PLANT SCIENCE TODAY
ISSN 2348-1900 (online)
Vol 11(sp4): 01–15
https://doi.org/10.14719/pst.5506

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RESEARCH ARTICLE

Prevalence of groundnut root rot and antifungal potential of wild mushroom extracts against *Macrophomina phaseolina*

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OPEN ACCESS

ARTICLE HISTORY
Received: 03 October 2024
Accepted: 02 November 2024
Available online
Version 1.0 : 25 December 2024

Check for updates

Additional information
Peer review: Publisher thanks Sectional Editor and the other anonymous reviewers for their contribution to the peer review of this work.

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CITE THIS ARTICLE
Vijayaragavi K, Revathy N, Yesuraja I, Chandramani P, Merina PK, Mareeswari P. Prevalence of groundnut root rot and antifungal potential of wild mushroom extracts against *Macrophomina phaseolina*. Plant Science Today.2024;11(sp4);01-15. https://doi.org/10.14719/pst.5506

Abstract

Macrophomina phaseolina is an important pathogen threatening groundnut production, with climate change-induced variations in temperature and precipitation resulting in considerable yield reductions. This research provides a comprehensive evaluation of the root rot prevalence and severity in the primary groundnut-cultivating areas of Tamil Nadu. Findings indicate an incidence range of 32.71% to 85.66%. Pathogenic variability testing identified GMP-3 as the most virulent strain. Morphological differences and sporulation patterns were observed and *M. phaseolina* isolates were confirmed through ITS 1 and ITS 4-sequencing, yielding Polymerase chain reaction amplicons of approximately 560 bp, which were submitted to Gene Bank. Additionally, a survey was conducted to collect various wild mushroom species from different regions in Tamil Nadu. Phenotypic and morphological characterization and molecular confirmation of the mushroom isolates were performed. The efficacy of these isolates against *M. phaseolina* was evaluated. The *Tricholoma equestre* (AWM-4) isolate demonstrated the highest inhibition in dual culture assays at 74.33%. Mushroom extracts prepared using various polar and nonpolar solvents were tested using the poisoned food technique to assess the inhibition percentage of *M. phaseolina* mycelial growth. Methanol extracts exhibited the greatest reduction in mycelial growth, with an inhibition rate of 75.56%. Gas Chromatography-Mass Spectrometry (GC-MS) analysis was performed to identify the compounds with active antifungal properties.

Keywords

active compounds; antifungal activity; molecular; morphological; survey

Introduction

The groundnut (*Arachis hypogaea* L.), also known as the "king of oilseeds," "wonder nut" or the "poor man's cashew nut," is a significant oilseed crop from the *Fabaceae* family." It is used as both a staple food and a cash crop. Groundnut cultivation covers around 32.7 Mha globally, producing 53.9 Mt, with an average productivity of 1,648 kg per hectare (1). Groundnut is primarily grown in the Indian states of Andhra Pradesh, Gujarat, Karnataka, Rajasthan, Tamil Nadu, Maharashtra, and Telangana (2). It was noted that Tamil Nadu cultivates groundnut on 338,300 hectares, yielding approximately 783,200 metric tonnes (3). Major groundnut-producing districts in Tamil Nadu include Cuddalore, Kancheepuram, Tiruvannamalai, Tiruvallur, Vellore, and Villupuram.

Groundnut crops face considerable challenges from various foliar and soil-borne diseases, including leaf spot, rust, stem rot, and root rot. Among these, groundnut root rot, caused by the soil-borne fungus *Macrophomina phaseolina*, is particularly devastating, with potential yield losses of up to 60% (4). *M. phaseolina* can infect groundnut plants at any growth stage, producing microsclerotia that persist in the soil and infect plants under favorable conditions. Symptoms include leaf yellowing, wilting, and plant death (5).

The environmental risks associated with chemical disease management underscore the need for alternative approaches to disease control. Consequently, wild mushroom extracts from *Ganoderma lucidum* and *Tricholoma* spp were used. presents a promising alternative to chemical fungicides. Research has demonstrated that wild mushroom extracts possess antifungal activities that are effective against a broad spectrum of phytopathogens. As macrofungi, mushrooms produce bioactive compounds such as alkaloids, terpenoids, polysaccharides, and phenolics, which exhibit significant antimicrobial properties (6). These compounds may inhibit pathogens indirectly or directly through antimicrobial action and nutrient competition. Previous studies have shown that mushrooms possess antimicrobial potential against various fungal and bacterial plant pathogens, including *Macrophomina phaseolina*, *Fusarium* spp., *Xanthomonas* spp., and *Ralstonia solanacearum* (7).

Extracts from wild mushroom species, obtained using both polar and nonpolar solvents, can be analyzed by GC-MS to identify active antifungal compounds, offering a natural mechanism for pathogen defense (8). This study aims to explore the antifungal properties of ten wild mushroom species collected from their natural habitats as a potential biocontrol solution against *M. phaseolina*-induced groundnut root rot, offering an effective, natural alternative for disease control.

Materials and Methods

Survey on the incidence of groundnut root rot and isolation of M. phaseolina

A roving survey was conducted to evaluate the incidence the root rot disease in several districts of Tamil Nadu during the groundnut growing season, specifically in Madurai, Dindigul, Namakkal, Erode, Salem, Cuddalore, Trichy, and Perambalur. Disease incidence data were recorded from four randomly marked plots in each field, with each plot covering an average area of ten square meters. The number of infected plants was compared to the total number of plants within each quadrant to calculate incidence.

$$\text{Percent Disease incidence} = \frac{\text{Number of plants infected}}{\text{Total no. of plants observed}} \text{X } 100$$

(Eqn.1) (9)

The pathogen *M. phaseolina* was isolated from the infected root tissues using the tissue isolation methods on potato dextrose agar (PDA) media (10). The affected root portions were sliced into small pieces with a sterilized blade, then dipped in 0.1% sodium hypochlorite for one minute, followed by three rinses in sterile distilled water to drain the solution completely. These root segments were then plated on the PDA medium and incubated for 7-10 days at 28 ± 2°C. A pathogenicity test was conducted in pot culture to determine the virulent isolates of *M. phaseolina*.

Morphology and molecular level identification of M. phaseolina

The pathogen characteristics were studied under *in vitro* conditions. A mycelial disc from a 5–7-day-old culture was placed on PDA in a Petri dish and incubated at 28 ± 2 °C for 7–10 days. To observe the mycelial structure of *M. phaseolina*, microscopic examination at 40x magnification and SEM analysis were conducted. Seven-day-old cultures of *M. phaseolina* were mounted on an aluminum stub using double-sided adhesive tape and sputter-coated with gold particles. These particles were ionized using an ion coater before SEM analysis. Images were captured using a CARL ZEISS EVO 18 SEM instrument at 2.0 KX magnification.

10-day-old mycelial cultures were ground using a pestle and mortar in cetyltrimethylammonium bromide (CTAB) buffer for genomic DNA isolation. A 500 µL portion of the ground mixture was transferred to a fresh 1.5 ml tube and incubated in a hot water bath for 30 minutes at 60 °C. After incubation, the mixture was centrifuged at 10,000 rpm for 10 minutes, and the supernatant was collected in a new tube. A 24:1 ratio of chloroform to alcohol was added to the supernatant and centrifuged for 15 minutes at 12,000 rpm. Ice-cold isopropanol was added, and the mixture was stored overnight at -20 °C. The resulting pellet was collected by centrifugation at 12,000 rpm for 15 minutes, washed with 75% ethanol, and air-dried for 30 minutes. The pellet was dissolved in 40–50 µL of TE buffer and stored at -20 °C for future use.

