districts. Prior to sampling, surface litter was removed, and soil samples were collected from a depth of 10–15 cm using a sterile soil auger (Chowhan *et al.*, 2023). At each location, multiple subsamples were collected randomly and combined to form a composite sample. Approximately 50 g of soil was collected in sterile polyethylene bags, transported to the laboratory and stored at 4 °C until further processing. Soil samples were air-dried, sieved through a 2 mm sieve and used for microbial isolation.

### 2.2 Isolation and Purification of Bacteria

One gram of the soil sample was transferred into 9 ml of sterile distilled water and shaken thoroughly to obtain a homogeneous suspension ($10^{-1}$ dilution). Serial dilutions were prepared up to $10^{-6}$ by transferring 1 ml of suspension into test tubes containing 9 ml of sterile distilled water. From appropriate dilutions ($10^{-4}$-$10^{-6}$), 0.1 ml of the suspension was spread onto sterile Nutrient Agar (NA) plates for the isolation of bacteria. The plates were incubated at 28 ± 2°C for 24–48 h. After incubation, distinct colonies were observed and purified for further processing (Ibrahim *et al.*, 2021).

The selected colonies were purified using the streak plate method on NA plates. A sterile inoculation loop was used to streak individual colonies using the quadrant streak technique. The plates were then incubated at 28 ± 2 °C and colonies with uniform morphology were obtained. The purified isolates were maintained on agar slants and stored at 4°C for further characterisation and screening studies (Anzum *et al.*, 2022).

### 2.3 Morphological Characterisation

Purified bacterial isolates were subjected to morphological characterisation to study their colony morphology. The isolates were streaked on Nutrient Agar (NA) for the growth of *Bacillus* spp. and incubated at 28 ± 2 °C. After incubation, colonies were examined visually and cultural characteristics were recorded. Observations were recorded after 24-48 h of incubation for *Bacillus* spp. Colony features, including colony colour, colony surface, colony type, elevation, pigmentation, shape and sporulation, were used for preliminary identification of *Bacillus* isolates (Devi *et al.*, 2023).

### 2.4 Biochemical Characterisation

To determine the metabolic and enzymatic characteristics of the isolated *Bacillus* strains, a series of standard biochemical tests were carried out following the procedures described in Bergey's Manual of Determinative Bacteriology (Holt *et al.*, 1994) and other standard microbiological protocols.

#### 2.4.1 Gram Staining

Gram staining was performed to determine the Gram reaction and cellular morphology of the isolates. A thin smear of bacterial culture was prepared on a clean glass slide, heat-fixed, and stained sequentially with crystal violet, iodine, ethyl alcohol and safranin. The stained slides were examined under a compound microscope using oil immersion (100x) to determine the Gram reaction and morphology of the bacterial cells (Aehsas *et al.*, 2025).

#### 2.4.2 Catalase Test

A loopful of bacterial culture was placed on a clean glass slide and a drop of 3% hydrogen peroxide ($H_2O_2$) was added. The appearance of effervescence (bubbling) indicated a positive reaction, confirming the production of the enzyme catalase, which decomposes hydrogen peroxide into water and oxygen (Mwamburi *et al.*, 2024).

#### 2.4.3 Oxidase Test

The oxidase test was performed using an oxidase reagent (tetramethyl-p-phenylenediamine). A small portion of a bacterial colony was smeared on filter paper impregnated with the reagent. The development of a deep purple colour within 30 seconds indicated the presence of the enzyme cytochrome c oxidase (Mwamburi *et al.*, 2024).

### 2.4.4 Starch Hydrolysis Test

Isolates were streaked onto starch agar plates and incubated at 30 ± 2 °C for 48 hours. After incubation, the plates were flooded with iodine solution. The appearance of a clear halo around the colony indicated a positive reaction for starch hydrolysis due to amylase activity (Uddin *et al.*, 2025).

### 2.4.5 Citrate Utilisation Test

Simmons' citrate agar slants were inoculated with the test strains and incubated at 30 °C for 48–72 hours. A colour change of the medium from green to blue indicated the utilisation of citrate as the sole carbon source (Simmons, 1926).

