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In vitro efficacy of organic amendments and biocontrol agents against *Sclerotium rolfsii* causing groundnut stem rot disease

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Abstract

Groundnut is a crucial oil seed crop cultivated worldwide and often referred to as the "King of Oil Seeds." However, its productivity is significantly reduced by various biotic and abiotic stresses. Among these, soil-borne fungal infections, particularly stem rot disease caused by *Sclerotium rolfsii* Sacc., pose a major threat, potentially leading to yield losses of up to 80 percent. In this study, stem rot-infected samples were collected from five major groundnut-growing districts in Tamil Nadu, India, and nine isolates of *S. rolfsii* were obtained. Based on pathogenicity tests, the most virulent isolate was identified and characterized at the molecular level. The pathogen produces a resting structure called sclerotia, which survives in soil for many years. Considering the ill effects of chemical methods of management, the present study is focused on non-chemical methods using organic amendments and biocontrol agents against the pathogen. Six amendments, *viz.,* groundnut cake, neem cake, castor cake, cotton cake, sesame cake, and cow manure, were tested against S. *rolfsii* under *in vitro* conditions at two concentrations (5 % and 10 %). Among these, sesame cake exhibited the highest inhibition of 48.36 percent and 63.80 percent at 5 percent and 10 percent concentrations, respectively. Through GC-MS analysis, the bioactive compounds, *viz*., 9,12-Octadecadienoic acid (Z, Z) - (100%) and 9-Octadecenoic acid (E) - (76.13%), responsible for pathogen inhibition were identified. Furthermore, rhizospheric bacterial biocontrol agents were evaluated against *S. rolfsii*, which revealed that isolate B_2 showed maximum inhibition (79.48%). The bacterial isolate B_2 was molecularly characterized and confirmed as *Bacillus subtilis* (PP882830).

Keywords

groundnut; *Sclerotium rolfsii* ; stem rot; organic amendments; biocontrol agents

Introduction

Groundnut (*Arachis hypogea* L.) is an important oilseed crop also known as wonder nut, poor men's cashew nut, earth nut, goober pea, monkey nut, and pig nut (1), which is mostly grown in tropical as well as subtropical countries of the world. It is also called the 'King of oil seeds' because of its massive uses. Groundnut is the world's third most important oilseed and thirteenth most important food crop. Groundnuts are high in energy (567 calories per 100g), contain 45–50% oil, 27–33% easily digested protein, 18% carbohydrates, and minerals like calcium, magnesium, and iron, as

well as vitamins B1, B2, and niacin. Mixed glycerides and a high percentage of unsaturated fatty acids, particularly oleic acid (50–65%) and linoleic acid (18–30%) constitute groundnut oil (2). India stands as the world's second-largest groundnut producer, following China. With approximately 25% of the world's land area, India contributes 19% of the global groundnut output. Worldwide, groundnut cultivation spans 327 lakh hectares, yielding 539 lakh tonnes of produce, with an average productivity of 1648 kg per hectare (3). Groundnut pod yield faces significant threats from various biotic factors, particularly foliar and soilborne diseases, as highlighted by (4). Among these, soil -borne diseases emerge as primary constraints, notably caused by pathogens like *Aspergillus niger*, *Sclerotium rolfsii,* and *Rhizactonia bataticola*, resulting in collar rot, stem rot, and root rot, respectively (5). Stem rot disease inflicts substantial yield losses, up to 80% as reported by (6). Pod yield was normally reduced by 10% to 25%, but under severe disease conditions, yield losses might reach up to 80% (7). All stages of groundnut plants are affected by *S. rolfsii*, including the seed germination stage, causing pre-emergence rot, and young plants causing stem rot. In grown-up plants, the infected plant exhibits yellowing and wilting with white mycelial growth in the stem near soil level and mustard color sclerotia (8). The traditional chemical management methods provide instant relief but leave behind chemical residues in the soil, leading to long-term soil pollution. As an alternative to chemical fungicides, organic amendments, and biocontrol agents can be used for the management of stem rot pathogens. Since the organic amendments and biocontrol agents enhance soil health, microbial activity, and nutrient availability. It also induces systemic resistance and promotes a stronger root system and plant vigour, which makes groundnuts more resilient to infection. The main objectives of this study are: i) Isolation and molecular characterization of the pathogen. ii) *In vitro* evaluation of organic amendments and biocontrol agents against the stem rot pathogen.

Materials and Methods

Chemicals and reagents

Potato Dextrose Agar medium and Nutrient Agar medium were used for the isolation of pathogen and biocontrol agents, respectively. For the molecular characterisation of the pathogen, the chemicals used were CTAB (Cetyl Trimethyl Ammonium Bromide) buffer, Phenol-chloroform mixture, Isopropanol, and Ethanol. HPLC grade methanol was used in GCMS analysis. Catalase, Glucose Phosphate Peptone broth, Starch Agar media, Simmon's citrate agar media, Casein agar media, Nutrient gelatin agar media, and Chrome-Azurol S agar media were used for biochemical characterization of effective biocontrol agent. For the molecular characterization of bacterial biocontrol agents, Butanol, TE (Tris-Hcl, EDTA) buffer, and Lysozyme were used.

Survey and collection of diseased samples

A roving field study was conducted to ascertain the occurrence of stem rot disease on groundnut in many farmer's fields in the major groundnut-growing regions of Tamil Nadu, India. To determine the disease incidence in each field, two diagonal transects in the form of an "X" were cut across the field, and the plants were examined visually. The survey was carried out in five districts of Tamil Nadu, India, *viz.,* Coimbatore, Cuddalore, Trichy, Salem, and Namakkal, known as important groundnut growing areas in the state. Nine samples infected with *Sclerotium* were taken during the study to isolate the pathogen. 2 samples were taken each from the Coimbatore and Salem districts, 3 samples from Namakkal district, and 1 sample each from Trichy and Cuddalore districts, were collected during the survey. (9) used the following formula to determine the incidence of disease in different fields.

$$\text{Disease incidence} = \frac{\text{Number of diseased plants}}{\text{Total number of plants}} \times 100$$

Isolation of pathogen

The pathogen was isolated from stem rot-infected groundnut plants collected from various locations with typical symptoms using the standard tissue isolation approach (10). The surface was sterilized following the guidelines provided by (11). The samples were cut into small pieces and aseptically positioned equally apart on a Petri plate that had previously been filled with Potato Dextrose Agar (PDA) medium, which was treated with 500 ppm of streptomycin to inhibit the growth of bacteria. For three to five days, the inoculation plates were incubated at 28 ± 1 °C and 60 ± 5% relative humidity. Periodically, the plates were examined for hyphal development. Hyphal tip culture was used to preserve the pure culture (12). The pathogen's pure culture was kept on PDA slants for future research. Nine isolates of *Sclerotium* were isolated and named SR1, SR2, SR3, SR4, SR5, SR6, SR7, SR8, and SR9. The pathogen's cultural and morphological characteristics were investigated by (13).

Morphological characterization of S. rolfsii

Nine isolates of *S. rolfsii* (SR1, SR2, SR3, SR4, SR5, SR6, SR7, SR8, and SR9) were analyzed in terms of their morphological and cultural characteristics. Small mycelial discs with a diameter of 5 mm were cut from the edges of colonies that were actively growing and positioned in the middle of the PDA plate. Three replications were imposed, and the discs were cultured for three days at room temperature (28 ± 1 ° C). The colony characteristics of each isolate, shape, and color of the sclerotia were also studied.