The PCR amplification of the fungal DNA was performed using ITS 1 (5'-TCCGTAGGTGAACCTCCG-3') and ITS 4 (5'-TCCTCCGCTTATGATATGC-3') primers, following a standardized protocol (11). The PCR products were loaded into wells with a DNA ladder as a marker. The gel was run at 5–8 V/cm until the bromophenol blue dye had migrated three-quarters of the gel, and the results were observed under UV light using gel documentation equipment. Bio Kart India Private Limited, Bangalore, sequenced the PCR products. The sequences were analyzed using the NCBI BLAST database, and new sequences were submitted to GenBank. Alignment and phylogenetic analysis were performed using MEGA 11 and iTOL software (12).

Collection, isolation, and identification of wild mushrooms

Healthy wild mushroom specimens were collected from

their natural habitat. Tissue isolation was performed on the PDA medium. A mature, disease-free basidiocarp was surface-sterilized with 70% ethanol, split vertically, and a small tissue piece from the stipe-pileus junction was further immersed in 70% ethanol for 10 seconds, followed by rinsing with distilled water. Excess moisture was removed with tissue paper. The tissue was then placed on PDA and incubated at 28 °C until the fungal mycelium covered the entire plate. The single hyphal tip method obtained Pure cultures by transferring the mycelium to new plates (13).

Molecular characterization of wild mushroom species

The fungal mycelium was ground using a pestle and mortar in a CTAB buffer. The homogenized solution of 500 µl was poured into the tube (1.5 ml). It was kept for 30 minutes at 65 °C in a hot water bath. After incubation, tubes were centrifuged at 10,000 rpm for 10 minutes, and then the supernatant was transferred to the new tube. An equal volume of chloroform and alcohol was mixed in a 24:1 ratio and subjected to centrifugation at 12,000 rpm for 15 minutes. An equal amount of isopropanol was added to the supernatant, and the tube was stored at -20 °C overnight. The following day, the sample was centrifuged at 10,000 rpm for 15 minutes. The resulting pellet was washed with 75% ethanol, air-dried briefly, and dissolved in 50 µL of TE buffer for storage at -20 °C.

PCR amplification was carried out using the ITS 1 (5'-TCCGTAGGTGAACCTCCG-3') and ITS 4 (5'-TCCTCCGCTTATTGATATGC-3') primers (11). The reaction mixture included template DNA, Milli-Q water, master mix, and primers. A 1.5% agarose gel was prepared in 100 ml of 1X Tris-acetate-EDTA (TAE) buffer, stained with ethidium bromide, and poured onto a casting tray. DNA samples were loaded into the wells, and electrophoresis was conducted at 5-8 V/cm until the dye bands had migrated adequately. Bands were visualized using a Gel Documentation system under UV light. Bio Kart India Private Limited, Bangalore, sequenced the PCR products. The sequences were verified using the BLAST tool in the NCBI database and submitted to GenBank, and a phylogenetic tree was constructed with MEGA 11 software to identify sequence similarity (14).

Preparation of crude extract of wild mushroom species using different solvents

The collected wild mushroom samples were shade-dried for 24 hours at 40 °C and then pulverized into a fine powder using a Willey mill. Ten grams of the mushroom powder was mixed with 100 mL of the respective solvents (polar – methanol, acetone, ethanol, and acetonitrile; non-polar – hexane, ethyl acetate, chloroform, and acetic acid). The mixture was soaked overnight in solvent with occasional stirring, and the extract was obtained using rotary evaporation at 45 rpm at 60 °C.

Testing the efficacy of wild mushroom species against M. phaseolina

Dual culture assay: The dual culture method was employed to observe the inhibition percentage of wild mushroom isolates against *M. phaseolina* using a standardized assay method (15). A 9 mm culture disc of actively growing wild mushroom isolates and the virulent *M. phaseolina* isolate GMP-3 were placed on opposite sides of the same diagonal line on the PDA medium, 1 cm from the edge. The control plate was inoculated only with the virulent pathogen. All inoculated plates were incubated at 28 ± 2 °C for 10-15 days. Pathogen radial growth inhibition was calculated using the following formula:

$$PI = C - T/T \times 100 \quad \text{.....(Eqn.2) (16)}$$

Where, C- pathogen growth (cm) in control

T- pathogen growth (cm) in treatment

Observation on Scanning Electron Microscope (SEM):

Samples from the interaction zone in the dual culture assay were collected and fixed in a 3:1 mixture of glutaraldehyde and osmium tetroxide vapor, then air-dried for 48 hours (17). Following preparation, the samples were gold-coated by sputtering and analyzed using a SEM (CAREL ZEISS EVO 18 model) at the ACIC Instrumentation Center, St. Joseph's College, Trichy.

Poisoned food technique: The PDA medium was used to analyze the reduction in fungal growth of *M. phaseolina* using the solvent extracts of wild mushroom species as per standardized protocol (15). A 200-300 µL portion of the mushroom extract was applied to the surface of the PDA to form a thin layer. The Petri plate containing sterile water was used as the control. The plates were inoculated with a seven-day-old culture of *M. phaseolina* and then incubated at 28 ± 2°C for one week. Pathogen growth was measured on both the treatment and control plates. The percent inhibition of mycelial growth was calculated using the formula.

$$PI = C - T/T \times 100 \quad \text{.....(Eqn.3) (16)}$$

Where, C = growth of pathogen mycelium (cm) in control

T = growth of pathogen mycelium (cm) in treatment

Sample preparation of methanol extract of mushroom isolates for GC-MS analysis

To prepare the methanol extract, 10 grams of the powdered mushroom sample were soaked in 100 ml of methanol and left overnight. The solvent was then filtered using Whatman No. 1 filter paper to remove impurities that might interfere with the reaction after the reflux process. A rotary evaporator set at 60 °C and 45 rpm concentrated the solution by evaporation until the solvent was completely evaporated. The final extract was diluted with methanol and stored at -4°C for future use (18).

GC-MS analysis for secondary metabolites extracted using methanol solvent

The Agilent GC-MS 5975C with a Triple-Axis Detector and an autosampler was used for the GC-MS analysis. The fused silica capillary column employed was 30 m in length, 0.25 mm in diameter, with a film thickness of 0.25 µM. Helium was used as the carrier gas at a flow rate of 1.51 mL/min during the first minute. In EI mode, electron impact was applied at 70 eV, with a scanning range for the mass-to-charge ratio from 40 to 700. The sample solution was introduced with a 1:10 injection ratio and a volume of 1 µL. The injector temperature was set to 250 °C. The oven started at 70 °C for 3 minutes, ramped at 14 °C/min to 250 ° C, and the total running time was 40 minutes. The crude extracts were identified based on the peak by comparing the retention times to standards. The resulting mass spectra were matched to the NIST Mass Spectral Library, with a match acceptance of 80% or higher (19).

Statistical Analysis

The data were analyzed using SPSS software, which applied an analysis of variance (ANOVA). The least significant differences (LSD) were calculated between treatments at the 5% significance level. Three replications were conducted for each treatment to ensure the reliability and accuracy of the results.

Results

Survey on groundnut root rot incidence and isolation of M. phaseolina

A roving survey was conducted in major groundnut-growing areas of Tamil Nadu, including Cuddalore, Dindigul, Erode, Madurai, Namakkal, Perambalur, Salem, and Tiruvannamalai. The incidence in the surveyed areas ranged from 32.71% to 85.66%, with the highest infection rate recorded in Kallandiri, Madurai (85.66%), followed by Thatchur, Tiruvannamalai (79.30%). The lowest incidence was observed in Anandagoundempalayam, Namakkal (32.71%) (Table 1.).