### 2.4.6 Nitrate Reduction Test

The nitrate reduction test was carried out using nitrate broth inoculated with bacterial isolates and incubated for 48 hours. After incubation, sulfanilic acid and α-naphthylamine reagents were added. The development of a red colour indicated the reduction of nitrate to nitrite (Uddin *et al.*, 2025).

### 2.4.7 Indole Test

Isolates were grown in tryptone broth at 30 °C for 48 hours. After incubation, Kovac's reagent was added. The formation of a cherry-red layer on the surface indicated a positive indole reaction (Jurry *et al.*, 2026).

### 2.4.8 Methyl Red (MR) Test

The isolates were inoculated into MR-VP broth and incubated for 48 hours. After incubation, five drops of methyl red indicator were added. The development of a red colour indicated a positive methyl red reaction (Aehsas *et al.*, 2025).

### 2.5 Isolation of *Sclerotium rolfsii*

The pathogen was isolated from diseased groundnut plants exhibiting typical stem rot symptoms using a tissue segment method (Meena *et al.*, 2024). Infected stem portions were cut into small segments, surface sterilised with 0.1% sodium hypochlorite for 30 seconds and rinsed thoroughly with sterile distilled water. The sterilised segments were aseptically transferred onto streptomycin-amended potato dextrose agar (PDA) plates and incubated at 28 ± 2°C for five days. The emerging white mycelial growth was purified by the single-hyphal-tip method (Windels *et al.*, 1988).

### 2.6 Dual Culture Assay

The antagonistic potential of *Bacillus* isolates against *Sclerotium rolfsii* was assessed *In vitro* using the dual culture technique as described by Dennis and Webster (1971). Seven-day-old cultures of the pathogen and individual *Bacillus* isolates were placed on opposite sides of PDA plates. Plates inoculated exclusively with the pathogen served as the control. Each treatment was maintained in triplicate and the plates were incubated at 28 ± 2°C. The radial growth of *S. rolfsii* was measured in millimetres and the percentage inhibition of mycelial growth was calculated using the formula proposed by Pandey and Upadhyay (2000).

$$\text{Per cent inhibition (I)} = [(C - T)/C] \times 100$$

Where,

I = Per cent inhibition of mycelial growth of the test pathogen
C = Radial growth of the pathogen in control (mm)
T = Radial growth of the pathogen in dual culture with *Bacillus* (mm)

### 2.7 Molecular Characterisation

Based on the results of the dual culture assay, five promising *Bacillus* isolates (BaLDP, BaPRG, BaVPD, BaMPY, and BaPDR) exhibiting superior antagonistic activity against *Sclerotium rolfsii* were selected for molecular characterisation through 16S rRNA gene sequencing. Pure cultures of *Bacillus* spp. were maintained on nutrient agar slants at 4°C for molecular analysis. Genomic DNA was extracted from 24 h cultures using the CTAB method and its quality was assessed by agarose gel electrophoresis and spectrophotometry (Bakri, 2023). The 16S rRNA gene was amplified using universal primers 27F and 1492R in a 25 µL PCR mixture. PCR conditions included initial denaturation at 94 °C, followed by 30–35 cycles of denaturation, annealing (52–55°C) and extension, with a final extension at 72°C (Koilybayeva *et al.*, 2023). Amplified products were confirmed on a 1–1.5% agarose gel. PCR products were purified and subjected to Sanger sequencing. The obtained sequences were edited and aligned using BioEdit/Chromas. Sequence similarity was determined using NCBI BLAST for identification. Phylogenetic analysis was performed using ClustalW/MUSCLE and the Neighbour-Joining method with bootstrap analysis (Kumar *et al.*, 2024). Identification was confirmed based on ≥97–100% similarity with reference sequences.

