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Identification, Characterization and In silico analysis of fungal species from plant pathogens

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Abstract

The study aimed to identify, characterize and analyze fungal species associated with two economically significant plant species, *Pongamia pinnata* and *Asclepias syriaca*. Through molecular technique, fungal isolates were identified.The study employed DNA sequencing and phylogenetic analysis to classify the fungal isolates accurately. Furthermore, in silico analyses provided insights into the pathogenicity mechanisms and genetic diversity of the identified fungal species. Functional annotation and pathway analysis elucidated metabolic pathways associated with pathogenesis and host interactions. Overall, our integrated approach provides a comprehensive understand of fungal diversity, evolution and pathogenicity in *P.pinnata* and *A.syriaca*. These findings lay the foundation of future research aimed at developing sustainable strategies for disease management and crop improvement in agricultural systems.

Keywords : *Asclepias syriaca*, Fungal species, Molecular techniques, Phylogenetic analysis, *Pongamia pinnata*.

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Introduction

The study of plant diseases, including their causative agents, diagnostics, physiological consequences, population dynamics, and management, is known as plant pathology. (John, 1977). One of the super biotic components that cause disastrous harvest sicknesses is growth-based plant contaminations. One of the vitally biotic components that cause horrendous harvest illnesses is parasite-based plant contaminations. Anthracnose, scourge, ulcer, damping off, dieback, nerve, leaf spot, fine buildup, rust, root decay, scab, and shrivel are the most pervasive infections welcomed on by plant pathogenic parasite (Hariharan, Ganeshamoorthy , & Kandeeparoopan, 2021). One of India's brightest and richest trees is the "Pongam Tree." In science, the tree is known as "*Pongamia pinnata*." The "Pongam Tree" is painted red throughout the months of March and April as the buds turn into wilting, new leaves. A week or two after the leaves start to become mature, the tree turns a lovely bright lime green. Each leaflet has a slender stem and the leaves range in length from 15 to 30 cm (Yadhav, Jain, Alok, Prajapati, & Verma, 2011). Research on the signs and seriousness of *Pongamia pinnata* L. leaf spot illness was finished at the nurseries of the Bangladesh Timberland Exploration Organization (BFRI), Aronnak Nursery, and the Foundation of Ranger service and Ecological Sciences, College of Chittagong (IFESCU). The IFESCU nursery had the greatest infection rate and illness index, followed by BFRI, while Aronnak nursery had the lowest. From the afflicted plant components, the related organism of *P. pinnata* leaf spot disease was isolated, and the pathogenicity of the isolated fungus was established (Mridha, Jabbar, M.K., & *et al.*, 2007). The dogbane family Apocynaceae (previously Asclepiadaceae) includes around 140 species of herbaceous perennial flowering plants of the genus Asclepias, or milkweed. Many milkweeds are grown as ornamentals, and they may be found all across North and South America (Petruzzello, 2020, February 26).

Materials and Methods

Sample Collection

Tainted leaf tests *Pongamia pinnata* and *Asclepias syriaca* were collected (Vellore District, Tamil Nadu) and then sterilized by using chemical sterilizing agents.

Identify fungi

The tainted leaf pieces were cut into little pieces with sterile edges. The growth was developed on Potato Dextrose Agar (PDA). The subsequent organisms were purged on Potato Dextrose Agar Medium utilizing the Hypha tips technique, and each isolated fungus was subsequently sub cultured on an agar plate for additional research. The fungi that were isolated were identified by both morphological and microscopical techniques.

Morphological Identification

The fungal morphology was studied microscopically. Two slides have been taken, and both slides show a tiny part of dark green fungus and white fungus on each slide. Add a few drops of lactophenol blue to both slides. View it under a compound microscope.