Identification of virulent isolate of S. rolfsii

The purpose of the experiment was to identify the virulent *Sclerotium* isolates on the groundnut variety CO 2, which is susceptible to stem rot. According to (14), *Sclerotium* isolates were mass multiplied in the sand-maize medium. CO 2 seeds were surface sterilized with a 1% sodium hypochlorite solution for 1 minute and washed three times with sterile distilled water. The potting mixture was made and sterilized following (14) recommendations. After sterilization, the potting mixture was transferred into uniformly

sized pots with *S. rolfsii* multiplied sand-maize media in a 19:1 ratio. Surface-sterilized seeds were then seeded at a depth of one centimetre and kept in a glasshouse with sufficient care to reduce infection by other pathogens. A pot without the test fungus was kept as a control. To identify the virulent isolate, various observations like Germination percent, Number of days for symptom expression, and Percent Disease incidence were observed for each isolate.

Molecular characterization of pathogen

Based on the pathogenicity studies, a virulent *Sclerotium* isolate has been identified and it was molecularly characterized. The CTAB (Cetyl Trimethyl Ammonium Bromide) extraction procedure was used to isolate the genomic DNA (15). Fungal mycelia of 500 mg from seven-day-old *S. rolfsii* grown on potato dextrose agar medium were ground in a pestle and mortar with three milliliters of CTAB buffer, then the mixture was incubated for thirty minutes at 65 °C. Following incubation, 700 µl of phenol:chloroform (25:24) was added. The mixture was vortexed for one minute, and then it was centrifuged for ten minutes at 12,000 rpm. After collecting the supernatant, DNA was precipitated using an equivalent volume of ice-cold isopropanol at -20 ℃ for 60 minutes. The mixture was then centrifuged for 15 minutes at 13,000 rpm. Following a 70% ethanol rinse and air drying, the DNA pellet was re-suspended in 40 microliters of double-sterile water. The primers ITS 1 and ITS 4 were used to carry out the PCR assay. Direct sequencing in both directions was done on the 550 bp amplified PCR products. After the representative sequence was added to GenBank, a similarity search was conducted using nBLAST. With MEGA11 software, the ITS sequencing data were aligned, and a phylogenetic tree based on the Neighbour-Joining [NJ] approach was constructed (16).

In vitro evaluation of organic amendments against Sclerotium rolfsii

In a 250 ml conical flask, fifty grams of finely ground powder of six organic amendments (groundnut cake, neem cake, castor cake, cow manure, cotton cake, and sesame cake) were taken separately. The amendments were then allowed to decompose for fifteen days, after which the extract was filtered with muslin cloth and autoclaved for twenty minutes at 1.2 kg/cm pressure, which was considered a 100% concentration (standard solution) (17). To obtain the required concentrations of 5% and 10%, 5 ml and 10 ml of the standard solution of organic extracts were added separately to 95 ml and 90 ml of melted sterilized PDA media respectively in conical flasks aseptically at the time of pouring the medium. After solidification, a 5 mm mycelial disc from a 7-day-old, actively growing *S. rolfsii* fungal culture was placed in the center of the Petri plates with three replications. Using the (18) formula, the percentage of radial growth over the control was used to express the effectiveness of organic amendments.

$$I = [(C-T)/C] * 100$$

Where, I = Percentage of mycelial growth inhibition, C = Mycelial colony's average diameter in the control treatment (mm), T = Mycelial colony's average diameter in the treated plate (mm)

GCMS analysis of sesame cake extract

Sesame cake was crushed to a fine powder with an average diameter of 0.2 mm using a mortar and pestle. Then, 0.5 g of sesame powder was dissolved in 20 mL of HPLC grade methanol, vortexed, placed in a sonicator for 15 minutes, and then centrifuged for 15 minutes at 500 rpm. Subsequently, the mixture was passed through Whatman filter paper No. 1 (19). The acquired sample was analyzed using a GCMS (Model: ISQ 7610 GC-MS, Make: USA, Company: ThermoFisher) with a DB-5ms column length of 30 m, 0.25 mm internal diameter, and 0.25 micron film thickness. Helium served as a carrier gas. The oven temperature was set to 50°C for one minute, then increased to 10°C/min until it reached 300°C for one minute, with an injection port temperature of 250°C. The chemicals were identified based on the retention durations. Different substances were measured and identified according to their respective molecular masses and peak areas. The identified compounds were verified by comparing their peak spectra with mass spectra obtained from NIST20 library databases.

Isolation of bacterial strains from the rhizosphere region

Using the serial dilution and plating method, seven bacterial isolates were isolated from the soil taken from the rhizosphere area of healthy groundnut plants. One gram of soil was mixed with 10 ml of sterile water, and the mixture was serially diluted to 10^{-5} and 10^{-6} dilutions to isolate bacterial strains from soil samples. Then, 200 µl of dilution was poured on a Nutrient agar plate, and the plates were kept for 2 days at 28°C. Following incubation, individual colonies were subcultured onto new NA plates to produce a pure culture, which was then utilized to examine the distinct isolate's morphological characteristics.

In vitro evaluation of bacterial antagonist against S. rolfsii

The dual culture method (20) was used to assess the antagonistic activity of newly isolated seven bacterial isolates and *Bacillus subtilis* (Bbv 57) maintained in the Department of Plant Pathology at TNAU, Coimbatore, Tamil Nadu, India, against *S. rolfsii*. Mycelial discs with a diameter of 5 mm, obtained from a four-day-old test pathogen culture were placed on one side of a Petri plate and a one-day-old bacterial culture was streaked equally apart on sterile petri plates with PDA medium. After that, the petri dishes were incubated at 28 ± 2°C. For each treatment, three replications were maintained. Appropriate controls were maintained with the pathogen alone. After recording the full growth in the control plate, the growth of antagonists, the pathogen, and the zone of inhibition were recorded. The formula provided by (18) was used to compute the percentage inhibition of the test pathogen's mycelial growth.

$$I = [(C-T)/C] * 100$$

Where:I = Percentage of mycelial growth inhibition C = Mycelial colony's average diameter in the control treatment (mm), T = Mycelial colony's average diameter in the treated plate (mm)

Biochemical characterization of effective bacterial antagonist

The effective bacterial isolate identified from the dual culture technique was confirmed by a variety of biochemical tests.

Catalase test (21)

The bacterial colony was added to a drop of 3% H_2O_2 that had been placed on a sterile glass slide. The production of gas bubbles was interpreted as a positive reaction.

Methyl Red – Voges Proskauer test (22)

Pure bacterial culture was added to glucose phosphate peptone broth tubes and cultured for 2-3 days at 30±10°C. After adding a few drops of methyl red reagent to the broth, the test was considered successful when a red color appeared.

Starch hydrolysis test (23)

On a starch agar plate, a loopful of bacterial growth was streaked, and the plate was incubated for 48 hours at 37°C. Iodine solution was poured onto the plate following a two-day incubation period. When starch is present, iodine becomes blue or black. The presence of hydrolyzed starch was indicated by a clear zone surrounding the bacterial growth.

Citrate utilization test (21)

On Simmon's citrate agar plate, a loopful of bacterial growth was streaked, and the mixture was incubated for 24 hours at 37°C. The organism's use of citrate as a carbon source resulted in the formation of a blue color, which was interpreted as a positive reaction, and a green color as a negative one.

Casein hydrolysis test (23)

After streaking the bacterial isolate on a casein agar plate, it was incubated at 30±10° C for five to seven days. The hydrolysis of casein is indicated by the presence of a clear zone beneath and surrounding the colony, which is seen as a positive result.

Gelatin liquefaction test (24)

Liquid cultures of test organisms were stabbed into nutrient gelatin agar test tubes. The tubes were kept at 30±10° C for two days, and then they were refrigerated for thirty minutes. The bacteria that tested positive for this test produced enough gelatinase to dissolve the medium, which did not solidify even after being refrigerated.