### 2.8 Scanning Electron Microscopy

The selected *Bacillus isolate* (BaLDP), which exhibited the highest inhibitory activity against *Sclerotium rolfsii* in the dual culture assay, was subjected to scanning electron microscopy (SEM) to investigate its cellular morphology and surface architecture. Freshly

harvested bacterial cells were fixed with 2.5% glutaraldehyde, followed by dehydration through a graded ethanol series. The dehydrated specimens were mounted onto SEM stubs and coated with a thin conductive layer prior to microscopic examination. The samples were examined using a JEOL JSM-IT200 scanning electron microscope operated at an accelerating voltage of 15.0 kV, with a working distance of 28.1 mm and a magnification of 9,500x. The morphological characteristics, including cell shape, surface texture and cellular arrangement, were documented through representative micrographs.

### 2.9 Statistical Analysis

All experimental trials were performed in triplicate using a completely randomised design (CRD). The results were presented as mean values, accompanied by the standard error of the difference (SED) and critical difference (CD) at the 5% probability level. Statistical analysis was carried out using one-way analysis of variance (ANOVA) to assess the significance of differences among the treatments, following the procedure described by Gomez and Gomez (1984).

## 3. Results and Discussion

### 3.1 Morphological Characterisation of *Bacillus* Isolates

All fifteen bacterial isolates obtained from rhizosphere and forest soils exhibited considerable variation in their colony morphological characteristics (Table 1 & Fig. 1). Among the rhizosphere isolates, BaVLG and BaPRG produced creamy white colonies with an oval shape, whereas BaPRD, BaVPD and BaKRP exhibited white colonies with irregular or round forms. BaLDP and BaSRP showed dull white to yellowish white colonies with round morphology, while BaKPN and BaMPY recorded dull white to creamy white colonies with oval to irregular shapes. Most of these isolates possessed smooth colony surfaces, with either a shiny or slimy texture, and margins varied from serrated to non-serrated, while elevations ranged from flat to raised and convex forms.



**Fig. 1. *Bacillus* isolates obtained from the collected soil samples**

Among the forest soil isolates, BaOOT, BaSMD and BaPDR showed yellowish white to white colonies with oval to irregular shapes, whereas BaNGL, BaKVL and BaVML exhibited dull white to creamy white colonies with predominantly oval or round morphology. The colony surface was generally smooth with slight variations, and elevation varied from flat to raised and convex. Pigmentation among all isolates ranged from yellow and light yellow to orange and pink, with orange pigmentation being more prominent in BaLDP, BaSMD and BaPDR, while pink pigmentation was observed in BaPRD, BaSRP, BaKPN, BaKVL and BaVML.

The observed diversity in colony morphology among the isolates indicates the phenotypic variability commonly associated with soil-derived *Bacillus* species. Members of this genus typically produce white to cream-coloured colonies with smooth or irregular surfaces, although variations in pigmentation, shape and elevation may occur depending on species and environmental conditions (Aloo *et al.*, 2022). Similar variability among *Bacillus* isolates from soil has been reported, where phenotypic differences were influenced by nutrient availability and ecological conditions (Zhang *et al.*, 2023).

The rod-shaped cells and the presence of endospores in all isolates further support their identification as *Bacillus*. Endospore formation enables survival under adverse conditions such as desiccation, nutrient limitation and temperature fluctuations (El-Saadony *et al.*, 2022). The diversity among isolates may also be attributed to rhizosphere interactions, where root exudates support diverse microbial populations, including beneficial *Bacillus* species (Singh *et al.*, 2023).

### 3.2 Biochemical Characterisation of Bacterial Isolates

The bacterial isolates were subjected to biochemical characterisation to determine their metabolic and enzymatic properties. A total of fifteen *Bacillus* isolates were evaluated using standard biochemical tests, including Gram staining, catalase test, oxidase test, starch hydrolysis test, citrate utilisation test, nitrate reduction test, indole test and methyl red test. The results of these biochemical tests are presented in Fig. 2.

