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RESEARCH ARTICLE

Exploring the integrated strategies for enhanced stem rot disease management in groundnut

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Abstract

Groundnut (*Arachis hypogaea* L.), a significant edible oilseed crop, suffers yield reductions due to stem rot incited by *Sclerotium rolfsii* Sacc. A 2022 survey across three major groundnut-growing districts of Tamil Nadu revealed stem rot incidences ranging from 16.57 % to 39.55 %, with the highest occurrence recorded in the Kalpadai region of Kallakurichi. A total of 10 *S. rolfsii* isolates were obtained and pathogenicity assays identified the Sr3 isolate as the most virulent, causing 44.85 % disease incidence. Morphological and molecular characterization, including ITS-based analysis, confirmed the pathogen as *S. rolfsii* (OQ5681821). Principal Component Analysis (PCA) demonstrated that pathogenic variability negatively impacted yield traits such as seed length, plant height and shelling percentage. Native biocontrol agents *Trichoderma asperellum* Tr4 (OQ581458) and *Bacillus licheniformis* B5 (OQ195791) exhibited significant antagonistic effects, inhibiting *S. rolfsii* mycelial growth by 88.88 % and 70.55 % respectively. Additionally, vermicompost and neem cake extract (20 % concentration) suppressed pathogen growth by 88.22 % and 78.01 % respectively, using the poisoned food technique. Compatibility studies confirmed that *T. asperellum* Tr4 and *B. licheniformis* B5 were mutually compatible as well as with vermicompost and neem cake. Combined treatments of bioagents with vermicompost and neem cake in pot and field trials effectively reduced stem rot incidence and improved yield parameters in groundnut. This study offers valuable insights into *S. rolfsii* pathogenesis and highlights sustainable, eco-friendly strategy for managing stem rot, thereby enhancing groundnut productivity.

Keywords: bioagents; FE-SEM; groundnut; neem cake; PCA; vermicompost

Introduction

Groundnut (*Arachis hypogaea* L.), a major oilseed of the Leguminosae family native to South America, is broadly cultivated throughout warm and humid climatic conditions. It is often called "the king of oilseeds" due to its high oil and protein content. Major producers include India, China and the United States, where groundnut serves as a crucial food and cash crop (1). India leads in groundnut cultivation area and is the second-largest producer globally after China, with a production of 68.57 lakh tonnes and an average yield of 1562 kg per hectare (2). The genus *Arachis* includes 69 species native to South America, with its origins believed to lie in central Brazil. In India, groundnut cultivation is primarily concentrated in the states of Karnataka, Gujarat, Andhra Pradesh, Tamil Nadu and Maharashtra.

Despite its year-round cultivation potential, groundnut faces many challenges from diseases like seedling rot, rust, stem rot, leaf spot and pod rot. Among these, *S. rolfsii* is a major pathogen causing stem rot, leading to significant yield losses (3). *S. rolfsii* affects over 500 species, with yield losses ranging from 25 % to 80 %. The fungus attacks stem bases, causing yellowing, wilting and brown stem rot, accompanied by white mycelial growth. It produces brown to black sclerotia, serving as resting structures that survive in the soil for years (4). Advanced microscopy techniques such as light microscopy and scanning electron microscopy (SEM) have been used to identify sclerotia-producing organisms, including *S. rolfsii*. Koch's postulates have been validated through pathogenicity tests for *S. rolfsii* (5). PCA has been employed to refine the study of pathogenic variability, focusing on key growth and yield contributors (6).

Despite the limited comprehensive understanding of *Sclerotium* diversity, significant efforts have been made in this area. Molecular techniques, particularly internal transcribed spacer (ITS) region sequencing are instrumental in elucidating the genetic similarities and phylogenetic relationships of *S. rolfsii* (7). The long-term effectiveness of fungicides for managing stem rot is constrained by issues such as cost, environmental and health hazards, residual longevity and resistance development. Therefore, there is an urgent necessity for another substitute, sustainable strategies for disease control. Although individual

biocontrol agents have demonstrated efficacy against soil-borne pathogens, their inconsistent performance is often attributed to limited adaptability to diverse soil environments (8). Recent studies highlighted the improved efficacy of combining biocontrol agents with organic amendments, offering broader-spectrum activity, greater reliability and enhanced disease suppression (9). This study aimed to investigate the disease incidence, pathogenicity and their influence on plant biometrics, as well as the morphological and molecular characterization of *S. rolfsii* in groundnut crops in Tamil Nadu, India. Moreover, the study examined the combined application of fungal and bacterial bioagents along with organic soil amendments to establish sustainable, eco-friendly strategies for managing stem rot in groundnut.

Materials and Methods

In vitro and *in vivo* investigations were carried out during 2022-2023 at the Department of Plant Pathology, Annamalai University, located in Chidambaram, Tamil Nadu, India (latitude 11.3908° N, longitude 79.7148° E).

Survey, isolation and identification of groundnut stem rot pathogen

In 2022, an intensive roving survey assessed groundnut stem rot disease incidence in the Kallakurichi, Cuddalore and Villupuram districts of Tamil Nadu. The disease incidence percentage was calculated using the formula:

Disease incidence (%) = (No. of plants infected / Total number of plants observed) × 100

Pathogen isolation was conducted using the tissue segment technique, where infected stem sections were placed on Potato Dextrose Agar (PDA) amended with 0.1 % streptomycin in sterile 90 mm Petri dishes. The cultures were incubated at 27±2°C for 4 to 5 days. A total of ten isolates were collected and purified using single hyphal tip method and these were labelled as Sr1 to Sr10. The cultural and morphological traits of the pathogen were confirmed using light microscopy (Olympus CX23).

Evaluation of pathogenic variability and its impact on groundnut yield traits

A pathogenicity assay was performed to assess the aggressiveness of *S. rolfsii* isolates on the susceptible groundnut variety TMV 8. Ten isolates were propagated in a sand-maize medium following the methodology described previously (10). After sterilization, seeds were sown in pots (25 cm × 30 cm) containing 10 to 20 g of *S. rolfsii* inoculum, with control pots sown without inoculum. Thirty days after sowing (DAS), typical wilting symptoms were observed (11). Eight biometric components were calculated to assess the pathogenicity of the ten isolates: leaf length (LL), pod length (PL), leaf width (LW), pod weight (PW), seed length (SL), seed weight (SW), plant height (PH) and shelling percentage (SP) (weight of seeds (kernels) / total weight of pods × 100). The isolate with the highest virulence was further selected for investigation.

Cultural and morphological characterization of *S. rolfsii* isolates

The virulent Sr3 isolate was cultured on PDA to perform cultural, morphological and sclerotial characterization, with incubation at 25 ± 2 °C for 7 to 10 days. Sclerotial and mycelial features were analyzed using a 10x stereoscope (Zeiss, Göttingen, Germany) equipped with an Art Cam 300 MI digital camera (Artray, Tokyo, Japan). Additionally, Field Emission Scanning Electron Microscopy (FE-SEM) in conjuction with Energy Dispersive X-ray Spectroscopy (EDAX) was employed for detailed identification of the isolate. Mycelium and sclerotia were fixed for 24 hr at 28 °C in a 0.005 M phosphate buffer containing 4 % glutaraldehyde on aluminium stubs, which were subsequently coated with chromium using a carbon polaron E-500 sputter coater. Observations were conducted with the CARL ZEISS-SIGMA 300 FE-SEM at magnifications up to 823x. This analysis was carried out at the Central Instrumentation Service Laboratory (CSIL), Department of Physics, Annamalai University yielding ultra-high-resolution images of mycelia and sclerotia.

Molecular conformation of *S. rolfsii* Sr3 isolate

Genomic DNA was sourced from the mycelial mat of the Sr3 isolate, which was cultured for seven days in Potato Dextrose Broth (PDB). The collected fungal biomass was lyophilized and pulverized in liquid nitrogen prior to DNA extraction using the CTAB (Cetyl Trimethyl Ammonium Bromide) protocol. The ITS region, encompassing the 5.8S rDNA gene was amplified using the universal primer pair ITS1 and ITS4. A 25 µL PCR mixture was prepared incorporating 1× PCR buffer, 200 µM of each dNTP, 1.5 mM $MgCl_2$, 0.4 µM of each primer, 1 U of Taq DNA polymerase and approximately 50 ng of template genomic DNA. Thermal cycling conditions included an initial denaturation step at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 1 min. The process concluded with a final extension at 72 °C for 10 min. PCR amplicons were electrophoresed on a 1.2 % agarose gel, stained with ethidium bromide and visualized under ultraviolet light. Sequencing of the amplified products was outsourced to Medauxin (Bangalore) and sequence similarity was determined using BLASTn in the NCBI database. The partial 5.8S rDNA sequence was deposited in GenBank and an accession number was obtained. Phylogenetic analysis was carried out using MEGA version 11 based on the neighbour-joining algorithm, with multiple sequence alignment initially performed using Clustal X v1.83. The analysis incorporated five ITS sequences-four obtained from NCBI and one from the current study. *Alternaria solani* (OQ568225) served as the outgroup and a bootstrap analysis with 1000 replicates was used to evaluate branch support.

