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RESEARCH ARTICLE

Functional proteomics in groundnut: Unveiling resistance mechanisms against stem rot using seaweed and bioinoculant

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Abstract

Sclerotium rolfsii, a soil-borne fungal pathogen with a broad host range, poses a major threat to groundnut cultivation by causing stem rot disease, leading to significant yield losses. In this study, *S. rolfsii* and five *Trichoderma* spp. isolates were isolated. PCR amplification using universal fungal primers viz., ITS-1 and ITS-4 confirmed the identity of *S. rolfsii* via BLAST analysis, which was further validated using species-specific primers SR1-F and SR1-R. The sequence was submitted to GenBank under the accession number MZ920141. Antagonistic potential of five *Trichoderma* isolates was assessed, among which *Trichoderma asperellum* (Tr1) exhibited the highest mycelial inhibition (73.81 %) in dual culture and up to 89.11 % inhibition in poisoned food assays. Volatile metabolites from Tr1 significantly suppressed mycelial growth (67.56 %) and sclerotial production (91.22 %). Molecular identification of Tr1 via ITS and TEF1 gene sequencing confirmed it as *T. asperellum*, with GenBank accession number OL872253. Additionally, solvent extracts of marine macroalgae, particularly *Sargassum wightii* (10 %), showed potent antifungal activity (87.56 % inhibition). A pot culture study combining Tr1 and *S. wightii* extract significantly reduced stem rot incidence (84.93 %) and improved plant growth as well as yield parameters. Protein profiling using 2D-PAGE and MALDI-TOF analysis revealed unique expression of defense-related proteins such as Peptidyl-prolyl cis-trans isomerase, bHLH145 and 1, 8-cineole synthase in treated plants. Functional analysis indicated their involvement in auxin transport, transcriptional regulation and secondary metabolite biosynthesis, contributing to plant immune responses. These findings highlight the synergistic potential of Tr1 and marine macroalgal extracts in sustainable management of *S. rolfsii*, while proteomic insights provide a molecular basis for induced resistance in groundnut.

Keywords: brown seaweed; groundnut; proteomics; S. *rolfsii*; *T. asperellum*

Introduction

Groundnut (*Arachis hypogaea* L.), a commercially important oilseed crop, is cultivated widely in tropical and subtropical regions. Renowned for its high nutritional value and versatility, groundnut serves as a critical source of protein, oil and fodder (1). However, the productivity of groundnut is severely threatened by numerous diseases, among which stem rot caused by the soil-borne fungal pathogen *S. rolfsii* is one of the most destructive (2). This pathogen has a wide host range and thrives under diverse environmental conditions, making its management particularly challenging. The disease often results in substantial yield losses, adversely affecting both production and farmer income. Traditional methods for controlling *S. rolfsii*, such as chemical fungicides, have limitations due to environmental concerns, pathogen resistance and negative impacts on soil health and non-target organisms (3). Consequently, there is a growing emphasis on eco-friendly and sustainable alternatives such as the use of biological control agents and natural products derived from marine and terrestrial resources (4).

T. asperellum, a well-known fungal biocontrol agent has demonstrated efficacy in managing a broad range of soil-borne pathogens. Its antagonistic mechanisms include competition for nutrients, mycoparasitism, production of antifungal secondary metabolites and induction of resistance in plants (5). Additionally, marine macroalgae particularly brown seaweeds like *S. wightii* are emerging as promising natural resources for plant disease manage -ment (6). Rich in bioactive compounds such as polysaccharides, phenolics and terpenoids, these seaweeds exhibit antimicrobial properties and enhance plant

growth and immunity (7). The combination of *T. asperellum* and *S. wightii* presents a novel strategy to combat *S. rolfsii*-induced stem rot in groundnut while promoting environmental sustainability.

Understanding the molecular dynamics of host-pathogen interactions and the contribution of biocontrol agents in inducing plant defense mechanisms is crucial for developing effective management strategies (8). Proteomic analysis has emerged as a powerful approach to elucidate these interactions by identifying differentially expressed proteins involved in plant defense responses. By using advanced proteomic approaches like two-dimensional electrophoresis (2D-PAGE) and mass spectrometry (MALDI-TOF MS) techniques, researchers can uncover key proteins and pathways associated with resistance mechanisms (9).

This study investigates the efficacy of extract of *S. wightii* and *T. asperellum* in suppressing *S. rolfsii* and enhancing groundnut resistance under both *in vitro* and *in vivo* conditions. Furthermore, it aims to unravel the molecular underpinnings of this enhanced resistance by profiling differentially expressed proteins in the treated plants. The findings of our research not only contribute to our understanding of plant defense mechanisms but also provide insights into the potential application of biocontrol agents and marine resources in sustainable agriculture.

Materials and methods

Isolation and identification of stem rot pathogen

S. rolfsii, the causative agent of stem rot in groundnut was isolated from diseased plant tissues collected in Aalathur, Perambalur district, Tamil Nadu using standardized protocols (10). Morphological identification was performed, followed by molecular characterization using Internal Transcribed Spacer (ITS) primer pairs ITS1 (5′-CTT GGT CAT TTA GAG GAA GTA A-3′) and ITS4 (5′-TCC TCC GTT ATT GAT ATG C-3′) through PCR (polymerase chain reaction) (10). PCR was initiated with a denaturation step at 94 °C for one minute, followed by 30 repeated cycles comprising denaturation (94 °C, 1 min), primer annealing (58 °C, 1 min) and DNA synthesis (72 °C, 1 min). To complete the amplification process, a final elongation step was carried out at 72 °C for five minutes. The amplified DNA fragments were resolved on a 1.2 % agarose gel, treated with ethidium bromide for staining and visualized under ultraviolet illumination using a transilluminator. The PCR products were then sequenced and identified through comparison with sequences in the NCBI database. For the precise molecular identification of *S. rolfsii*, the species-specific primer pair SR1-F (5′-GCCGTTTGTGTTGCATTTAC-3′) and SR1-R (5′-TTCGAAAGTGCGTGTGTCAG-3′) (11) was utilized. These primers enable the accurate differentiation of *S. rolfsii* from other fungal species, ensuring specificity in diagnostic applications.

Isolation of fungal biocontrol agents

Five native fungal isolates of *Trichoderma* spp. were isolated from the rhizosphere microbiota of Parigam village, Kallakurichi district, Tamil Nadu using standardized protocols (12). Morphological identification was done using a stereomicroscope at the Molecular Lab, Department of Plant Pathology, Annamalai University.

In vitro evaluation of fungal antagonists against *S. rolfsii*

The inhibitory effect of five different *Trichoderma* species on *S. rolfsii* isolate was assessed through a dual culture assay. Seven-day-old cultures of both the *S. rolfsii* and *Trichoderma* spp. were inoculated at opposite peripheries of Potato Dextrose Agar (PDA) plates, with culture plates without *Trichoderma* serving as controls. The study was carried out in triplicate, with plates incubated at 28 °C. The radial expansion of *S. rolfsii* was measured to evaluate the suppressive activity of the *Trichoderma* species.

To assess the impact of volatile compounds, the inverted plate assay was performed. The antagonists and pathogen were inoculated separately onto PDA plates and positioned face-to-face in an inverted manner. The plates were closed tightly with adhesive tape or parafilm to prevent direct contact and incubated at 28 ± 1 °C. The reduction in *S. rolfsii* mycelial growth and sclerotia production was recorded and the inhibitory effect of volatile compounds was analyzed.

Furthermore, the poisoned food method was employed to assess the impact of non-volatile compounds produced by Trichoderma. The isolates were inoculated in PD broth (Potato Dextrose Broth) for 15 days at 28 ± 2 °C and the culture filtrate was obtained through vacuum filtration using a bacteriological filter. The sterile filtrates were incorporated into molten PDA at concentrations of 5 %, 10 %, 15 % and 20 %, poured into Petri dishes (15 mL per plate) and allowed to solidify. A five-day-old *S. rolfsii* mycelial disc was placed at the centre of each plate, with PDA plates without *Trichoderma* filtrate serving as controls. Three replications were maintained and pathogen growth was measured after five days of incubation.