Molecular Identification

Utilizing a sterile surgical blade, a fresh fungal sample was moved to a 1.5 ml eppondrof microcentrifuge tube. 100µl of phosphate cushioned saline (PBS) was added, trailed by 700µl of lysis cradle (TE Buffer: 9.34 µl, 10% SDS: 600 µl, Proteinase: k-60 µl) and mixed sterilized 0.5-1 mm glass dots. The blend was vortexed for five minutes at a high velocity in a homogenizer. For 60 minutes, the sample were kept at 55°C in a dry shower hatchery. Add 500µl of phenol to the eppendrof microcentrifuge tube following 60 minutes. Twice or thrice, reverse the cylinders. For ten minutes, the samples were centrifuged at 10,000 rpm. Subsequent to gathering the supernatant, phenol, chloroform, and isoamyl

alcohol (25:24:1) were joined in an equivalent volume. For five minutes, rearrange the cylinders. The samples were centrifuged again for ten minutes at surrounding temperature at 12,000 rpm. Again, the supernatant was gathered and 400 µl of chloroform was added to the eppendrof tube. Twice through the tubes invert well. The materials were centrifuged again for ten minutes at surrounding temperature and 10,000 rpm. In the wake of gathering the supernatant, a comparable measure of super cold isopropanol was added. The samples were kept at - 20°C for one to two hours. To remove the DNA, the samples were centrifuged for 20 minutes at 10,000 rpm. After the supernatant was tapped, 500µl of 70% ethanol was utilized to wash the DNA pellet. After air drying, the DNA pellet was disintegrated in 200µl of TE cradle (10 mM Tris-HCl pH 8, 1 M EDTA)(Aamir, Sutar, & Baghela, 2015).

Agarose gel electrophoresis

Prepare the 1X TAE buffer with 1% of the agar solution. As the agarose dissolves fully, boil it to ensure that no visible agarose particles are left in suspension. Place the comb in the proper location on the gel tray after sealing the gel casting tray onto both slides. Pour the agarose mixture into the gel tray with the comb after the gel temperature reaches around 40°C. Then, add the ethidium bromide. Remove the seal from either side of the tray without disrupting the gel after the agarose has fully solidified. With the well on the cathode side, keep the gel in the tank that holds 1X TAE buffer. Maintaining the tank's buffer level above the gel tray is important. The gel is now ready for loading; just move the comb gently so as not to damage the wells. Before adding the sample, make sure the power cables are connected between the power pack and the electrophoresis tank. Stuff the well with a 10µl gel loading dye mixture. Turn on the power pack after loading, then set the volume to 50V. After the dye has reached 3/4 of the gel, continue the electrophoresis. Using a UV transilluminator, the gel was visible (Sambrook & Russell, 2001).

DNA sequencing

For a chosen strain, a total volume of 25µl of Polymerase Chain Reaction (PCR) was carried out in a T-100 Thermal Cycler, TTC100. In each PCR tube, the PCR mixture contains 3µl autoclaved water, 1µl of each primer, and 100µl of Master Mix Primers NS1 (GTAGTCATATGCTTGTCTC) and NS4 (CTTCCGTCAATTCCTTTAAG), each containing 19 and 20 bases, were used. A five-minute initial denaturation at 95°C was followed by thirty cycles of 30 seconds at 94°C, 30 seconds at 57°C, 90 seconds at 72°C, and a final extension of 10 minutes at 72°C for DNA amplification. Following purification and analysis on a 1% (w/v) agarose gel, the amplified product was utilised for DNA sequencing.

Phylogenetic analysis

Utilizing the Impact search program, the NS1 and NS4 DNA arrangements of the segregates were contrasted and those found in GenBank data sets to recognize expected homologous groupings of the as of late sequenced contagious species.To reduce the number of gaps that may be inferred, the sequences of closely related fungus that were recovered from Genbank were aligned. Clustal W software was used for multiple alignments while aligning the sequence. To determine the dependability level for the tree's nodes, 1,000 replications served as the bootsteap. Every sequence produced for this investigation was added to the NCBI GenBank database (Tamura , *et al*., 2011).

ExPASy-ProtParam tool

Protein physicochemical properties were assessed utilizing the ProtParam program

(www.expasy.org/tools/protparam.html) for speculative proteins. Protein arrangements or SWISS/TrEMBL IDs can be utilized to submit question proteins. The isoelectric point and molecular weight (pI/MW), level of every amino acid, elimination coefficient (EC), insecurity record (II), aliphatic file (artificial intelligence), and great normal of hydrophobicity (GRAVY) are all directly computed numbers that are provided by the server (Bidkar, Thakur, Bolshette, & Gogoi, 2014).