Isolation of *Trichoderma* spp. and *Bacillus* spp. isolates

Ten isolates of *Trichoderma* spp. and *Bacillus* spp. were obtained from native rhizospheric soils across Kallakurichi, Cuddalore and Villupuram districts in Tamil Nadu using the dilution plate method on *Trichoderma* Selective Medium (TSM) and nutrient agar medium (12). Plates were incubated at 28±2°C for 72-96 hr. Fungal isolates were purified using the hyphal tip method and bacterial isolates were purified using the streak plate method to obtain contaminant-free pure cultures.

In vitro evaluation of *Trichoderma* spp. and *Bacillus* spp. isolates against Sr3 isolate

The antagonistic potential of ten isolates of *Trichoderma* spp. and *Bacillus* spp. against the virulent Sr3 isolate of the pathogen was evaluated using dual culture method. An actively growing 9 mm mycelial disc of Sr3 was positioned 1 cm from the margin of a PDA plate and on the opposite side, a 9 mm disc of the fungal antagonist was placed. Similarly for *Bacillus* spp., a loopful of

bacterial culture was smeared 1 cm distant from the plate edge that was plated with the pathogen Sr3 isolate as described previously. Control plates were maintained with the pathogen alone. Each treatment was carried out in triplicate and the plates were maintained at 28 ± 2 °C for a period of seven days. The percentage of mycelial inhibition was calculated using the formula described previously (13). The most effective *Trichoderma* and *Bacillus* isolates exhibiting maximum inhibition were selected for further studies.

Molecular characterization of *Trichoderma* spp. Tr4 and *Bacillus* spp. B5 isolates

The molecular identification of the virulent isolates of *Trichoderma* was performed using sequencing of the ITS regions 1 and 4, following the procedure previously described for Sr3. The purified fungal isolate was maintained on PDA medium and used for further research. Similarly, the virulent isolate of *Bacillus* was cultured on Luria Bertani (LB) medium (Hi Media Labs Private Ltd., Mumbai, India). The plates were allowed to incubate at 36 °C for 34 -48 hr, after which they were re-streaked onto fresh LB agar plates. A single bacterial colony was selected and transferred to LB broth for genomic DNA extraction. PCR was performed using the universal primers 27F and 1492R. The primers used were 27F - 5′ (AGA GTT TGA TCM TGG CTC AG) 3′ and 1492R - 5′ (TAC GGY TAC CTT GTT ACG ACT T) 3′. The gene sequence of 16S rRNA were analyzed and compared to existing sequences in GenBank for species identification. A phylogenetic tree was generated using MEGA 11, employing the neighbour joining method with sequence alignment performed using Clustal X v.1.83. The analysis included the isolates from the present study along with four GenBank reference strains. *Ampelomyces quisqualis* (HQ108051.1) and *Streptomyces venezuelae* (AB184836) were used as outgroups for the fungal and bacterial isolates respectively. Branch support was evaluated using 1000 bootstrap replications.

In vitro evaluation of organic amendments against the virulent pathogen (Sr3 isolate)

The poisoned food technique was used to evaluate their antagonistic activity against Sr3 isolate. Vermicompost was sourced from the Department of Agricultural Microbiology, Annamalai University, which was prepared using *Eisenia fetida* fed with cow manure, vegetable peels, rice husk ash and soil after 45 days of composting. 10 g of vermicompost and neem cake were suspended in 90 mL of 0.85 % NaCl, shaken at 100 rpm at 25 ± 2 °C for 1 hr. Further, it was extracted using Whatman® filter paper No. 1, following the procedure outlined previously (14). PDA plates were amended with 5 %, 10 %, 15 % or 20 % extracts of each. Further, it was inoculated with a 9 mm mycelial disc of Sr3, while unamended PDA served as the control. Treatments and control were conducted in five replicates. The percentage of fungal inhibition was calculated using the formula outlined earlier (13).

Compatibility between fungal and bacterial antagonists and organic amendments

The compatibility of the effective fungal (Tr4) and bacterial (B5) bioagents with neem cake and vermicompost was assessed using the poisoned food technique. Neem cake and vermicompost extracts (20 % concentration) were incorporated into PDA, King's B and Nutrient agar media. Later, 15 mL of the amended medium was provided into sterile Petri dishes. A 9 mm mycelial disc of Tr4 was positioned at the edge of the plate and B5 was streaked along the opposite edge. Each treatment was replicated thrice, with three independent plates per replication. Growth was assessed under incubation at ambient room temperature and compatibility was categorized as compatible (+) or incompatible (-).

Pot culture experiment

In 2023, the pot experiment was performed using the susceptible groundnut cultivar TMV 8, comprising 12 treatments, each with three replications and six pots per replication. Talc-based formulations of Tr4 and B5 were prepared according to the procedure described previously (15). Plastic pots (25 cm × 30 cm) were filled with a 5 kg of pot mixture with 1:1:1 ratio of sterilized sand, garden soil and farmyard manure (FYM). The virulent *S. rolfsii* isolate (Sr3) was introduced into the soil using the soil inoculation method by incorporating 10–20 g of sand-maize inoculum per pot prior to sowing. The treatments were as follows: T1 (ST (seed treatment) with *T. asperellum* (Tr4) @ 4 g/kg of seeds + SA (soil application) of *T. asperellum* (Tr4) @ 50 g/pot at the time of sowing (TOS) and 30 days after sowing (DAS)); T2 (ST with *B. licheniformis* (B5) @ 10 g/kg of seeds + SA of *B. licheniformis* (B5) @ 50 g/pot at TOS and 30 DAS); T3 (ST with neem cake extract at 10 % at TOS + SA of neem cake @ 5 g/kg of soil at TOS); T4 (ST with vermicompost extract at 10 % at TOS + SA of vermicompost at 10 g/kg of soil at TOS); T5 (T1 + T2); T6 (T1 + T3); T7 (T1 + T4); T8 (T2 + T3); T9 (T2 + T4); T10 (T1 + T2 + T3 + T4); T11 (ST using carbendazim 50 % WP @ 2g/kg of seeds); T12 (inoculated control) and T13 (healthy control). Stem rot incidence was observed at 30, 60, 90 and 105 DAS and indicated as disease incidence (%). Additionally, growth attributes such as plant height, root length, biomass, number of pods per plant and percentage increase over the control were measured for all treatments (16).

Field experiment

In 2023, a field experiment was carried out at Kalpadai village (11.7836° N, 78.7913° E) in Kallakurichi district, Tamil Nadu, to assess the efficacy of bioformulations and organic amendments on stem rot disease incidence. The study employed a randomized block design (RBD) and was replicated three times. Treatments included: T1 (ST with *T. asperellum* (Tr4) @ 4 g/kg + SA at 1.25 kg/acre); T2 (ST with *B. licheniformis* (B5) at 10 g/kg + SA at 1.5 kg/acre); T3 (ST with neem cake extract at 10 % + SA at 250 kg/acre); T4 (ST with vermicompost extract at 10 % + SA at 450 kg/acre); T5 (T1 + T2); T6 (T1 + T3); T7 (T1 + T4); T8 (T2 + T3); T9 (T2 + T4); T10 (T1 + T2 + T3 + T4); T11 (ST using carbendazim 50 % WP @ 2 g/kg) and T12 (untreated control). Stem rot incidence and yield attributes were recorded as described earlier for pot study.

Data analysis

Analysis of variance (ANOVA) for both a completely randomized design and RBD was performed using OPSTAT software, with a significance level of 5 % along with DMRT analysis was performed using WASP 2.0 (17). Data analysis and visualization were carried out in R Studio 4.4.1 with the dataset cleaned for missing values and outliers. PCA was performed using the 'FactoMineR' and 'factoextra' packages. To assess correlations and detect multicollinearity among variables, a correlation matrix was generated using Pearson's correlation coefficients and visualized with the 'corrplot' package. The resulting heatmap highlighted significant correlations and patterns of multicollinearity (18).

Results and Discussion

Survey, isolation and identification of groundnut stem rot pathogen

Groundnut stem rot disease incidence across the surveyed regions of Kallakurichi, Cuddalore and Villupuram districts in Tamil Nadu varied between 16.57 % and 39.55 %. The highest incidence of 39.55 % was recorded in Kalpadai village of Kallakurichi district, as presented in Table 1. The cultural and morphological aspects of these isolates were isolated and assessed using light microscopy (Supplementary Table 1, Supplementary Fig. 1). These findings were in consistent with previous reports of stem rot disease prevalence in Karnataka, where incidence rates ranged from 10.71-18.50 % (19). Similar trend was observed in a disease survey across six groundnut-growing districts of Tamil Nadu, where Kallakurichi had the highest incidence of 39.03 % (20).