In all methods, the percentage inhibition of *S. rolfsii* growth was determined using the standard formula: Inhibition (%) (I) = [(C - T) / C] × 100, where, I indicates the percentage of pathogen growth inhibition, C represents the radial growth observed in the control and T refers to the radial growth measured in the treated plates (13).

Molecular characterization of fungal biocontrol agent

Molecular characterization of *Trichoderma* spp. was performed using ITS primer pairs, as described for *S. rolfsii*. Additionally, for the identification of *T. asperellum*, the species-specific primer pair T2AF (5′-CTCTGCCGTTGACT GTGAACG-3′) and T2AR (5′-CGATAGTGGGGTTGCCG TCAA-3′) (14) was used to amplify the translation elongation factor 1 (TEF1) gene. The application of these primers enables accurate and reliable molecular characterization of the fungal species.

Collection of marine macro algae

Marine macroalgae specimens encompassing red, brown and green seaweed groups were systematically collected from diverse coastal ecosystems along the eastern coast of Tamil Nadu, specifically from Kanyakumari, Pamban, Mandapam, Velankanni and Rameshwaram to facilitate a comprehensive biodiversity assessment (Table 1). The collected samples were thoroughly rinsed in fresh water to remove debris, sand and any extraneous materials; subsequently preserved using both wet and dry preservation methods (15). Species identification was conducted through detailed morphological and anatomical examinations, following standard taxonomic keys and reference guides (16).

Table 1. Marine macro algae collected from various seashores of Tamil Nadu.

Sl.No	Marine Macro algae(Seaweeds)	Common name or Group of Macro Algae	Place of collection
1	*Dictyota dichotoma*	Brown algae	Kanyakumari
2	*S. wightii*	Brown algae	Pamban
3	*Padina gymnospora*	Brown algae	Pamban
4	*Hydroclathrus hornemannii*	Brown algae	Pamban
5	*Turbinaria conoides*	Brown algae	Rameshwaram
6	*Caulerpa scalpelliformis*	Green algae	Pamban
7	*Chaetomorpha antennina*	Green algae	Mandapam
8	*Enteromorpha intestinalis*	Green algae	Velankanni
9	*U. lactuca*	Green algae	Rameshwaram
10	*Halimeda gracilis*	Green algae	Puducherry
11	*Acanthophora spicifera*	Red algae	Mandapam
12	*Gracilaria salicornia*	Red algae	Pamban
13	*Jania rubens*	Red algae	Puducherry
14	*Kappaphycus alvarezii*	Red algae	Mandapam
15	*Hypnea musciformis*	Red algae	Mandapam

In vitro evaluation of marine macro algal extracts against *S. rolfsii*

Solvent extracts were prepared using Soxhlet extractor from five marine macroalgae representing red, brown and green groups, namely *S. wightii*, *Gracilaria corticata*, *Ulva lactuca*, *Padina tetrastromatica* and *Turbinaria ornata*. Each extraction involved soaking 20 g of powdered macroalgae in 100 mL of methanol. The extracts were further filtered through a 0.45 µm Millipore filter to remove any dust and particulate matter (17). The fungicidal property of the extract was assessed against *S. rolfsii* using the poison food technique. PDA supplemented with macroalgal extracts at concentrations of 2.5 %, 5 % and 10 %, were poured into sterile Petri dishes respectively. The pH of the medium was measured prior to inoculation and any deviations resulting from the addition of extracts were corrected using 1N NaOH or 1N HCl to maintain an optimal range of 5.5 - 6.0 for fungal growth. To initiate the assay, a culture disc of *S. rolfsii* was placed at the centre of each petri dish. Each treatment was performed in triplicate. Plates containing plain PDA medium, without macroalgal extracts, served as the negative control. All samples were incubated at a temperature of 28 ± 2 °C and fungal growth was assessed by measuring the colony diameter after a five-day incubation period. The percentage of mycelial growth inhibition was calculated using Vincent's formula to assess the efficacy of the macroalgal extracts (18).

Pot culture assay

A pot experiment was conducted using the susceptible groundnut cultivar VRI 2 to evaluate the effects of different treatments on stem rot incidence and plant growth parameters. The experiment was arranged in CRD (Completely Randomized Design) comprised of six treatments, each replicated three times and three pots per replication. Talc-based formulations of Tr1 were prepared (20). Plastic pots were filled with a sterilized 1:1:1 mixture of sand, garden soil and FYM (farmyard manure). The *S. rolfsii* inoculum (sand - maize medium) were inoculated prior to sowing. The treatments included T1 (seed treatment with Tr1 at 4 g/kg of seeds combined with soil application of Tr1 at 50 g/ pot at the time of sowing (TOS) and 45 days after sowing (DAS), T2 (seed treatment (ST) with *S. wightii* extract at 2 % concentration along with soil application of *S. wightii* as a powdered form at 100 g/pot at TOS and 45 DAS), T3 (combined application of T1 and T2), T4 (seed treatment with Carbendazim 50 % WP at 2 g/kg of seeds), T5 (inoculated control without any treatment) and T6 (healthy control without inoculation or treatment). Stem rot incidence was recorded at the susceptible stage of the crop (70 DAS) and expressed as percentage disease incidence. Additionally, plant growth parameters including root length, plant height, biomass, number of pods per plant and percentage increase over the control were measured for all treatments (21).

Plant sample collection for proteomic assay

Stem samples were collected from the collar region, as *S. rolfsii* primarily colonizes the stem base near the soil surface. The pathogen's mycelia emerge from soilborne sclerotia and directly invade this region, making it the critical site of infection. Sampling from this area enables the investigation of early host-pathogen interactions and defense responses such as lignin deposition and pathogenesis-related (PR) protein expression at the infection front, providing key insights into disease progression and host defense mechanisms (19). To analyze these responses, plants were subjected to T3 (bioagent and macroalgal extract treatment) and T5 (untreated healthy control) were selected and kept separately. Stem samples, (2 - 3 cm segments) were collected two days post-final treatment, rinsed with distilled water, blotted dry and immediately stored at -80 °C in a dry ice box for further protein extraction and analysis.

Proteomic analysis

Protein extraction

Stem segments were ground with liquid nitrogen and 2 g of powder were homogenized in a 20 % TCA-acetone solution, then centrifuged at 12000 rpm for 15 - 20 min. Pellets were washed with ethanol and acetone, lyophilized. The dried pellet (30 mg) was reconstituted in 502 µl of rehydration solution comprising urea (7 M), CHAPS (2%), thiourea (2 M) and DTT (0.2 %). The mixture was centrifuged at 15000 rpm for 20 min, followed by a 1 -hr incubation at 37 °C. The resulting supernatant was carefully collected, stored at -80 °C for later use in 2D-PAGE analysis and used for protein quantification.

Two-Dimensional Polyacrylamide Gel Electrophoresis (2D-PAGE)

First-Dimension - Isoelectric Focusing (IEF)

The protein extracts were dissolved in a buffer solution comprising high concentrations of urea (7 M) and thiourea (2 M), with 2 % CHAPS as a detergent, 0.5 % ampholytes to aid in pH stabilization, 50 mM DTT as a reducing agent and a minimal amount of bromophenol blue for tracking. To allow for consistent protein uptake, IPG strips spanning a pH range of 3 - 10 were incubated overnight with the sample through passive rehydration. IEF was carried out using a stepwise voltage gradient, starting at 50 V and gradually increasing to 8000 V, accumulating 50000 - 60000 Vh to achieve efficient protein separation based on their isoelectric points. Upon completion, the IPG strips were either stored at -20 °C for later use or immediately processed for equilibration.