M. phaseolina was isolated from infected root samples using the standard tissue segment method. Ten isolates were collected from different locations and labeled GMP-1, GMP-2, GMP-3, GMP-4, GMP-5, GMP-6, GMP-7, GMP-8, GMP-9, and GMP-10. The mycelium exhibited blackish to grayish fluffy growth, producing black sclerotia. To ensure purity, the isolates were subcultured for further studies. Pathogenicity tests under pot culture conditions revealed that all ten isolates exhibited typical symptoms similar to those observed in the field survey. Among them, GMP-3 was identified as the most virulent isolate and was selected for subsequent *in vitro* assays.

Morphological and molecular confirmation of M. phaseolina

The morphological characteristics, such as mycelial color, growth pattern, and colony type, were observed for all the isolates. The isolates GMP-1 and GMP-10 exhibited black and fluffy mycelial growth, while GMP-3 and GMP-5 produced dark black and fluffy mycelial growth. The isolates GMP-2, GMP-4, and GMP-6 showed blackish-white and fluffy mycelial growth. The GMP-7 and GMP-9 isolates displayed grey and fluffy mycelial growth. The isolate GMP -8 exhibited blackish-grey, fluffy mycelial growth. The mycelial and sclerotial structures were observed under a microscope (Fig. 1). The characteristics of the mycelium were also examined using Scanning Electron Microscopy (SEM). Furthermore, the virulent isolate GMP-3 was observed under SEM, revealing that the mycelium was septate, thick, branched, and interconnected (Fig. 2).

The PCR was performed for each isolate using ITS-1 and ITS-4 primers. A 550-650 bp band was obtained and sequenced during agarose gel electrophoresis. The sequenced isolate rDNA homology was analyzed using the BLAST tool from the NCBI database for the virulent isolate GMP-3. The sequences showed 96% identity to *M. phaseolina.* The resulting sequence was submitted to GenBank with the accession number PQ113793. A dendrogram was generated by comparing isolate GMP-3 with existing database isolates using the neighbor-joining method. The phylogenetic tree of *M. phaseolina* and its related sequences were constructed based on the internal transcribed spacer (ITS) rRNA gene (Fig. 3).

Collection and phenotypic character of wild mushroom species

The collected wild mushroom species varied in phenotypic characteristics, including colour differences (white, yellow,

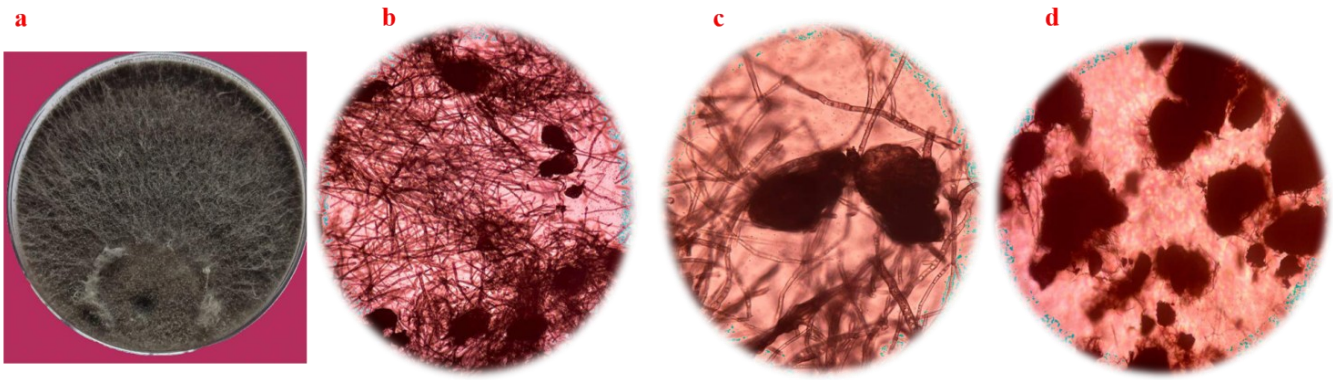


Fig. 1. Morphological characterization of M. *phaseolina*. a) Culture plate of M. *phaseolina* (GMP-3) b) hyphal branching of mycelium at 10X magnification c) formation of pycnidia in 40 X magnification d) irregular sclerotia bodies.

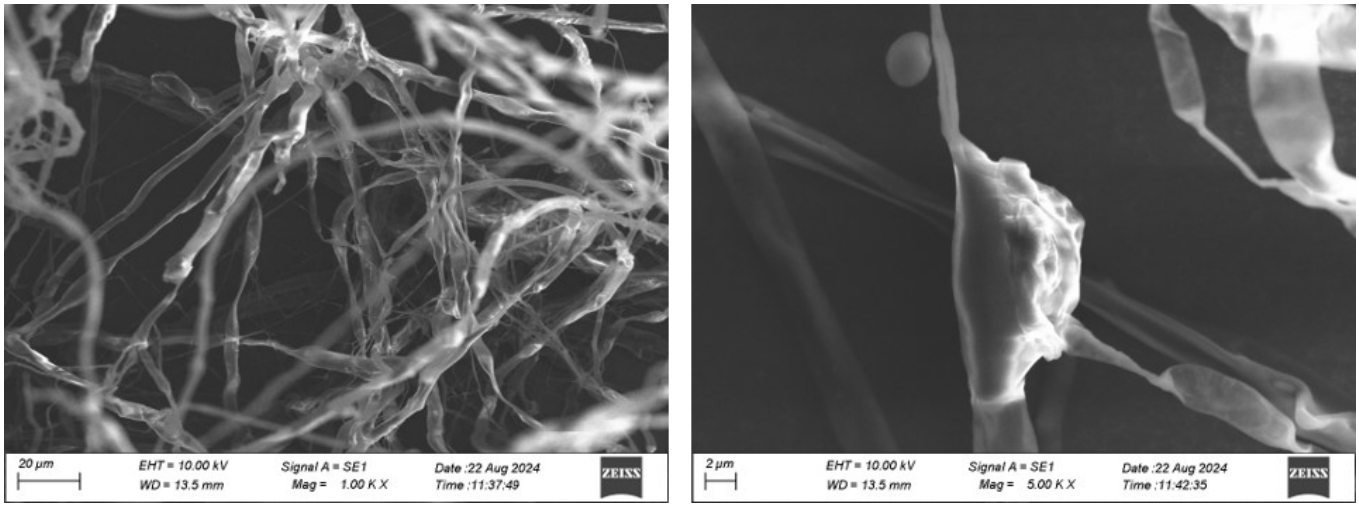


Fig. 2. Scanning electron microscope view of M. *phaseolina* (GMP-3) shows the network of mycelium and pycnidia formation.