Evaluation of pathogenic variability and its impact on growth parameters

Under artificial inoculation, the Sr3 isolate exhibited the highest disease incidence in overall mean at 44.85 %, whereas Sr9 showed the lowest at 16.61 % (Table 2). Our study was supported by an earlier study that utilized the TMV-2 groundnut variety to assess the pathogenicity of ten *S. rolfsii* isolates (19). Similarly, researchers identified the most virulent isolate among fifty tested, which presented the highest disease incidence (%) (21). Significant variability was observed across all isolates, while SKT showed the greatest virulence (21). In our study, Sr3 notably affected key biometric traits such as SL, LL, SP and PH (Table 3, Supplementary Table 2a). These findings align with a previous study that highlighted the impact of pathogenic variability on yield and plant architectural characteristics such as LL and PH (23). PCA indicated

Table 1. Isolation and identification of groundnut stem rot pathogens from major groundnut growing districts of Tamil Nadu

S. No.	Districts	Name of the village	Isolates	Soil type	Variety	Water source	Disease incidence (%)
1	Kallakurichi	Pottiyam	Sr1	Red loam	TMV 2	Irrigated	37.06^{b} (36.67)
2		Kallipattu	Sr2	Black soil	VRI 2	Rainfed	26.55^{e} (30.86)
3		Kalpadai	Sr3	Red loam	Local	Rainfed	39.55^{a} (38.96)
4		Vadakkanandal	Sr4	Red loam	VRI 4	Irrigated	23.54^{f} (29.02)
5	Cuddalore	Puduchatram	Sr5	Sandy loam	TMV 9	Rainfed	31.01^{c} (33.83)
6		Adoor agaram	Sr6	Sandy loam	TMV 6	Rainfed	28.22^{d} (32.08)
7		Killai	Sr7	Sandy loam	VRI 3	Rainfed	34.09^{bc} (35.72)
8		Pinnathur	Sr8	Sandy loam	Local	Rainfed	18.11^{h} (25.37)
9	Villupuram	Surapattu	Sr9	Red soil	Local	Rainfed	16.57^{i} (24.01)
10		Kakkanur	Sr10	Red soil	ALR 1	Irrigated	20.07^{g} (26.61)
					SED		0.628
					CD ($p \leq 0.05$)		1.331

Means of three replicates were analyzed using ANOVA and treatment differences were compared using DMRT at $P \leq 0.05$.

Table 2. Pathogenicity of ten *S. rolfsii* isolates on groundnut (variety TMV 8) under pot culture conditions

Isolates	Stem rot incidence (%)				Mean
	25 DAS	50 DAS	75 DAS	At harvest	
Sr1	28.51^{b} (32.27)	35.66^{ab} (38.63)	45.06^{b} (42.28)	54.67^{b} (45.94)	40.97^{b} (39.79)
Sr2	15.18^{g} (23.33)	20.03^{e} (26.64)	27.06^{f} (29.79)	36.10^{e} (36.29)	24.59^{e} (29.72)
Sr3	32.95^{a} (35.03)	38.07^{a} (38.09)	49.61^{a} (44.77)	58.80^{a} (50.07)	44.85^{a} (42.04)
Sr4	12.21^{h} (20.45)	18.20^{ef} (25.25)	23.98^{f} (29.31)	32.05^{ef} (34.47)	21.61^{ef} (27.70)
Sr5	20.03^{e} (26.58)	26.49^{cd} (30.97)	36.42^{d} (37.11)	44.06^{cd} (41.58)	31.75^{cd} (34.29)
Sr6	10.34^{i} (18.75)	15.56^{f} (23.23)	20.03^{g} (26.58)	30.55^{fg} (33.55)	19.12 (25.29)
Sr7	26.05^{c} (30.48)	32.09^{b} (34.50)	43.35^{bc} (41.17)	50.55^{bc} (45.31)	38.01^{bc} (38.06)
Sr8	17.23^{f} (24.52)	23.80^{d} (28.95)	32.45^{e} (32.60)	40.09^{d} (38.92)	28.39^{d} (32.19)
Sr9	08.56^{j} (17.56)	11.90^{g} (20.17)	17.55^{h} (24.76)	28.44^{g} (32.22)	16.61^{g} (24.05)
Sr10	23.47^{d} (28.97)	29.06^{bc} (32.62)	40.36^{cd} (39.44)	47.04^{c} (43.30)	34.98^{c} (36.25)
Healthy control	0.00	0.00	0.00	0.00	-
SED	0.528	0.612	0.806	0.944	0.721
CD (P = 0.05)	1.117	1.295	1.706	1.999	1.526

Means of three replicates were analyzed using ANOVA and treatment differences were compared using DMRT at P ≤ 0.05

DAS - Days after sowing

Table 3. Impact of pathogenic variability among ten *S. rolfsii* isolates on biometric parameters

Isolates	LL (cm)	LW (cm)	PL (cm)	PW (cm)	SL (cm)	SW (g)	PH (cm)	SP (%)
Sr1	6.14^{b} (14.34)	3.02^{b} (10.00)	3.07^{b} (10.09)	8.54^{b} (16.99)	0.81^{b} (5.16)	30.45^{b} (33.49)	41.23^{b} (39.94)	38.14^{b} (38.13)
Sr2	8.11^{de} (16.54)	3.67^{d} (11.04)	3.69^{d} (11.05)	20.22^{de} (26.72)	1.40^{d} (6.79)	44.36^{f} (41.76)	57.13^{f} (49.09)	56.78^{f} (48.89)
Sr3	4.62^{a} (12.41)	2.47^{a} (8.47)	2.54^{a} (8.60)	6.78^{a} (15.09)	0.38^{a} (3.53)	28.10^{a} (31.37)	36.10^{a} (36.92)	34.91^{a} (35.79)
Sr4	8.83^{e} (17.28)	3.88^{de} (11.36)	3.81^{de} (11.25)	24.19^{e} (29.46)	1.61^{de} (7.28)	47.50^{g} (43.56)	63.16^{g} (52.63)	59.74^{g} (50.61)
Sr5	7.06^{bcd} (15.40)	3.46^{bcd} (10.72)	3.45^{c} (10.70)	15.66^{bcd} (23.31)	1.12^{c} (6.07)	37.24^{d} (37.60)	49.31^{d} (44.60)	47.10^{d} (43.33)
Sr6	7.90^{d} (16.32)	3.51^{cd} (10.79)	3.56^{cd} (10.32)	18.22^{d} (25.26)	1.29^{cd} (6.52)	40.22^{e} (39.36)	55.21^{e} (47.99)	52.03^{e} (46.16)
Sr7	6.98^{bc} (15.31)	3.33^{bc} (10.51)	3.11^{bc} (10.32)	11.34^{bc} (19.67)	0.99^{bc} (5.71)	34.56^{c} (36.00)	45.56^{c} (42.68)	42.21^{c} (40.51)
Sr8	9.94^{fg} (18.37)	3.97^{ef} (11.49)	4.11^{f} (11.69)	30.45^{fg} (33.49)	1.97^{f} (8.06)	56.19^{i} (48.55)	74.23^{i} (59.49)	67.14^{i} (55.02)
Sr9	10.53^{g} (18.93)	4.12^{f} (11.71)	4.21^{fg} (11.84)	33.12^{g} (35.13)	2.13^{fg} (8.39)	61.33^{j} (51.54)	78.70^{j} (62.51)	73.11^{j} (58.76)
Sr10	9.12^{f} (17.57)	3.81^{def} (11.25)	3.94^{def} (11.44)	27.55^{f} (31.66)	1.83^{def} (7.77)	50.74^{h} (45.42)	68.14^{h} (55.63)	63.15^{h} (52.62)
Healthy control	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
CD (P = 0.05)	1.92	0.65	0.66	2.94	0.58	2.54	9.46	7.54
SE (d)	0.915	0.311	0.317	1.402	1.037	1.212	4.504	3.590

Means of three replicates were analyzed using ANOVA and treatment differences were compared using DMRT at P ≤ 0.05

LL - leaf length; LW - leaf width; PL - pod length; PW - pod weight; SL - seed length; SW - seed weight; PH - plant height; SP - shelling percentage.

that Principal Component 1 (PC1) accounted for most of the variance driven by SL, LL, SP and PH. High positive correlations were identified between SW and PW, whereas LW and PW displayed negative correlations (Supplementary Table 2b, Fig. 1a). These results are consistent with those reported by earlier researchers who observed significant variability among isolates in their PCA of morphological traits under pathogenicity tests (24). Furthermore, the correlation matrix demonstrated strong positive relationships among most variables, with correlation coefficients frequently exceeding 0.90. Notable correlations included LL and PL (0.98), PW and PH (1.00) and SL and SP (0.99). The lowest correlation was observed between LW and PW (0.89) (Fig. 1b). A similar relationship between these variables was also reported by an earlier study (22).

