PLANT SCIENCE TODAY
Vol x(x): xx-xx
https://doi.org/10.14719/pst.10371

eISSN 2348-1900

RESEARCH ARTICLE

Managing groundnut dry root rot with organic amendments and rhizosphere antagonists

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Received: 30 June 2025; Accepted: 10 September 2025; Available online: Version 1.0: 23 September 2025

Cite this article: Rajinimala N, Kanmani S, Thilagavathi R, Elanchezhyan K, Leninraja D, Muthukumar A, Kavitha K, Sheela J. Managing groundnut dry root rot with organic amendments and rhizosphere antagonists. Plant Science Today (Early Access). https:/doi.org/10.14719/pst.10371

Abstract

Groundnut (*Arachis hypogaea* L.) is an important oilseed crop which is primarily grown in tropical and subtropical regions. Dry root rot caused by soil borne fungus *Macrophomina phaseolina* is the most destructive and widespread disease worldwide. For better management of this disease, rhizosphere antagonists and organic amendments were employed both *in vitro* and *in vivo* studies. Seven rhizosphere antagonists (*Trichoderma* sp. - Tsp 1, Tsp 2, Tsp 3, Tsp 4, *Bacillus* sp. - Bsp 1, Bsp 2 and *Pseudomonas* sp.) were isolated from rhizosphere regions of healthy groundnut plants and tested *in vitro* against *M. phaseolina* by dual culture method. Among the antagonists tested, Tsp1 recorded maximum mycelial inhibition of 67.81 %. The best performing antagonists Tsp1 was molecularly characterised and confirmed as *Trichoderma virens*. Among the seven different organic amendments tested, neem cake showed maximum mycelial inhibition of 52.21 % and 52.50 % at 10 % and 15 % concentrations respectively. The effective rhizosphere antagonists and organic amendments were tested against *M. phaseolina* under pot culture and field condition. In pot culture experiment, soil application of *T. virens* (5 g/pot) + neem cake (5 g/pot) was effective in managing the dry root rot disease with lowest disease incidence (22.21 %) against inoculated control (88.88 %). In field experiment, soil application of *T. virens* (2.5 kg/ha) + neem cake (150 kg/ha) recorded lowest disease incidence of 10.32 % and highest yield of 3200 kg/ha against untreated control (58.57 %, 900 kg/ha respectively).

Keywords: *Bacillus*; dry root rot; *Macrophomina*; oilcakes; *Trichoderma*

Introduction

A. hypogaea L. is an important oil seed crop which is cultivated for consumptive use as food and oil. It is the fourth most important source of edible oil and third most source of vegetable protein in the world, which contains 40 %- 49 % of oil and 26 % of protein. In addition to this, it is a rich source of calcium, phosphorus, iron, zinc and boron and has a high calorific value. Groundnut shells can be used as fuel, fillers in cattle feed, activated carbon and compost preparation etc. Groundnut haulms can be used as fodder. After extraction of oil, the groundnut cake can be used as livestock feed. Being legume, it helps to fix nitrogen in the soil which is valuable for cropping system. Every part of groundnut crop is having commercial value. Groundnut is originated in Northwest Argentina region in South America and spread by Portuguese from Brazil to West Africa and then to Southwestern India in 16th century. It is presently cultivated in more than 108 countries.

During 2017-18, area under groundnut cultivation in India is 4.91 million hectares accounting to 9.18 million tonnes of production. The average productivity of the groundnut in India is about 1893 kg ha^{-1}. In Tamil Nadu, 0.33 million hectares of area comes under groundnut cultivation accounting to 0.97 million tonnes of production. The average productivity of groundnut in Tamil Nadu is about 2914 kg ha^{-1} (1). Export of groundnut from India to world market ranges up to 489187 MT worth of 3298.31 crores during the year 2018-19 (1).

Groundnut production is affected both by biotic and abiotic stresses during different growth stages of crop. Many fungi, bacteria, virus and nematodes are causing biotic stresses which leads to reduction in groundnut production (2). Soil borne fungi are causing serious yield losses in groundnut (3).

Among the different soil borne fungi, *M. phaseolina* which causes dry root rot disease is the most devastating pathogen of groundnut. This disease has been found to cause major losses

from seedling stage to maturity of the crop with disease incidence of 29.30 % and yield loss of 435 kg ha^{-1}. It causes 100 % loss in highly susceptible plants under hot and dry conditions (4). Dry root rot infected groundnut plants show yellowing and drooping of the leaves. Roots of the infected plants show black lesions and bark shredding, which can be easily pulled out due to the rotting of lateral and finer roots (5). *M. phaseolina* has the host range of more than 500 plant species. This pathogen survives well in high temperature and low moisture levels as micro-sclerotia in the soil and plant debris. In plants, the pathogen produces dark mycelia and numerous black sclerotia (6). Pycnidial or conidial stage of the pathogen is often seen on the diseased portions.

To combat the massive yield loss caused by this disease, there is a need to formulate better management strategy by using organic amendments and rhizosphere antagonists. With this view, the present study has been executed.

Materials and Methods

Isolation, purification and maintenance of the pathogen

Dry root rot infected ground nut root samples were collected from VOC Agricultural College and Research Institute, Killikulam. Isolation of the pathogen was done on the PDA medium from dry root rot affected roots of the groundnut plant by using tissue segmentation method (7). Diseased roots were washed with the running tap water. Then, inside the laminar airflow chamber 2-5 mm size of diseased root tissues along with some healthy tissues were sliced out with the help of a sterilized blade. To avoid the surface contaminants, tissues were surface sterilized with 0.1 % mercuric chloride for about 30 sec. After surface sterilization the tissues were washed three times with sterile water to remove the traces of mercuric chloride and blot dried in sterile blotter paper. The diseased root tissues were then transferred aseptically to the sterilized PDA medium and incubated at 28 ± 3 °C for 6 to 7 days. Mycelial growth of the pathogen from the infected tissues was observed periodically. For maintenance of pure culture, fungal hyphae grown from the infected tissues were sub-cultured by transferring it into fresh sterilized PDA slants by single hyphal tip method. Then the culture was stored at 5 °C-6 °C.

Isolation of antagonistic organism from the rhizosphere region of groundnut plants

Rhizosphere soil samples were collected from the groundnut fields of Tirunelveli, Thoothukudi, Tenkasi and Kanyakumari districts of Tamil Nadu. The biocontrol agents like fungal and bacterial antagonists were isolated from the collected rhizosphere soil samples. For the isolation of rhizosphere antagonists, 10 g of rhizosphere soil sample was transferred into 250 mL conical flask containing 100 mL of sterilized distilled water. The antagonists were isolated from the soil suspension by serial dilution technique (8). Then the soil suspension serially diluted from 10^{-1} to 10^{-7}. One mL dilution aliquot from 10^{-5} and 10^{-6} dilutions were added into both Nutrient Agar and King's B medium in the sterile Petri plates. One mL of aliquot solution was pipetted out from 10^{-3} dilution and poured in sterile Petri plates containing *Trichoderma* selective medium by spread plate method. For antagonistic fungi, plates were incubated at room temperature (28 ± 2 °C) for seven days and for antagonistic bacteria, plates were incubated for 24 hr. A total of three bacterial isolates and four fungal isolates were obtained. Pure culture of these isolates was stored in -4 °C. The details of rhizosphere antagonists are listed in Table 1.

Effect of rhizosphere antagonist on the growth of *M. phaseolina* under *in vitro*

The effect of fungal antagonists (RAF1, RAF2, RAF3 and RAF4) and bacterial antagonists (RAB1, RAB2, RAB3) against *M. phaseolina* were tested by dual culture technique. In case of antagonistic fungi, a 9 mm mycelial disc of seven days old culture of *M. phaseolina* was placed at one edge of the sterilized Petri plate containing molten PDA medium and 9 mm disc of fungal antagonist was placed at the opposite edge of the same Petri plate. For antagonistic bacteria, actively growing mycelial disc of 9 mm size of *M. phaseolina* was kept at one edge of the sterile Petri plate and actively growing bacterial cultures were streaked at the opposite edge of the same Petri plate. For each treatment, three replications were maintained along with control. The inoculated plates were incubated at room temperature (28 ± 2 °C) for seven days. The observation on the radial growth of the pathogen was noted for each treatment along with control. The inhibition per cent over control was calculated by using following formula.

$$I = \frac{C - T}{C} \times 100$$

Where,

I = Per cent inhibition over control

C = Growth in control

T= Growth in treatment

Biochemical characterization of rhizosphere antagonists

Biochemical tests were carried out to identify the plant growth promoting microbes. The biochemical tests conducted under laboratory conditions were described below (9).

Gram staining

A thin layer of bacterial colony was smeared with small amount of water on the glass slide. The bacterial smear was air dried and heat fixed. The smear was first coated with 2-3 drops of crystal violet for 30 sec and slightly rinsed in distilled water. Gram's iodine was added to the smear and left for 30 sec and washed slightly with distilled water. Then the bacterium was decolorized using 95 % ethyl alcohol. Then the counter stain safranin was applied to the smear for 30 sec and gently washed with tap water, air dried and observed microscopically using oil immersion at 100× magnification (10).