Equilibration of IPG Strips

Following isoelectric focusing, the IPG strips underwent two-step equilibration to stabilize the proteins prior to SDS-PAGE. The initial equilibration buffer was composed of high concentrations of urea (6 M) and SDS (2 %) for protein denaturation, along with 50 mM Tris-HCl (pH 8.8) to maintain pH stability, 30 % glycerol to prevent protein aggregation and 1 % DTT to reduce disulfide linkages and ensure proper unfolding of proteins. The second equilibration step was conducted using Buffer II, which contained the same composition as Buffer I but replaced 1 % DTT with 2.5 % iodoacetamide, allowing alkylation of free thiol groups to prevent reformation of disulfide bonds. Each equilibration step was carried out for 15 min to ensure proper protein stabilization before proceeding to SDS-PAGE.

Second-Dimension: SDS-PAGE

Glass plates and spacers were thoroughly cleaned with distilled water and ethanol before assembling the gel casting unit. A 12 - 15 % acrylamide separating gel was prepared, poured between the plates, overlaid with isopropanol to prevent dehydration and allowed to polymerize for two hr. After polymerization, the gel was rinsed with running buffer to remove any residual isopropanol. The equilibrated IPG strips were carefully placed onto the SDS-PAGE gel and secured with 2 mL of molten agarose solution containing 0.1 % bromophenol blue to ensure firm adherence and smooth electrophoretic migration. Protein separation was carried out by electrophoresis, starting at a constant current of 15 mA per gel for the initial 30 min and then increased to 25 mA per gel until the tracking dye migrated to the bottom, allowing effective resolution based on molecular weight.

Protein visualization

After fixing for one hr, gels were washed twice with 30 % ethanol and once with deionized water. The gels were stained overnight using a Coomassie Brilliant Blue G-250 solution (Bio-Rad), formulated with methanol, acetic acid and distilled water in a ratio of 4:1:5, along with 0.1 % of the dye. This staining protocol enabled clear detection of protein bands with reduced background interference. The gels were then destained with a solution of 40 % methanol, 10 % acetic acid and 50 % distilled water until proteins were clearly visible.

Gel image analysis

Gels were scanned using Gel documentation system, Gel Doc XR (BioRad Laboratories, Hercules, CA) and analyzed using PD Quest software, version 8.0.1 (BioRad Hercules, CA).

Protein identification

Protein digestion

Among the identified proteins, three spots from treated samples and one from the healthy control with highest molecular weight (kDa) were selected, excised using a Proteome Work gel cutter (Bio-Rad) and matched across gels with the Classic Match tool for verification. Gel fragments containing the protein spots were manually removed and subjected to enzymatic digestion using trypsin (6 ng/µL) in 25 µL of 50 mM ammonium bicarbonate. The digestion was performed at 37 °C for approximately five hr using an automated digestion and spotting system (ProPrep, Genomic Solutions, Ann Arbor, MI) (20). Peptides were extracted in two rounds: the first using 30 µL of 1 % formic acid and the second with either 1 % formic acid or a 50 % acetonitrile solution in 12 µL. All peptide extracts were pooled and stored in a PCR plate at 4 °C for downstream analysis.

Mass Spectrometry

Once completely dried, the sample was analyzed using the ULTRAFLEX III MALDI-TOF mass spectrometry platform to capture peptide mass and fragmentation spectra. The obtained spectral profiles were then interpreted and refined using Flex Analysis software tools.

Protein Interaction and Functional Analysis

Protein selection and network construction

The proteins PIN1-2 (Peptidyl-prolyl cis-trans isomerase), BHLH145 (Transcription factor) and TPS27 (1,8-cineole synthase) were analyzed using the STRING database (v11.5) with *Digitalis lanata*, *Arabidopsis thaliana*, *Nicotiana suaveolens* as the reference organism. A confidence score of ≥0.7 was applied to ensure high-confidence interactions. Protein-protein interaction networks were generated and visualized in STRING and further refined using Cytoscape (v3.9.1). Functional clusters were identified through STRING's clustering algorithm and annotated with Gene Ontology (GO) terms and KEGG pathways.

Functional annotation and validation

Interactions were categorized as physical or functional and associated biological processes were inferred from GO and KEGG data. Literature mining was performed to validate the functional roles of PIN1-2 (auxin transport), BHLH145 (transcriptional regulation) and TPS27 (terpenoid biosynthesis).

Results and Discussions

Molecular identification of *S. rolfsii*

PCR amplification of the 18S rRNA from the *S. rolfsii* isolate using the universal primers ITS-1 and ITS-4 generated an amplicon of approximately 563 bp. Partial sequencing of the amplified product confirmed the isolate's identity as *S. rolfsii* (Teleomorph: *Agroathelia rolfsii*), exhibiting 95 % nucleotide sequence similarity with *S. rolfsii* reference sequences in the NCBI database through BLAST analysis (Supplementary Fig. 1-3). The sequenced isolate has been deposited in GenBank under the accession number MZ920141.Furthermore, amplification using the species-specific primers SR1-F and SR1-

R resulted in a distinct amplicon of 534 bp, providing conclusive confirmation of the molecular identity of *S. rolfsii*. The high specificity of these primers facilitated precise differentiation of *S. rolfsii* from other fungal species, emphasizing their reliability for accurate species-level identification in molecular diagnostics. The genetic variability of *S. rolfsii* using ITS primers had produced amplicon sizes ranging from 490 to 699 bp (21). The amplification of 18S rDNA regions using ITS primers, resulted in a 550 to 570 bp amplicon and showed 99 % homology with *S. rolfsii* (22). Our results are consistent with the previous findings (11) where they also used *S. rolfsii*-specific primers SCR-F and SCR-R for PCR amplification and successfully obtained a 540-bp product encompassing portions of the ITS1 and ITS2 regions as well as the full 5.8S rDNA subunit. These findings further confirm the reliability of these primers for detecting *S. rolfsii*. The phylogeny of the sclerotium been studied with 98 % bootstrap support (23). Based on ITS sequencing, six *S. rolfsii* isolates have been confirmed (24). *In vitro* evaluation of fungal antagonists against *S. rolfsii*

Among the five *Trichoderma* isolates evaluated using the dual culture technique, Tr1 exhibited the highest antagonistic activity against *S. rolfsii*, achieving 73.81 % mycelial inhibition, followed by Tr4 with 70.02 % inhibition compared to the control (Table 2, Supplementary Fig. 4a). These findings align with a recent study (25) where similar approach was utilized for the assessment of five *Trichoderma* isolates (FAVF335, FAVF340, FAVF345, FAVF349 and FAVF351); reported a 56 % to 71 % *S. rolfsii* mycelial inhibition. *Trichoderma* isolate NM082 demonstrated strong antagonistic potential, exhibiting an inhibition rate of 86 %, which was significantly higher than that of other tested strains (26).

Table 2. *In vitro* efficacy of *Trichoderma* spp. against *S. rolfsii* (Dual culture technique).

Sl. No	Isolates	Mycelia growth (cm) (5 DAI*)		% inhibition overcontrol
		Trichoderma spp.	*S. rolfsii*	
1	Tr-1	6.66^{a}	2.34^{a} (8.79)	73.81
2	Tr-2	5.48^{d}	3.52^{d} (10.81)	60.28
3	Tr-3	5.62^{c}	3.38^{c} (10.59)	62.56
4	Tr-4	6.33^{b}	2.67^{b} (9.40)	70.02
5	Tr-5	4.00^{e}	5.00^{g} (12.91)	56.47
6	Control	-	9.00^{h} (17.45)	-

DAI - Days after inoculation.

In this study, five culture filtrates of *Trichoderma* spp. were assessed for their antagonistic activity against *S. rolfsii* at varying concentrations using poisoned food technique. Among the tested filtrates, Tr1 at a 20 % concentration demonstrated the highest inhibition rate of 89.11 %, resulting in minimal mycelial growth of 0.98 cm, while the control exhibited extensive mycelial growth of 9.00 cm (Table 3, Supplementary Fig. 4b). The findings are consistent with previous report (24), which evaluated various *Trichoderma* species at a 20 % concentration against *S. rolfsii* mycelial growth. The highest inhibition (71.85%) with isolate TvG1, while the lowest inhibition (61.11%) was observed with isolate ThrG4.