Cultural and morphological characteristics of Sr3 isolate

All the isolates exhibited distinct whitish, hairy mycelial growth. Among them, Sr3 isolate demonstrated the fastest mycelial growth under *in vitro*. It was further examined under a stereoscope to observe its well-known virulent mycelial and sclerotial characteristics (Supplementary Fig. 2). The process of sclerotial development unfolds using FE-SEM in three distinct stages: a) initiation of sclerotial formation; b) subsequent developmental phase and c) attainment of maturity, as depicted in Fig. 2. Consistent morphological variations of *Sclerotium* have been documented globally as reported earlier (23). Aerial mycelium typically appears white with strands exhibiting a fan-shaped expansion. The mycelium produces nearly spherical, shiny-surfaced sclerotia ranging in diameter from 0.7-1.5 mm (24). Sclerotial characteristics typically manifest as dark brown to black with a scattered and peripheral arrangement of sclerotial bodies on the culture media, which may vary between 7 to 15 days after incubation (25).

Molecular confirmation of *S. rolfsii* Sr3 isolate

Based on 18S rDNA sequencing, the virulent Sr3 isolate was identified as *Sclerotium rolfsii* (teleomorph: *Athelia rolfsii*), with a 461bp sequence (Accession No. OQ568182). Phylogenetic analysis confirmed the identity of the isolate by clustering it within a common clade with *A. rolfsii* sequences from GenBank, as shown in Supplementary Fig. 3. Specifically, the isolate Sr3 clustered closely with *A. rolfsii* (OQ172303) with 90 % bootstrap support, confirming its identity as a strain of *A. rolfsii*, distinct from the outgroup *Alternaria solani* (OQ568225). These results are in line with previous research which reported PCR amplification of *S. rolfsii* with a 461bp product showing 100 % similarity to GenBank sequences (29). Similarly, researchers analysed the genetic diversity of ten *S. rolfsii* isolates using ITS primers, obtaining band sizes between 450-700bp (30). A phylogenetic tree was then constructed for *S. rolfsii* using MEGA11, demonstrating genetic similarities.

In vitro evaluation of *Trichoderma* spp. and *Bacillus* spp. isolates against Sr3 isolate

The antagonistic potential of ten *Trichoderma* spp. and *Bacillus* spp. isolates was evaluated against the virulent Sr3 isolate using the dual culture assay, as summarized in Table 4. Among the isolates, *Trichoderma spp.* Tr4 and *Bacillus spp.* B5 demonstrated the highest efficacy, achieving 88.88 % and 70.55 % inhibition of Sr3 mycelial growth respectively (Supplementary Fig. 4). The high inhibition observed with Tr4 isolate aligns with previous reports which found similar *Trichoderma* isolates to be effective in suppressing *S. rolfsii* growth (11). The mycoparasitic nature of *Trichoderma* involves direct attack on *S. rolfsii* mycelium, producing lytic enzymes that degrade the pathogen's cell wall (26). *Bacillus* spp., including strains F-1 and R-11, showed strong antifungal activity (> 10 mm inhibition zones), likely owing to the presence of both volatile and non-volatile metabolites like lipopeptides and antibiotics. They also disrupt fungal growth by altering mycelial structure and reducing sclerotium formation, essential for *S. rolfsii* survival (27).

Molecular characterization of Tr4 and B5 isolates

PCR amplification of genomic DNA from Tr4 and B5 was carried out using universal primers. The amplification yielded amplicons of 515bp and 1156bp respectively. Sequence analysis was

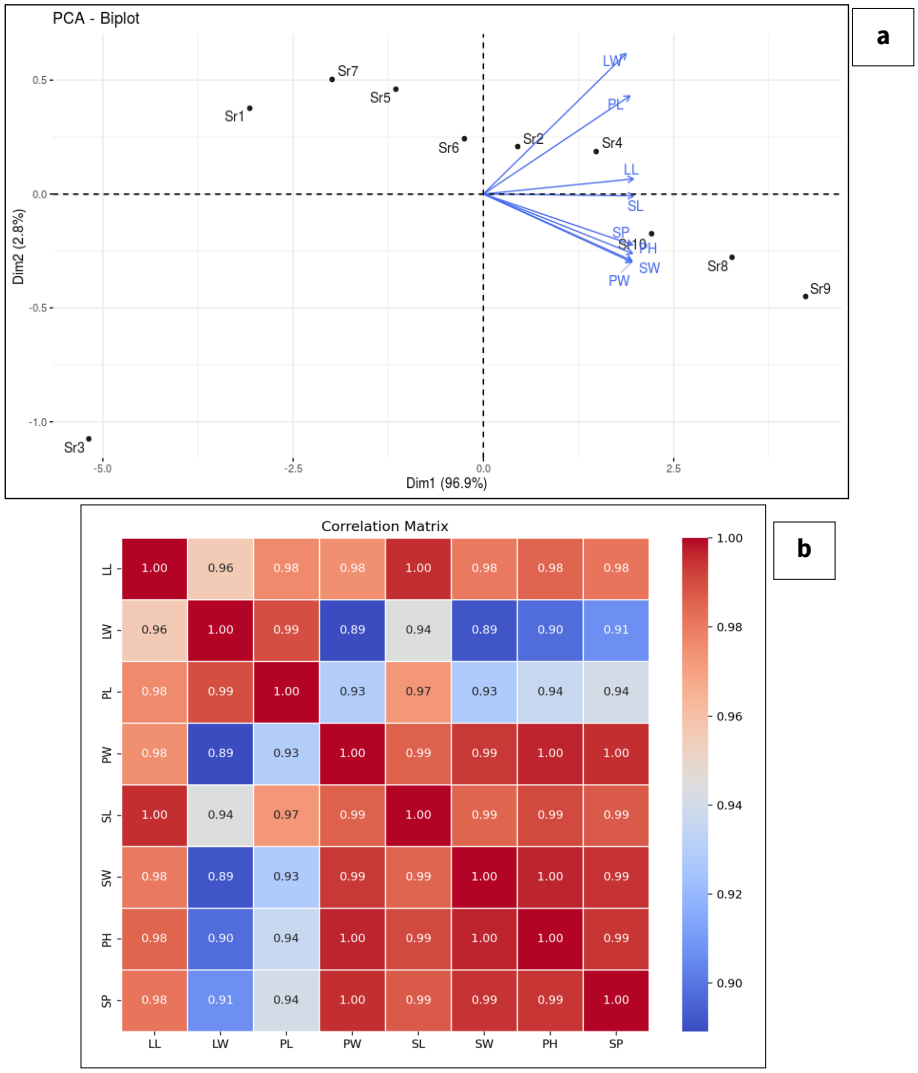

Fig. 1. Effect of pathogenic diversity among *S. rolfsii* isolates on biometric parameters: (a) PCA biplot representing all ten isolates (Sr1-Sr10), with vectors corresponding to biometric parameters (PCs); (b) correlation matrix illustrating the relationships among the seven component variables.