Mannitol fermentation test (25)

To the nutrient agar medium, 0.5–1% mannitol was added. Phenol-Red is the pH indicator used which is red at neutral pH and turns yellow at pH below 6.8. Additionally, when the pH increased from neutral, it would turn pink. On the medium, the test bacterial culture was streaked and incubated overnight at 35–37°C. If the color changed from red to yellow, it could indicate a positive result.

Siderophore production test (26)

On a Chrome Azurol S (CAS) agar plate, a loopful of bacterial growth was streaked, and the plate was incubated at 37°C for 48 hours. Examine the CAS agar plates for color changes after the two-day incubation period. The formation of a yellow color zone around the colony was indicated as positive for Siderophore production.

Molecular characterization of effective bacterial antagonist

The effective bacterial isolate identified from the dual culture test was confirmed at the molecular level. Genomic DNA was extracted from bacterial isolates that had been cultured for three days at 30°C in nutrient broth, which was centrifuged at 7000 rpm for 10 min. The supernatant was discarded, and to the bacterial pellet, 500µl butanol and 1ml TE buffer were added and centrifuged at 7000 rpm for 10 min. The lipid layer was removed, and 1ml TE buffer was added and centrifuged at 7000 rpm for 10 min. After centrifugation, 1ml TE buffer and 100µl lysozyme were added, which was incubated at room temperature for 5 min. After incubation, 200 µl of 5M NaCl was added and kept in a water bath at 60°C for 10 min. Following incubation, 700 µl of phenol: chloroform (25:24) was added, and the mixture was vortexed for one minute and then it was centrifuged for twenty minutes at 7000 rpm. After collecting the supernatant, DNA was precipitated using an equivalent volume of ice-cold isopropanol at -20 °C for 60 minutes. The mixture was then centrifuged for 10 minutes at 10,000 rpm. Following a 70% ethanol rinse and air drying, the DNA pellet was re-suspended in 40 microliters of double-sterile water. Using universal primers, the 16S rRNA gene was amplified. Sanger sequencing was performed after the PCR products were electrophoresed in 1.5% TAE-agarose gel. The US National Center for Biotechnology Information (NCBI) provided the BLAST sequence analysis tool, which was used to evaluate the nucleotide sequences that were obtained. A rhizobacterial isolate identity was determined by calculating its similarity percentage to the top-hit taxon. Accession number was obtained for the 16S rDNA sequences that were deposited in GenBank. To create a phylogenetic tree, the sequences were aligned using the MEGA11 (Molecular Evolutionary Genetics Analysis version 11.0) software (16).

Statistical analysis

All experimental data were statistically analyzed using AGRES software, with a focus on performing analysis of variance (ANOVA) to evaluate treatment differences. The ANOVA method enabled the identification of significant variations among the treatments, ensuring that observed effects were statistically validated. Significance was determined at a 0.05% probability level, ensuring the robustness of the results. The use of AGRES software provided accurate and reliable statistical analysis, contributing to the study's overall validity.

Results and Discussion

Survey for incidence of stem rot disease

The occurrence of stem rot in five major groundnut-growing regions in Tamil Nadu, India, was assessed in groundnut fields. The symptoms of stem rot disease

include yellowing of the leaves, a decrease in vigour, poor root growth, rotting at the stem area, and early mortality. White, dense, fluffy, aerial, cotton-like mycelium was seen surrounding the stem and root area. Additionally, deep brown, spherical, or round sclerotia bodies were formed and adhered to the infected stem (Fig. 1). The symptoms observed in the present study coincide with the report of (27). Many crop plants from different taxa have been reported to be infected by S. *rolfsii*, and the symptoms of the infection are almost similar in all species (28). According to the report, stem rot disease was widespread throughout the main groundnut-growing districts of Tamil Nadu. In the present study, the survey report revealed that the disease incidence ranged from 7.87 to 22.56 percent, with the minimum in Mathur village of Trichy district and the maximum in Maniyanur village of Namakkal district of Tamil Nadu (Table 1). Nine distinct *S. rolfsii* isolates were obtained from the infected samples collected from the surveyed areas.

Fig. 1. Symptoms of stem rot of groundnut observed during survey

Morphological characterization

The occurrence of morphological variations in *Sclerotium* isolates infecting groundnuts was studied. In the current experiment, the *Sclerotium* isolates revealed two types of colony morphology, *viz*., fluffy and compact. Six isolates (SR1, SR2, SR4, SR5, SR7, and SR9) showed compact growth, while three isolates (SR3, SR6, and SR8) exhibited fluffy growth. There was a variation in the days required to cover the full Petri plates. Among the nine isolates, two isolates (SR3, SR9) took three days, four isolates (SR1, SR5, SR6, SR8) took four days, and three isolates (SR2, SR4, and SR7) took five days to attain full growth in the Petri plates. However, the growth rate of *S. rolfsii* (mm/day) from two days after inoculation varied between 20.65mm to 30.46mm (Table 2, Fig. 2). Similar morphological diversities were observed in *S. rolfsii* by several scientists from various places (29-33).

Pathogenicity and virulent studies for S. rolfsii

To find out the virulent isolate, all nine *Sclerotium* isolates (SR1, SR2, SR3, SR4, SR5, SR6, SR7, SR8, and SR9) collected from five districts of Tamil Nadu were evaluated for pathogenicity on CO 2, a susceptible groundnut variety. All nine isolates cause infection on the susceptible groundnut variety and produce stem rot symptoms as in the field. Infected plant samples were also taken from each isolate, and the pathogen was successfully re-isolated. Among the nine isolates, the virulent isolate is also identified based on the pathogenicity test. It is noticed that isolate SR3 is the most virulent isolate because of the maximum disease severity index of 56.96 with the lowest germination percentage,

Table 1. Survey of stem rot disease incidence in major groundnut-growing regions of Tamil Nadu.

District	Place	Latitude	Longitude	Isolate	*Disease incidence (%)
Coimbatore	TNAU	11.012265°	76.935498°	SR1	8.97^{h} (3.07)
	Aliyar	10.433337°	77.133335°	SR2	9.56^{f} (3.17)
Namakkal	Maniyanur	11.287229°	77.955255°	SR3	22.56^{a} (4.80)
	Koothampoondi	11.320601°	78.022214°	SR4	15.46^{d} (3.99)
	Sarkarserukali	11.277792°	78.005905°	SR5	13.84^{e} (3.78)
Salem	Pillanallur	11.427836°	78.130965°	SR6	9.24^{g} (3.12)
	Palamedu	11.441424°	78.071077°	SR7	16.76^{c} (4.15)
Trichy	Mathur	10.725268°	78.581295°	SR8	7.87^{i} (2.89)
Cuddalore	Virudachalam	11.516197°	79.326634°	SR2	17.27^{b} (4.21)

*Mean of three replications. The means followed by a common letter are not significantly different at the 5% level by DMRT. Values in the parentheses are square root transformed values.

Table 2. Morphological characterization of *S. rolfsii* isolates collected from major groundnut- growing regions of Tamil Nadu.

Isolate	Colony type	*Growth rate (mm/day) from 2nd DAI	Days taken to reach the periphery of the plate	Sclerotia colour
SR1	Compact	20.65^{i} (4.59)	4	Light brown
SR2	Compact	22.86^{h} (4.83)	5	Light brown
SR3	Fluffy	30.46^{a} (5.56)	3	Reddish brown
SR4	Compact	26.74^{f} (5.21)	5	Light brown
SR5	Compact	28.83^{c} (5.41)	4	Light brown
SR6	Fluffy	29.72^{b} (5.49)	4	Reddish brown
SR7	Compact	25.85^{g} (5.13)	5	Light brown
SR8	Fluffy	27.86^{d} (5.32)	4	Dark brown
SR9	Compact	27.73^{e} (5.31)	3	Dark brown
SE (d)		0.008		
CD (0.05%)		0.017		

*Mean of three replications. The means followed by a common letter are not significantly different at the 5% level by DMRT. Values in the parentheses are square root transformed values.