A volatile compounds production of *Trichoderma* spp. isolates was assessed against *S. rolfsii* using inverted plate assay. Toxic volatile compounds from Trichoderma spp. reduced the radial growth and sclerotial production of *S. rolfsii*. The minimum mycelial growth of 2.92 cm and least sclerotial numbers (28 Number/plate) was observed the isolate Tr1 with 67.56 % and 91.22 % inhibition over control (Table 4, Supplementary Fig. 4c). The volatile compounds from *Trichoderma* species inhibited *S. rolfsii* mycelial growth and sclerotial formation, with *T. viride* being the most effective, reducing mycelial growth by 51.11 % and sclerotia production by 95.90 %.

Molecular identification of *T. asperellum* (Tr1)

The 18S rRNA gene of the *T. asperellum* (Tr1) isolate was successfully amplified using the universal primers ITS-1 and ITS -4, yielding a 632 bp amplicon. Partial sequencing of the amplified product confirmed the isolate's identity as

Table 4. Efficacy of volatile compounds produced by *Trichoderma* spp.against *S. rolfsii* (Inverted plate assay).

Sl. No	Isolates	Mycelial growth of *S. rolfsii* (cm) 5DAI	% inhibition over control	No. of sclerotia produced 15 DAI	% inhibition overcontrol
1	Tr1	2.92^{a} (9.86)	67.56	28^{a}	91.22
2	Tr2	4.67^{d} (12.48)	48.11	186^{d}	41.70
3	Tr3	3.70^{c} (11.09)	58.89	137^{c}	57.05
4	Tr4	3.38^{b} (10.59)	62.44	97^{b}	69.60
5	Tr5	5.31^{e} (13.33)	41.00	279^{e}	12.54
6	Control	9.00^{f} (17.46)	-	319^{f}	-

Mean of three replications. Values in the column followed by same superscript letters do not differ significantly at 5% level by Duncan's multiple range test(DMRT).

Table 3. *In vitro* evaluation of various *Trichoderma* spp. isolates against *S. rolfsii* (Poisoned food technique).

Sl. No	Isolates	Mycelial growth (cm) (5DAI)							
		5 %	% inhibition over control	10 %	% inhibition over control	15 %	% inhibition over control	20 %	% inhibition over control
1	Tr-1	3.02^{a} (10.00)	66.45	2.40^{a}(8.90)	73.34	1.81^{a}(7.72)	79.89	0.98^{a}(5.67)	89.11
2	Tr-2	6.00^{d} (14.17)	33.34	5.28^{d} (13.29)	41.33	4.94^{e} (12.84)	45.11	4.31^{e} (11.98)	52.11
3	Tr-3	5.05^{c} (12.99)	43.89	4.49^{c} (12.23)	50.11	4.02^{d} (11.57)	55.33	3.59^{d} (10.93)	60.11
4	Tr-4	4.43^{b} (12.14)	50.78	3.56^{b} (10.88)	60.44	2.73^{b}(9.51)	67.45	1.96^{b}(8.04)	78.22
5	Tr-5	6.00^{d} (14.17)	33.34	5.28^{d} (13.29)	41.33	4.94^{e} (12.84)	45.11	4.31^{e} (11.98)	52.11
6	Control	9.00							

Mean of three replications. Values in the column followed by same superscript letters do not differ significantly at 5 % level by Duncan's multiple range test(DMRT).

T. asperellum, exhibiting 99.83 % nucleotide sequence similarity with *T. asperellum* reference sequences in the NCBI database through BLAST analysis. The sequenced isolate was subsequently deposited in GenBank under the accession number OL872253. Additionally, amplification of the *TEF1* gene using the species-specific primers T2AF and T2AR resulted in a distinct amplicon of ~500 bp, further validating the molecular identity of *T. asperellum* (Supplementary Fig. 5, 6). The successful amplification underscores the high specificity and efficiency of these primers, demonstrating their reliability for precise species identification and molecular characterization of *T. asperellum*.

T. asperellum isolates were amplified using ITS primers, yielded PCR products in the range of 500 to 600 bp (28), to which our findings are consistent with. Further validation of these findings emphasizes the reliability of ITS-based molecular identification for *Trichoderma* species, reinforcing its effectiveness as a diagnostic tool (29).A 507 bp amplicon has been found out, confirming the reliability of TEF1 gene amplification using the species-specific primers T2AF and T2AR, which supports their effectiveness as robust molecular markers for the accurate identification of *Trichoderma asperellum* (14); to which the current results are allign with.

In vitro evaluation of marine macro algal extracts against *S. rolfsii*

All five solvent extracts of marine macroalgae were tested against *S. rolfsii* at three different concentrations using poisoned food technique. Among them, *S. wightii* at a high concentration (10 %) demonstrated the most significant reduction in mycelial growth to 1.12 cm, achieving an inhibition rate of 87.56 %. This was closely followed by *U. lactuca* at 15 %, which exhibited a percent inhibition of 84.12 % (Table 5, Supplementary Fig. 7). The predominant reduction in *S. rolfsii* mycelial growth was correlated with the higher concentration of brown seaweed extract. The efficacy of seaweed liquid fertilizers (SLF) derived from *T. ornata* and *Ulva reticulata* against the soil-borne pathogen *S. rolfsii* was evaluated and shown to have mycelial inhibition zones after 72 hr of incubation, with a 15 mm zone at 80 % SLF and an 18 mm zone at 100 % SLF (30); present findings also substantiate this. *Fusarium oxysporum* mycelial growth was inhibited by 100 % using brown algal extract from *Turbinaria coenoides* for up to six days after inoculation (31). Under *in vitro* conditions, the red macroalga *Cystocera humilis* inhibited *Fusarium culmorum* mycelial growth by 100 % (32). The green macroalga *U. lactuca* exhibited the highest antifungal activity against *Aspergillus fumigatus* and *Aspergillus niger*, with mycelial growth reduced to 1.0 cm and 0.9 cm respectively (33). Macroalgae contain bioactive secondary metabolites including terpenes, phenols, alkaloids, fatty acids and polysaccharides, which can significantly reduce mycelial growth of *S. rolfsii* under *in vitro* (32).

Pot culture assay

This study assessed the combined efficacy of a marine macroalgal extract and a biocontrol agent in mitigating stem rot incidence and enhancing the growth performance of groundnut under controlled pot culture conditions (Table 6, Supplementary Fig. 8). Results indicated a significant reduction in disease incidence across all treatments. Notably, treatment T3 - comprising seed treatment (ST) with *T. asperellum* (Tr1) at 4 g/kg of seeds, along with soil application (SA) of Tr1 at 50 g/pot at the TOS and 45 DAS, in addition with seed treatment using *S. wightii* extract (2 % concentration) and soil application of powdered *S. wightii* at 100 g/pot at TOS and 45 DAS, demonstrated the highest disease suppression, with an 84.93 % reduction in stem rot incidence. Furthermore, this treatment significantly enhanced key agronomic parameters, including germination percentage (97.93 %), shoot length (49.79 cm), root length (22.52 cm), total biomass accumulation (48.04 g/plant) and pod yield (63.68 g/plant). In contrast, the inoculated control exhibited substantially lower values for these parameters, recording a

Table 5. *In vitro* evaluation of various macro algal extracts against *S. rolfsii* (Poisoned food technique).