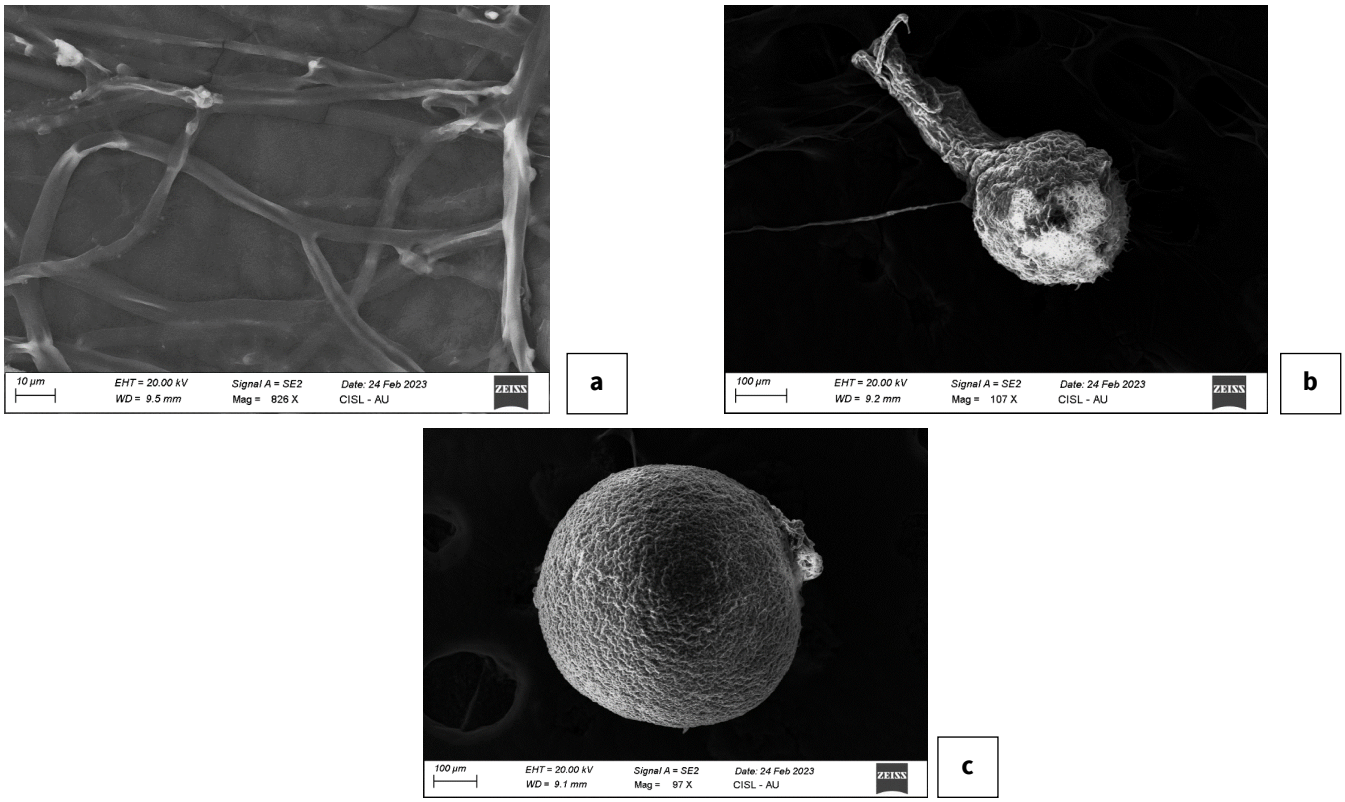

Fig. 2. FE-SEM images of *S. rolfsii* (Sr3). The process of sclerotial development unfolds in three distinct stages: (a) initiation of sclerotial formation; (b) subsequent developmental phase; (c) attainment of maturity.

Table 4. *In vitro* evaluation of *Trichoderma* spp. and *Bacillus* spp. isolates against *S. rolfsii* (Sr3) using the dual culture technique

S. No.	Isolates of *Trichoderma* spp.	Mycelial growth of *S. rolfsii* (cm) (5 DAI)	Percentage of Inhibition over control	Isolates of *Bacillus* spp.	Mycelial growth of *S. rolfsii* (cm) (5 DAI)	Percentage of Inhibition over control
1	Tr1	3.28^{e} (10.43)	63.55	B1	7.12^{ij} (15.47)	20.88
2	Tr2	4.91^{i} (12.80)	45.44	B2	6.43^{gh} (14.68)	28.55
3	Tr3	2.22^{cd} (8.56)	75.33	B3	3.78^{c} (11.21)	58.00
4	Tr4	4.55^{g} (12.31)	49.44	B4	4.35^{d} (12.03)	51.66
5	Tr5	1.00^{a} (5.73)	88.88	B5	5.98^{g} (14.15)	33.55
6	Tr6	1.97^{c} (8.06)	78.11	B6	6.95^{i} (15.28)	22.77
7	Tr7	3.90^{f} (11.38)	56.66	B5	2.65^{a} (9.36)	70.55
8	Tr8	4.33^{g} (12.01)	51.88	B8	5.45^{f} (13.50)	39.44
9	Tr9	1.41^{b} (6.81)	84.33	B9	4.80^{e} (12.65)	46.66
10	Tr10	2.86^{d} (9.73)	68.22	B10	3.34^{b} (10.53)	62.88
11	Control	9.00^{h} (17.45)	-	Control	9.00^{k} (17.45)	-
SED		0.102	-	SED	0.140	-
CD (P = 0.05)		0.213	-	CD (P = 0.05)	0.291	-

Means of three replicates were analyzed using ANOVA and treatment differences were compared using DMRT at P ≤ 0.05.

DAI - Days after inoculation

performed to assess genetic similarities and coherence of Tr4 and B5 through a BLAST search on the NCBI database. The analysis revealed that the Tr4 and B5 isolates shared more than 99 % and 100 % nucleotide identity respectively. Partial sequence data were submitted to GenBank, where accession numbers OQ581458 and OR195791 were assigned to *T. asperellum* and *B. licheniformis*. Phylogenetic analysis confirmed the identities of the isolates Tr4 and B5 by clustering them within well-supported clades alongside reference sequences from GenBank. The isolate Tr4 (OQ581458.1) grouped with *Trichoderma asperellum* strains (PV470997.1 and PV413029.1) with 99 % bootstrap support, confirming its identity as *T. asperellum*, distinct from the outgroup *Ampelomyces quisqualis* (HQ108051.1). Similarly, the isolate B5 (OR195791.1) clustered with *B. licheniformis* (PQ780529.1 and PV471946.1) with 51 % bootstrap support, verifying it as a strain of *B. licheniformis*, distinct from the outgroup *S. venezuelae* (AB184836.1) (Supplementary Fig. 5). Molecular identification is crucial for accurately distinguishing species within complex genera like *Trichoderma*, where morphological features often overlap and are not sufficient for precise classification. In line with previous studies PCR-based identification of *Trichoderma* isolates from the shallot rhizosphere successfully differentiated *T. asperellum* from other *Trichoderma* species based on distinct amplicon sizes (28). Similarly, the 18S rRNA gene sequence analysis of *T. asperellum* yielded a 100 % similarity score with known sequences, confirming the molecular characterization of this biocontrol agent (29). The bacterial agent *B. licheniformis* was identified through 16S rRNA sequencing, showing 99.17 % similarity with other *B. licheniformis* strains in the EZBio-Cloud database. A phylogenetic tree was constructed using MEGA11 which illustrated the evolutionary relationships of the isolates (30).

In vitro evaluation of organic amendments against Sr3 isolate

The antimicrobial activity of organic amendments including neem cake extract and vermicompost extract was assessed against Sr3 isolate using the poisoned food technique. Vermicompost extract at 20 % concentration demonstrated the highest efficacy, inhibiting mycelial growth by 88.22 % followed by neem cake extract at 20 % concentration, which achieved 78.01 % inhibition compared to the control (Table 5, Supplementary Fig. 6). The *in vitro* inhibitory effects of vermicompost extracts observed in this study align with findings from a previous study that reported mycelial inhibition rates of 54.2 %, 61.7 %, 65.2 %, 74.0 % and 87.0 % against various soil-borne fungal pathogens (36). Similarly, researchers demonstrated even higher suppression rates of 90.6 %, 80.2 % and 65.1 % at 100 % concentration of vermicompost extracts, further supporting its potential as a natural biocontrol agent (37). Furthermore, vermicompost originally having bioactive principles responsible for antifungal activity (31-34) (Supplementary Table 3). The effectiveness of neem cake filtrates observed in our study, with a maximum suppression of 1.29 cm at 15 % concentration, is consistent with the findings of earlier researchers, who also reported significant fungal growth inhibition by neem cake (38).

Compatibility between fungal and bacterial antagonists and organic amendments

The *in vitro* compatibility of fungal and bacterial antagonists with selected organic amendments was first evaluated, followed by pot and field trials. The results showed no inhibition of growth of the biocontrol agents when combined with neem cake and vermicompost, indicating compatibility between Tr4 and B5 with these amendments (Supplementary Table 4, Supplementary Fig. 7). Furthermore, the consortial effect of vermicompost, *Bacillus* spp. and *Trichoderma* spp. has been demonstrated in previous studies, highlighting their high compatibility and synergistic potential in disease suppression (35). Similarly, compatibility between antagonistic organisms and neem cake was also evident, as reported by previous researchers where the combined effect notably enhanced the control of soil-borne fungal pathogens (40).

Table 5. Evaluating the efficacy of organic amendments on the mycelial growth of *S. rolfsii* (Sr3) by poison food technique

S. No.	Source	Mycelial growth (cm)							
		5 %	Percentage of inhibition over control	10 %	Percentage of inhibition over control	15 %	Percentage of inhibition over control	20 %	Percentage of inhibition over control
	Vermicompost	5.95[a] (14.11)	33.88	4.21[a] (11.84)	53.22	2.01[a] (8.15)	77.66	1.06[a] (2.22)	88.22
	Neem cake	7.52[b] (15.91)	16.44	4.50[b] (12.24)	50.00	3.20[b] (10.30)	64.44	1.98[b] (5.76)	78.01
	Control	9.00	0.00	9.00	0.00	9.00	0.00	9.00	0.00
	SED	1.037	-	0.115	-	0.063	-	0.059	-
	CD (P = 0.05)	2.284	-	0.253	-	0.140	-	0.129	-

Pot culture experiment

Treatment T10, which included the ST and SA of *T. asperellum* (Tr4), *B. licheniformis* (B5), neem cake and vermicompost extracts, resulted in a significant reduction in stem rot incidence of 74.53 % on 105 DAS compared to the untreated control. This treatment also enhanced plant growth, with improved parameters such as PH (55.10 cm), root length (19.42 cm) and biomass (27.72 g/plant) (Table 6, Fig. 3, 4 Supplementary Fig. 8). These findings align with previous research which reported that neem cake effectively inhibited *Fusarium oxysporum* f. sp. *cumini* both *in vitro* and *in vivo*, with mustard cake showing similar results (41). This supports the growing recognition of organic amendments and biocontrol agents as effective tools for managing soil-borne fungal diseases. In accordance with earlier studies, bioagents play a vital role in reducing fungal disease incidence across various crops (42). The results indicate that while *T. asperellum* T13 effectively controlled *A. rolfsii* in controlled conditions, its combination with vermicompost consistently improved disease suppression. Alone, *T. asperellum* T13 may not provide adequate control due to strain-specific interactions (36). In a similar vein, organic amendments such as neem, mustard and sesame cakes significantly reduced disease incidence compared to untreated controls (37). These results emphasize the effectiveness of integrated approaches involving biocontrol agents and organic amendments in managing soil-borne fungal diseases and promoting plant growth.