Garnier Ostoporpe Robson

In light of 17-amino-acid arrangement windows, the GOR procedure examinations groupings to predict alpha helix, beta sheet, turn, or arbitrary curl auxiliary construction at each spot. Four 17-by-20-inch scoring lattices with sections addressing the log-odd score-a proportion of the probability of finding a specific amino corrosive at every area in the 17-buildup grouping were given in the underlying clarification of the methodology. The chances of the focal, 10th amino acid being in a helical, sheet, turn, or loop compliance are addressed by the four lattices. Because sequences in turn areas vary greatly, the turn matrix was removed in later iterations of the algorithm. The methodology that expected something like two bordering buildups for a beta sheet and no less than four coterminous deposits to score as alpha helices to classify the district as helical was believed to be awesome (Hait, 2022).

Extraction of secondary metabolites:

For 21 days, an isolated fungus was cultured in a 250 mL conical flask filled with potato dextrose broth. To extract the metabolites from the culture filtrate, an identical volume of ethyl acetate (1:1) was added. Subsequently, the ethyl acetate-containing culture filtrate was moved to a separating funnel. In addition, it was left for 24 hours to extract the metabolites since the chemicals are better extracted when the culture filtrates are shaken in between with a solvent. To obtain the pure extract, the secondary metabolites of the culture filtrate that were on the upper layer of the separation funnel were collected and filtered.by extracting the secondary metabolites with the addition of 8–10 mL of ethyl acetate. The resultant suspension underwent filtering and purification. To evaporate the solvent, the filtered suspension was put into a watch glass and allowed to air dry for four hours. After scraping the metabolic extract from the watch glass, it was gathered in an eppendorf tube for further use.

Phytochemical screening of fungal extracts:

Test for alkaloids (Wagner's test)

Following the disintegration of potassium iodide (2 g) and iodine (1.27 g) in 5 mL of distilled water, the solution was diluted to 100 mL using distilled water. A small amount of this solution was added to the filter, and the presence of alkaloids is shown by brown-colored precipitate.

Test for flavonoids (Alkaline reagent test)

Two ml of extract were mixed with two to three drops of sodium hydroxide. A few drops of diluted HCL were added, and although the mixture first had a strong yellow hue, it eventually became colourless, suggesting the presence of flavonoids.

Test for saponin (Foam test)

The extract was combined with an equal amount of distilled water, agitated well, and left undisturbed for two to five minutes. The presence of saponins is indicated if the foam continues.