Fig. 2. Morphological characterization of different isolates of *S. rolfsii.*

which requires a lesser number of days for symptom development (Table 3, Fig. 3). The results are in line with the findings of (34) who showed the pathogenicity of *S. rolfsii* isolates isolated from groundnut, tomato, and taro on groundnut and observed variance in their virulence levels.

Molecular characterization of a virulent isolate

Using universal primers, the ITS region of the whole genomic DNA of the virulent *Sclerotium* isolate SR3 was amplified by PCR to a size of 550 bp amplicons by using ITS-

Table 3. Pathogenicity and virulent studies on various isolates of *S. rolfsii* .

Treatment	Name of the treatment	*Germination %	No of days for symptom expression	*Disease severity index
T_1	SR1	57.39^{b} (7.60)	47	48.29^{f} (6.98)
T_2	SR2	63.58^{e} (8.00)	43	47.39^{g} (6.92)
T_3	SR3	55.35^{a} (7.47)	34	56.96^{a} (7.58)
T_4	SR4	64.58^{f} (8.06)	38	52.97^{c} (7.31)
T_5	SR5	58.29^{c} (7.66)	36	49.64^{e} (7.08)
T_6	SR6	65.68^{g} (8.13)	49	51.76^{d} (7.22)
T_7	SR7	59.39^{d} (7.73)	47	42.67^{i} (6.57)
T_8	SR8	57.39^{b} (7.60)	48	44.86^{h} (6.73)
T_9	SR9	68.39^{h} (8.30)	42	54.98^{b} (7.44)
T_{10}	Control	100^{i} (10.02)	55	38.58^{j} (6.25)
SE(d)		0.007		0.008
CD (0.05%)		0.016		0.017

*Mean of three replications. The means followed by a common letter are not significantly different at the 5% level by DMRT. Values in the parentheses are square root transformed values.

Fig. 3. Pathogenicity and virulent studies for *S. rolfsii.*

1F/ITS-4R. The ITS region nucleotide sequences of the *Sclerotium* isolates that infected groundnuts were compared to those of the isolates from the NCBI database infecting other crop species. There was a 98.06% similarity between the sequence and *Agroathelia rolfsii*. The sequence was then submitted to the NCBI Genbank and assigned an accession number (PP839816) (Fig. 4). (35) reported that the genomic DNA of *S. rolfsii* was extracted by the CTAB method.

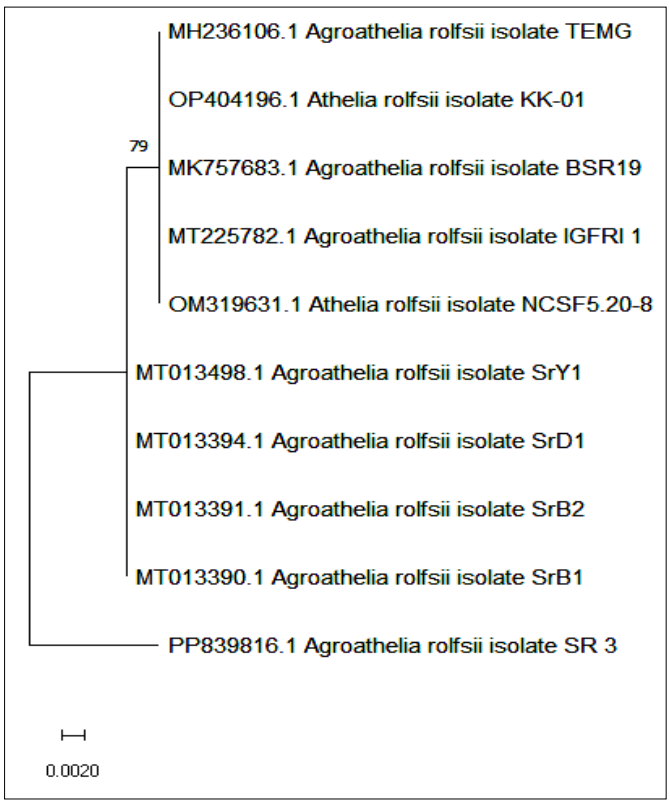


Fig. 4. Phylogenetic tree of *Sclerotium rolfsii* isolate SR3.

Evaluation of organic amendments against S. rolfsii

In the present study, six different organic amendments *viz.,* groundnut cake, neem cake, castor cake, cow manure, cotton cake, and sesame cake, were tested for antifungal activity against *S. rolfsii.* The results revealed that the minimum mycelial growth of 46.47 mm and 32.58 mm was obtained in sesame cake extract, with the maximum growth inhibition of 48.36 percent and 63.80 percent over control at 5 percent and 10 percent concentrations, respectively. The next best organic amendments were Groundnut cake, which was followed by Neem cake and Castor cake. Cow manure was found to be the least effective among the organic amendments tested (Table 4, 5, Fig. 5a, 5b). These results are following (36) who reported that Mustard oil cake (at a concentration of 2 percent) and sesame oil cake (at a concentration of 3 percent) were found to be highly

Table 4. Radial growth of mycelium (mm) at different concentrations of organic amendments.

S. No	Treatment	*Radial growth of mycelium (mm)	
		Concentration 5%	Concentration 10%
T_{-1}	Control	90.00^{d} (9.51)	90.00^{e} (9.51)
T_2	Groundnut cake	75.65^{b} (8.72)	63.34^{c} (7.98)
T_3	Neem cake	84.56^{b} (9.22)	82.38^{d} (9.22)
T_4	Castor cake	83.28^{c} (9.15)	78.28^{b} (8.87)
T_5	Cow manure	84.38^{c} (9.21)	83.23^{d} (9.15)
T_6	Cotton cake	84.59^{c} (9.22)	81.37^{d} (9.22)
T_7	Sesame cake	46.47^{a} (6.85)	32.58^{a} (5.75)
SE(d)		0.755	0.755
CD (0.05%)		1.621	1.621

*Mean of three replications. The means followed by a common letter are not significantly different at 5% level by DMRT. Values in the parentheses are square root transformed values.

Table 5. Percentage reduction in the growth of mycelium over control using different organic amendments.

S. No	Treatment	*Percentage reduction over control	
		Concentration 5%	Concentration 10%
T_{-1}	Control	0.00^{e} (0.70)	0.00^{f} (0.70)
T_2	Groundnut cake	15.94^{b} (4.05)	29.62^{b} (5.48)
T_3	Neem cake	6.04^{c} (2.55)	6.04^{d} (2.55)
T_4	Castor cake	7.46^{c} (2.82)	13.02^{c} (3.67)
T_5	Cow manure	6.24^{d} (2.59)	7.46^{d} (2.82)
T_6	Cotton cake	6.01^{d} (2.55)	6.01^{e} (2.34)
T_7	Sesame cake	48.36^{a} (6.98)	63.80^{a} (8.01)
SE(d)		1.209	0.776
CD (0.05%)		2.595	1.665

*Mean of three replications. The means followed by a common letter are not significantly different at 5% level by DMRT. Values in the parentheses are square root transformed values.