Field experiment

The combined treatment (T10) of *T. asperellum* (Tr4), *B. licheniformis* (B5), neem cake and vermicompost extracts with ST and SA significantly reduced stem rot disease and enhanced plant growth, yielding 734 kg/acre, 39 pods/plant, 57.78 cm plant height and 39.22 g/plant of biomass (Table 7, Fig. 5, 6, Supplementary Fig. 9) which is followed by T11 (ST with carbendazim 50 % WP @ 2 g/kg) recorded 690 kg/acre, 36 pods/plant, 55.09 cm plant height and 36.00 g/plant of biomass. This is consistent with earlier research, which found better control of stem rot when combining *T. longibrachiatum* and *T. asperellum* (44). Further, researchers found that raw oil cakes (neem, madhuca and simarouba) reduced *Alternaria* leaf spot severity in chilli, emphasizing the potential of organic amendments for disease management (38). The application of bacterial consortia (*Priestia endophytica*, *B. licheniformis* and *P. flexa*) significantly promoted plant growth, as supported by earlier research (39). In line with a previous study, combining vermicompost and mealworm frass reduced *R. solani* incidence, fostering better growth (40). These results highlight the efficacy of integrated treatments combining organic amendments and microbial inoculants.

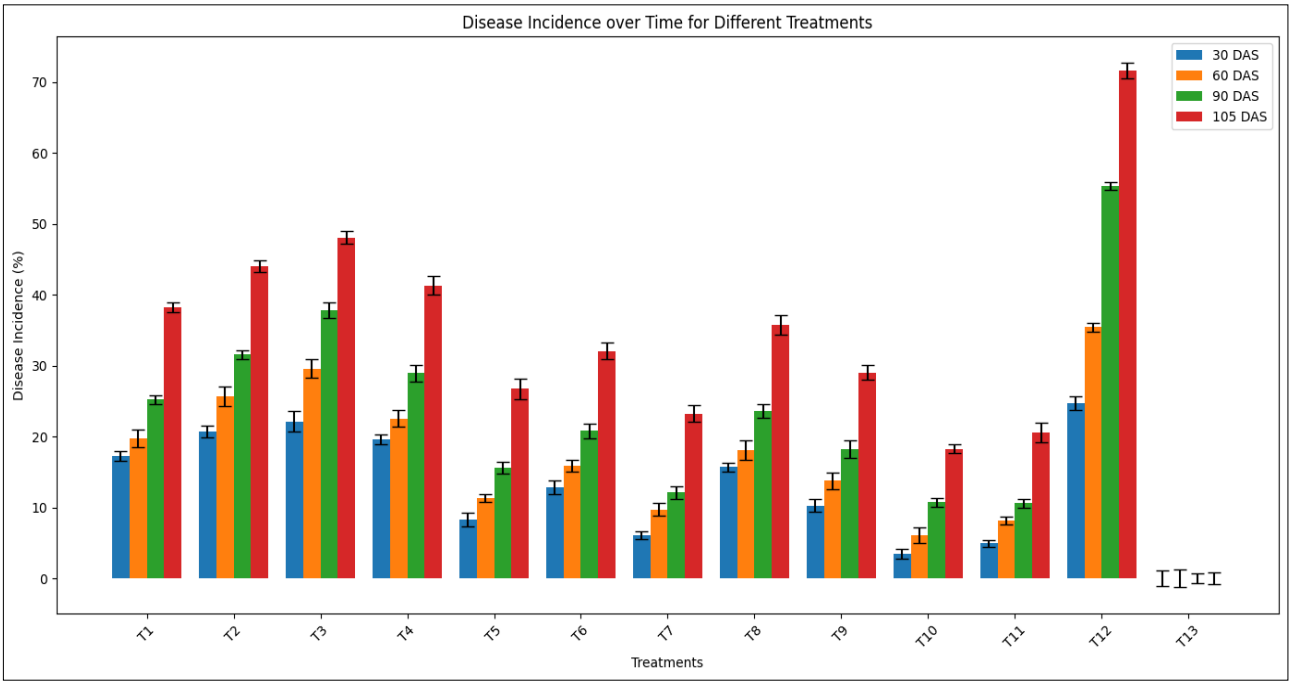

Fig. 3. Efficacy of biocontrol agents and organic amendments on stem rot disease incidence in groundnut (*Arachis hypogaea* L. var. TMV 8) under pot culture conditions.

Table 6. Effect of biocontrol agents and organic amendments against stem rot incidence in groundnut under pot culture condition

		Disease incidence (%)								Growth and yield attributes				
Tr. No.	Germination (%)	30 DAS	Percentage disease reduction over control	60 DAS	Percentage disease reduction over control	90 DAS	Percentage disease reduction over control	At harvest (105 DAS)	Percentage disease reduction over control	Plant height (cm)	Root length (cm)	Biomass (g/ plant)	No. of pods / plant	Percentage increase over control
T1	82.11^{h}	17.22^{h} (24.51)	30.19	19.75^{h} (26.38)	44.20	25.18^{h} (30.11)	54.46	38.22^{h} (38.18)	46.58	32.90^{f}	12.85^{e}	20.04^{e}	14.20^{h}	59.29
T2	78.90^{j}	20.67^{j} (27.04)	16.21	25.67^{j} (30.44)	27.48	31.52^{j} (34.15)	43.00	44.02^{j} (41.56)	38.47	28.77^{g}	09.18^{fg}	16.33^{g}	11.23^{j}	48.53
T3	76.22^{k}	22.09^{k} (28.02)	10.45	29.57^{k} (32.93)	16.46	37.80^{k} (37.93)	31.64	48.07^{k} (43.89)	32.81	26.90^{gh}	08.27^{g}	14.90^{h}	09.78^{k}	40.89
T4	80.25^{i}	19.55^{i} (26.23)	20.75	22.50^{j} (28.31)	36.44	28.90^{j} (32.51)	47.73	41.27^{i} (39.97)	42.38	30.03^{fg}	10.05^{f}	19.76^{f}	12.90^{i}	55.19
T5	90.45^{d}	8.22^{d} (16.66)	66.68	11.34^{d} (19.67)	67.96	15.61^{d} (23.27)	71.77	26.72^{d} (31.12)	62.65	43.22^{d}	16.03^{c}	23.89^{c}	21.45^{d}	73.05
T6	85.70^{f}	12.89^{f} (21.03)	47.75	15.88^{f} (23.48)	55.14	20.77^{f} (27.11)	62.44	32.07^{f} (34.49)	55.17	38.45^{e}	14.06^{d}	21.07^{d}	16.00^{f}	63.87
T7	92.08^{c}	6.07^{c} (14.26)	75.39	9.67^{c} (18.11)	72.68	12.10^{c} (20.35)	78.11	23.19^{c} (28.78)	67.58	46.80^{c}	17.52^{b}	24.15^{bc}	24.89^{c}	76.77
T8	83.49^{g}	15.67^{g} (23.31)	36.48	18.04^{g} (25.13)	49.03	23.60^{g} (29.06)	57.23	35.69^{g} (36.68)	50.11	35.05^{ef}	13.78^{de}	20.82^{de}	14.35^{g}	59.72
T9	87.21^{e}	10.22^{e} (18.64)	58.57	13.77^{e} (21.77)	61.10	18.20^{e} (25.25)	67.08	29.02^{e} (32.59)	59.44	41.94^{de}	15.34^{cd}	22.06^{cd}	18.40^{e}	68.58
T10	96.55^{a} `	3.45^{a} (10.68)	86.01	6.09^{a} (14.26)	82.79	10.70^{a} (19.12)	80.65	18.22^{a} (25.26)	74.53	55.10^{a}	19.42^{a}	27.72^{a}	29.36^{a}	80.31
T11	94.20^{b}	4.90^{b} (12.78)	80.13	8.17^{b} (16.60)	76.92	10.57^{b} (18.97)	80.88	20.54^{b} (26.94)	71.27	50.78^{b}	18.76^{ab}	25.07^{b}	26.50^{b}	78.18
T12	69.56^{m}	24.67^{l} (29.78)	-	35.40^{l} (36.51)	-	55.30^{l} (48.04)	-	71.55^{l} (57.76)	-	22.10^{i}	07.22^{h}	12.69^{i}	07.22^{l}	19.94
T13	73.29^{l}	0.00	-	0.00	-	0.00	-	0.00	-	15.09^{j}	05.05^{i}	09.70^{j}	05.78^{m}	-
SED	1.61	0.33	-	0.42	-	0.54	-	0.76	-	0.77	0.32	0.43	0.40	-
CD (P = 0.05)	3.36	0.69	-	0.88	-	1.13	-	1.59	-	1.60	0.67	0.91	0.83	-