**Table 1. Morphological characterization of *Bacillus* spp.**

| S.No. | Soil | Locality | Isolate | Colony colour | Colony type | Colony surface | Elevation | Pigmentation | Shape | Sporulation |
|---|---|---|---|---|---|---|---|---|---|---|
| 1. | Rhizosphere | Velliangadu | BaVLG | Creamy white | Oval | Smooth, non-serrated | Raised | Yellow | Rod | Present |
| 2. | | Perundurai | BaPRD | White | Irregular | Smooth and shiny, non-serrated | Flat | Pink | Rod | Present |
| 3. | | Ladapuram | BaLDP | Dull white | Round | Smooth and slimy, serrated | Raised | Orange | Rod | Present |
| 4. | | Surapattu | BaSRP | Yellowish white | Round | Smooth, serrated | Convex | Pink | Rod | Present |
| 5. | | Parigam | BaPRG | Creamy white | Oval | Smooth, serrated | Flat | Yellow | Rod | Present |
| 6. | | Vallampadugai | BaVPD | White | Round | Smooth and slimy, serrated | Convex | Light yellow | Rod | Present |
| 7. | | Keelpennathur | BaKPN | Dull white | Oval | Smooth and shiny, non-serrated | Flat | Pink | Rod | Present |
| 8. | | Kurinjipadi | BaKRP | White | Irregular | Smooth and slimy, serrated | Raised | Light yellow | Rod | Present |
| 9. | | Melapuliyur | BaMPY | Creamy white | Irregular | Smooth, non-serrated | Flat | Yellow | Rod | Present |
| 10. | Forest | Ooty | BaOOT | Yellowish white | Oval | Smooth and slimy, slightly serrated | Flat | Light yellow | Rod | Present |
| 11. | | Semmedu | BaSMD | Yellowish white | Irregular | Smooth, serrated | Raised | Orange | Rod | Present |
| 12. | | Nagalur | BaNGL | Dull white | Oval | Smooth, serrated | Flat | Yellow | Rod | Present |
| 13. | | Kavalur | BaKVL | Creamy white | Oval | Smooth, non-serrated | Convex | Pink | Rod | Present |
| 14. | | Pudur | BaPDR | White | Irregular | Smooth, serrated | Raised | Orange | Rod | Present |
| 15. | | Vellimalai | BaVML | White | Round | Smooth, serrated | Flat | Pink | Rod | Present |