Test for tannins (Ferric Chloride test)
1ml of the extract was mixed with two millilitres of a 5% neutral ferric chloride solution; the extract's dark blue colouring suggested the presence of tannins and chemicals.
Test for phenols (Lead tetra acetic acid test)
After adding 0.5 mL of extract to one millilitre of lead tetra acetate solution, a precipitate formed, signifying the presence of phenolic chemicals.
Test for triterpenoids (Salkowski's test)
After treating the extract with chloroform, it is filtered and a few drops of concentrated sulfuric acid are added. After that, it is shook and left to stand. The presence of triterpenoids is indicated by the emergence of reddish-brown or golden yellow colors.
Test for proteins and aminoacids:
The extract was dissolved in 10 ml of distilled water, then the filtrate was passed through Whatmann No. 1 filter paper and tested for amino acids and proteins.
Biuret Test
Few ml of extract was treated with 2% Copper Sulphate Solution. To this 1 ml of Ethanol (95%) was added followed by excess of Potassium hydroxide pellets. Pink color in the ethanolic layer indicates the presence of proteins and amino acids.
Gas Chromatography and Mass Spectrometer (GC-MS) spectral analysis
Sample preparation is the first step in the gas chromatography-mass spectrometry (GC-MS) analysis process. Here, the sample is extracted using the proper solvents or procedures. The extracted material is then introduced into the gas chromatography (GC) system, where it passes through a chromatographic column and is separated according to its chemical characteristics. The molecules that have been isolated are subsequently broken up into distinct ions in the mass spectrometer by ionisation. Following the detection of these ions, a mass spectrum is produced by measuring their abundance. Compound identification is accomplished by database matching or by comparing the mass spectra to those of recognised standards. Using internal or external standards, or by integrating peak areas, quantification is carried out. Specialised software suites are used for thorough data analysis (Adams, 2007).
Protein extraction for characterization of Isolated fungi
A 1 g fungus sample was taken by using a scalpel and mixed in 50 ml of a conical flask. The conical flask contains 50 ml of potato dextrose broth. Add an equal volume of extraction buffer (20 mM Tris-HCl in 1X PBS) to the conical flask. Centrifuge the sample at 5000 rpm for 10 min. Dispose of the pellet from the sample. Add an equivalent volume of ice-cold acetone to the supernatant. Incubate the solution at room temperature for 15–20 minutes. Again, centrifuge the sample at maximum speed for 15 minutes. Dispose the supernatant from the sample. Wash the pellet by using ice-cold acetone to remove pigment. Reconstruct the pellet by adding Phosphate Buffer Saline (PBS).
Sodium Dodecyl Sulphate
Place the two glass plates within the casting frames and assemble the casting frames on the casting stands. In a small beaker, prepare the separating gel according to the above instructions. Thoroughly yet gently swirl the mixture. The separating gel solution should be pipetted into the space between the glass plates in the proper amount. Fill the space with water (either isopropanol) until it overflows in order to make the top of the separating gel horizontal. Give it 20 to 30 minutes to gel. Once the remaining water has been discarded, the separating gel will be visible. As previously indicated, prepare the stacking gel in a small beaker. The stacking gel should be pipetted above the separating gel until it overflows. Take care not to trap any air bubbles behind the teeth while inserting the well-forming comb. It gelled in 20 to 30 minutes. Verify that the stacking gel has fully gelled, then remove the comb. At the point when the cradle surface in the external chamber arrives at the important level, eliminate the glass plates from the projecting edge and spot them in the cell buffer dam. Then, at that point, pour the running buffer — otherwise called the electrophoresis buffer — into the inward chamber.
Before loading the sample, prepare the 1X running buffer from the 10X and fill the casting tray. The microfuge tubes with labels. Transfer 10ul of every protein sample into the appropriate tube. To each, add 10 ul of sample loading buffer, then heat for 3 to 5 minutes at 100°C.Fill the wells with 20ul of sample and 10ul of marker. In order for the protein to migrate towards the anode, attach electrodes. Use 100 to 200V to run the gel. Regarding the overall running time, stop the SDS-PAGE running when the protein marker's downward sign almost touches the glass plate's foot line. The run duration will vary depending on the gel and buffer composition's % cross-linking. Cut off the electrodes, remove the gel sandwich from the tank, and discard the electrophoresis solution. Take off the gel tray and place it into the staining tray. Fill gel with staining solution. Slide the gel-coated plate upside down into the staining solution, then slowly let the gel "float" off the plate into the solution. For fifteen to thirty minutes, cover with plastic wrap and gently stir the gel on the gel rocker. To get rid of extra stain, remove the staining solution and rinse the gel with ddH2O. For ten to fifteen minutes, add the destaining solution and stir on the gel rocker. Once the right degree of destaining is reached, change the destaining solution and agitate and kept for further use.

Results and Discussion

Molecular Identification
Pongamia pinnata and *Ascleipas syriaca* were identified by their morphological traits. The isolated fungus was examined morphologically under a microscope Figure (1,2).

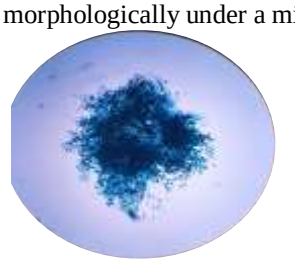


Fig. 1 Micrscopiv view of *Pongamia pinnata*

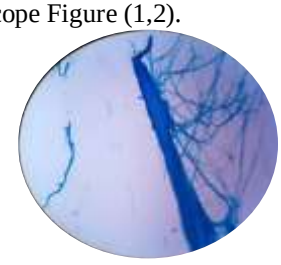


Fig. 2 Microscopic view of *Asclepias syriaca*

DNA extraction from fungal plants
DNA was extracted directly from the fungus plates using the Phenol:Chloroform:Isoamyl alcohol technique on a representative sample of fungal spores (Figure 3). Using primers unique to the fungal NS1 and NS4 regions—which are useful for differentiating between fungal species—the recovered DNA was subjected to PCR analysis. In previous research, fungal DNA (from the Ascomycete Aspergillus fumigatus) was extracted by sonication at 55 °C in CTAB buffer, either with or without glass beads. In the same study, other techniques for extracting DNA were examined,

including vortexing and glass beads, which could extract DNA in two to three hours (J, R, T, R, & D).DNA extraction from commercial kits, such as the Qiagen® Plant Mini Kit, takes around an hour (A. M. M., H, G, & A, 2019).