Means of three replicates were analyzed using ANOVA and treatment differences were compared using DMRT at P ≤ 0.05

Table 7. Effect of biocontrol agents and organic amendments against stem rot incidence in groundnut under field condition

Tr. No.	Germination (%)	Disease incidence (%)								Growth and yield attributes				
		30 DAS	Percentage disease reduction over control	60 DAS	Percentage disease reduction over control	90 DAS	Percentage disease reduction over control	At harvest (105 DAS)	Percentage disease reduction over control	Plant height (cm)	Biomass (g/ plant)	No. of pods / plant	Yield (kg/ac)	Percentage increase over control
T1	82.10^{g}	22.17^{h} (28.08)	23.99	27.60^{h} (31.69)	19.20	28.50^{h} (32.26)	30.30	29.44^{h} (32.85)	49.63	37.80^{h}	21.15^{f}	20.00^{h}	536^{f}	42.55
T2	78.39^{h}	25.06^{j} (30.03)	14.08	29.55^{j} (32.92)	13.49	31.78^{j} (34.31)	22.27	32.67^{j} (34.86)	44.10	30.14^{j}	15.22^{h}	16.00^{j}	503^{gh}	25.24
T3	75.08^{i}	27.97^{k} (31.92)	04.11	31.09^{k} (33.88)	08.98	33.10^{k} (35.11)	19.29	34.22^{k} (35.80)	41.45	27.33^{k}	12.88^{i}	13.00	477^{h}	21.17
T4	81.72^{gh}	23.40^{j} (28.92)	19.78	28.34^{i} (32.16)	17.03	30.41^{i} (33.46)	25.62	31.22^{i} (33.96)	46.58	34.67^{i}	19.05^{g}	18.00^{i}	515^{g}	26.99
T5	88.58^{d}	11.90^{d} (20.17)	59.20	13.75^{d} (21.76)	59.74	17.29^{d} (24.57)	57.71	22.30^{d} (28.17)	61.84	48.57^{d}	30.11^{c}	33.00^{d}	603^{d}	37.64
T6	85.34^{e}	17.85^{f} (24.99)	38.80	21.45^{f} (27.58)	37.20	23.65^{f} (29.09)	42.16	26.65^{f} (31.08)	54.40	43.83^{f}	25.10^{e}	25.00^{f}	564^{e}	33.33
T7	90.81^{c}	9.29^{c} (17.74)	68.15	11.98^{c} (20.24)	64.92	15.90^{c} (23.49)	61.11	19.80^{c} (26.42)	66.12	51.45^{c}	33.51^{bc}	35.00^{c}	641^{c}	41.34
T8	84.30^{f}	20.18^{g} (26.69)	30.81	24.66^{g} (29.77)	27.81	26.81^{g} (31.18)	34.43	28.51^{g} (32.27)	51.22	39.01^{g}	23.04^{ef}	22.00^{g}	552^{ef}	31.88
T9	87.01^{de}	14.43^{e} (22.32)	50.53	17.84^{e} (24.98)	47.77	20.63^{e} (27.01)	49.54	25.09^{e} (30.05)	57.07	46.98^{e}	28.47^{d}	28.00^{e}	588de	36.05
T10	95.39^{a}	3.55^{a} (10.84)	87.82	7.22^{a} (15.56)	78.86	11.10^{a} (19.42)	72.85	14.09^{a} (22.04)	75.89	57.78^{a}	39.22^{a}	39 .00^{e}	734^{a}	48.77
T11	93.22^{b}	6.20^{b} (14.41)	78.74	9.89^{b} (18.32)	71.04	13.78^{b} (21.79)	66.29	17.77^{b} (24.93)	69.59	55.09^{b}	35.03^{b}	36.00^{b}	690^{b}	45.50
T12	71.10^{j}	29.17^{l} (32.68)	-	34.16^{l} (35.76)	-	40.89^{l} (39.75)	-	58.45^{l} (49.86)	-	23.08^{l}	10.06^{j}	09.00	376^{i}	-
SED	1.66	0.38	-	0.47	-	0.52	-	0.57	-	0.87	0.56	0.57	11.22	-
CD (P =0.05)	3.47	0.80	-	0.98	-	1.10	-	1.20	-	1.82	1.17	1.20	23.43	-

Means of three replicates were analyzed using ANOVA and treatment differences were compared using DMRT at P ≤ 0.05

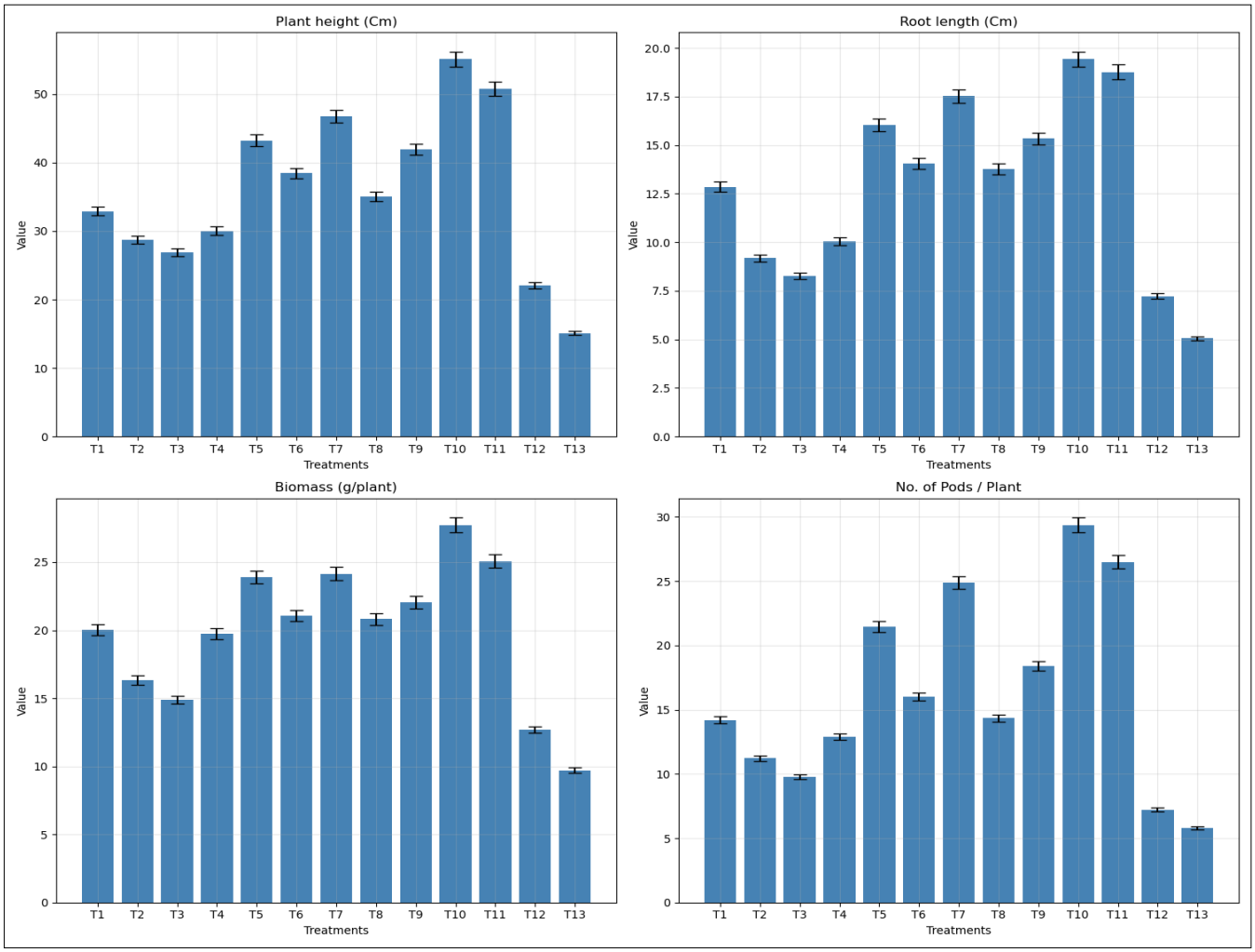

Fig. 4. Efficacy of biocontrol agents and organic amendments on growth and yield attributes on stem rot infected groundnut (var. TMV 8) plants under pot culture conditions.