Table 1. Details of Rhizosphere antagonists collected from different Districts of Tamil Nadu

Sl. no.	Places of collection	District	State	Isolate number*
1	Pudur	Thoothukudi	Tamil Nadu	RAF1
2	Serndhamaram	Tirunelveli	Tamil Nadu	RAF2
3	Thovalai	Kanyakumari	Tamil Nadu	RAF3
4	Aralvaimozhi	Kanyakumari	Tamil Nadu	RAF4
5	Surandai	Tenkasi	Tamil Nadu	RAB1
6	Pudur	Thoothukudi	Tamil Nadu	RAB2
7	Thovalai	Kanyakumari	Tamil Nadu	RAB3

*RAF - Rhizosphere Antagonistic Fungi

*RAB -Rhizosphere Antagonistic Bacteria

Catalase

The bacterial cultures were streaked on the nutrient agar medium. After 48 hr of incubation, 3-4 drops of 3 % hydrogen peroxide was added on the plates. Appearance of effervescence indicated the positive reaction for catalase test (11).

Amylase test

Amylase test was done by using nutrient agar medium containing 2 % soluble starch. The bacterial cultures were streaked on the medium. Starch hydrolysis was tested after 48 hr of incubation by flooding the agar surface with Lugol's iodine solution. Formation of clear colourless zone around the bacterial growth in contrast to the blue background of the medium indicated positive reaction.

Citrate utilization

The bacterial cultures were streaked on the surface of Simmons citrate agar slant and incubated at room temperature at 28 ± 2 °C. Colour change of medium from green to blue colour indicated that the citrate was utilized by bacteria whereas the original green colour indicated the absence of citrate utilization (11).

Ammonia production

Bacterial cultures were inoculated in test tubes containing peptone water and incubated at room temperature for three days. After incubation, Nessler's reagent was added in the test tube. Change of colour from brown indicated positive result (12).

Phosphate solubilization

The bacterial cultures were streaked on the Pikovskaya's agar medium and incubated at 30 °C for 3 days. Formation of clear zone around the bacterial colonies indicated positive reaction (13).

Methyl red test

Test tubes containing methyl red broth were sterilized and inoculated with bacterial cultures and incubated at 28 °C-30 °C for two days. After incubation, five drops of methyl red indicator were added to each tube and shaken gently. Production of red colour in the broth indicated positive reaction.

Urease

The bacterial isolates were streaked onto the urease medium and incubated for two days. After incubation, change of colour in the medium was observed. The positive reaction was confirmed by change of colour from yellow to pink.

Molecular identification of rhizosphere fungal antagonists

Isolation of Genomic DNA

Genomic DNA was isolated from the effective rhizosphere fungal isolates (RAF1 and RAF2) (14). One gram of seven days old fresh mycelia of GM2 isolate was ground using pestle and mortar with CTAB buffer [10 mM Tris (pH: 8.5)], 250 mM EDTA and 0.5 % SDS, NaCl 100 mM. The mixture was transferred into 2 mL Eppendorf tube and incubated at 60 °C for 1 hr. Then the mixture was centrifuged at 12000 rpm for 15 min. The supernatant was transferred into a fresh Eppendorf tube and equal volume of chloroform:isoamyl alcohol (24:1) was added and centrifuged at 12000 rpm for 15 min. The aqueous phase was then transferred into another fresh Eppendorf tube and 50 µL of 100 % chilled ethanol was added and centrifuged at 12000 rpm for 15 min. This process pelletizes the DNA and the pellet was washed with 70 % ethanol by centrifugation. The pellet was air dried and suspended with 50 µL of TE buffer (pH 8.0). The quality of isolated DNA was checked by loading 2.5 µL of DNA in 0.8 % agarose gel electrophoresis.

ITS sequencing of rhizosphere fungal antagonists

PCR reactions were performed in a total volume of 50 µL containing 25 µL of master mix using Emerald Amp® GT PCR master mix, 19 µL of de-ionized water, 2 µL of forward primer, 2 µL of reverse primer and 2 µL of template DNA. PCR reaction was done using genomic DNA of rhizosphere fungal isolates viz. RAF1 and RAF2 as template. The intermediate 5.8S ribosome gene along with ITS1 and ITS2 regions were amplified using the ITS1 and ITS4 primers with PCR conditions of initial denaturing at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30 sec, annealing at 59 °C for 30 sec, extension at 70 °C for 2 min and final extension at 72 °C for 7 min. The reactions were carried out in Eppendorf tube master cycle gradient PCR machine. The PCR products were resolved by electrophoresis in 1 % agarose gel and sent for sequencing at Eurofins Genomics India Pvt. Ltd., Bangalore.

The primers used for amplification of ITS region were,

ITS 1- 5' TCCGTAGGTGAACCTGCGG 3' (forward primer)

ITS 4 - 5' TCCTCCGCTTATTGATATGC 3' (reverse primer)

Molecular identification of rhizosphere bacterial antagonists

Extraction of genomic DNA

For the extraction of genomic DNA, 24 hr old bacterial culture was taken in 2 mL centrifuge tube which was first centrifuged at 10000 rpm for 10 min. The pellet obtained was resuspended with 800 µL of TE buffer and vortexed for 5 min. To the sample, 45 µL of 10 % sodium dodecyl sulphate and 5 µL of Lysozyme were added and kept for incubation at 65 °C. After 1 hr of incubation, the sample was centrifuged for 5 min at 10000 rpm. The supernatant was transferred into fresh Eppondorf tube. Mixture of chloroform and isoamyl alcohol (24:1) was supplemented to the sample, gently flipped and kept for centrifugation at 12000 rpm for 10 min. The aqueous phase was transferred to another fresh Eppendorf tube and 100 µL of 5 M sodium acetate was added. Then equal amount of ice-cold propanol was added and stored at -20 °C for 30 min which was followed by centrifugation at 14000 rpm for 10 min. Supernatant was discarded and 500 µL of 70 % ethanol was added to the pellet centrifuged for 5 min and air dried at room temperature. Supernatant was discarded and pellet was retained. Into the pellet 500 µL of 70 % ethanol was added and centrifuged for 5 min and air dried at room temperature. 50 µL of TE buffer was added to the DNA pellet and stored at -20 °C. The quality of the genomic DNA was checked through 0.8 % agarose gel electrophoresis.

16S rDNA sequencing of rhizosphere bacterial antagonists

A PCR mixture of 50 µL volume was prepared using genomic DNA of bacteria as the template. EmeraldAmp GT PCR master mix and universal primers were used for amplification of 16S regions. 27F and 1115r were the forward and reverse primers used. Initial denaturation temperature of 94 °C for 5 min, denaturation temperature of 94 °C for 30 sec, annealing temperature of 50 °C for 30 sec and extension temperature of 72 °C for 2 min were set and a reaction of 35 cycles was carried out in Eppendorf master cycler gradient PCR machine. After the completion of PCR reaction, the product was tested by gel electrophoresis using 1 % agarose gel. Then the PCR products were sent for sequencing at

Eurofins genomics India Pvt. Ltd., Bangalore.

The primers used were -

27F - 5'AGAGTTTGATCTGGCTCAG 3' (Forward primer)

1115r - 5'AGGGTTGCGCTCGTTG 3' (Reverse primer)

Formulation of rhizosphere antagonist

Preparation of talc-based formulations of antagonistic bacteria: Talc-based formulation of effective rhizosphere bacterial antagonists against *M. phaseolina* was prepared. One loopful of the bacterial isolate was taken and inoculated into the nutrient broth and flasks were placed in the shaker with 150 rpm for 72 hr at room temperature to hasten the bacterial growth. After incubation, the broth having 9×10^8 Cfu/mL was used to prepare talc-based formulation. For this, 1 kg of talc powder, 15 g calcium carbonate (to neutralize pH) and 10 g of CMC (as adhesive) were added to 400 mL of bacterial suspension under sterile condition. Then the mixture was shade dried to bring down the moisture level to 20 %. Finally, the talc formulation was packed into polythene bags and used for pot culture experiment (15).

Preparation of talc-based formulation of antagonistic fungi: Talc-based formulations of effective rhizosphere fungal antagonists (RAF1 and RAF2) against *M. phaseolina* were prepared for soil application. Ten days old culture of fungal antagonists were inoculated into molasses yeast medium and allowed to grow at room temperature for ten days. After ten days, the fungal population of 1 X 10^7 Cfu/mL was added with 1 kg of talc powder and then the mixture was air dried to reduce moisture content and packed in polythene bags. This talc-based formulation was employed for the control of dry root rot pathogen.