Fig. 3 DNA extraction by using P:C:I method

Phylogenetic analysis

The Genebank lists the sequence as Fusarium proliferatum and Trichoderma. The NS1 and NS4 sequences were checked for sequence identity in the GenBank DNA database using the BlastN (NCBI) search engine. The Blast results revealed that the NS1 and NS4 sequences of endophytic fungi had 99–100% similarity with the current sequences of the NCBI database after the sequences of closely related fungal strains were obtained from the database (Figure 4). A phylogenetic tree was subsequently created using the molecular evolutionary genetics analysis (MEGA 5) software after the sequences had been modified (using BioEdit software) and aligned (using Clustal W software) Figure (5). Trichoderma and Fusarium proliferatum were shown to be tightly associated with the sequence generated from the endophytic fungus. A phylogenetic analysis of the ABC proteins extracted from the genomes of 27 fungal species from 18 orders, which correspond to 5 fungal phyla, revealed the most important groupings. According to our research, some ABC protein subfamilies have undergone significant group-specific evolution, while others have remained remarkably conserved in fungi. The amount of ABC proteins in the genomes of the various fungal phyla also varied significantly; the yeasts S. cerevisiae and S. pombe have notably low amounts of these proteins. (Kovalchuk, A., *et al.*, 2010).

Expasy tool:

The Nucleotide sequence of *Trichoderma* fungus was translated to a protein sequence using expasy tool. By using the protein sequence, protparam has been identifiedThe different physical and chemical characteristics of a certain protein maintained in Swiss-Prot for the protein sequence are displayed in the Protparam tool Figure (6).

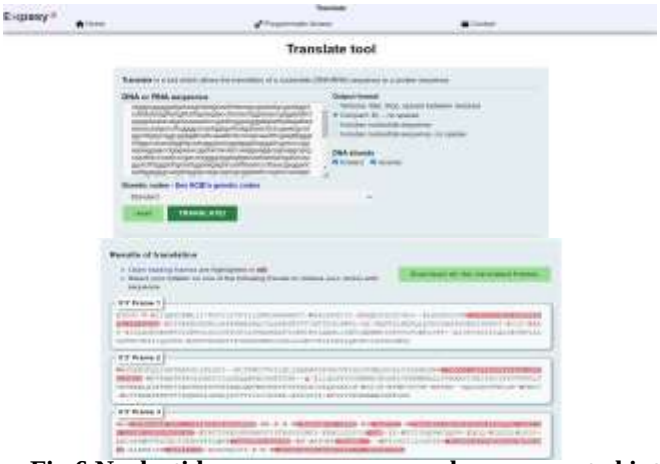


Fig 6.Nucleotide sequence sequences been converted into protein sequence

Scan prosite tool:

Proteins can be scanned for matches against the PROSITE collection of motifs by using the expasy tool's protein sequence Figure (7).

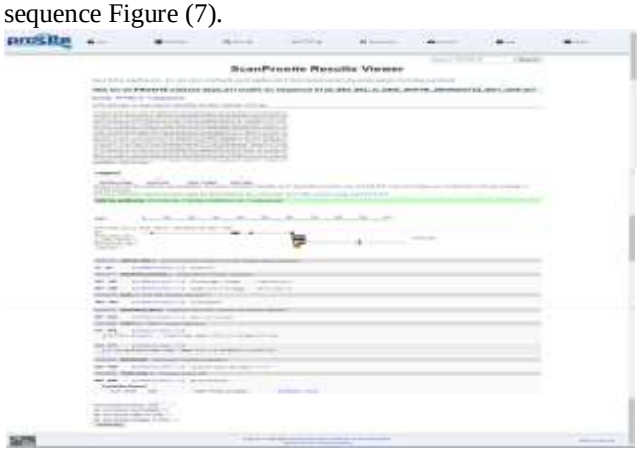


Fig 7. Scan prosite analysis

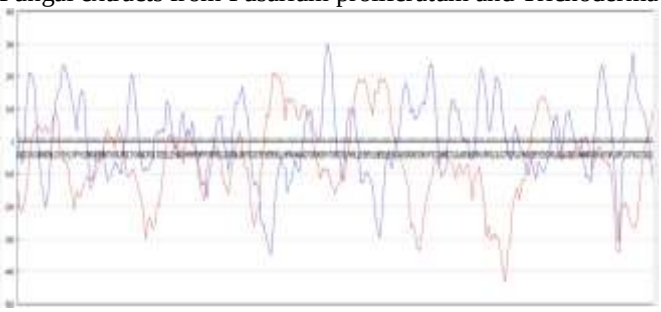
Garnier-Osguthorpe-Robson

To generate the output shown (Graph 1), paste the protein sequence that was obtained in the FASTA format into the URL provided. The fraction of coils, beta-strands and helices is determined and identified