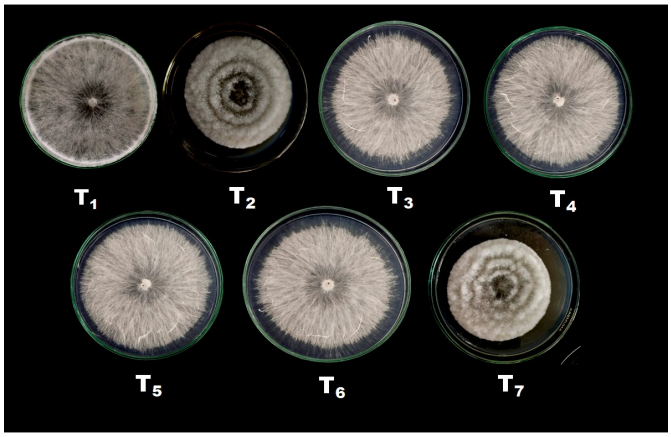


Fig. 5a. *In vitro* evaluation of organic amendments against *S. rolfsii* at 5% concentration.

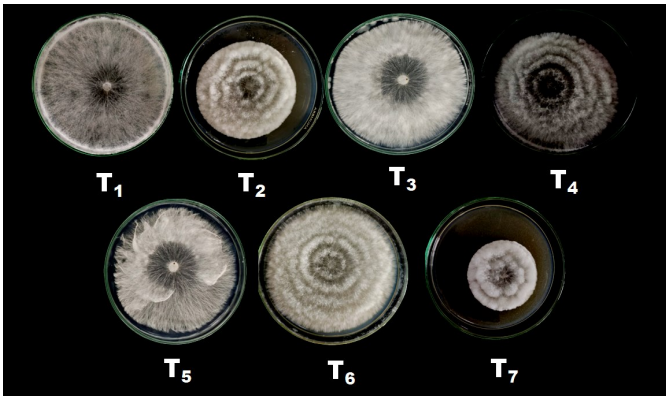


Fig. 5b. *In vitro* evaluation of organic amendments against *S. rolfsii* at 10% concentration.

inhibitive, with respective inhibitions of 47.40 percent and 38.51 percent. Mustard oil cake (2 percent concentration) and sesame oil cake (3 percent concentration) both showed moderately higher sclerotia inhibition. The mycelial growth reduction is increased when the concentration is increasing due to the higher production of phenolic compounds and alkaloids. Higher concentrations of organic materials can create a physical barrier that restricts the mycelium's ability to spread through the substrate, limiting its growth.

The findings of this study demonstrate the significant environmental benefits of organic amendments over chemical fungicides. By reducing chemical pollution, enhancing soil health, conserving biodiversity, and promoting sustainable agriculture, organic amendments offer an eco-friendlier approach to disease management (37). While they may not always provide immediate control, integrating organic amendments with other sustainable practices can effectively mitigate plant diseases while minimizing harm to the environment.

GCMS analysis of sesame cake extract

Following a GC-MS analysis of the Sesame cake methanolic extract, a set of 33 compounds was found. These compounds are shown in Table 6 along with their retention time, molecular weight, formula, and percent area. Fig. 6 shows the GC-MS chromatogram of the Sesame cake methanolic extract. The two most prevalent compounds among the 33 GCMS-analyzed compounds were 9,12-Octadecadienoic acid (Z, Z)-(100%) and 9-Octadecenoic acid (E)-(76.13%). The GC-MS analysis indicated the existence of the metabolic active chemicals listed above, which contributed to fungicidal activity against *S. rolfsii*. (38) published similar findings, revealing that fatty acid methyl esters such as hexadecanoic acid ethyl ester,9,12-octadecadienoic acid (Z, Z)-,9-octadecenoic acid, (E)-, and octadecanoic acid have antifungal action against *S. rolfsii*. Important bioactive compounds that have antifungal action against *S. rolfsii* were also discovered by (39). These compounds include n-Hexadecanoic acid, Hexadecanoic acid, ethyl ester, Octadecadienoic acid (Z, Z)-, 9-Octadecenoic acid, (E)-, and Octadecanoic acid.

Isolation and characterization of various bacterial isolates

Table 6. GCMS analysis of Sesame cake extract.

S. No	Retention time	Area %	Compound name	Molecular weight (g/mol)	Molecular formula
1	5.2692	0.41	Butanoic acid, pentyl ester	58.24	$C_9H_{18}O_2$
2	5.3238	2.11	1,2-Cyclopentanedione	98.1	$C_5H_6O_2$
3	6.3143	16.95	2-Hydroxy-gamma-butyrolactone	102.09	$C_4H_6O_3$
4	7.1482	1.44	3(2H)-Furanone, 4-hydroxy-5-methyl-	114.1	$C_5H_6O_3$
5	7.6726	7.95	Maltol	126.11	$C_6H_6O_3$
6	7.8765	1.47	Cyclopentanol	86.13	$C_5H_{10}O$
7	11.0919	0.67	Phenol, m-tert-butyl-	150.22	$C_{10}H_{14}O$
8	11.8493	4.23	2,4,4-trimethylpentyl ethylphosphonofluoridate	196.23	$C_{10}H_{22}FO_2P$

9	13.3678	0.89	.alpha.-D-Digitoxopyranose	148.16	$C_6H_{12}O_4$
10	13.4807	11.02	1,3-Propanediol, 2-ethyl-2-(hydroxymethyl)-	134.17	$C_6H_{14}O_3$
11	13.8376	0.71	2-Butenoic acid, 1-methylethyl ester	128.169	$C_7H_{12}O_2$
12	14.1107	1.55	Dodecanoic acid	200.32	$C_{12}H_{24}O_2$
13	14.4603	0.24	Hexanoic acid, 2-isopropyl-2-methyl-5-oxo-, methyl ester	200.27	$C_{11}H_{20}O_3$
14	14.6023	0.59	Diethyl Phthalate	222.24	$C_{12}H_{14}O_4$
15	15.2250	13.39	3-Deoxy-d-mannoic lactone	162.14	$C_6H_{10}O_5$
16	16.3539	0.53	Tetradecanoic acid	228.37	$C_{14}H_{28}O_2$
17	18.0799	2.48	Hexadecanoic acid, methyl ester	270.5	$C_{17}H_{34}O_2$
18	18.1309	0.27	7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8- dione	276.4	$C_{17}H_{24}O_3$
19	18.4514	27.33	n-Hexadecanoic acid	256.42	$C_{16}H_{32}O_2$
20	18.7427	0.28	Hexadecanoic acid, ethyl ester	284.5	$C_{18}H_{36}O_2$
21	19.7222	7.47	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	294.5	$C_{19}H_{34}O_2$
22	19.7732	7.89	9-Octadecenoic acid (Z)-, methyl ester	296.5	$C_{19}H_{36}O_2$
23	19.9917	1.92	Methyl stearate	298.5	$C_{19}H_{38}O_2$
24	20.1447	100.00	9,12-Octadecadienoic acid (Z,Z)-	280.4	$C_{18}H_{32}O_2$
25	20.1847	76.13	9-Octadecenoic acid, (E)-	282.5	$C_{18}H_{34}O_2$
26	20.3377	13.05	Octadecanoic acid	284.5	$C_{18}H_{36}O_2$
27	21.7615	0.66	9,12-Octadecadienoyl chloride, (Z,Z)-	298.9	$C_{18}H_{31}C_{lO}$
28	21.7979	0.61	trans-9-Octadecenoic acid, pentyl ester	352.6	$C_{23}H_{44}O_2$
29	22.9887	1.83	9-Octadecenoic acid (Z)-, oxiranylmethyl ester	338.5	$C_{21}H_{38}O_3$
30	23.2581	3.72	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester	330.5	$C_{19}H_{38}O_4$
31	24.3688	1.03	Cinnamoylechinadiol, TMS	343.34	$C_{27}H_{40}O_4Si$
32	24.6601	45.66	9-Octadecenoic acid (Z)-, 2-hydroxy-1- (hydroxymethyl)ethyl ester	358.6	$C_{21}H_{40}O_4$
33	24.8203	3.36	Octadecanoic acid, 2,3-dihydroxypropyl ester	358.6	$C_{21}H_{42}O_4$

A total of seven distinct bacterial colonies were isolated from the groundnut rhizosphere on Nutrient Agar media using serial dilution and plating methods. Morphological characters like form, margin, surface, and chromogenesis were studied for these seven bacterial isolates (Table 7, Fig. 7). It was a well-known fact that rhizospheric bacteria were excellent agents to control soil-borne plant pathogens. Rhizospheric isolates like *Bacillus*, *Pseudomonas*, *Serratia,* and *Arthrobacter* have been proven to be best in controlling fungal diseases (40).