Sl. No	Treatment	Mycelial growth (cm) (5DAI)					
		2.5 %	% inhibition over control	5 %	% inhibition over control	10 %	% inhibition over control
1	*D. dichotoma*	4.53^{f} (12.29)	49.67	3.92^{g} (11.42)	56.45	3.46^{g} (10.71)	61.56
2	*Sargassum wightii*	2.86^{a} (9.74)	68.23	1.39^{a} (6.77)	84.56	1.12^{a} (6.08)	87.56
3	*P. gymnospora*	3.19^{b} (10.28)	64.56	2.36^{b} (8.84)	73.78	1.43^{bc} (6.87)	84.12
4	*H.hornemannii*	5.69^{i} (13.80)	36.78	5.02^{k} (12.94)	44.23	4.79^{jk} (12.64)	46.78
5	*T. conoides*	3.40^{bc} (10.51)	62.22	2.96^{c} (9.91)	67.12	2.10^{cd} (8.33)	76.67
6	*C. scalpelliformis*	5.89^{j} (14.05)	34.56	5.63^{l} (13.72)	37.45	5.00^{k} (12.92)	44.44
7	*C. antennina*	5.26^{hi} (13.26)	41.56	4.91^{ijk} (12.80)	45.67	4.53^{j} (12.29)	49.67
8	*E.intestinalis*	4.10^{de} (11.67)	54.45	3.62^{de} (10.97)	59.78	2.86^{de} (9.73)	68.23
9	*U. lactuca*	3.39^{bcd} (10.67)	62.33	2.48^{bc} (9.05)	72.45	1.39^{b} (6.77)	84.56
10	*H. gracilis*	4.47^{ef} (12.21)	50.32	3.86^{f} (11.33)	57.12	3.13^{e} (10.19)	65.23
11	*A. spicifera*	4.87^{g} (12.74)	45.89	4.42^{i} (12.14)	50.89	3.91^{h} (11.41)	56.56
12	*G. salicornia*	4.02^{d} (11.27)	55.34	3.37^{d} (10.58)	62.56	2.08^{c} (8.30)	76.89
13	*J. rubens*	5.27^{h} (13.06)	41.45	4.87^{ij} (12.74)	45.87	4.33^{i} (12.01)	51.89
14	*K. alvarezii*	4.56^{fg} (12.33)	49.33	3.98^{gh} (11.51)	55.78	3.24^{f} (10.36)	64.00
15	*H. musciformis*	4.19^{e} (11.81)	53.44	3.65^{def} (11.02)	59.45	2.78^{d} (9.60)	69.12
16	Control	9.00^{k}	-	9.00^{m}	-	9.00^{l}	-

Table 6. Combined applications of *S. wightii* and *T. asperellum* Tr1 on growth parameters and stem rot incidence of groundnut in pot culture conditions.

Name of the treatments	Germination (%)	Shoot length (cm)	Root length (cm)	Biomass (g/plant)	Pod Yield (g/Plant)	Stem rot incidence(%)	% disease reduction over control (70 DAS)
T_1: ST of *T. asperellum* (Tr1) @ 4g/kg of seeds + SA of *T. asperellum* (Tr1) @ 50g/Pot on TOS & 45DAS	91.50^{bc}(73.64)	42.18^{c}	19.46^{c}	44.47^{bc}	57.65^{b}	18.46^{c}(25.43)	75.28
T_2: ST of *S. wightii* extract @ 2 % conc. + SA of *S. wightii* @ 100g/ Pot on TOS & 45 DAS	93.37^{b}(75.26)	45.03^{b}	20.21^{b}	45.14^{ab}	59.37^{ab}	15.58^{b}(23.24)	79.13
T_3: ST of *S. wightii* extract @ 2 % conc. + SA of *S. wightii* @ 100g/ Pot on TOS & 45 DAS +ST of *T. asperellum* (Tr1) @ 4g/kg of seeds + SA of *T. asperellum* (Tr1) @ 50g/Pot on TOS & 45DAS	97.93(83.05)	49.79^{a}	22.52^{a}	48.04^{a}	63.68^{a}	11.25^{a}(19.60)	84.93
T_4: Seed treatment with Carbendazim 50% WP @ 2g/Kg of seeds onTOS + Soil drenching @ 0.1% 45DAS	84.16^{c} (66.70)	30.08^{d}	13.33^{d}	33.34^{c}	41.29^{c}	29.47^{d} (32.87)	60.53
T_5: Healthy control	82.07^{d} (64.95)	26.74^{e}	11.48^{e}	30.09^{d}	37.97^{d}	0.00^{a}(0.28)	100
T_6: Inoculated control	68.39^{e}(55.81)	19.60^{f}	9.72^{f}	26.40^{e}	20.79^{e}	74.67^{e} (59.82)	-

Mean of three replications. Values in the column followed by same superscript letters do not differ significantly at 5% level by Duncan's multiple range test (DMRT).

germination percentage of 68.39 %, shoot length of 19.60 cm, root length of 9.72 cm, total biomass of 26.40 g/plant and pod yield of 20.79 g/plant. A significant reduction of 82.67 % was obtained in stem rot incidence in groundnut under controlled conditions following the combined application of seed treatment and soil inoculation with *Trichoderma longibrachiatum* and *T. asperellum* (34). A chitin-enriched *Trichoderma* bioformulation significantly enhanced seedling vigour in groundnut, resulting in 100 % germination, increased shoot (27.67 cm) and root lengths (26.56 cm), higher biomass accumulation (31.20 g) and a markedly improved vigour index (5423.33) indicating the growth-promoting potential of fortified biocontrol agents (35). Foliar application of macroalgal extracts in groundnut substantially improved yield-related traits including pod yield (1463 kg/ha), shelling percentage (67.86 %) and number of pods per plant (16.37); underscoring the bio-stimulant effects of marine algal formulations (36) and was reported, complementing the above findings.

Defense protein induction in groundnut plants triggered by brown seaweed and fungal antagonist

Proteins were extracted from groundnut stems treated with T3 (macroalgal extract + fungal bioagent) and T5 (healthy control) samples, separated using 2D-PAGE and identified via MALDI-TOF analysis.

Profiling of protein spots by 2D-PAGE analysis

Protein profiling of groundnut stem collected from treated plants (*S. wightii* + *T. asperellum* (Tr1) + *S. rolfsii*) and un-treated (healthy plants) and were examined by 2D-PAGE analysis. A total of 64 protein spots were identified, among them 26 spots were unique to treated plants (mentioned as TA), 24 to healthy control (mentioned as CA) and 14 were common to both groups (mentioned as CT). Protein profiling of the sample pH ranged from 4 to 7, with molecular size varying between 10 to 170 kDa (Fig. 1-3). Proteomic analysis using two-dimensional PAGE is an effective method for identifying protein expression patterns in various plant tissues under biotic and abiotic stress conditions (37). For analyzing the expression patterns of proteins in different peanut varieties using MALDI-TOF analysis, 45 up-regulated and novel protein spots were selected from 2D PAGE and it was identified that 28 were putative proteins from resistant and susceptible peanut varieties, compared to controls (38). Twenty protein profiles showing varietal differences in groundnut seeds have been identified (39) and more than 250 leaf proteins including numerous PR proteins have been identified in groundnut plants (40).

Protein identification by using MALDI-TOF

Four prominently expressed proteins with significant expression differences were excised, identified using MALDI-TOF mass spectrometry and analyzed with Mascot software (Fig. 4). Proteins associated with plant defense such as Peptidyl-prolyl cis -trans isomerase, Transcription factor bHLH145 and 1,8-cineole synthase were detected exclusively in treated plants, whereas antiviral protein was identified in healthy control plants. The functions, molecular weights (kDa), scores, accession numbers and protein lengths are presented in Table 7. Actin (spot number 109) and tubulin (spot numbers 81 and 274) protein profiles were identified from peanut leaf proteome analysis (41). Actin plays a key role in cell division, cytoplasm streaming, organelle movement and cell shape determination, while tubulin, a major component of microtubules, binds to GTP (Guanosine-5'-triphosphate). Using the MASCOT database, two purified proteins: a 41 kDa peptide [MLVESEGR], with a maximum score of 44 from *Medicago truncatula* (Q1T5F9_MEDTR) and a 39 kDa peptide [AAFLNNDYTK] with a maximum score of 96 from *Hypericum perforatum* (gi|57868106), were identified.