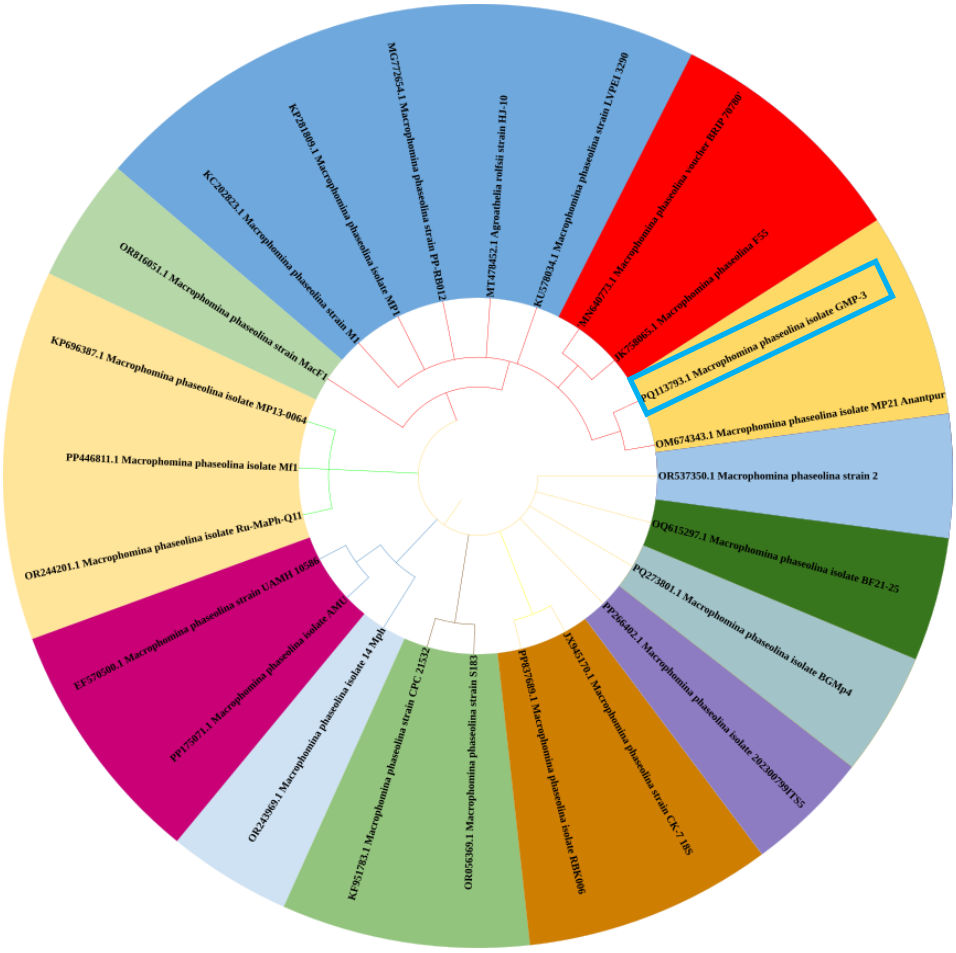


Fig. 3. A phylogenetic tree generated based on the internal transcribed region in M. *phaseolina.* It was built using the neighbor-joining method in Mega 11 software. Using iTol, a circular diagram with color differences among sequences was created. *Agroathelia* rolfsii is used as an outgroup

and brown) and shapes (globose, bulbous, umbrella, cylindrical, and ear-shaped) (Table 2., Fig. 4).

Morphological characteristics of wild mushroom isolates

The colony color, mycelial development, and growth rate were examined for each of the ten isolates. The isolates took 14-21 days to cover the 9 cm plate. The isolates MWM -1, and KWM-7 showed white, cottony growth, OWM-2, and OWM-3 showed white, fluffy growth, PWM-5, and KWM-6 showed white, thin, and thick layered growth, and KWM-9, MWM-10 showed white, thread-like growth. The colony color of the isolates varied from bright to dull white. The isolate MWM-1 showed pure white, OWM-2, and PWM-3 showed dull- white, OWM-3, and KWM-6 showed bright white, AWM-4 and KWM-7 showed creamy white, DWM-8 showed pale white, and KWM-9 and MWM-10 showed white -colour mycelium (Supplementary Fig. 1).

Molecular characterization of wild mushroom isolates

The molecular confirmation of the wild mushroom isolates

Table 1. Survey on root rot disease incidence in groundnut growing areas of Tamil Nadu

Place of collection	Name of Isolate	District	Geo co ordinates		Disease Incidence* (%)
			Latitude(°N)	**Longitude**(°E)	
Pottikampatti	GMP-1	Dindigul	10.557859	77.704193	43.99[f] (41.55)
Velampalayam	GMP-2	Erode	11.288244	77.439539	65.71[c] (54.16)
Kallandiri	GMP-3	Madurai	10.230109	77.455141	85.66[a] (67.75)
Malayalathanpatti	GMP-4		9.9653661	78.205844	52.72[e] (46.56)
Agaram sigoor	GMP-5	Perambalur	11.264316	78.859573	58.67[d] (50.00)
Thatchur	GMP-6	Tiruvannamalai	12.464531	79.273529	79.30[b] (62.94)
Mandakolathur	GMP-7		12.465603	79.243579	41.70[g] (40.22)
Annamalaipatti	GMP-8	Salem	11.572288	78.038573	37.46[h] (37.68)
Anandagoundem palayam	GMP-9	Namakkal	11.526049	78.112371	32.71[i] (34.88)
Pudhukoorapettai	GMP-10	Cuddalore	11.516449	79.357841	40.71[g] (39.64)
CD (*P*=0.05)					**1.161**

* The average disease incidence values were calculated by observing three different areas in the field. The arcsine transformation values are mentioned in the parentheses. The critical difference is calculated at the 5% significance level.

Table 2. Phenotypic characters of wild mushroom species.

S. No	Place of collection	Name of isolate code	Morphological characters
1	Madurai	MWM-1	White colour, bulbous at base, enlarged cap
2	Oddanchatram	OWM -2	Light yellow color, umbrella cap, spots are present on the cap, ring present on the stem
3	Oddanchatram	OWM -3	Whitish yellow colour, resembles like oyster mushroom
4	RRS Aruppukottai	AWM -4	Yellow color in appearance, enlarged cap
5	Pachalur hills	PWM -5	Pure whitish color, arranged in stacks in between the barks
6	Kodaikannal	KWM-6	The upper surface is dark brown to pale grey color, wavy layers on the surface, margins are surrounded by white color, brown spores appear dusty
7	Kodaikannal	KWM -7	White in color, smaller in size of cap and stem
8	Dindigul	DWM -8	White color, cylindrical in shape, rough surface, small stipe attached to the tree
9	Kodaikannal	KWM -9	Light brown coloured spines are present on the surface, cylindrical to globose-shaped
10	Madurai	MWM -10	Brown color, ear-like shaped, gelatinous fruit bodies, arranged in clusters

The appearance of ten different wild mushroom species was observed based on the mushroom's colour and shape.

Fig. 4. Survey on the different wild mushroom species from various hilly and plains of regions of Tamil Nadu.

was done by extracting DNA from the isolates using the CTAB method. The primers ITS 1 and ITS 4 were used in the PCR reaction, and the products were subjected to agarose gel electrophoresis. An amplicon size of approximately 500 -600 bp was obtained. The PCR products were then sequenced, and the sequences were analyzed for rDNA homology using the NCBI BLAST program. The sequence results showed that the isolates *Calocybe indica* (MWM-1), *Tricholoma equestre* (AWM-4), and *Ganoderma applanatum* (KWM-6) exhibited 94.49%, 95.98%, and 95.00% homology, respectively, with the accession numbers PQ114533, PQ113734, and PQ113735. A phylogenetic tree was constructed using MEGA 11 software (Fig. 5).

Testing efficacy of wild mushroom species against M. phaseolina under in vitro assay