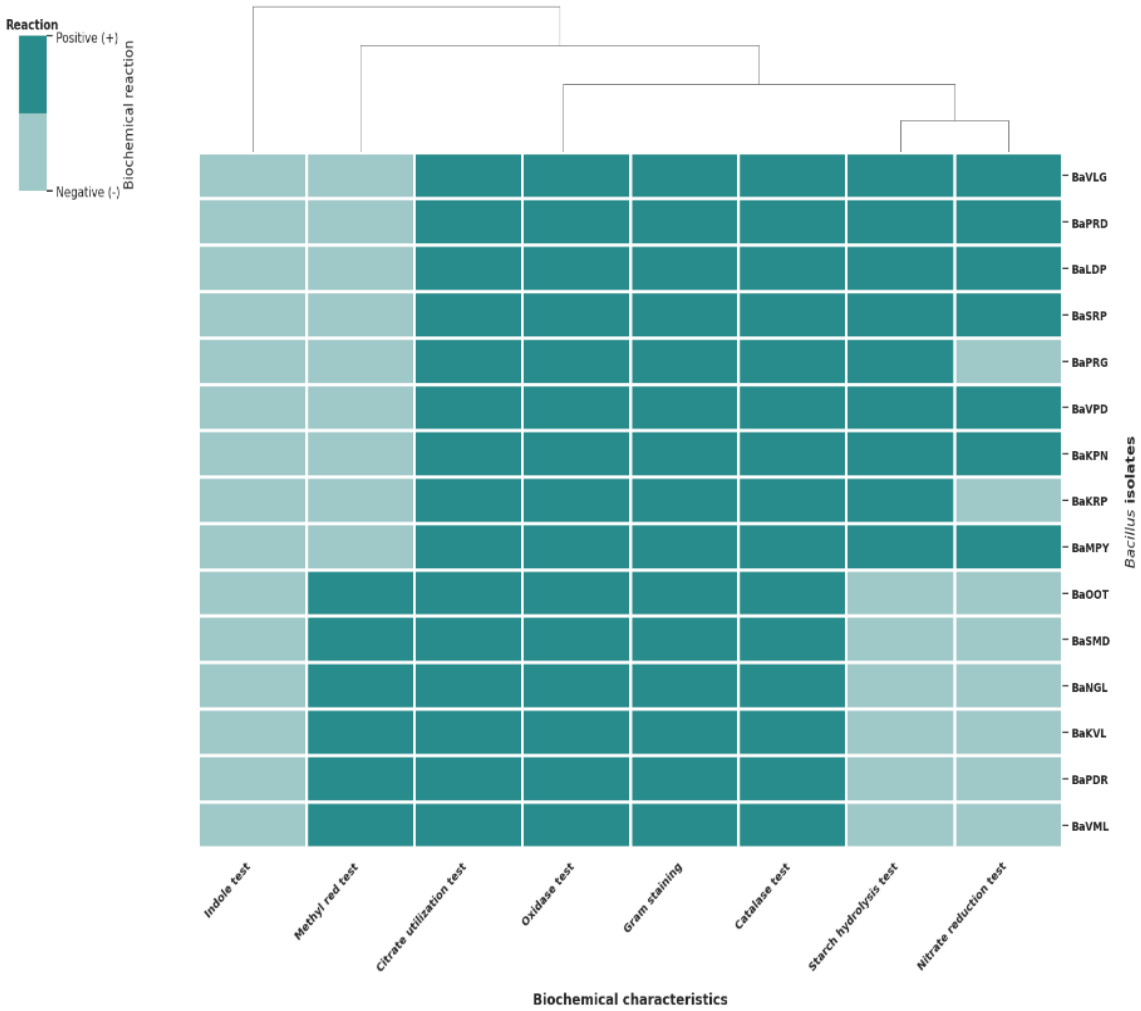


**Fig. 2. Hierarchical cluster heatmap of biochemical characteristics of *Bacillus* isolates**

Biochemical characterisation of the fifteen *Bacillus* isolates revealed variations in their metabolic and enzymatic properties. All isolates exhibited Gram-positive reactions and positive results for catalase, oxidase and citrate utilisation tests. Starch hydrolysis was positive in nine isolates (60.00%), while the remaining six isolates (40.00%) showed negative reactions. Similarly, nitrate reduction was observed in nine isolates (60.00%), whereas six isolates (40.00%) were negative. All fifteen isolates showed negative reactions for indole production. In contrast, the methyl red test was positive in six isolates (40.00%) and negative in the remaining nine isolates (60.00%). These variations in biochemical characteristics demonstrated phenotypic diversity among the soil-derived *Bacillus* isolates.

Biochemical characterisation is widely used for the preliminary identification and differentiation of bacterial isolates based on their metabolic and enzymatic activities. The Gram-positive reaction observed in the present study indicates the presence of a thick peptidoglycan layer in the bacterial cell wall, a characteristic feature of many soil bacteria, including *Bacillus*. *Bacillus* species are typically Gram-positive, rod-shaped bacteria, as reported in earlier studies (Barka *et al.*, 2016; Golnari *et al.*, 2024).

The catalase activity observed among the isolates indicates the production of the enzyme catalase, which decomposes hydrogen peroxide into water and oxygen, protecting cells from oxidative damage. Such catalase-positive reactions are common among aerobic soil microorganisms (Barka *et al.*, 2016; Golnari *et al.*, 2024; Sagar *et al.*, 2024). Similarly, oxidase-positive reactions confirm the presence of cytochrome c oxidase, which is involved in aerobic respiration (Afridi *et al.*, 2024; Amin *et al.*, 2025).

The ability of the isolates to hydrolyse starch suggests the production of extracellular amylases (Hasanin & Abdella, 2025; Wang *et al.*, 2025). Citrate utilisation indicates the presence of citrate permease for metabolic activity under nutrient-limited conditions (Lata & Gond, 2025; Pandey *et al.*, 2024). Most isolates showed nitrate reduction, reflecting their role in nitrogen metabolism (Acevedo-Barrios *et al.*, 2024; Golnari *et al.*, 2024). All isolates were negative for indole and methyl red tests, indicating the absence of tryptophanase activity and differences in fermentation pathways (Hafezi & Khamar, 2024; Lata & Gond, 2025). Overall, the observed traits are consistent with typical *Bacillus* characteristics.