Protein Interaction and Functional Analysis

PIN1-2, bHLH145 and TPS27 revealed key roles in distinct yet interconnected pathways (Fig. 5). PIN1-2 is linked to auxin transport, suggesting involvement in plant developmental processes (43). BHLH145 acts as a transcriptional regulator, forming connections with other transcription factors to mediate gene expression (44). TPS27 interacts with multiple terpenoid synthase proteins (e.g., TPS14, TPS24), highlighting its role in terpenoid and other secondary metabolites biosynthesis (45). The interactions between these proteins suggest a potential coordination between hormone signaling, transcriptional regulation and secondary metabolite biosynthesis, which may be critical for plant growth and stress responses. Recent studies have provided valuable insights into the roles of PIN1-2, BHLH145 and TPS27 in plant immune development and stress

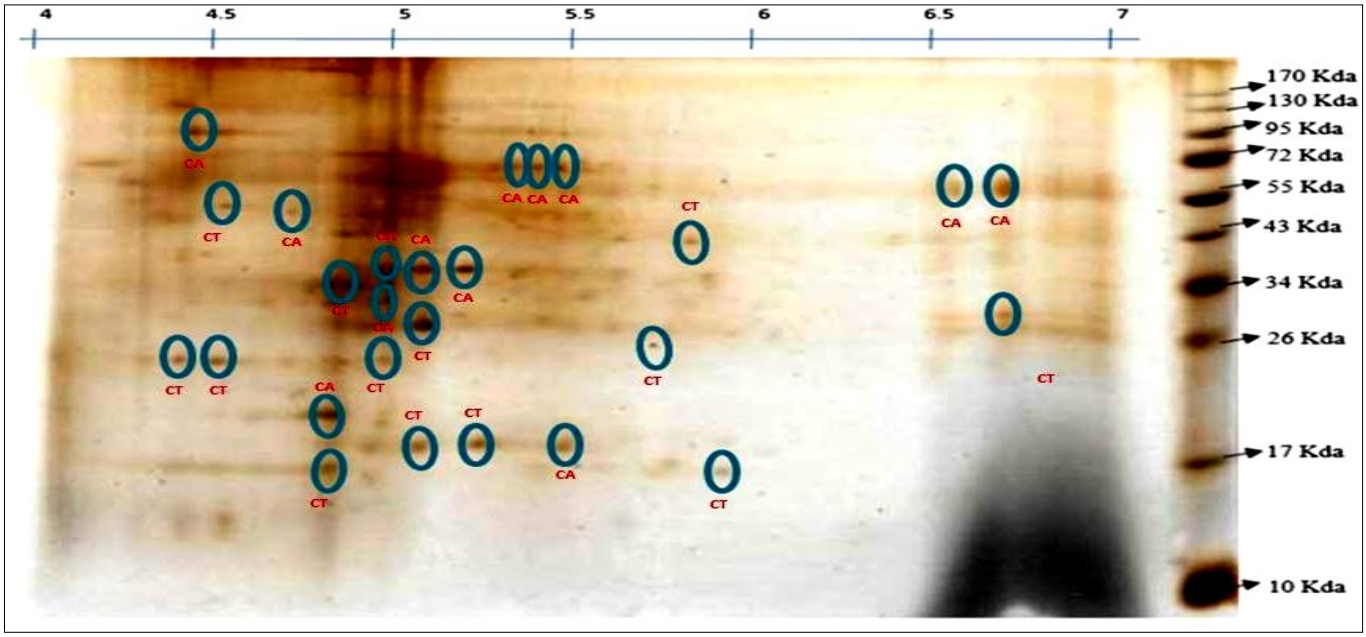


Fig. 1. Proteome analysis of T5 treated groundnut stem sample, **CT** – spots present in control and treated plants, **CA** – spots present only in control plants and **CT** – spots present only in treated plants.

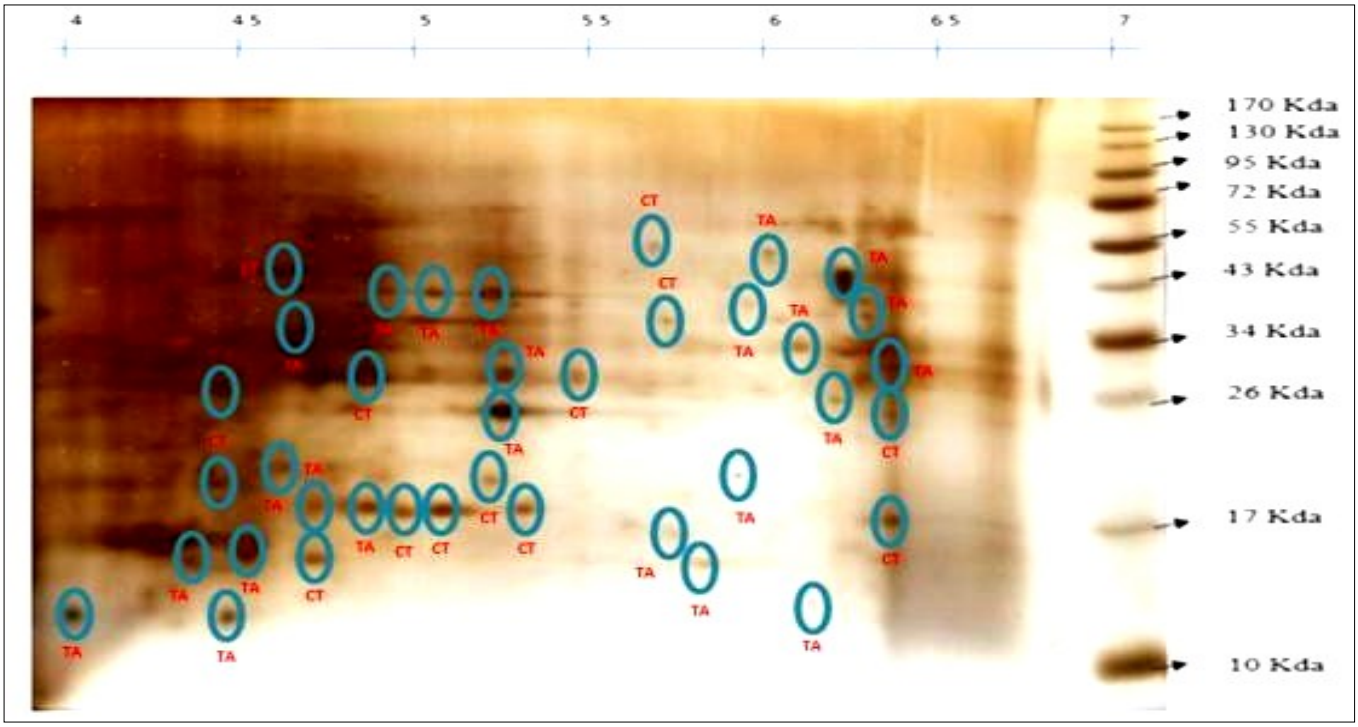


Fig. 2. Proteome analysis of T3 treated groundnut stem sample, **CT** - spots present in control and Treated plants, **CA** - spots present only in control plants and **CT** - spots present only in treated plants.