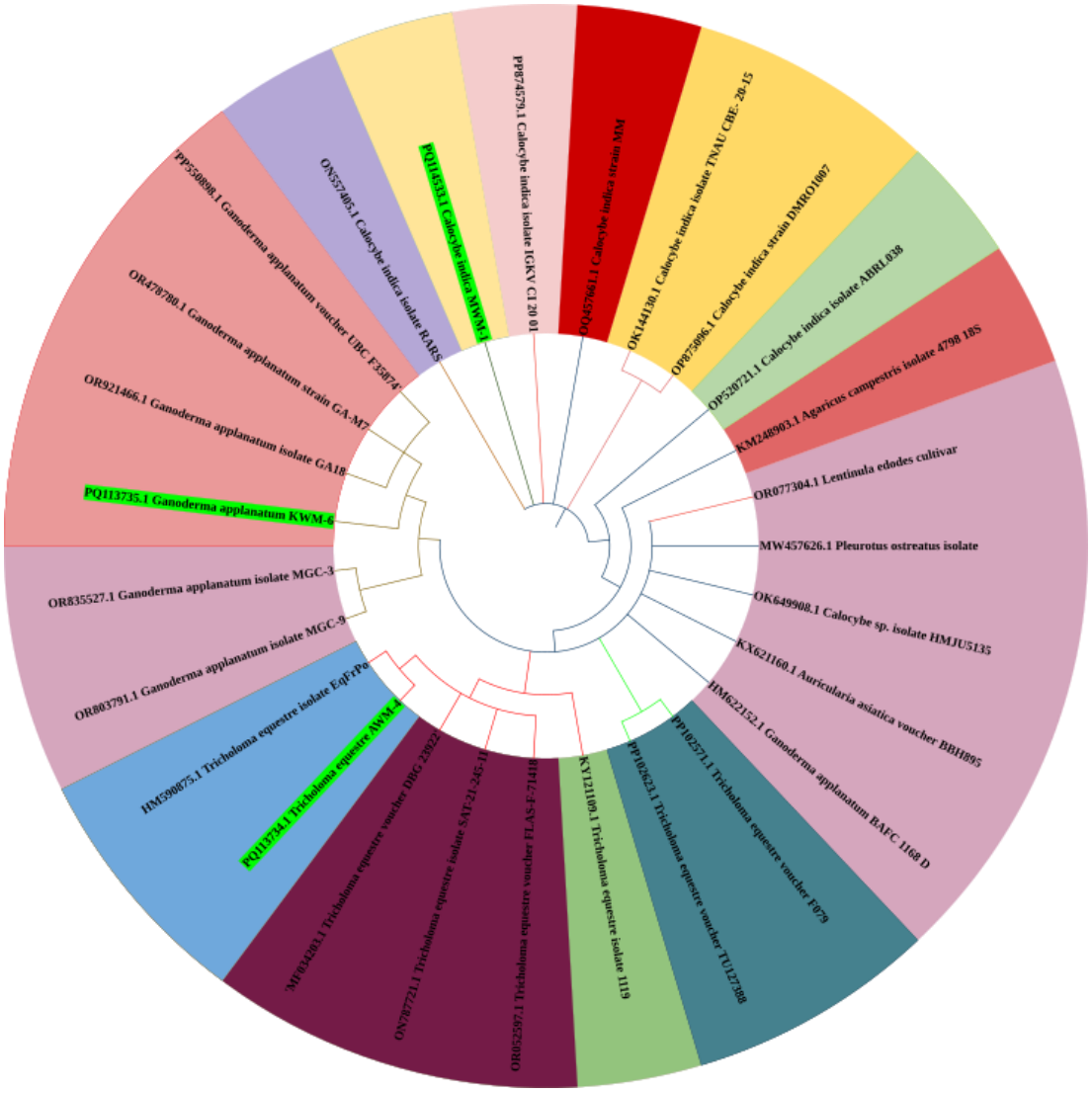
Dual culture assay: Ten isolates, namely MWM-1, OWM-2, OWM-3, AWM-4, PWM-5, KWM-6, KWM-7, DWM-8, KWM-9, and MWM-10, were tested against a virulent isolate of *M. phaseolina* (GMP-3). The isolates AWM-4 and KWM-6 reduced the mycelial growth to 2.30 cm and 2.76 cm, respectively, showing the maximum percentage inhibition over the control at 74.33% and 69.34%. The least percentage of inhibition over the control was observed in PWM-5 (37.21%). The other isolates, including MWM-1, OWM-2, OWM-3, KWM-7, DWM-8, KWM-9, and MWM-10, exhibited 63.67%, 58.45%, 53.78%, 43.00%, 45.11%, 60.45%, and 49.67% inhibition over the control, respectively (Table 3., Fig. 6).

Observation on Scanning Electron Microscope: The antagonistic effect of *T. equestre* (AWM-4) against the pathogen was analyzed using SEM. The results revealed that the mycelium of *T. equestre* deformed the mycelium of *M. phaseolina* (Fig. 7).

Poisoned food technique (polar solvent): Different polar solvents, such as methanol, ethanol, acetone, and acetonitrile, were used to prepare mushroom extracts from mushroom isolates AWM-4, KWM-6, MWM-1, OWM-2, and KWM-9. Among these, the methanol extract of AWM-4 showed the maximum reduction in mycelial growth, measuring 2.20 cm (75.56%), followed by KWM-6 at 3.29 cm (63.45%). The least reduction was observed in KWM-9, with a growth of 6.37 cm (29.22%). Other solvents, such as ethanol, acetone, and acetonitrile, showed lower inhibition rates than the methanol extract. In comparison, the methanol extract of AWM-4 exhibited the highest inhibition, followed by KWM-6, against *M. phaseolina* (Table 4., Supplementary Fig. 2).

Poisoned food technique (non-polar solvent): The non-polar solvents, including hexane, ethyl acetate, chloroform, and acetic acid, were examined. The hexane extract of AWM-4 showed the maximum reduction in mycelial growth at 2.37 cm (73.67%), followed by KWM-6 at 3.73 cm (58.55%). The least reduction in mycelial growth was observed in KWM-9 at 6.78 cm (24.67%). The ethyl acetate, chloroform, and acetic acid extracts exhibited lower inhibition percentages. The hexane extract of AWM-4 demonstrated the highest reduction, while KWM-6 also exhibited significant inhibition against *M. phaseolina* compared to the remaining solvent extracts.

Overall, the hexane extract of AWM-4 showed the highest



inhibition, followed by

Fig. 5. A phylogenetic tree was generated using sequences of isolates of wild mushroom species (*Tricholoma equestre, Ganoderma appalanatum, Calocybe indica).* The isolates of this study were highlighted in green. It was built using the neighbor-joining method in Mega 11 software. A Circular diagram was created using iTol. *Pleurotus* sp., *Agaricus* sp., and *Lentinula* sp. are used as outgroups.

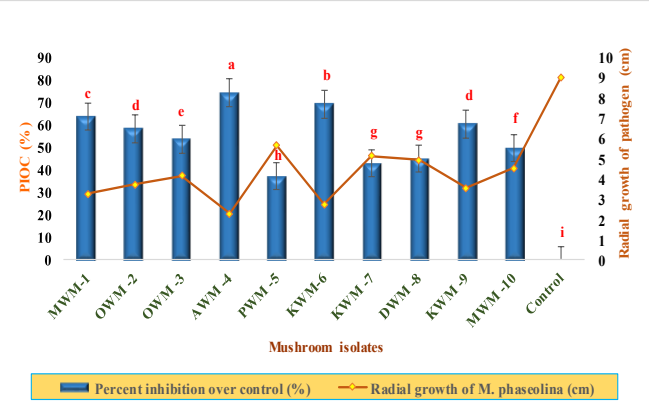


Fig. 6. Antagonistic activity of ten wild mushroom isolates against M. *phaseolina* (GMP-3) using dual culture assay. Each value is the mean of three replications, and error bars are the standard deviation obtained per treatment. The least significant difference is calculated by 5 %.

KWM-6, which also demonstrated inhibition against *M. phaseolina*. (Table 5., Supplementary Fig. 3). Finally, the methanol extracts of AWM-4 and KWM-9 showed the maximum reduction compared to other isolates and solvents**.**

GC- MS analysis of extracts of Tricholama equestre and Ganoderma applanatum

The active compounds of wild mushroom isolates AWM-4 and KWM-6 were analyzed using GC-MS. *T. equestre* contained compounds such as p-Hydroxynorephedrine, 5,5-Dimethylimidazolidin-2,4-diimine, 9-Amino-Pupukeanane, 9,12-Octadecadienoic acid, Carbamic acid, Butanoic acid, D-Gluconic acid delta-lactone, 2,3-Dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one, Hexadecanoic acid, and Psilocin. The maximum peak area was observed for 9,12-Octadecadienoic acid (7.72%), followed by 9-Amino-Pupukeanane (3.38%), and the smallest peak area for D-Gluconic acid, delta-lactone (0.32%) (Table 6).