Efficacy of organic amendments against the growth of *M. phaseolina*

Preparation of aqueous extracts of various organic amendments: The antifungal activity of seven different organic amendments viz. castor cake, coconut cake, cotton cake, neem cake, sesame cake, mixture cake (cotton cake and sesame cake) and FYM cake was tested against groundnut dry root rot pathogen *M. phaseolina* through poisoned food technique at 10 % and 15 % concentrations. The aqueous extracts of different amendments mentioned above were obtained by suspending 40 g of finely grinded oil cake in 150 mL of sterile distilled water and soaked for ten days. After 10 days, the contents were filtered through three layered muslin cloth. Then the extracts were centrifuged at 10000 rpm for 15 min and supernatant obtained was used as standard solution (100 %).

Testing the inhibitory activity of oil cake extracts on the growth of *M. phaseolina* under *in vitro*: Each organic extracts were taken at 10 mL and 15 mL quantities and mixed with 90 mL and 85 mL molten PDA medium to get 10 % and 15 % concentrations. The flasks containing media were then sterilized by autoclaving at 15 psi for 15 min. Then the poisoned media were poured into the Petri plates and inoculated with fresh culture discs of *M. phaseolina* and incubated at room temperature for seven days. Petri plates containing PDA alone served as control. Three replications were maintained for each treatment. Mycelial growth of the pathogen was observed and noted with which the mycelia inhibition percentage was calculated using Vincent's formula (16)

$$I = \frac{C - T}{C} \times 100$$

Where,

I = Percent inhibition over control

C = Growth in control

T= Growth in treatment

Efficacy of potential rhizosphere antagonist and organic amendments against *M. phaseolina* under pot culture condition

The effective rhizosphere antagonist and organic amendments were tested against *M. phaseolina* under pot culture. The pot culture experiments were conducted with most popular high yielding groundnut Cv. VRI 8 by artificial inoculation of *M. phaseolina* in soil. The pathogen was mass multiplied in sand maize media and mixed with sterile potting mixture at 10 g/pot containing 5 kg of soil. In each pot, five groundnut seeds were sown. All the treatments were applied at 30 days after sowing. Three replications were maintained for each treatment and inoculated, uninoculated control was also maintained. Completely Randomized Design (CRD) is used for this experiment. The treatment details are listed in the Table 2.

In the pot culture experiment groundnut root rot disease incidence was calculated by using the following formula

$$\text{Percent disease incidence} = \frac{\text{Number of plants infected}}{\text{Total number of plants observed}} \times 100$$

Efficacy of potential rhizosphere antagonist and organic amendments against *M. phaseolina* under field condition

The effective rhizosphere antagonists and organic amendments tested under pot culture condition were also tested against *M. phaseolina* under field condition. The field experiment was conducted at V.O.C AC & RI, Killikulam with groundnut Cv. VRI 8. All the treatments were applied immediately after the appearance of symptom (30 days after sowing). Three replications were maintained for each treatment and uninoculated control was also maintained. Treatment details are given in Table 3.

In the above experiment, groundnut root rot disease incidence was recorded by using following formula,

$$\text{Percent disease incidence} = \frac{\text{Number of plants infected}}{\text{Total number of plants observed}} \times 100$$

Table 2. Treatment details for pot culture experiment

Treatment no.	Treatments
T1	Soil application with *T. virens* @ 5g/pot
T2	Soil application with *Trichoderma asperellum* @ 5g/pot
T3	Soil application with *Bacillus subtilis* @ 5g/pot
T4	Soil application of neem cake @ 5g/pot
T5	Soil application of *T. virens* @ 5g/pot + neem cake @ 5g/pot
T6	Soil application of *T. asperellum* @ 5g/pot + neem cake @ 5g/pot
T7	Soil application of *B. subtilis* @ 5g/pot + neem cake @ 5g/pot
T8	Soil drenching with Carbendazim 50 WP @ 0.1 %
T9	Inoculated control

Table 3. Treatment details of field experiment

Treatment no.	Treatments
T1	Soil application with *T. virens* @ 2.5 kg/ha
T2	Soil application with *T. asperellum* @ 2.5 kg/ha
T3	Soil application with *B. subtilis* @ 2.5 kg/ha
T4	Soil application of neem cake @ 150 kg/ ha
T5	Soil application of *T. virens* @ 2.5 kg/ha + neem cake @ 150 kg/ ha
T6	Soil application of *T. asperellum* @ 2.5 kg/ha + neem cake @ 150 kg/ ha
T7	Soil application of *B. subtilis* @ 5g/pot + neem cake @150 kg/ha
T8	Soil drenching with Carbendazim 50 WP @ 0.1 %
T9	Untreated control

Statistical analysis

Lab experiments were carried out under Completely Randomized Block Design (CRBD). The data were recorded in a Microsoft Excel spread sheet and analyses were done by the AGRES standard error and significant difference between values were determined using Duncan's multiple range test (p= 0.05). The graphs and the other figures are presented with the help of the MS Excel program.

Results and Discussion

Symptomatology

Dry root rot affected groundnut plants showed dark lesions around the stem and collar portion during initial stage. When the affected plants were uprooted, the root portions were severely disintegrated and prominent black coloured pycnidia were found. In later stage, infected plants showed wilting and premature dying (Fig. 1 & 2) the dry root rot infected groundnut plants showed necrotic lesions on stem and shredding, rotting on the taproot, along with the presence of numerous sclerotia on the infected kernels (17). Similar types of symptoms in charcoal rot-affected pulse plants were observed with the presence of dark, minute pinhead sclerotia on root portions (18).

Isolation of groundnut dry root pathogen *M. phaseolina*

Groundnut dry root rot pathogen was isolated from diseased groundnut root samples collected from Agricultural College and Research Institute, Killikulam by tissue segment method aseptically and pure culture was maintained. Similarly, *M. phaseolina* from different plant species like castor, sesame, sunflower, cotton, peanut was isolated which exhibited typical dry root rot symptoms (19). Also, twenty isolates of *M. phaseolina* were isolated from the chickpea plants collected from ten different states of India (20).

Morphological identification of the pathogen

In the present study, the pathogen was identified as *M. phaseolina* based on the colony colour, texture, mycelial branching pattern, number of sclerotia and growth rate. *M. phaseolina* showed cottony, aerial, appressed type of intense, black-coloured mycelial growth, which produced hyaline septate mycelia and a greater number of sclerotia (Fig. 3). Identical results were reported previously by studying the phenotypic characters of ten isolates of *M. phaseolina* from maize plants and concluded that this pathogen produced, coloured mycelium and profuse aerial mycelia with abundant sclerotial production (17).

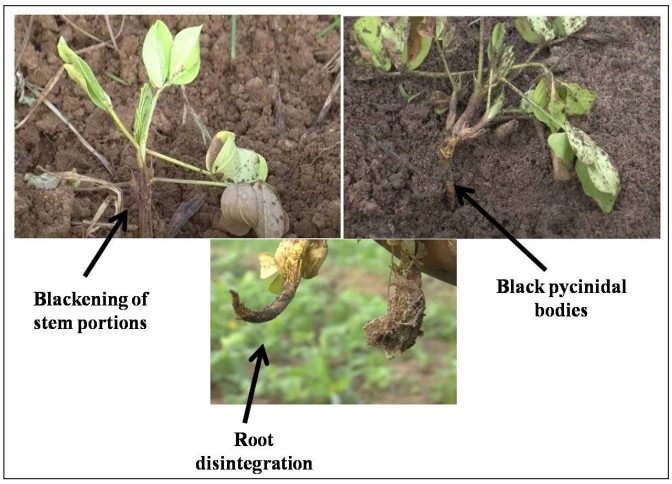


Fig. 1. Symptoms on the dry root rot infected groundnut plants.

Fig. 2. Complete wilting of the dry root rot infected plant.

Isolation of rhizosphere antagonists

Four isolates of fungal antagonists (RAF1, RAF2, RAF3 and RAF4) and three isolates of bacterial antagonists (RAB1, RAB2 and RAB3) were isolated from the rhizosphere region of healthy native groundnut soils collected from Thoothukudi, Tirunelveli and Kanyakumari districts of Tamil Nadu. The isolated rhizosphere antagonists were identified based on their morphology and the results are appended in Table 4.

Table 4. Details of rhizosphere antagonists

Sl. no.	Isolate no.	Isolate name
1	RAF1	*Trichoderma* sp. (Tsp1)
2	RAF2	*Trichoderma* sp. (Tsp2)
3	RAF3	*Trichoderma* sp. (Tsp3)
4	RAF4	*Trichoderma* sp. (Tsp4)
5	RAB1	*Bacillus* sp. (Bsp1)
6	RAB2	*Bacillus* sp. (Bsp2)
7	RAB3	*Pseudomonas* sp.