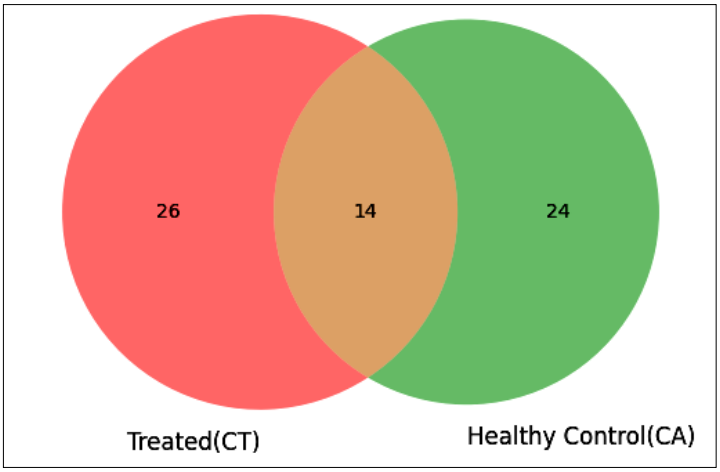
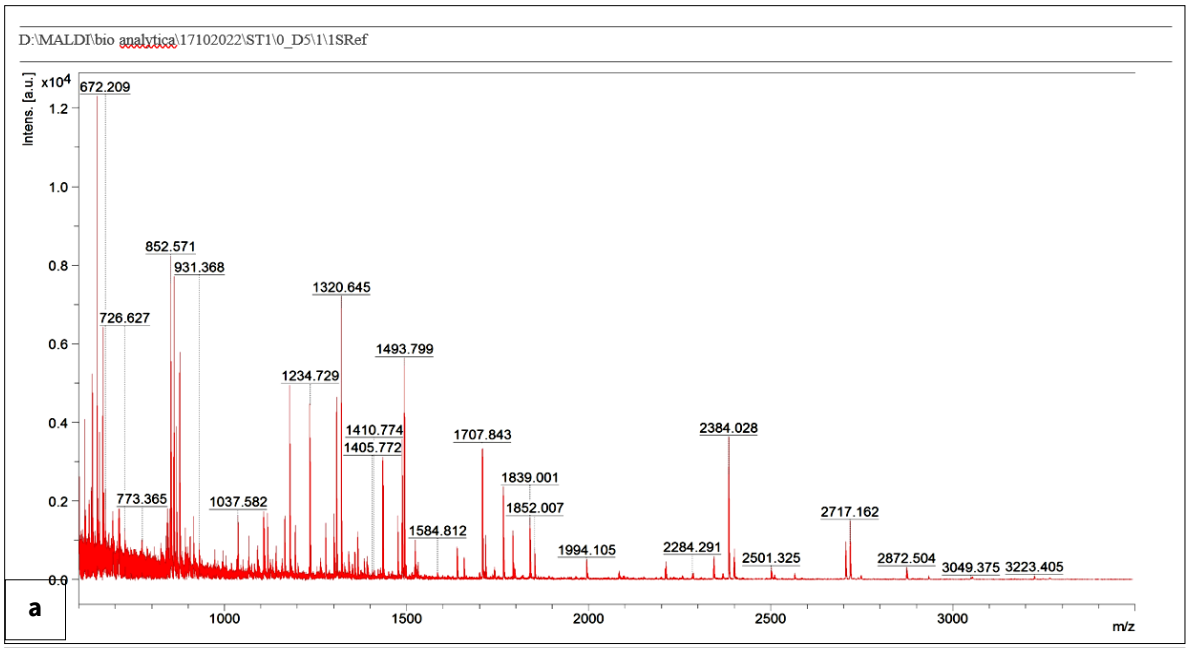


Fig. 3. Venn diagram approach of differentially expressed proteins in treated, both and heathy control plants.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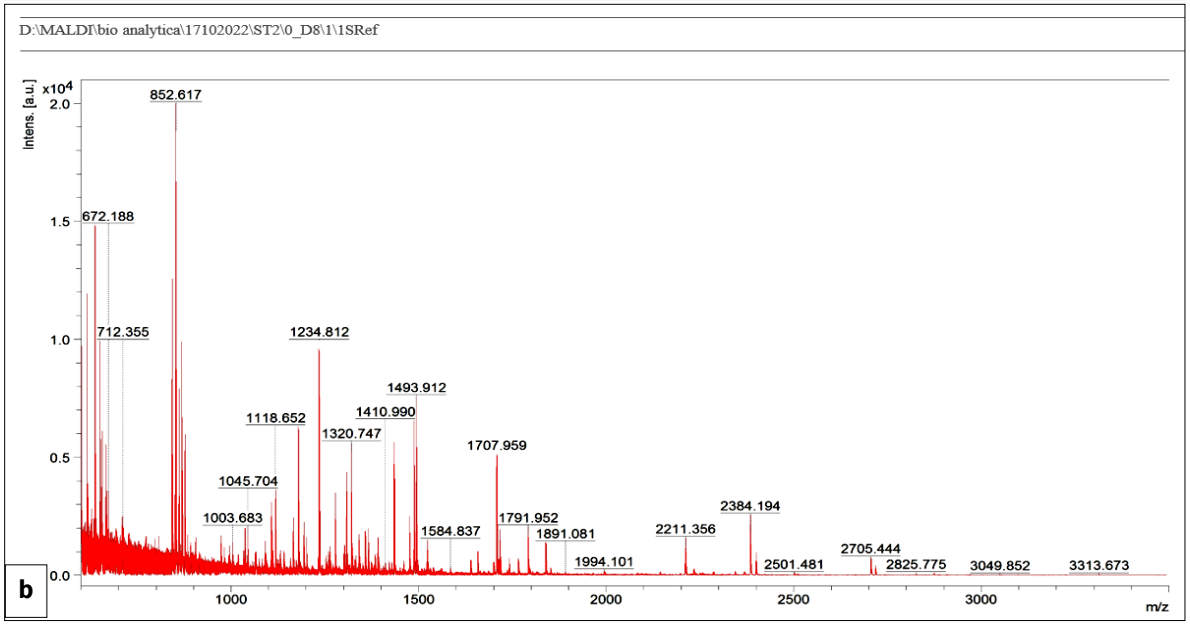
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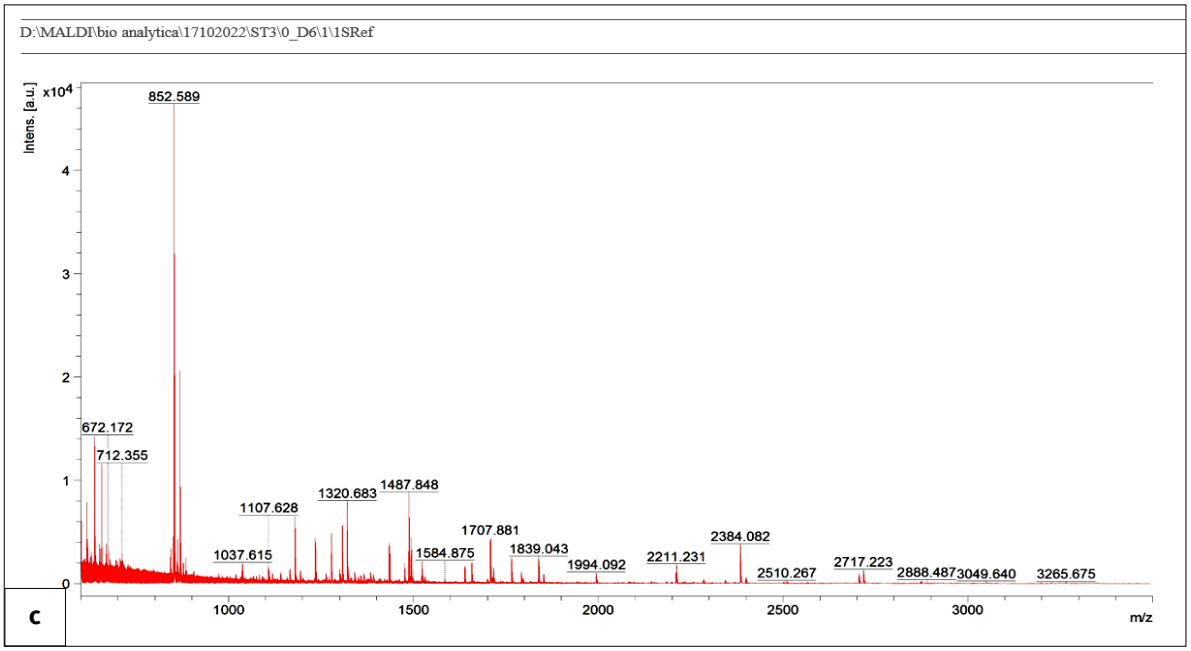
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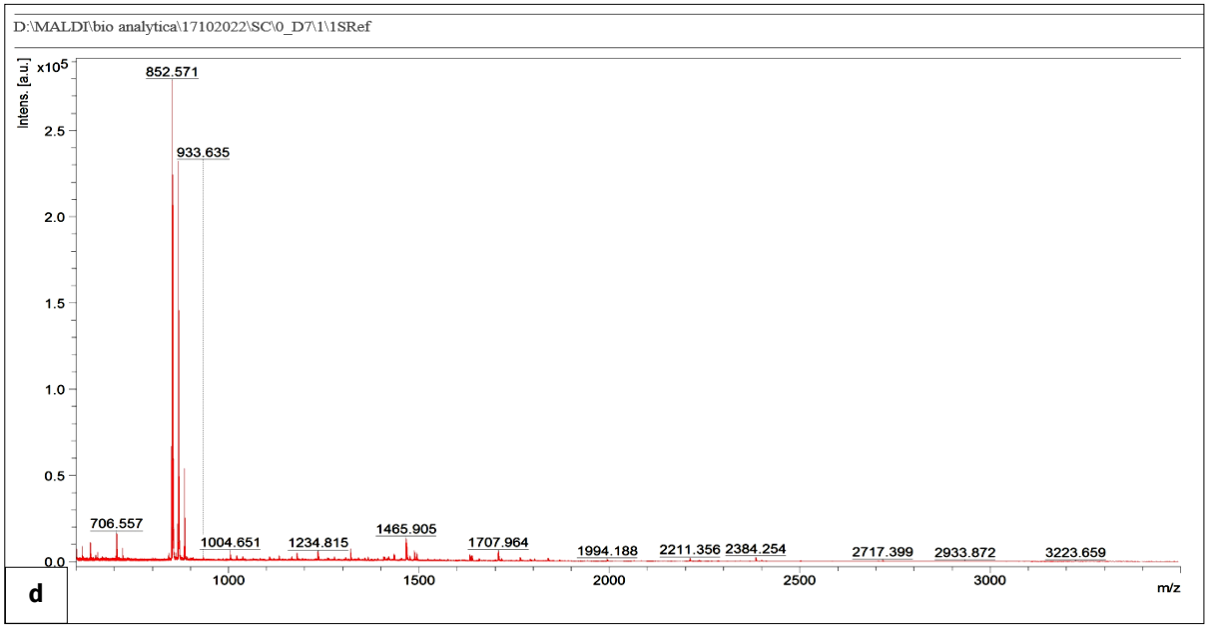