G. applanatum contained compounds such as 10-Undecenoic acid, Benzaldehyde methylamine, Benzenedicarboxylic acid, 1,3,5-Trisilacyclohexane, 9,12-Octadecadienoic acid, 10-Undecenoyl chloride, 13-Hexyloxacyclotridec-10-en-2-one, Cyclodecene, n-Hexadecanoic acid, and Ricinoleic acid. The highest peak area was observed for 9,12-Octadecadienoic acid (37.89%),

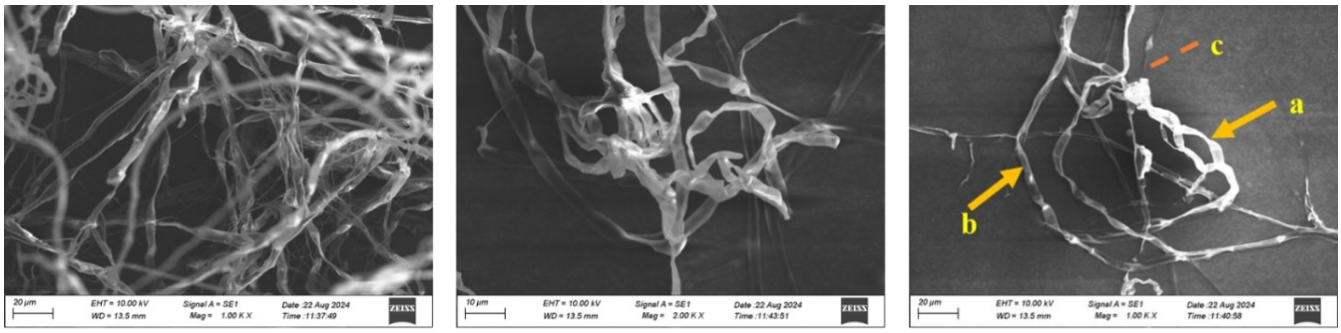


Fig. 7. Scanning electron microscope view of M. *phaseolina* (GMP-3), *Tricholama equestre* and interactive region of pathogen and mushroom. A) Septate mycelial network of hypha of *M. phaseolina*; B) Branching of mycelium of *T. equestre*; C) a- Mycelium of *T. equestre*, b- Mycelium of *M. phaseolina*, c- Mycelium of *T. equestre* deformed the mycelium of *M. phaseolina*.

Table 3. Effect of wild mushroom isolates against mycelial growth of *M. phaseolina* by using dual culture technique.

S. No	Isolate	Radial growth of pathogen* (cm)	Percent inhibition over control *(%)
1	MWM-1	3.27^{c}	63.67^{c}
2	OWM -2	3.74^{d}	58.45^{d}
3	OWM -3	4.16^{e}	53.78^{e}
4	AWM -4	2.30^{a}	74.33^{a}
5	PWM -5	5.65^{h}	37.21^{h}
6	KWM-6	2.76^{b}	69.34^{b}
7	KWM -7	5.13^{g}	43.00^{g}
8	DWM -8	4.94^{g}	45.11^{g}
9	KWM -9	3.56^{d}	60.45^{d}
10	MWM -10	4.53^{f}	49.67^{f}
11	Control	9.00^{i}	0.00^{i}
	CD (P=0.05%)	0.213	1.443

* The mean of three replications calculated the radial growth of pathogen and percent inhibition over control. The values in the parentheses were arcsine transformed values.

Table 4. Effect of mushroom extract against *M. phaseolina* using poisoned food technique (Polar solvent).

Mushroom Isolate	Methanol extract		Ethanol extract		Acetone extract		Acetonitrile extract	
	Diameter of mycelium* (cm)	Percent inhibition over control (%)	Diameter of mycelium * (cm)	Percent inhibition over control (%)	Diameter of mycelium * (cm))	Percent inhibition over control (%)	Diameter of mycelium * (cm)	Percent inhibition over control (%)
AWM -4	2.20	75.56^{a} (60.38)	2.50	72.21^{a} (58.20)	2.85	68.32^{a} (55.76)	3.17	53.59^{a} (64.78)
KWM-6	3.30	63.45^{b} (52.80)	3.87	57.00^{b} (49.03)	4.36	51.55^{c} (45.89)	4.68	48.00^{b} (43.85)

followed by Ricinoleic acid (10.81%), with the smallest peak area for 10-Undecenoic acid (0.04%)

MWM-1	4.15	53.89[c]	4.80	46.78[c]	5.45	39.44[d]	5.90	34.45[c]
OWM -2	5.62	37.56[e]	6.21	31.00[e]	4.02	55.30[b]	6.27	30.33[d]
KWM -9	6.37	(29.22[f]	6.98	22.45[f]	7.27	19.24[f]	7.81	13.21[f]
Solvent	4.95	45.00[d] (43.13)	5.60	37.78[d] (37.92)	6.01	33.23[e] (35.20)	6.48	28.00[e] (31.94)
Control	9.00	0.00	9.00	0.00	9.00	0.00	9.00	0.00
CD (*P*=0.05%)	**0.305**	**1.538**	**0.350**	**1.841**	**0.282**	**1.077**	**0.315**	**0.851**

The different polar solvent was used to observe the radial growth of *M. phaseolina* mycelium.
* Mean of three replications. The values in parentheses represent the arcsine transformed values. The critical difference has been determined at the 5% significance level.

Table 5. Effect of mushroom extract against *M. phaseolina* using poisoned food technique (non-polar solvent).

	Hexane extract		Ethyl acetate extract		Chloroform extract		Acetic acid extract	
Mushroom Isolate	**Diameter of myceli-um (cm)**	**Percent inhibition over con-trol (%)**	**Diameter of mycelium (cm)**	**Percent inhibition over con-trol (%)**	**Diameter of mycelium (cm)**	**Percent inhibition over con-trol (%)**	**Diameter of mycelium (cm)**	**Percent inhibition over con-trol (%)**
AWM -4	2.37	73.67[a] (59.12)	2.78	69.11[a] (56.23)	3.48	61.33[a] (51.54)	4.20	53.33[a] (46.90)
KWM-6	3.73	58.55[b] (49.92)	4.60	49.00[b] (44.42)	3.98	55.77[b] (48.31)	5.68	36.88[b] (37.39)
MWM-1	4.68	48.00[c] (43.84)	5.61	37.67[c] (37.85)	6.28	30.22[c] (33.34)	6.90	23.34[c] (28.88)
OWM -2	5.95	33.88[e] (35.59)	6.48	28.00[e] (31.94)	7.01	22.10[d] (28.04)	7.22	19.74[d] (26.37)
KWM -9	6.78	24.67[f] (29.77)	7.47	16.94[f] (24.30)	7.88	12.45[e] (20.65)	8.50	5.56[e] (13.63)
Solvent	5.46	39.33[d] (38.83)	6.07	32.55[d] (34.78)	6.62	26.43[dc] (30.93)	7.28	19.11[d] (25.92)
Control	9.00	0.00 (0.286)	9.00	0.00 (0.286)	9.00	0.00 (0.286)	9.00	0.00 (0.286)
CD (*P*=0.05%)	**0.274**	**0.902**	**0.392**	**1.057**	**0.500**	**0.879**	**0.207**	**0.722**

Different non-polar solvents were used to observe the radial growth of *M. phaseolina* mycelium.
*The treatments were based on the mean of three replications. The values in parentheses indicate the arcsine-transformed values and the critical difference was determined at the 5% significance level.

(Table 7). The metabolite enrichment ratio of AWM-4 and KWM-6 was analyzed (Fig. 8).