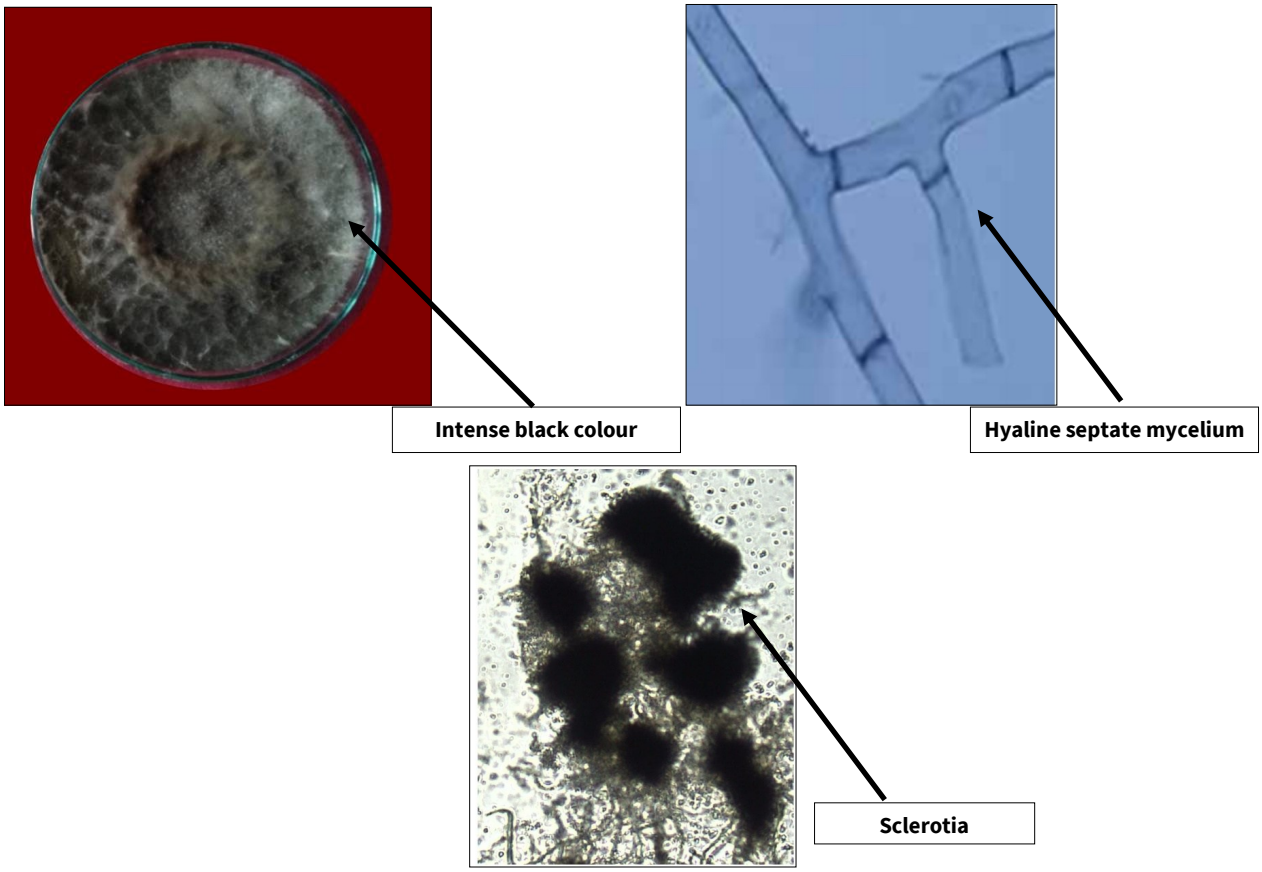


Fig. 3. Morphology of root rot pathogen *M. phaseolina*.

Biochemical characterization of rhizosphere antagonistic bacteria

The results of biochemical characterization of rhizosphere antagonistic bacteria are presented (Table 5, Fig. 4(a-g)).

Gram's staining

Among the three bacterial isolates Bsp1 and Bsp2 were confirmed as Gram positive bacteria whereas *Pseudomonas* sp. was confirmed as Gram negative bacteria.

Catalase

All the bacterial isolates viz. Bsp1, Bsp2 and *Pseudomonas* sp. were produced effervescence on the media when hydrogen peroxide was added over the isolates and confirmed the positive reaction to catalase activity (Fig. 4a).

Amylase

The bacterial isolates viz. Bsp1and Bsp2 were showed positive reaction by the formation of clear halo around their colonies reaction with iodine solution. The isolate *Pseudomonas* sp. did not produce clear halo around its colony hence it showed negative reaction (Fig. 4b).

Table 5. Biochemical tests for rhizosphere antagonistic microbes

Sl. no.	Biochemical tests	Bsp1	Bsp2	*Pseudomonas* sp.
1	Gram staining	+	+	-
2	Catalase	+	+	+
3	Amylase	+	+	-
4	Citrate utilization	+	+	+
5	Ammonia production	-	-	+
6	Phosphate solubilization	+	+	-
7	Methyl red test	-	-	-
8	Urease	-	-	-

Citrate utilization

All the bacterial isolates (Bsp1, Bsp2 and *Pseudomonas* sp.) were showed positive reaction by changing colour of the medium from green to blue (Fig. 4c).

Ammonia production

The bacterial isolate *Pseudomonas* sp. alone showed positive reaction by changing the colour of the broth form yellow to brown on addition of Nessler's reagent. The other two isolates (Bsp1 and Bsp2) showed negative reaction (Fig. 4d).

Phosphate solubilisation

The bacterial antagonists Bsp1 and Bsp2 showed positive reaction to this test by producing a clear halo on Pikovskaya's agar. The isolate of *Pseudomonas* sp. was showed negative reaction (Fig. 4e).

Methyl red test

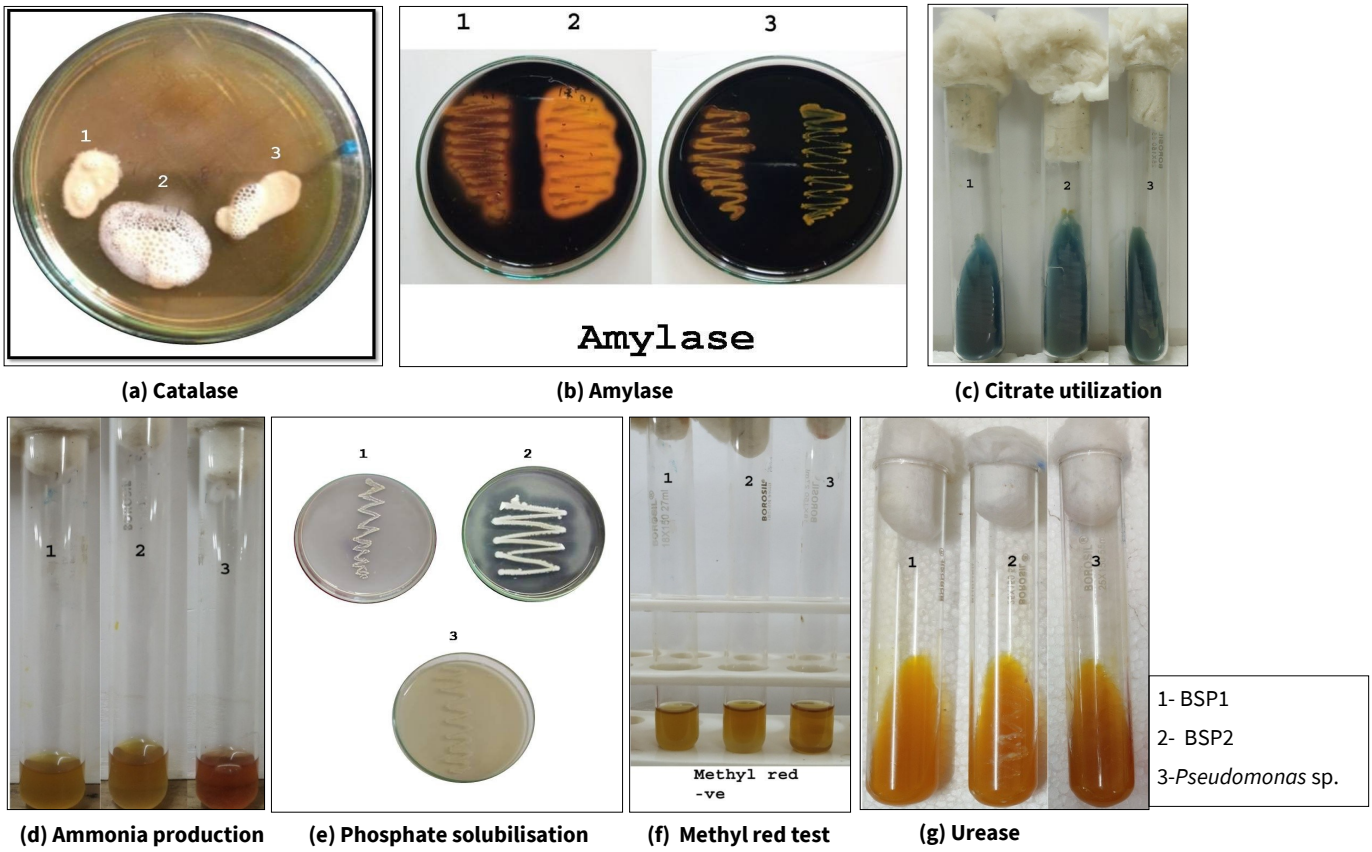
All the bacterial isolates (Bsp1, Bsp2 and *Pseudomonas* sp.) were showed negative result as there was no formation of red colour (Fig. 4f).