### 3.3 Dual Culture Assay

The antagonistic activity of fifteen *Bacillus* isolates against *Sclerotium rolfsii* was evaluated using the dual culture technique, revealing considerable variation in their inhibitory potential (Table 2). Among the isolates tested, BaLDP exhibited the highest antagonistic activity, recording the minimum mycelial growth of 30.02 mm and the maximum inhibition of 66.64% over the control. This was followed by BaMPY and BaPDR, which recorded 63.84% and 60.43% inhibition, respectively. BaPRG and BaVPD also showed considerable antagonistic activity, with 55.32% and 52.93% inhibition, respectively. Moderate inhibition was observed in BaKPN (47.37%), BaKRP (46.33%), BaOOT (44.87%) and BaPRD (44.18%). In contrast, BaSRP exhibited the lowest antagonistic activity, with 75.31 mm mycelial growth and 16.32% inhibition. The untreated control recorded the maximum mycelial growth of 90.00 mm.

**Table 2. Dual culture assay of *Bacilllus* isolates against *Sclerotium rolfsii***

| S. No. | Isolates | Mycelial growth (mm) | Percent inhibition over control |
|---|---|---|---|
| 1. | BaVLG | 72.23 (58.19) | 19.74 |
| 2. | BaPRD | 50.23 (45.13) | 44.18 |
| 3. | BaLDP | 30.02 (33.22) | 66.64 |
| 4. | BaSRP | 75.31 (60.20) | 16.32 |
| 5. | BaPRG | 40.21 (39.35) | 55.32 |
| 6. | BaVPD | 42.36 (40.60) | 52.93 |
| 7. | BaKPN | 47.36 (43.49) | 47.37 |
| 8. | BaKRP | 48.30 (44.02) | 46.33 |
| 9. | BaMPY | 32.54 (34.79) | 63.84 |
| 10. | BaOOT | 49.62 (44.79) | 44.87 |
| 11. | BaSMD | 69.34 (56.38) | 22.95 |
| 12. | BaNGL | 68.32 (55.74) | 24.09 |
| 13. | BaKVL | 72.36 (58.28) | 19.60 |
| 14. | BaPDR | 35.61 (36.63) | 60.43 |
| 15. | BaVML | 70.26 (56.95) | 21.93 |
| 16. | Control | 90.00 | -- |
| | S.Ed | 1.54 | |
| | CD | 2.89 | |

**Mean of three replications*

Similar findings were reported by Vamshi *et al.* (2025), who recorded a maximum inhibition of 58.22% by *Bacillus cereus* against *S. rolfsii* using the dual culture technique. Likewise, Vamshi *et al.* (2026) reported considerable variation in the antagonistic activity of different *Bacillus* species under varying $CO_2$ concentrations, with *B. tequilensis* exhibiting 68.32% inhibition at 700 ppm $CO_2$. Further, Kumar and Ramadevi (2024) reported a maximum inhibition of 61.70% by *Bacillus pumilus* isolate S5BC4 obtained from groundnut rhizosphere. The variation in antagonistic activity among the isolates may be attributed to differences in their physiological and biochemical characteristics. These differences could influence the production of antifungal metabolites and extracellular hydrolytic enzymes. Competition for nutrients and space may also contribute to the suppression of pathogen growth. Isolates exhibiting lower inhibition may possess comparatively limited antagonistic capabilities under the tested conditions. Such variations highlight the importance of screening diverse bacterial isolates to identify effective biocontrol candidates.