Discussion

A roving survey on *M. phaseolina* was conducted in major groundnut-cultivating regions of Tamil Nadu. The infection

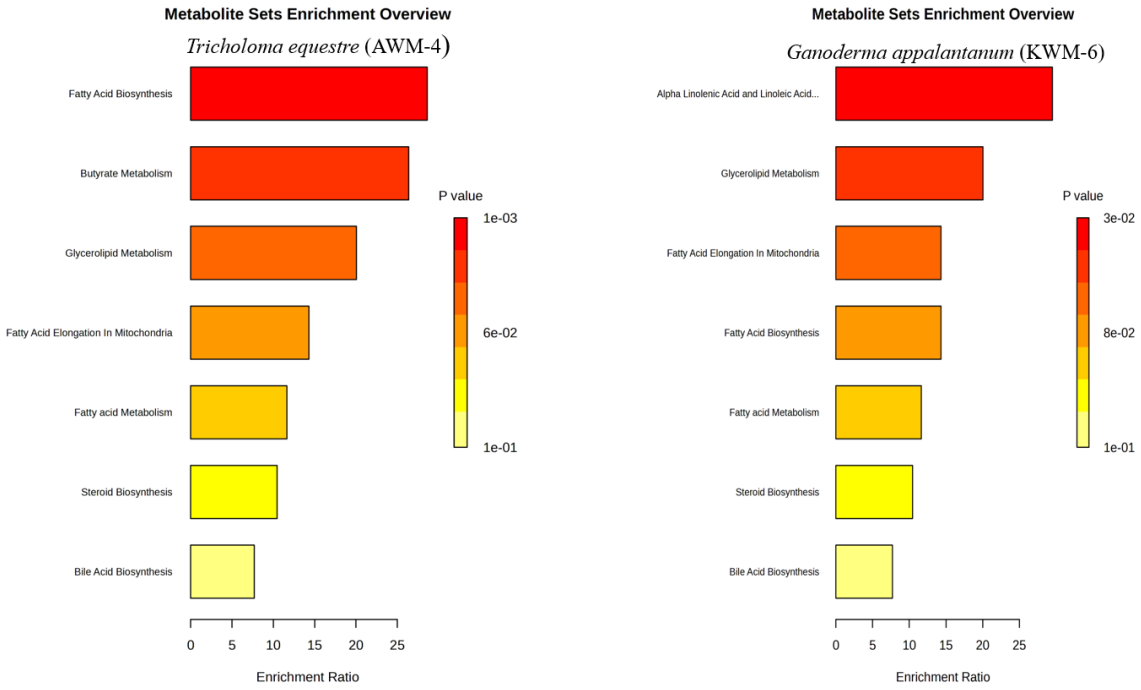


Fig. 8. The metabolite enrichment of *Tricholoma equestre* (AWM-4) and *Ganoderma appalanatum* (KWM-6) analyzed through Metabo Analyst software 6.0.

Table 6. GC-MS analysis for secondary metabolites of *Tricholoma equestre*

S. No	Name of the compound	Peak Area (%)	Retention Time	MW	Molecular formula	Specific role	References
1	p-Hydroxy-	0.49	1.245	167.21	$C_9H_{13}NO_2$	Antimicrobial activity	(32)
2	5,5-Dimethylimid-azolidin-2,4-	1.68	1.691	126.16	$C_5H_{10}N_4$	Antifungal activity	(33)
3	9-Amino-Pupukeanane	3.38	2.702	221.38	$C_{15}H_{27}N$	Antimicrobial and antibacte-rial activity	(34)
4	9,12-Octadecadi-enoic acid	7.72	2.900	280.45	$C_{18}H_{32}O_2$	Antimicrobial, antioxidant activity	(35)
5	Carbamic acid, methyl ester	0.39	3.548	61.04	CH_3NO_2	Antibacterial activity	(36)
6	Butanoic acid	0.88	3.739	88.11	$C_4H_8O_2$	Antimicrobial and Antibac-terial activity	(37)
7	2,3-Dihydro-3,5-dihydroxy-6-methyl-4H-	0.61	7.251	144.13	$C_6H_8O_4$	Antioxidant	(38)
8	D-Gluconic acid, delta-lactone	0.32	8.918	178.14	$C_6H_{10}O_6$	Antioxidant	(39)
9	n-Hexadecanoic acid	0.52	13.804	256.42	$C_{16}H_{32}O_2$	Antimicrobial activity	(40)
10	Psilocin	0.58	14.485	204.27	$C_{12}H_{16}N_2O$	Antimicrobial, antioxidant activity	(41)

The compounds in *Tricholoma equestre* were analyzed based on their percentage peak area, retention time, molecular weight, molecular formula and their role in inhibiting the pathogen.

frequency in the surveyed locations ranged from 32.71% to 85.66%. Previous reports of *M. phaseolina* in groundnut were noted in the Tiruvannamalai district (9) and Andhra Pradesh, particularly in Ananthapuram (20). A study found the prevalence of groundnut root rot in southern India, specifically in Tamil Nadu, Telangana, Karnataka, and Andhra Pradesh, with incidence rates between 8.06% and 20.61% (21). The *M. phaseolina* pathogen was isolated from affected tissues collected in the surveyed regions. The mycelial colony exhibited blackish to greyish fluffy growth and produced black sclerotia. A pathogenicity study was carried out using pot culture, and all isolates demonstrated their ability to cause disease by producing symptoms similar to those observed in the field. GMP-3

Table 7. GC-MS analysis for secondary metabolites of *Ganoderma applanatum*.

S. No	Name of the compound	Peak Area	Retention Time	MW (g/mole)	Molecular formula	Specific role	References
1	10-Undecenoic acid	0.04	17.836	184.28	$C_{11}H_2OO_2$	Antimicrobial	(42)
2	Benzaldehyde methylimine	0.09	18.075	119.16	C_8H_9N	Antifungal activity	(43)
3	1,2-Benzenedicarboxylic acid, dibutyl ester	0.56	31.339	278.35	$C_{16}H_{22}O_4$	Antifungal activity	(44)
4	1,3,5-Trisilacyclohexane	0.88	31.451	162.43	$C_3H_{18}Si_3$	Antimicrobial activity	(45)
5	n-Hexadecanoic acid	3.57	31.697	256.43	$C_{16}H_{32}O_2$	Antifungal activity	(46)
6	10-Undecenoyl chloride	1.50	32.278	202.72	$C_{11}H_{19}ClO$	Antimicrobial activity	(47)
7	13-Hexyloxacyclotridec-10-en-2-one	11.65	33.803	280.45	$C_{18}H_{32}O_2$	Antimicrobial and antioxidant activity	(48)
8	Cyclodecene	0.61	34.707	138.25	$C_{10}H_{18}$	Antibacterial activity	(49)
9	9,12-Octadecadienoic acid	37.89	35.991	280.45	$C_{18}H_{32}O_2$	Antifungal activity	(50)
10	Ricinoleic acid	10.81	40.274	298.46	$C_{18}H_{34}O_3$	Antifungal and antioxidant activi-	(51)

The compounds in *Ganoderma applanatum* were analysed based on their percentage peak area, retention time, molecular weight, molecular formula and their role in inhibiting the pathogen.

was the most virulent isolate, with a disease incidence of 92.10%. A further investigation documented the pathogenic heterogeneity of sixty isolates of *M. phaseolina* in soybeans from prominent agricultural districts in India (22).

The morphological characteristics, such as mycelial growth pattern, mycelial color, and sclerotia formation, were observed in all the isolates. The isolates exhibited fluffy and aerial mycelial growth. The mycelial color ranged from black to grey. The structure of sclerotia was analyzed microscopically, corroborating a prior description that specified its morphology as possessing thin walls with transparent hyphae, exhibiting a color spectrum from light brown to dark brown (23). The main hyphae extend from branches and typically form at right angles to the parent hyphae, becoming constricted at their initial growth point. Microsclerotia are mycelial structures with dense clusters, globular or irregular in shape. These structures start as light brown and darken to brown or black as they age. Pycnidia are black to brown, hard, spherical, or irregular, and possess an ostiolate (small opening) and beaked structure.