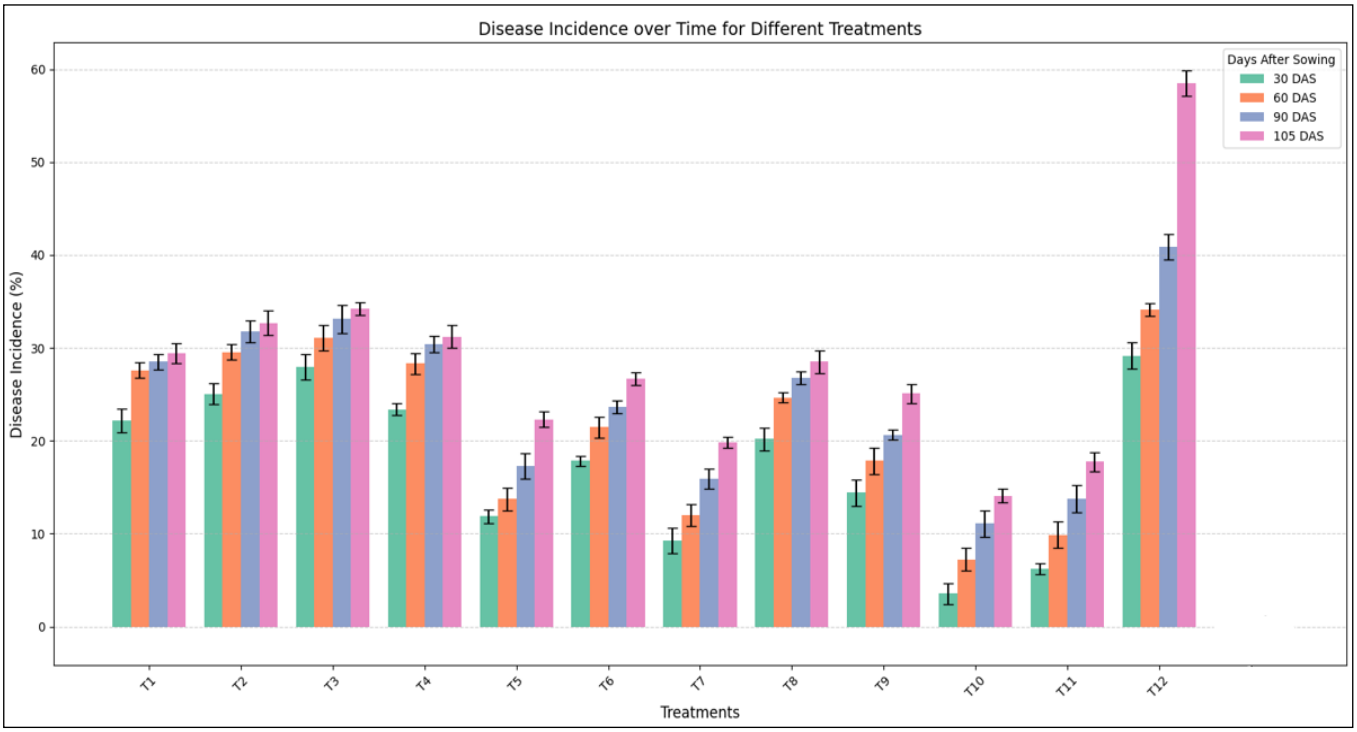

Fig. 5. Efficacy of biocontrol agents and organic amendments on stem rot disease incidence in groundnut (var. TMV 8) plants under field experiment.

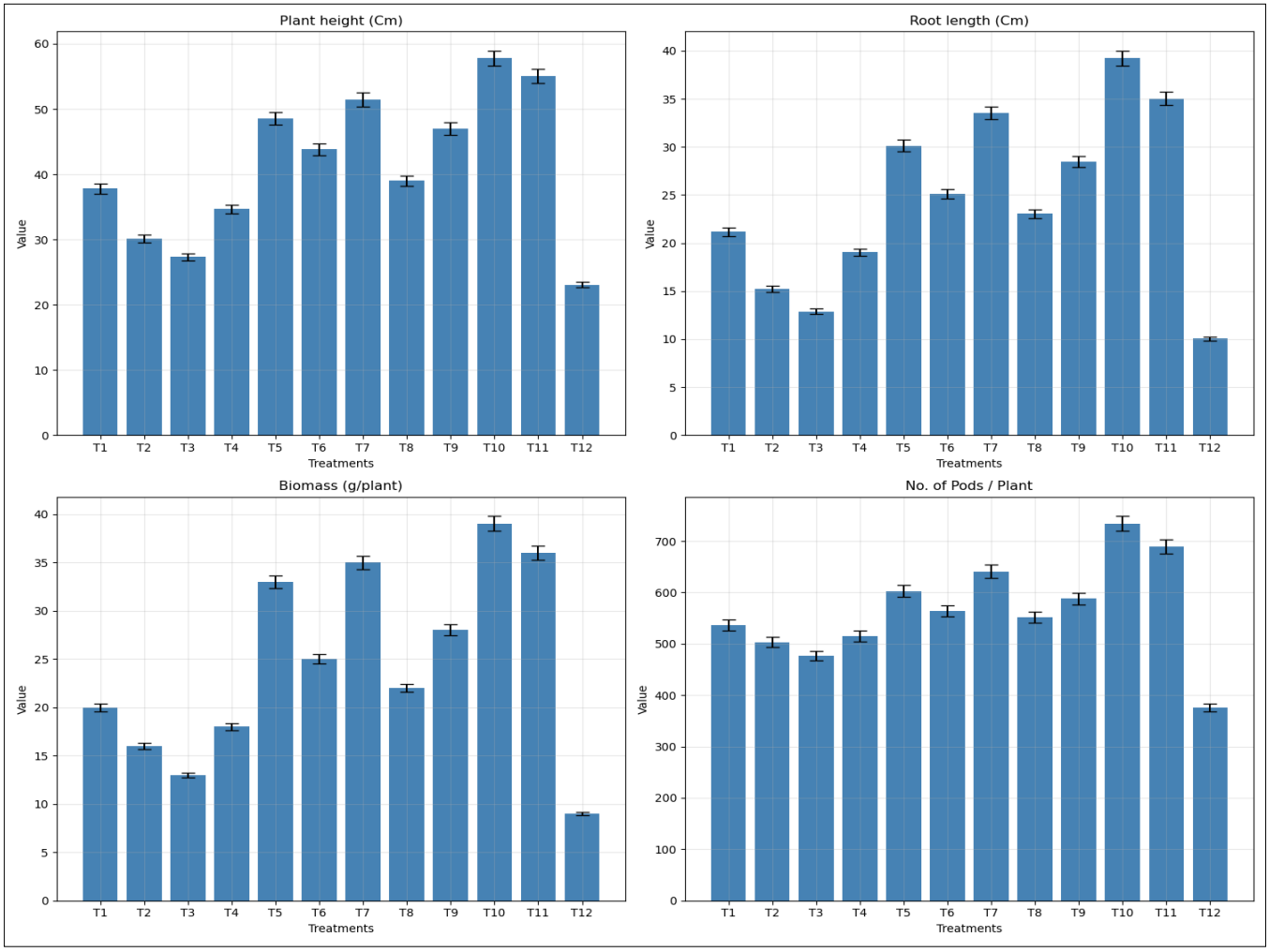

Fig. 6. Efficacy of biocontrol agents and organic amendments on growth and yield attributes on stem rot infected groundnut (var. TMV 8) plants under field experiment.

Conclusion

In conclusion, the combined application of *T. asperellum* (Tr4), *B. licheniformis* (B5), neem cake and vermicompost effectively reduced stem rot incidence in groundnuts while significantly improving plant growth and yield parameters. Molecular characterization validated the identity of the biocontrol agents and *in vitro* compatibility assays confirmed their synergistic interaction with organic amendments. This holistic, environmentally sustainable strategy offers a viable and effective solution for managing stem rot in groundnut cultivation.

Compliance with ethical standards

Conflict of interest: The authors have no conflicts of interest to declare.

Ethical issues: None

Declaration of generative AI and AI-assisted technologies in the writing process

Grammarly AI was used to improve the language and readability of the manuscript, with appropriate caution.

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Authors' contributions

In this study, RP and SRT conceptualized the research, designed the methodology and supervised data collection. JS conducted the investigations, performed statistical analyses and drafted the manuscript. DS and PA critically reviewed the manuscript for intellectual rigor. All authors made substantial contributions to ensure the accuracy and completeness of the study.

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