### 3.4 Molecular Characterisation

Based on their superior antagonistic activity against *Sclerotium rolfsii* in the dual culture assay, five promising *Bacillus* isolates, namely BaLDP, BaPRG, BaVPD, BaMPY and BaPDR, were selected for molecular characterisation and identification. The isolates were subjected to 16S rRNA gene sequencing and the obtained nucleotide sequences were compared with those available in the NCBI GenBank database using BLASTn analysis. The results revealed that isolates BaLDP, BaPRG and BaPDR exhibited the highest sequence similarity to *Bacillus subtilis*, whereas BaVPD and BaMPY showed the highest similarity to *B. velezensis* and *B. mycoides,* respectively. A phylogenetic tree was constructed using the Neighbour-Joining method in MEGA version 12 with 1,000 bootstrap replications. The selected isolates clustered with their respective closely related *Bacillus* reference sequences, supporting the BLASTn-based identification. The 16S rRNA gene sequences of BaLDP, BaPRG, BaVPD, BaMPY and BaPDR comprised 1,463, 1,292, 1,162, 1,296 and 1,103 bp, respectively. The 16S rRNA gene sequences of all five isolates were submitted to the NCBI GenBank database under accession numbers QB313449.1 (BaLDP), PZ868641.1 (BaPRG), PZ868636.1 (BaVPD), PZ868635.1 (BaMPY) and PZ840804.1 (BaPDR). All five isolates clustered with their respective closely related *Bacillus* reference sequences, supporting the BLASTn results; however, bootstrap values varied among the internal nodes (Fig. 3). Similar 16S rRNA-based identification and phylogenetic clustering of *Bacillus* isolates have been reported by Ye *et al.* (2023), Patel *et al.* (2023) and Zou *et al.* (2023). Biswas *et al.* (2023) and Sinha *et al.* (2023) also demonstrated the applicability of 16S rRNA sequencing for identifying plant-associated *Bacillus* species. Similarly, Rafiee *et al.* (2025) reported high sequence similarity of native *B. subtilis* strains through BLAST analysis and constructed a phylogenetic tree using the Neighbour-Joining method with 1,000 bootstrap replications. Chen *et al.* (2022) and Devindrappa *et al.* (2022) further highlighted the importance of molecular characterisation of beneficial rhizobacteria.

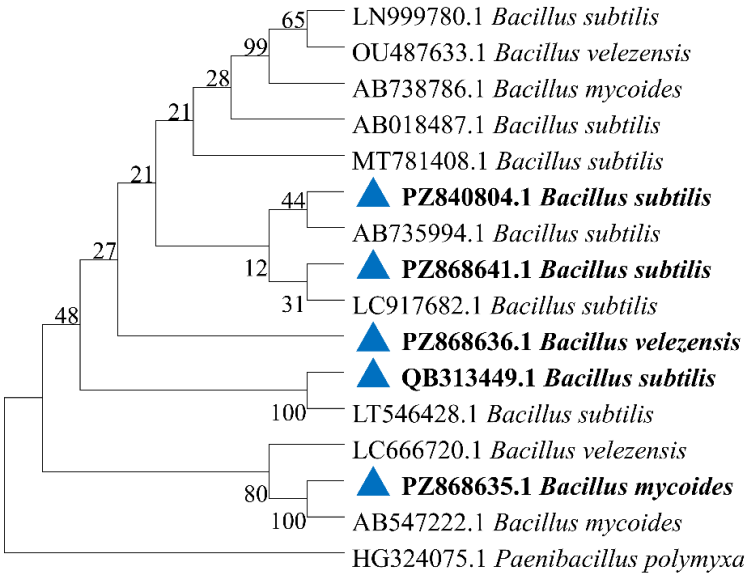


**Fig. 3. Phylogenetic tree based on 16S rRNA gene sequences showing the relationships between the *Bacillus* isolates and closely related reference sequences. The tree was constructed using the Neighbor-Joining method with 1,000 bootstrap replications**

### 3.5 Scanning Electron Microscopy

The SEM micrograph of the selected *Bacillus* isolate (BaLDP), recorded at 9,500x magnification with a 2 µm scale bar, revealed predominantly rod-shaped bacterial cells. The cells exhibited an elongated, bacillary morphology with rounded ends and a relatively smooth surface texture. Individual cells were clearly distinguishable, with variations in their orientation and arrangement, while some regions showed closely associated cells. These morphological features confirmed the characteristic rod-shaped cellular architecture of the selected *Bacillus* isolate. The observed bacilliform morphology is characteristic of members of the genus *Bacillus* (Fig. 4). Similar observations were reported by Sharma *et al.* (2024), who observed well-defined rod-shaped cells in untreated *Bacillus subtilis* through SEM analysis. However, exposure to iron ions resulted in noticeable changes in the cell surface, producing a denser and spongier appearance. Similarly, Hou *et al.* (2026) employed SEM to examine the cellular morphology of bacterial isolates and preliminarily characterised them as rod-shaped organisms. The present SEM observations therefore provide additional morphological evidence supporting the preliminary characterisation of the selected isolate as belonging to the genus *Bacillus*.