Urease

All the bacterial isolates (Bsp1, Bsp2 and *Pseudomonas* sp.) were showed negative result as there was no change in the colour of the medium from yellow to pink (Fig. 4g).

Evaluation of rhizosphere antagonists against pathogen under *in vitro*

By dual plate technique, the effect of antagonistic fungi viz. *Trichoderma* spp. (Tsp1, Tsp2, Tsp3, Tsp4) and three isolates of rhizosphere antagonistic bacteria viz. *Bacillus* sp. (Bsp1, Bsp2) and *Pseudomonas* sp. were tested against *M. phaseolina*. Among the rhizosphere antagonists tested, *Trichoderma* sp. (Tsp1) recorded highest mycelial inhibition percentage of 67.81 over



(a) Catalase **(b) Amylase** **(c) Citrate utilization**

(d) Ammonia production **(e) Phosphate solubilisation** **(f) Methyl red test** **(g) Urease**

Fig. 4(a-g). Biochemical characterization of bacterial antagonists.

control followed by *Trichoderma* sp. (Tsp2) and *Bacillus* sp. (Bsp2) exhibited the mycelial inhibition percentage of 64.99 and 61.11 respectively. *Pseudomonas* sp. recorded least mycelial inhibition percentage of 40.88 (Table 6, Fig. 5). The effective antagonistic organism *Trichoderma* sp. (Tsp1) can be used as alternate biocontrol agent to manage the ground nut root rot disease. Similar results were reported in a previous study, where *T. virens* exhibited 76.00 % mycelial inhibition of the charcoal rot pathogen of maize under *in vitro* (21).

Molecular identification of rhizosphere antagonistic fungi

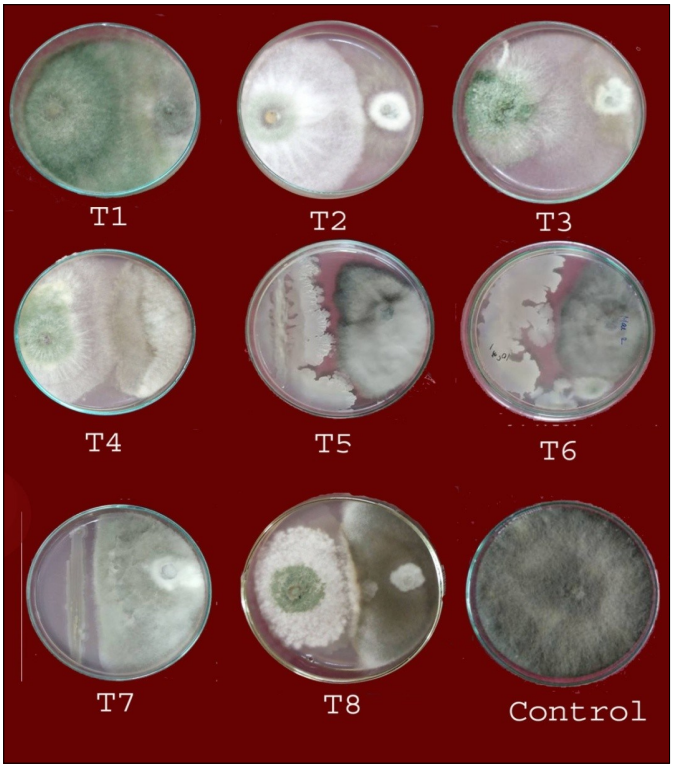
In the present investigation, ITS1 and ITS4 primers were used for the amplification of ITS region from effective fungal antagonists viz. Tsp1 and Tsp2. From the above experiment 590 bp size of amplicons were obtained for Tsp1 and Tsp2 respectively (Fig. 6). The amplicons were sequenced and analysed through blast which resulted Tsp1 as *T. virens* and Tsp2 as *T. asperellum*. The sequence of Tsp1 and Tsp2 isolates were published in NCBI database with the accession number of MW487249 and

Table 6. *In vitro* growth inhibition of *M. phaseolina* by rhizosphere antagonists

Treatment no.	Rhizosphere antagonists	*Mycelial growth of the pathogen (cm)	*Mycelial inhibition percentage over control (%)
T1	Tsp1	2.89	67.81 (55.43)[a]
T2	Tsp 2	3.15	64.99 (53.72)[b]
T3	Tsp 3	3.51	60.92 (51.30)[c]
T4	Tsp 4	4.82	46.22 (42.93)[e]
T5	Bsp1	4.40	51.11 (45.63)[d]
T6	Bsp 2	3.60	61.11 (51.41)[c]
T7	*Pseudomonas* sp.	5.26	40.88 (39.74)[g]
T8	*T. viride* (standard culture)	5.14	42.88 (40.90)[f]
T9	Control	9.00	0.00
	SE(d)	**0.09**	**0.31**
	CD (p = 0.05)	**0.25**	**0.65**

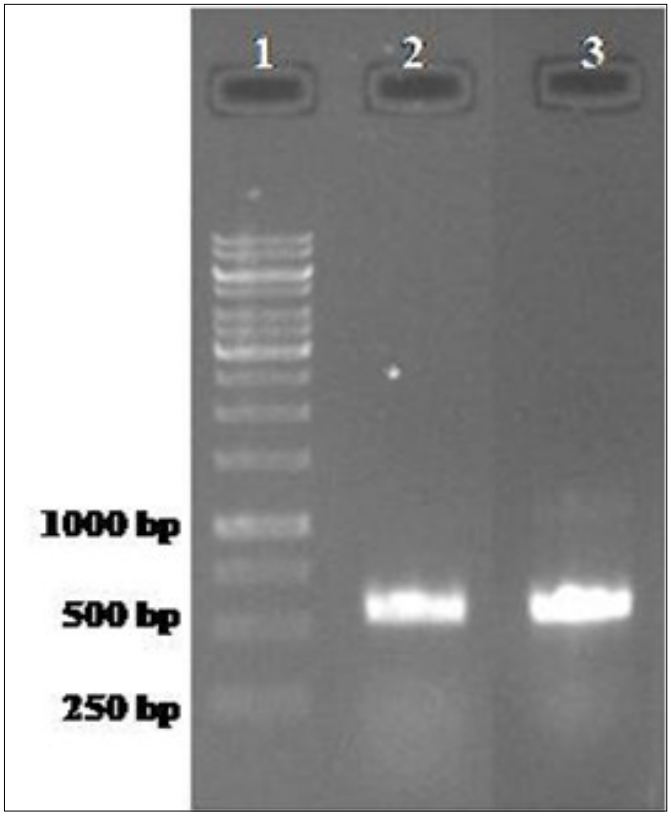
*Means of three replications

*Values in the parentheses are arcsine transformed values



T1 - Tsp1
T2 - Tsp2
T3 - Tsp3
T4 - Tsp4
T5 - Bsp1
T6 - Bsp2
T7 - *Pseudomonas* sp.
T8 - *T. viride*

Fig. 5. Effect of plant growth promoting rhizospheric microbes on the growth of *M. Phaseolina*.



Lane 1: Ladder (1kb)
Lane 2: Tsp1 isolate
Lane 3: Tsp2 isolate

Fig. 6. PCR amplification of ITS region of Tsp1 and Tsp2.

MW487267 respectively (Fig. 7-10). Similarly, several antagonistic fungi from healthy soils and further they amplified the ITS region with the amplicon size of 650 bp. They also sequenced the amplified regions and analysed through BLAST search. Based on this study, they confirmed the antagonistic fungi as *Trichoderma harzianum* (22).

Molecular identification of rhizosphere antagonistic bacteria

In the present study, 27F and 1115r primers were used for the amplification of 16S rDNA region from effective antagonistic bacteria Bsp2. From the above experiment, 1250 bp size of amplicons were obtained for Bsp2 (Fig. 11). The amplicon was then sequenced and BLAST analysed which resulted Bsp2 as *B. subtilis*. The sequence of Bsp2 isolate was published in the NCBI database with the accession number of MW494628 (Fig. 12 & 13). Similarly different bacterial antagonists were isolated and further amplified the 16S rDNA region with the amplicon size of 1500 bp which was then sequenced and analysed through BLAST and further confirmed the antagonistic bacteria as *B. subtilis* (23).

Effect of extracts of different organic amendments against the root rot disease under *in vitro*

Antifungal efficacy of seven different oil cakes against groundnut dry root rot pathogen *M. phaseolina* was tested at 10 % and 15 % concentration under *in vitro*. Among the different organic amendments tested, neem cake at 10 % and 15 % concentration reduced the mycelial growth of the pathogen to 52.21 % and 52.50 % respectively. Least mycelial inhibition of 8.88 % and 27.00 % were recorded in mixture cake at 10 % and 15 % concentration respectively (Table 7, Fig. 14 & 15). It has been reported that a 20 % concentration of neem cake extract effectively inhibited the mycelial growth of *M. phaseolina* (59.40 %) (24), which the current results agree with.