Evaluation of bacterial isolates against S. rolfsii

A total of seven biocontrol agents were tested for their efficacy in suppressing the mycelial growth of *S. rolfsii in vitro* in a dual culture assay. Among the various biocontrol agents tested, isolate B_2 was found effective in inhibiting the mycelial growth of *S. rolfsii* with a mean percentage inhibition of 79.48. The next best bacterial antagonistic isolate was B_3 (Table 8, Fig. 8). Several strains of *B. subtilis* are known to suppress plant pathogens and improve plant health (41-43). *Bacillus spp*. are capable of growing in di-

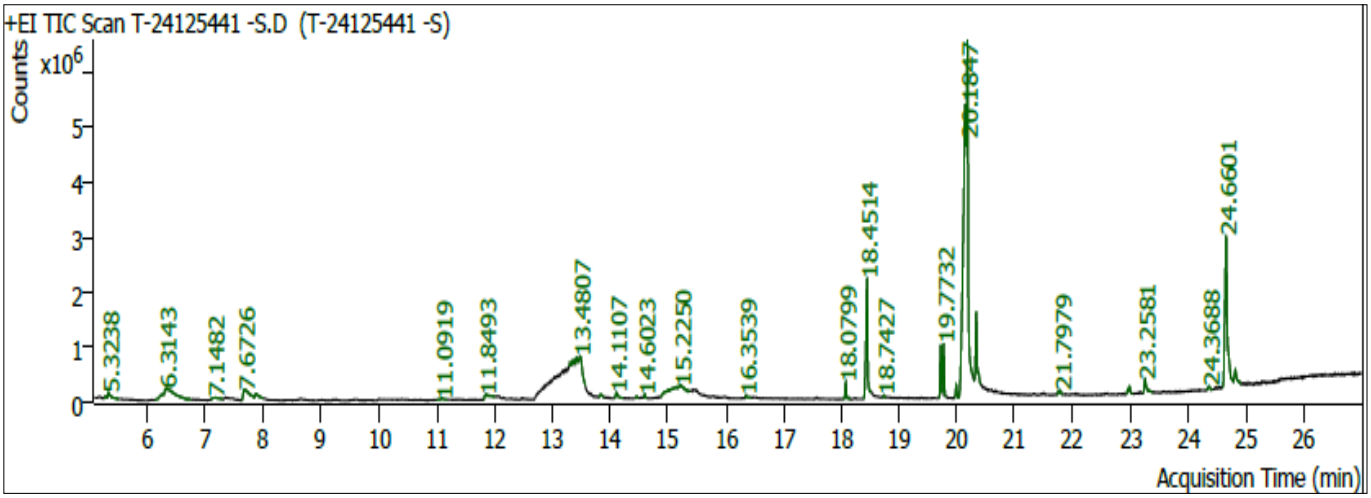


Fig. 6. GCMS analysis of sesame cake extract.

Table 7. Morphological characters of bacterial isolates.

Isolates	Form	Margin	Surface	Chromo genesis
B_1	Circular	Entire	Smooth	White
B_2	Circular	Entire	Smooth	White
B_3	Irregular	Undulated	Smooth and dull	Yellowish white
B_4	Circular	Entire	Smooth	White
B_5	Circular	Entire	Smooth	Transparent white
B_6	Circular	Entire	Smooth and glistening	White
B_7	Circular	Entire	Smooth	White

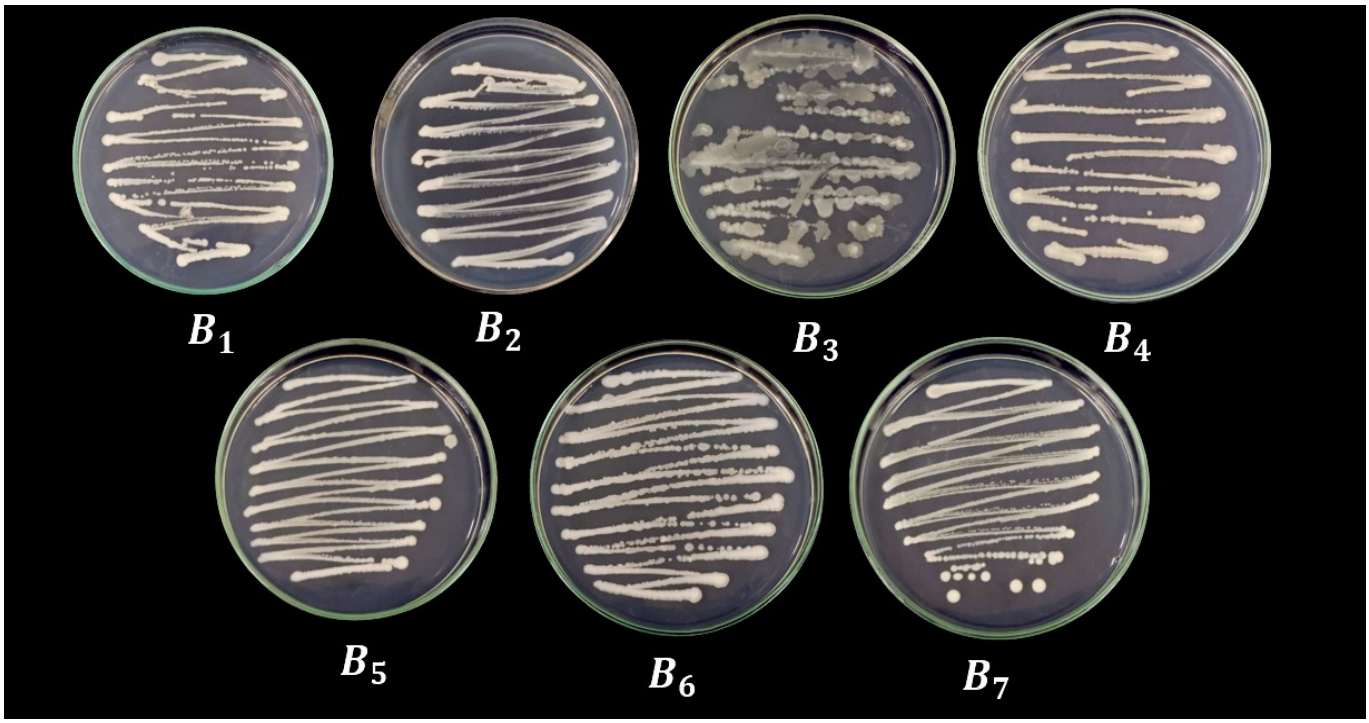


Fig. 7. Isolation of bacterial biocontrol agents from rhizosphere of healthy groundnut plants.

Table 8. *In vitro* evaluation of biocontrol agents against *S. rolfsii*.