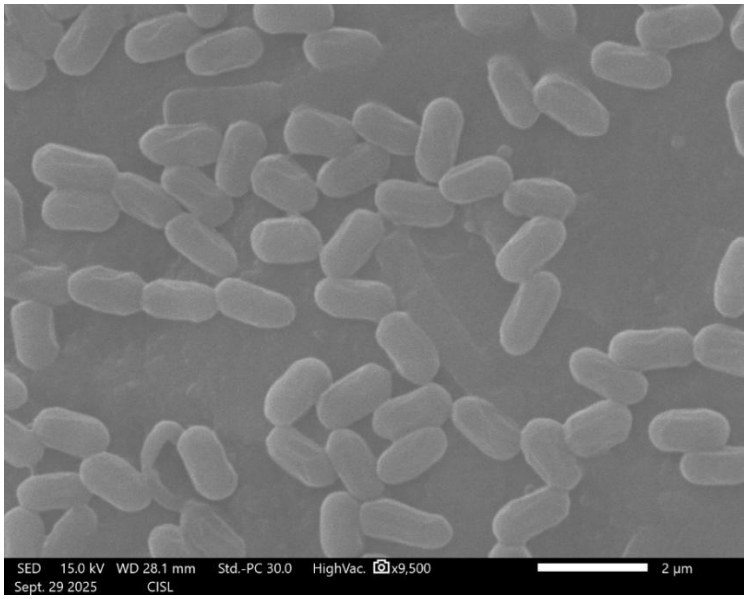


**Fig. 4. Scanning electron micrograph (SEM) of the selected *Bacillus* isolate (BaLDP) showing numerous rod-shaped bacterial cells with rounded ends, smooth surface morphology and varied cellular orientations. The micrograph was recorded at 9,500x magnification with a 2 µm scale bar (15 kV accelerating voltage)**

## 4. Conclusion

The present study revealed considerable diversity among fifteen *Bacillus* isolates obtained from groundnut rhizosphere and forest soils of Tamil Nadu. All isolates exhibited variations in their cultural, morphological and biochemical characteristics. Among the isolates, BaLDP, isolated from Ladapuram village in Perambalur district, Tamil Nadu, recorded the highest antagonistic activity (66.64%) against *Sclerotium rolfsii*. Molecular identification revealed the close association of BaLDP, BaPRG and BaPDR with *Bacillus subtilis*, BaVPD with *B. velezensis* and BaMPY with *B. mycoides*. Phylogenetic analysis further supported the molecular identification of the selected isolates. SEM analysis of BaLDP revealed characteristic rod-shaped cells with rounded ends and a smooth surface morphology. Overall, BaLDP was identified as a promising biocontrol candidate for the sustainable management of groundnut stem rot, requiring further validation under greenhouse and field conditions.

## 5. Limitations

The present study was confined to laboratory-based isolation, phenotypic characterisation and *In vitro* evaluation of the antagonistic potential of *Bacillus* isolates. Although five promising isolates were identified through 16S rRNA gene sequencing, the use of this marker alone may not provide sufficient resolution to distinguish closely related *Bacillus* species. Furthermore, antagonistic efficacy was assessed only under controlled laboratory conditions, without evaluating disease suppression in groundnut plants. Therefore, greenhouse and field experiments are necessary to validate the biocontrol potential and practical applicability of the selected isolates.

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## Declaration of AI Use

This manuscript was prepared through the intellectual contributions of the author(s) to the study design, data analysis, interpretation of results, and scholarly content. ChatGPT, Claude and Perplexity were used solely to assist with grammatical refinement and reference formatting during manuscript revision. The author(s) accept full responsibility for the accuracy, integrity, and final content of the manuscript.

## Competing Interests

Authors have declared that no competing interests exist.

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