Efficacy of potential rhizosphere antagonists and organic amendments against *M. phaseolina* under pot culture

Effective rhizosphere antagonists and organic amendments were tested against the dry root rot pathogen *M. phaseolina* under pot culture. Among the different treatments assessed, soil application of *T. virens* @ 5g/pot + neem cake @ 5g/pot was found to be effective by recording minimum disease incidence of 22.21 % against inoculated control (88.88 %) (Table 8).

Similarly soil application of *T. viride* + pungam cake showed least root rot disease incidence of 3.12 % followed by soil application of *T. viride* + neem + neem soil application of carbendazim were showed disease incidence of 5.62 and 6.87 per cent respectively (25)

Efficacy of potential rhizosphere antagonist and organic amendments against *M. phaseolina* under field condition

Effective rhizosphere antagonists and organic amendments were tested against the dry root rot pathogen *M. phaseolina* under field condition. Soil application of *T. virens* @ 2.5 kg/ha + Neem cake @ 150 kg/ha was recorded lowest disease incidence of 10.32 % and highest yield of 3200 kg/ha against control (58.57 %, 900 kg/ha respectively) (Table 9). Similarly, application of *T. asperellum* recorded lowest disease incidence (15 %) of maize charcoal rot against control (60 %) (26).

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Trichoderma virens clone SF_750 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer...	Trichoderma virens	667	667	88%	0.0	97.24%	621	MT530036.1
Trichoderma virens clone SF_586 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer...	Trichoderma virens	667	667	88%	0.0	97.24%	626	MT529862.1
Trichoderma virens clone SF_166 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer...	Trichoderma virens	667	667	88%	0.0	97.24%	624	MT529442.1
Trichoderma virens clone SF_136 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer...	Trichoderma virens	667	667	88%	0.0	97.24%	626	MT529406.1
Trichoderma virens clone SF_29 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer...	Trichoderma virens	667	667	88%	0.0	97.24%	636	MT529305.1
Trichoderma virens isolate KSSO1_2_6 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene...	Trichoderma virens	667	667	88%	0.0	97.24%	581	MN652840.1
Trichoderma virens isolate K_MISO2_2_14 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA g...	Trichoderma virens	667	667	88%	0.0	97.24%	579	MN652838.1
Trichoderma virens isolate W_MISO2_6_21 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA g...	Trichoderma virens	667	667	88%	0.0	97.24%	578	MN652837.1
Trichoderma virens isolate K_MISO2_2_1 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA ge...	Trichoderma virens	667	667	88%	0.0	97.24%	545	MN652800.1
Asterodon ferruginosus voucher research collection Farrer lab 258 internal transcribed spacer 1, partial sequence...	Asterodon ferrugi...	667	667	88%	0.0	97.24%	1151	MN644787.1
Trichoderma afroharzianum voucher research collection Farrer lab 251 internal transcribed spacer 1, partial seque...	Trichoderma afro...	667	667	88%	0.0	97.24%	1142	MN644782.1
Fusarium sporotrichioides voucher research collection Farrer lab 227 internal transcribed spacer 1, partial sequenc...	Fusarium sporotri...	667	667	88%	0.0	97.24%	1141	MN644771.1
Chromoclelsta sp. voucher research collection Farrer lab 226 internal transcribed spacer 1, partial sequence; 5.8S...	Chromoclelsta sp.	667	667	88%	0.0	97.24%	1149	MN644770.1

Fig. 7. NCBI BLAST analysis for ITS sequence of Tsp1.

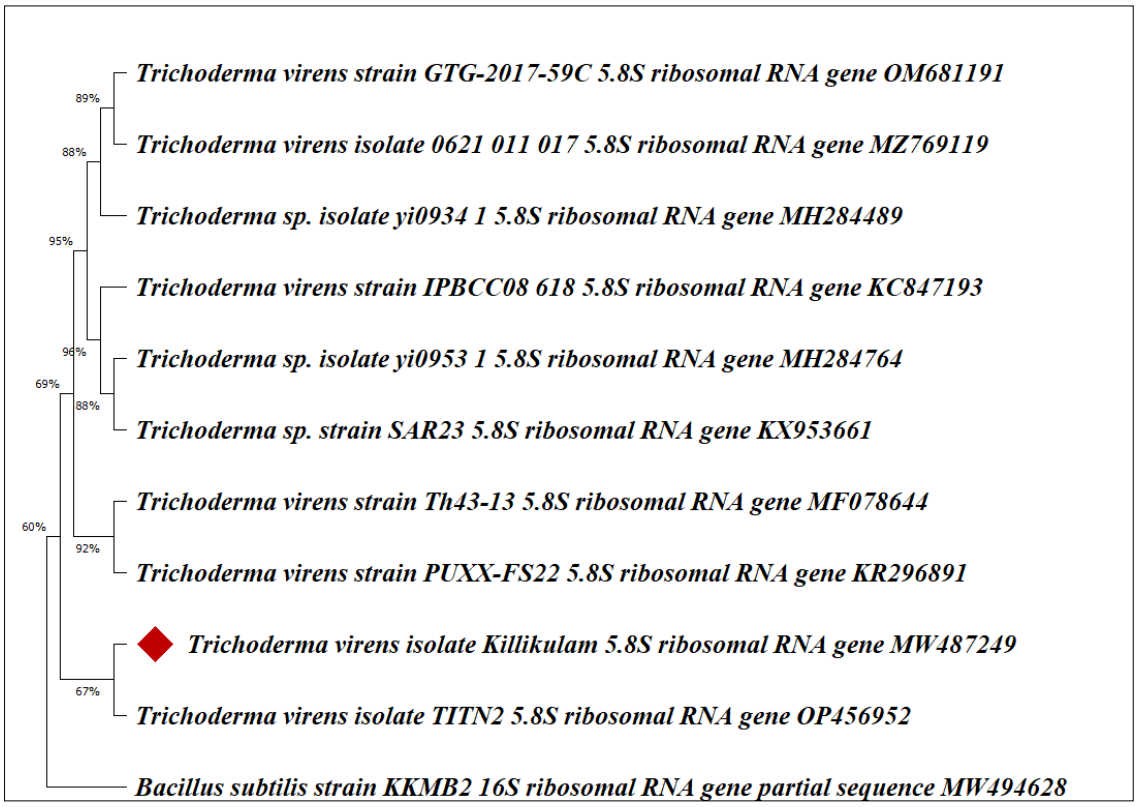


Fig. 8. Phylogenetic analysis of Tsp1.

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Trichoderma hamatum isolate HHFG12 small subunit ribosomal RNA gene, partial sequence; internal transcribed...	Trichoderma ham...	989	989	89%	0.0	98.23%	898	MT111894.1
Trichoderma asperellum strain NFML_CH10_No 18S ribosomal RNA gene, partial sequence; internal transcribed...	Trichoderma asp...	987	987	89%	0.0	98.40%	610	KM458788.1
Trichoderma asperellum strain TR15 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5...	Trichoderma asp...	987	987	89%	0.0	98.40%	751	KC859434.1
Trichoderma asperelloides isolate AMUTA-1 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA...	Trichoderma asp...	985	985	89%	0.0	98.23%	630	MH249249.1
Trichoderma asperellum isolate T1 small subunit ribosomal RNA gene, partial sequence; internal transcribed spac...	Trichoderma asp...	985	985	89%	0.0	98.40%	602	KY819794.1
Trichoderma asperellum clone SF_146 small subunit ribosomal RNA gene, partial sequence; internal transcribed s...	Trichoderma asp...	983	983	88%	0.0	98.39%	621	MT529422.1
Trichoderma yunnanense isolate Ty_Ludhiana internal transcribed spacer 1, partial sequence; 5.8S ribosomal RN...	Trichoderma yun...	983	983	90%	0.0	98.06%	671	MT306873.1
Trichoderma asperellum strain WZ-121 small subunit ribosomal RNA gene, partial sequence; internal transcribed...	Trichoderma asp...	983	983	88%	0.0	98.39%	606	MN872484.1
Trichoderma asperellum isolate T1 (Forward) internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA...	Trichoderma asp...	983	983	89%	0.0	98.23%	603	MK928414.1
Trichoderma sp. isolate SDAS203441 small subunit ribosomal RNA gene, partial sequence; internal transcribed s...	Trichoderma sp.	983	983	88%	0.0	98.56%	609	MK871238.1

Fig. 9. NCBI BLAST analysis for ITS sequence of Tsp2.