Treatment	*Mycelial diameter (mm)	*Inhibition zone (mm)	*Percent reduction over control
Control	90^{h} (9.51)	0^{d} (0.70)	0^{h} (0.70)
B_1	37.24^{fg} (6.14)	3.56^{c} (2.01)	58.62^{fg} (7.68)
B_2	18.46^{a} (4.35)	0^{d} (0.70)	79.48^{a} (8.94)
B_3	25.76^{b} (5.12)	0^{d} (0.70)	71.37^{b} (8.47)
B_4	38.14^{g} (6.21)	14.57^{a} (3.88)	57.62^{g} (7.62)
B_5	29.53^{d} (5.47)	10.35^{b} (3.29)	67.18^{d} (8.22)
B_6	32.97^{e} (5.78)	2.84^{c} (1.82)	63.36^{e} (7.99)
B_7	27.79^{c} (5.31)	3.56^{c} (2.01)	69.12^{c} (8.34)
Bbv 57	25.82^{b} (6.02)	0^{d} (0.70)	60.20^{f} (7.79)
SE(d)	0.769	0.544	0.769
CD (0.05%)	1.617	1.143	1.617

*Mean of three replications. The means followed by a common letter are not significantly different at 5% level by DMRT. Values in the parentheses are square root transformed values.

verse environments due to the production of endospores that can tolerate extreme pH, temperature, and osmotic conditions; therefore, they offer several advantages over other antagonistic microorganisms (44).

Bacillus strains also induce systemic resistance in plants via volatile organic compounds (45) and promote growth through phytohormones and extracellular enzymes (46, 47). They produce a range of secondary metabolites, including antibiotics and hydrolytic enzymes, enhancing their effectiveness as biological control agents against various pathogens (48, 49). Additionally, *Bacillus* spp. can compete with other soil microorganisms for nutrients and space due to its highly competitive saprophytic ability. All these mechanisms altogether contributed to the successful biocontrol agent.

Biochemical characterization of effective bacterial isolate

The effective biocontrol isolate B_2 was subjected to various biochemical tests. It showed positive results for the Catalase test, Methyl Red-Voges Proskauer test, Citrate hydrolysis test, Mannitol fermentation test, Gelatin hydrolysis test, and Siderophore production test. However, it was negative for the Starch hydrolysis test (Fig. 9). These results highlight the isolate's diverse enzymatic activities and metabolic capabilities, demonstrating its potential efficacy as a biocontrol agent. The positive tests suggest the isolate's ability to produce key enzymes and compounds, while the negative result for starch hydrolysis indicates a lack of amylase production. This study is following (50) who studied the biochemical characteristics of *Bacillus cereus.*

Molecular characterization of effective bacterial isolate

After performing PCR amplification of the 16S rDNA, amplicons of approximately 1500 bp were successfully obtained. The 16S rRNA gene sequences of the test rhizobacterial isolate were analyzed using NCBI-BLAST, revealing that the isolate, with GenBank accession no. PP882830, is identified as *B. subtilis.* The analysis showed a high similarity of

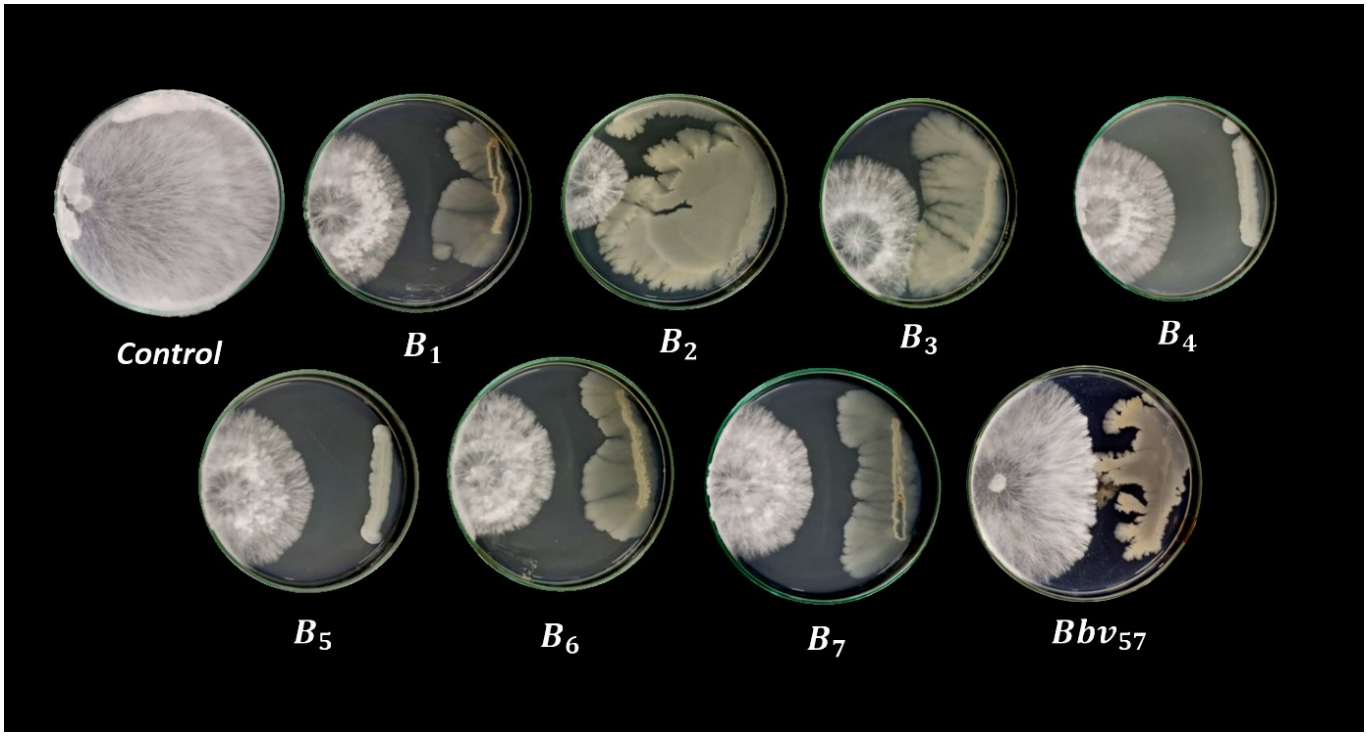
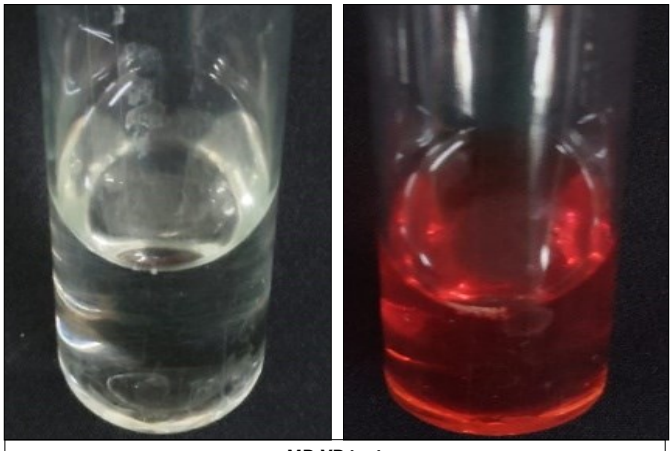


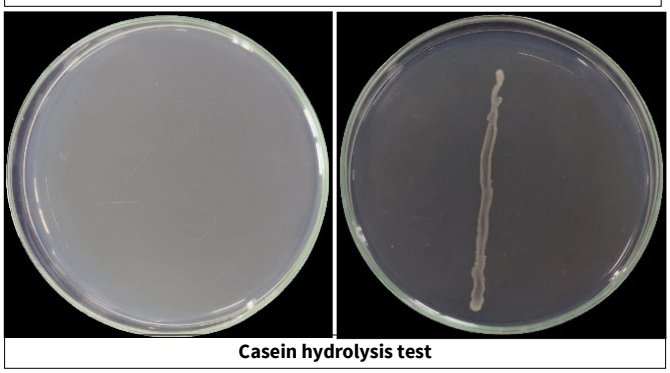
Fig. 8. *In vitro* evaluation of biocontrol agents against *S. rolfsii.*

99–100% with other *Bacillus* species, confirming its taxonomic classification. Additionally, a phylogenetic tree was constructed based on the 16S rRNA gene sequences to assess genetic relationships. This tree indicated significant genetic homogeneity among the *Bacillus* isolates, highlighting their close genetic relatedness (Fig. 10). The results underscore the isolate's strong resemblance to other *Bacillus* species and its placement within the *Bacillus* genus, providing a clearer understanding of its evolutionary background and confirming its identity through comprehensive molecular and phylogenetic analysis. This study follows (51) who isolated rhizospheric bacteria from tomatoes and got amplification at 1500 bp.