Fig. 4. MALDI TOF MS/MS analysis of differentially expressed proteins. **(a)** Stem treated 1 (ST1)**, (b)** Stem treated 2 (ST2)**, (c)** Stem treated 3 (ST3) and (**d**) Stem healthy control (SC).

Table 7. MALDI-TOF identification of proteins in groundnut plants under T3 and T5 treatments.

Treatment	Proteinspot ID	Molecular weight (kDa)	Accession No	Protein identity	Protein length	Function
T3	ST1	34	BH145_ARATH	Transcriptionfactor bHLH145 (*A. thaliana*)	311	Regulate secondary metabolite biosynthesis, including antimicrobial phytoalexins, flavonoids and alkaloids via DNA binding and transcriptional control
	ST2	71	CIN_NICSU	1,8-cineole synthase (*N. suaveolens*)	610	1,8-cineole synthase is an enzyme that catalyzes the 1,8-cineol, a monoterpenoid compound with antimicrobial properties
	ST3	12	PIN1_DIGLA	Peptidyl- prolyl cis-trans isomerase (*D. lanata*)	118	Protein folding, hormone signaling and stress responses (heat, salt, wounding, GA, IAA, BR) rely on peptidyl-prolyl cis-trans isomerases (PPIases). These enzymes are key to peptide bond isomerization, facilitating protein folding and regulation.
T5	SC	29	RIPS_PHYAM	Antiviral protein (*Phytolacca americana*)	261	Inhibits viral infection of plants and protein synthesis *in vitro*

(ST- Stem treated -T3 treatment, SC-Stem control -T5 treatment).

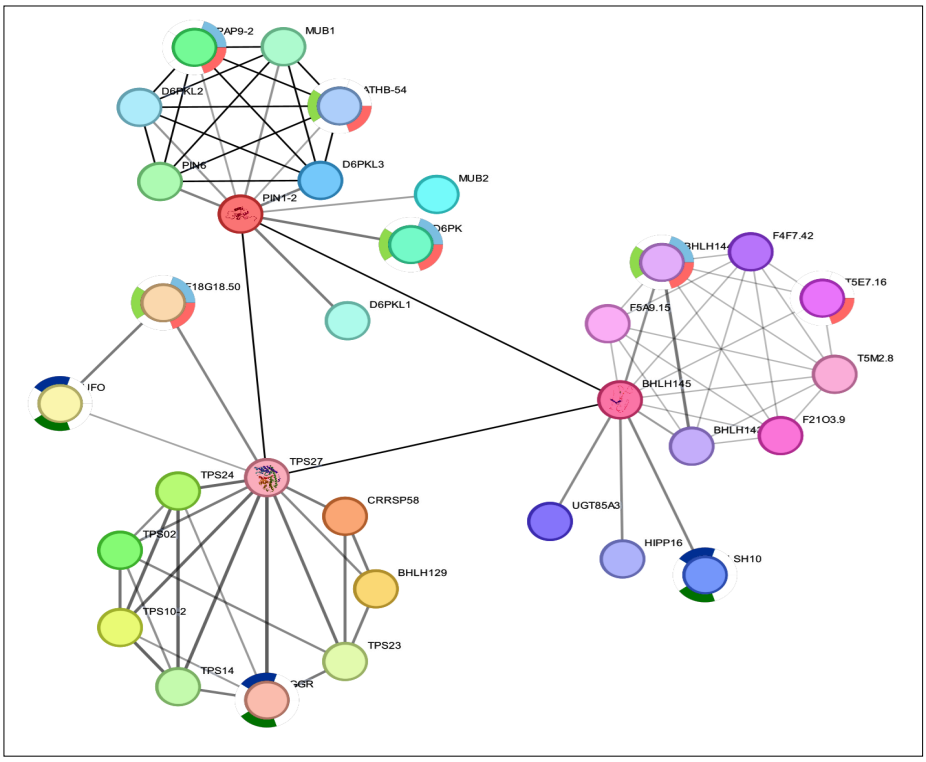

Fig. 5. Protein-protein interaction network of defense-related proteins PIN1-2, bHLH145 and TPS27.

responses. PIN1-2, a key auxin transporter, has been shown to interact with various proteins involved in proper protein folding, regulating hormone signaling pathways and stress response. These include responses to salt, heat, wounding, indole-3-acetic acid, gibberellic acid and brassinosteroid signaling (46). Auxin influences the endocytosis and trafficking of PIN proteins, thereby affecting their polarity and distribution within plant cells (47). BHLH145, a transcriptional regulator interacts with other transcription factors to modulate gene expression. Previous research has highlighted the role of bHLH transcription factors in regulating plant responses to environmental stress and developmental processes (48). TPS27, a 1,8-cineole synthase involved in terpenoid biosynthesis, interacts with multiple terpenoid synthase proteins, underscoring its role in the production of secondary metabolites (48). WRKY transcription factors play a crucial role in regulating specialized metabolism, particularly in the biosynthesis of plant-specialized metabolites (49). These findings suggest a coordinated interaction between hormone signaling, transcriptional regulation and secondary metabolite biosynthesis, which is critical for plant growth and defence responses.

Conclusion

This study highlights the efficacy of *T. asperellum* and *S. wightii* extracts in controlling *S. rolfsii* in groundnut. Proteomic analysis revealed enhanced defense responses in treated plants, underscoring the potential of integrated biocontrol strategies for sustainable agriculture against soil-borne pathogens.

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Authors' contributions

AP experimented the research work. CK contributed to the supervision of research work and drafted the manuscript. SJ and SRRR provided the initial guidance. VJ and SS provided expertise and critical manuscript review on molecular aspects. JS and NR helped to review the final manuscript.

Compliance with ethical standards

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

Ethical issues: None.

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

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

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