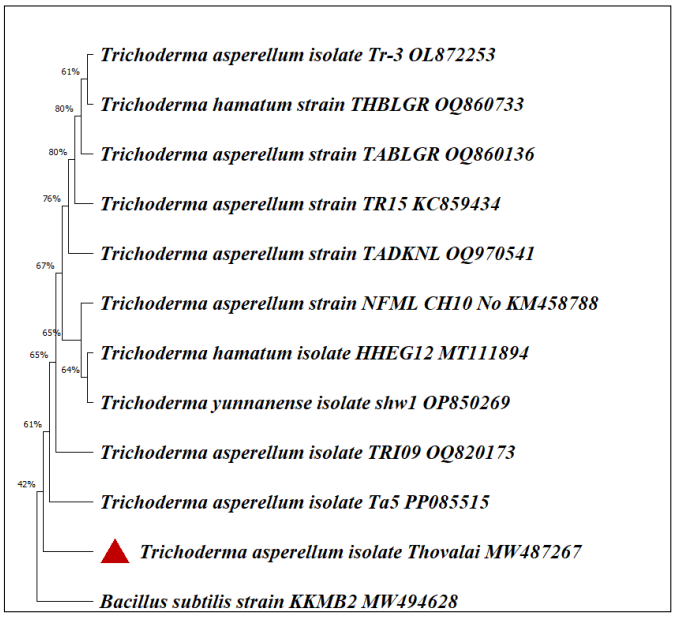


Fig. 10. Phylogenetic analysis of Tsp2.

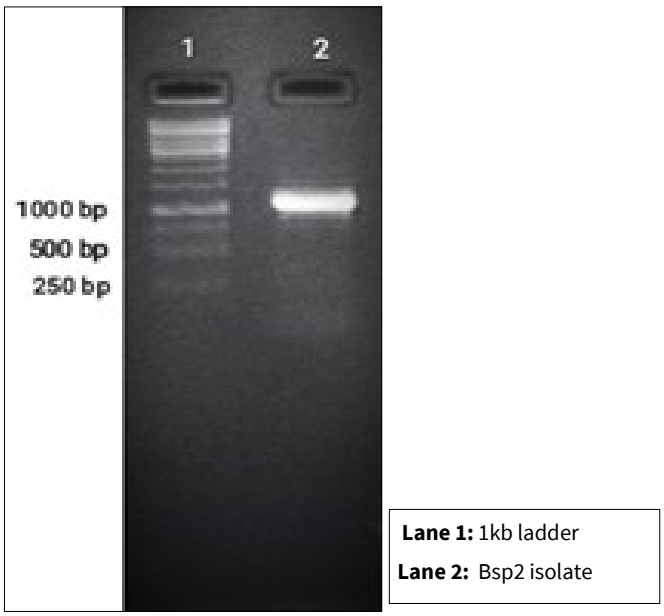


Fig. 11. PCR amplification of 16S rDNA of Bsp2.

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident
Bacillus sp. (in: Bacteria) strain DCAST-14 16S ribosomal RNA gene, partial sequence	Bacillus sp. (in: Bacteria)	532	532	34%	1e-146	95.00%
Bacillus subtilis strain MML2458 16S ribosomal RNA gene, partial sequence	Bacillus subtilis	531	531	34%	4e-146	94.99%
Bacillus sp. (in: Bacteria) strain MNBR 31 16S ribosomal RNA gene, partial sequence	Bacillus sp. (in: Bacteria)	529	529	33%	2e-145	95.48%
Bacillus amyloliquefaciens strain WZZ002 16S ribosomal RNA gene, partial sequence	Bacillus amyloliquefaciens	529	529	34%	2e-145	94.99%
Bacillus subtilis strain 264AY3 16S ribosomal RNA gene, partial sequence	Bacillus subtilis	529	529	34%	2e-145	94.72%
Bacillus amyloliquefaciens strain MR-AI 16S ribosomal RNA gene, partial sequence	Bacillus amyloliquefaciens	529	529	34%	2e-145	94.72%
Bacillus amyloliquefaciens strain EXWB3-03 16S ribosomal RNA gene, partial sequence	Bacillus amyloliquefaciens	529	529	34%	2e-145	94.72%
Bacillus amyloliquefaciens strain BV2007 16S ribosomal RNA gene, partial sequence	Bacillus amyloliquefaciens	527	527	33%	6e-145	95.21%

Fig. 12. NCBI BLAST analysis for 16S rDNA sequence of Bsp2.

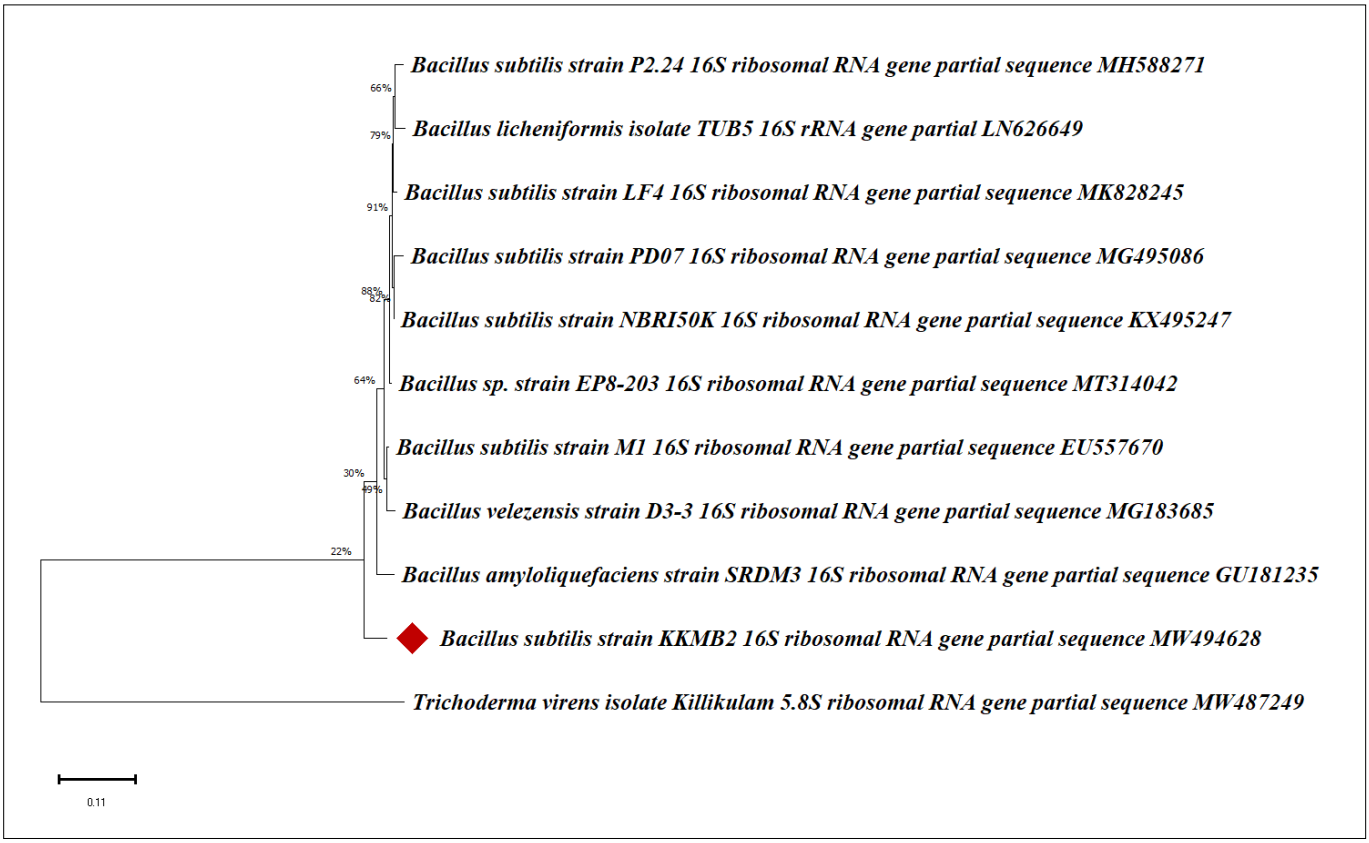


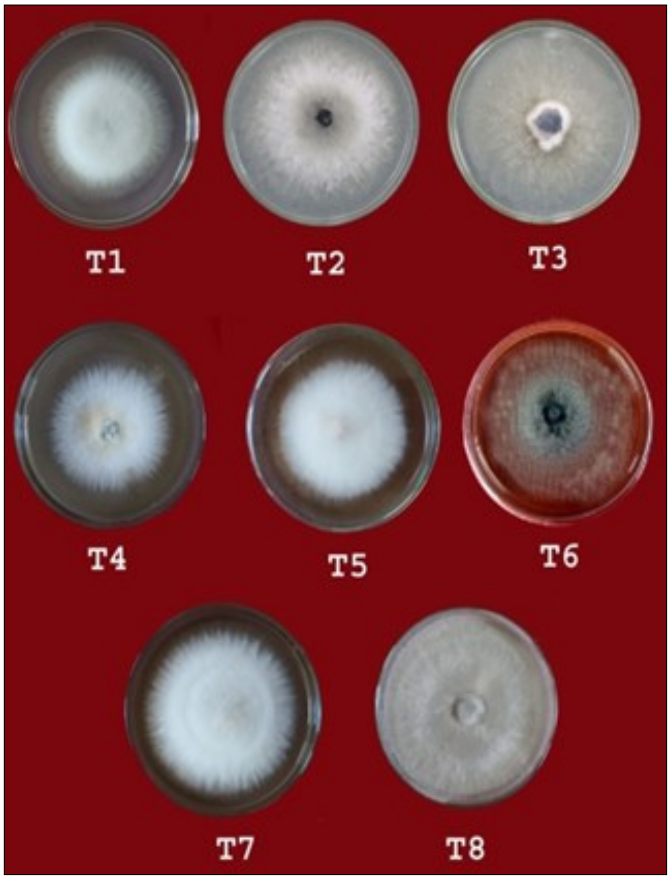
Fig. 13. Phylogenetic analysis of Bsp2.