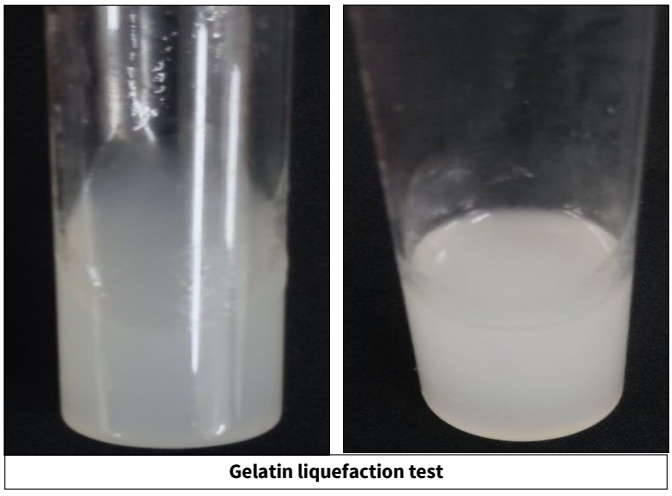
Catalase test



MR-VP test



Casein hydrolysis test



Gelatin liquefaction test

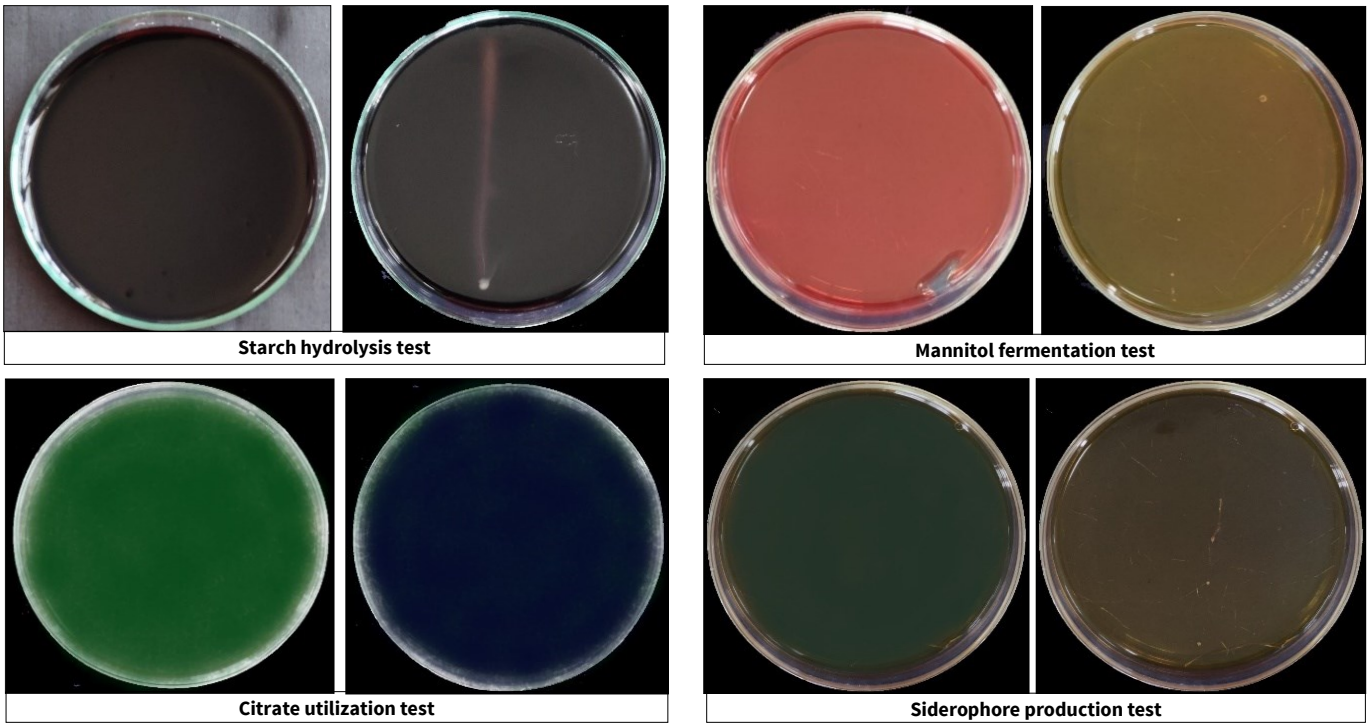


Fig. 9. Biochemical characterization of effective bacterial biocontrol isolate B_3 .

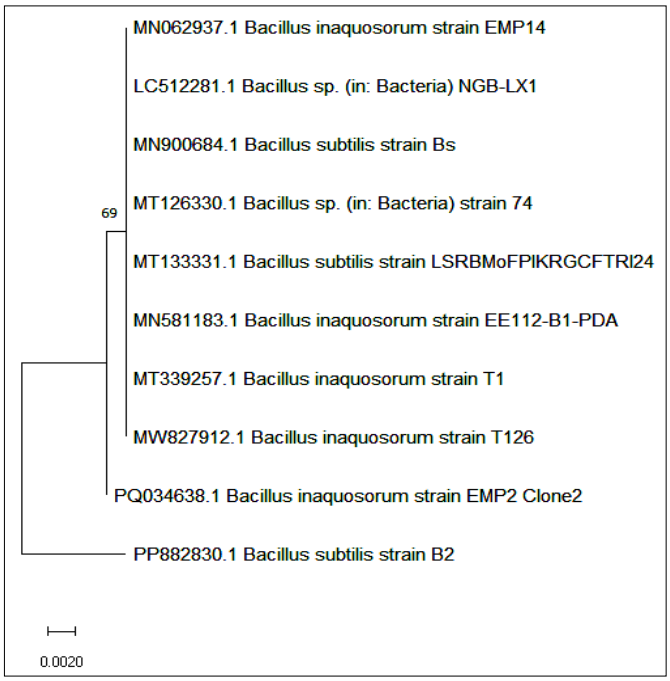


Fig. 10. Phylogenetic tree of effective bacterial isolate B_2.

Conclusion

In vitro evaluation of various organic amendments and biocontrol agents against *S. rolfsii*, the causative agent of stem rot in groundnut, revealed that sesame cake significantly inhibited the pathogen's growth, while *B. subtilis* (PP882830) exhibited strong antagonistic activity. These findings suggest that incorporating sesame cake and *B. subtilis* into groundnut cultivation could offer an effective, sustainable approach to managing stem rot disease. This integrated strategy reduces reliance on chemical fungicides and promotes environmental and human health. Further field studies are recommended to validate the *in vitro* results and optimize application methods for broader agricultural practices, potentially improving both groundnut yield and farmer's economic returns.

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Authors' contributions

BD conceptualized and drafted the manuscript. JS contributed to the supervision of research project and data analysis. NI provided expertise and critical manuscript review. The manuscript was revised and finalized by JS and RK. KN provided valuable insights. All authors have read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interests to declare.

Ethical issues: None.

AI Declaration

Grammarly AI tool was used to improve language and readability, with caution.

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