In another investigation, SEM was used to observe hyphal growth and sclerotia formation in *M. phaseolina*, which exhibited various developmental patterns, such as thickening, branching, and septation of the main hyphae (17). The SEM analysis of the virulent isolate GMP-3 revealed hyaline, septate, branching, and interconnected mycelium. To identify *M. phaseolina*, PCR was performed using ITS1 and ITS4 primers. Agarose gel electrophoresis showed a 560 bp amplification product. The NCBI BLAST results showed 96% identity to *M. phaseolina*. This sequence was submitted to GenBank (accession number PQ113793). A phylogenetic tree was constructed using MEGA 6 software. In another study, ITS1-F and ITS4-R primers were used to confirm *M. phaseolina*, and the NCBI GenBank database was used to identify the genomic product of the virulent isolate Mp3 as *M. phaseolina* (24).

The research also explores mushroom isolates with phenotypic diversity in morphology. The mushrooms exhibited a range of colors, including white, yellow, and various shades of brown. They had rounded, globose shapes and bulbous structures. An investigation of seven wild edible mushroom samples was also conducted, involving their collection, classification, and examination of morphological properties (25). These features included the shape, size, color, and structure of the pileus (cap), hymenium (spore-bearing surface), and spores. The samples were isolated using the tissue isolation method, and ten mushroom isolates were evaluated based on visual observations of their cultures, focusing on colony color and mycelial growth patterns.

The molecular confirmation of the ten isolates was performed by DNA extraction using the CTAB method. The extracted DNA was amplified using the ITS 1 and ITS 4 primers in a PCR reaction. The PCR reaction products were subsequently visualized using agarose gel electrophoresis, revealing amplicon bands of approximately 500-600 base pairs. The sequence results were analyzed using the BLAST tool against nucleotide databases in GenBank to determine their rDNA homology. This analysis revealed high levels of genetic similarity for three specific isolates. *C. indica* (MWM-1) showed 94.49% homology, *T. equestre* (AWM-4) displayed 95.98% homology and *G. applanatum* (KWM-6) exhibited 95.50% homology.

Ten isolates of mushroom species were tested for their

antagonistic activity against a virulent isolate of *M. phaseolina* (GMP-3). Among these, AWM-4 and KWM-6 exhibited inhibition rates of 74.33% and 69.34%, respectively. The PWM-5 isolate showed the least inhibition, with a rate of 37.21%. The inhibition of mycelial growth of *C. gloeosporioides* using five different mushrooms and reported that *G. lucidum* and *L. edodes* showed reductions of up to 68% and 65.3%, respectively was tested in one study (26). It was also reported that four *Pleurotus* species were tested against *Trichoderma harzianum*, *Verticillium* sp., and *Pythium* sp. The reduction percentages ranged from 46% to 56%, with the highest reduction (55.56%) observed in *P. salmoneostramineus* against *Verticillium* sp. The lowest reduction (46.15%) was recorded in *P. ostreatus* against *T. harzianum*. Using SEM, a dual culture assay observed the antagonistic effect of *T. equestre* (AWM-4) against *M. phaseolina*. The results showed that the mycelium of *T. equestre* caused lysis around the mycelium of *M. phaseolina*.

Methanol, ethanol, acetone, and acetonitrile were used as polar solvents to prepare mushroom extracts from isolates AWM-4, KWM-6, MWM-1, OWM-2, and KWM-9. The results showed that the methanol extract of AWM-4 exhibited the highest reduction at 75.56%, followed by KWM-6 with a reduction of 63.45%. KWM-9 showed the lowest inhibition rate at 29.22%. In one study, the methanol extract of the mushroom *S. ostrea* against the pathogens *B. cinerea, C. gloeosporioides*, and *C. miyabeanus* (27). It was further reported that the ethanolic extract of *A. dispansus* showed maximum inhibitory activity against *F. graminearum* and *S. sclerotium* (28).

Using non-polar solvents, mushroom extracts were prepared, and the hexane extract of AWM-4 showed the highest reduction at 73.67%. KWM-6 exhibited a reduction of 58.55%, while KWM-9 had the least reduction at 24.67%. The ethyl acetate extracts of *Pycnoporus sanguineus*, *Oudemansiella canarii*, *Climadocon pulcherrimus*, and *Agrocybe perfecta* were reported to inhibit the growth of the pathogen *C. krusei* (29). Another experiment demonstrated that diethyl ether extracts of mushrooms *G. lucidum* and *L. edodes* inhibited the growth of *C. gloeosporioides* (26).

The compounds in AWM-4 and KWM-6 mushroom isolates were analyzed using GC-MS. Isolate AWM-4 (*T. equestre*) contained several active compounds, with the highest peak area observed in 9,12-Octadecadienoic acid (7.72%), followed by 9-Amino-Pupukeanane (3.38%). The least peak area was observed in D-Gluconic acid, delta-lactone (0.32%). The analysis of the GC-MS profiles of two *Tricholoma* species and identified 31 compounds was also performed in another study (30). In *T. caligatum*, the most abundant compound was 2,5-dimethyl-1-H-indole (41.5%), followed by methyl cinnamate (39%).

Isolate KWM-6 exhibited more antifungal compounds, with the highest peak area recorded for 9,12-octadecadienoic acid (37.89%), followed by ricinoleic acid (10.81%), while the peak area for 10-Undecenoic acid was minimal (0.04%). Furthermore, the analysis of *G. applanatum* extract using gas chromatography-mass spectrometry to identify active compounds was also performed (31). The study detailed the peak numbers, retention times, and area percentages. The compounds with the highest peak areas were identified as ethenone, followed by octadecenoic acid.

Metabolic pathways identified in *T. equestre* (AWM-4) included fatty acid biosynthesis and elongation, glycerolipid metabolism, steroid biosynthesis, and butyrate metabolism. *G. applanatum* (KWM-6) was involved in specific pathways, including the metabolism of alpha-linolenic acid and linoleic acid.

Conclusion

The evaluation of mushroom extracts demonstrated their potential as effective antifungal agents against root rot pathogens, revealing a significant reduction in pathogen control. The GC-MS analysis of the extracts identified effective compounds, such as 9,12-Octadecadienoic acid, 9 -amino-pupukeanane, ricinoleic acid, and psilocin, with antimicrobial properties. These findings highlight that mushroom extracts could be a natural alternative to fungicides for controlling plant pathogens. Compared to synthetic fungicides, mushroom-based biocontrol agents offer several advantages, including environmental sustainability, lower toxicity levels, and reduced pathogen populations. However, standardizing bioactive compounds from mushroom extracts is challenging due to significant variations in species, growth conditions, and extraction methods, all of which contribute to inconsistent biocontrol efficacy. Nevertheless, large-scale field applications of bio-formulated mushroom extracts in the future can potentially improve disease management and promote plant growth more effectively.

Acknowledgments

The authors express their deep gratitude to the Department of Plant Pathology, Department of Agricultural Entomology, Department of Biotechnology, Centre of Excellence for Innovations, Agricultural College and Research Institute, Madurai and Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India, for providing the essential facilities to support the above research work.

Authors' Contributions

VK and RN framed the research idea and wrote the manuscript. RN and YI contributed ideas for structuring the tables and figures. CP and MPKS refined the manuscript, and MP revised it. RN, YI, CP, MPKS, and MP assisted in finalizing the manuscript.

Compliance with Ethical Standards

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

Ethical issues: None

Declaration of generative AI and AI-assisted

technologies in the writing process

While preparing the manuscript, the authors used Grammarly to improve the language and readability. After using this tool/ service, the authors reviewed and edited the content as needed and took full responsibility for the publication's content.

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