Table 7. Efficacy of extracts of organic amendments at 10 % and 15 % concentration against the growth of *M. phaseolina*

Treatment no.	Organic amendments	*Mycelial growth of the pathogen @ 10 % Conc. (cm)	*Per cent mycelial inhibition over control	*Mycelial growth of the pathogen @ 15 % Conc. (cm)	*Per cent mycelial inhibition over control
T1	Castor cake	4.76	47.03 (43.29)[b]	5.20	42.22 (40.39)[b]
T2	Coconut cake	7.96	11.48 (19.77)[d]	5.80	37.00 (36.66)[e]
T3	Cotton cake	8.17	9.25 (17.66)[e]	5.26	41.46 (40.04)[c]
T4	Neem cake	4.20	52.21 (46.27)[a]	4.26	52.50 (46.47)[a]
T5	Sesame cake	6.96	22.59 (28.36)[c]	5.80	35.00 (36.12)[f]
T6	Mixture cake	8.20	8.88 (17.25)[f]	6.60	27.00 (31.29)[g]
T7	Farmyard manure	8.16	9.25 (17.62)[e]	5.60	37.20 (37.62)[d]
T8	Control	9.00	-	9.00	-
SE(d)		**0.13**	**1.22**	**0.09**	**0.71**
CD(p=0.05)		**0.25**	**2.59**	**0.20**	**1.52**

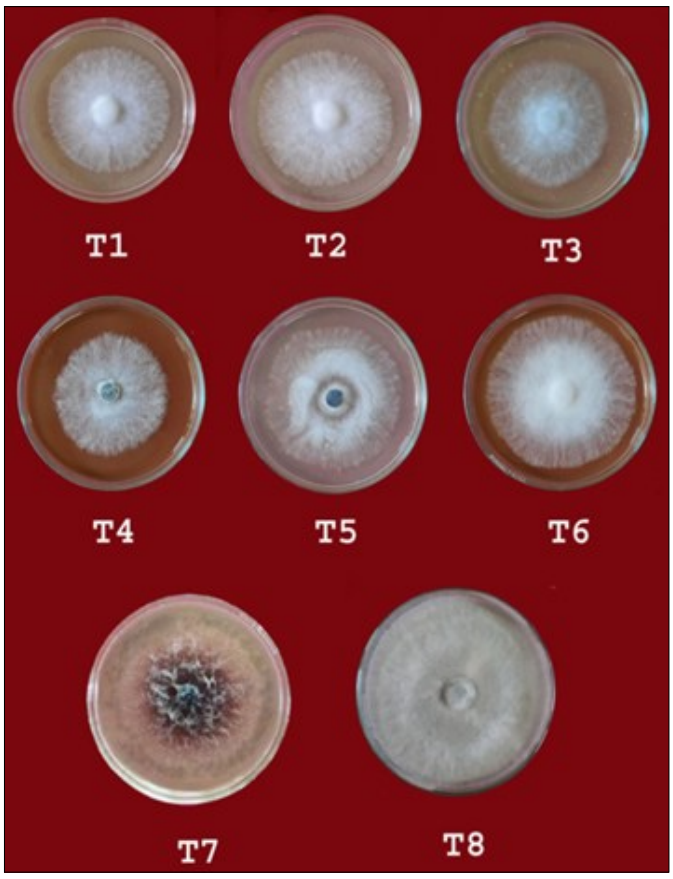
*Means of three replications

*Values in the parentheses are arcsine transformed values



T1 - Castor cake extract **T5-** Sesame cake extract
T2 - Coconut cake extract **T6-** Mixture cake extract
T3 - Cotton cake extract **T7-** FYM cake extract
T4 - Neem cake extract **T8-** Control

Fig. 14. *In vitro* assessment of aqueous extracts of different organic amendments (10 %) on the growth of *M. phaseolina*.



T1 - Castor cake extract **T5-** Sesame cake extract
T2 - Coconut cake extract **T6-** Mixture cake extract
T3 - Cotton cake extract **T7-** FYM cake extract
T4 - Neem cake extract **T8-** Control

Fig. 15. *In vitro* assessment of aqueous extracts of different organic amendments (15 %) on the growth of *M. phaseolina*.

Table 8. Efficacy of potential rhizosphere antagonists, organic amendments and fungicides against *M. phaseolina* under pot culture

Sl. no.	Treatments	* Disease incidence (%)	*Per cent disease reduction over control
T1	Soil application of *T. virens* @ 5g/pot	49.99[f]	43.75
T2	Soil application of *T. asperellum* @ 5g/pot	61.10[g]	31.25
T3	Soil application of *B. subtilis* @ 5g/pot	66.66[h]	25.00
T4	Soil application of neem cake @ 5g/pot as basal	44.44[e]	50.00
T5	Soil application of *T. virens* @ 5g/pot + neem cake @ 5g/pot	22.21[a]	75.00
T6	Soil application of *T. asperellum* @ 5g/pot + neem cake @ 5g/pot	33.33[c]	62.50
T7	Soil application of *B. subtilis* @ 5g/pot + neem cake @ 5g/pot	38.88[d]	56.25
T8	Soil drenching with Carbendazim 50 WP @ 0.1%	27.77[b]	69.00
T9	Inoculated control	88.88[i]	-
	CD (p = 0.05)	**21.76**	**14.92**
	SE(d)	**10.43**	**7.18**

*Means of three replications

*Values in the parentheses are arcsine transformed values

Table 9. Efficacy of potential rhizosphere antagonists and organic amendments against *M. phaseolina* under field condition

Sl. no.	Treatments	* Disease incidence (%)	*Per cent disease reduction over control	Yield (Kg/ha)
T1	Soil application of *T. virens* @ 2.5 kg/ha	26.62[f]	54.55 (47.61)[f]	1500[f]
T2	Soil application of *T. asperellum* @ 2.5 kg/ha	32.21[g]	45.00 (42.13)[g]	1420[g]
T3	Soil application of *B. subtilis* @ 2.5 kg/ha	33.63[h]	42.58 (40.73)[h]	1200[h]
T4	Soil application of neem cake @ 150 kg/ha as basal	25.58[e]	56.32 (48.63)[e]	1600[e]
T5	Soil application of *T. virens* @ 2.5 kg/ha + neem cake @ 150 kg/ha	10.32[a]	82.38 (65.18)[a]	3200[a]
T6	Soil application of *T. asperellum* 2.5 kg/ha + neem cake @ 150 kg/ha	18.32[c]	68.72 (54.78)[c]	2000[c]
T7	Soil application of *B. subtilis* 2.5 kg/ha + neem cake @ 150 kg/ha	23.87[d]	59.24 (50.32)[d]	1865[d]
T8	Soil drenching with Carbendazim 50 WP @ 0.1 %	12.56[b]	78.55 (62.41)[b]	2600[b]
T9	Untreated control	58.57[i]	-	900[i]
	CD (p=0.05)	**19.23**	**12.82**	**158.69**

*Means of three replications

*Values in the parentheses are arcsine transformed values

Conclusion

This study revealed the effect of anatagonists in combination with organic amendments for the management of dry root rot disease of groundnut. Application of biocontrol agent triggers the plant's natural defence mechanisms, making the plant more resistant to subsequent pathogen attacks. As well as it enhances the plant health and growth by improving nutrient absorption or producing plant growth-stimulating compounds. Oil cakes help to manage plant diseases by releasing compounds toxic to pathogens, promoting beneficial soil microbes, improving soil health through enhanced nutrient and water retention, besides acting as a natural bio-fumigant. Hence, application of *T. virens* @ 2.5 kg/ha + neem cake @ 150 kg/ha in groundnut reduced the root rot incidence, increased the yield and which is also cost effective.

Acknowledgements

The authors are thankful to The Professor and Head, Department of Plant Pathology, V.O. Chidambaranar Agricultural College and Research Institute, Killikulam for providing the required research facilities.

Authors' contributions

RN designed the study, did the *in vitro* assay and molecular identification of biocontrol agents. KS carried out pot culture experiments, TR carried out the field experiment, EK and LD assisted in field experiment, MA, KK and SJ compiled the manuscript. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: The authors declare that there is no conflict of interest among them.

Ethical issues: None

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