. **Graph 1. Garnier Osguthorpe Robson**

Phytochemical analysis:

Fungal extracts from Fusarium proliferatum and Trichoderma

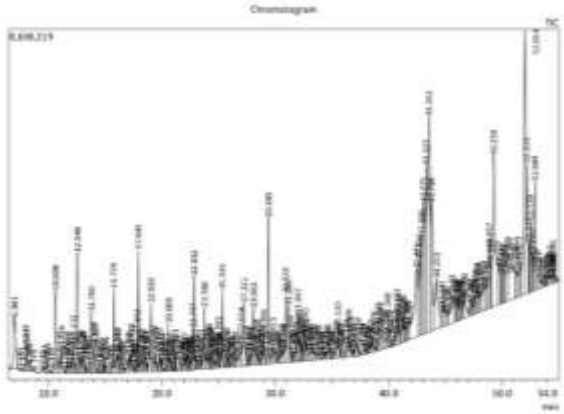


cultivated with ethyl acetate were subjected to qualitative analysis, which produced good findings for alkaloids, phenols, saponins, tannins, and triterpenoids. The variations in colour signify the existence or non-existence of phytochemical elements in fungal extracts Table (1). The fungal ethyl acetate extract contained alkaloids, phenols, flavonoids, tannins, and glycosides, according to the results of the qualitative phytochemical examination. But the fungal extract contained no saponins (Nameirakpam *et al*., 2012).

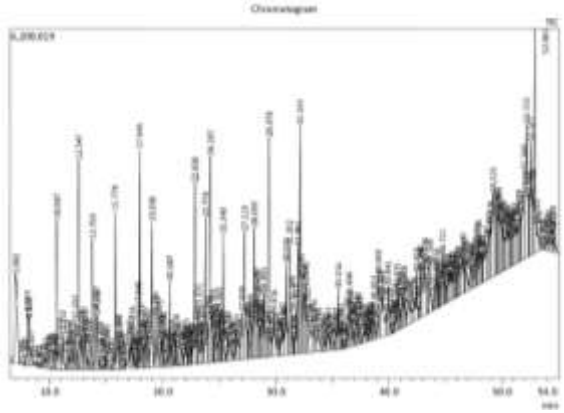
S. No.	**Phytochemical Test**	**Observation**	
		Fusarium proliferum	***Trichoderma***
1	Alkaloids	Presence	Presence
2	Flavonoids	Absence	Presence
3	Phenols	Presence	Absence
4	Protein and Amino acid	Absence	Absence
5	Saponin	Presence	Absence
6	Tannins	Presence	Presence
7	Triterpenoids	Presence	Presence

Table 1. A phytochemical analysis result indicates the presence of various compounds

Gas Chromatography and Mass Spectrometer (GC-MS) Spectral Analysis: The Gc-ms study showed that bioactive chemicals were created by Trichoderma ethyl acetate extracts. The collected peaks are displayed as a chromatogram (Graph 2), and the components that correspond to the peaks are listed in a table along with their structure, molecular weight, peak area percentage concentration, retention duration, and CAS value. The Chromatogram of Fusarium proliferatum predicts the presence of 4,6-Dimethyldodecane, 5-Isobutylnonane, 2,7-Dimethylundecane, 2,6,10-Trimethyldodecane, 6-Ethyl-2-methyloctane, 2-Methylundecane, 5-n-Butylnonane, n-Tetradecane, n-Pentadecane, 3-Trifluoroacetoxy-6-ethyldecane, 4,5-Dimethyl-2-undecene, 1-n-Nonyl iodide, 2-Bromononane, 5-(2-methylpropyl), 2,6-Dimethyldecane. The Gc-ms study showed that bioactive chemicals were created by Fusarium proliferum ethyl acetate extracts. The collected peaks are displayed as a chromatogram (Graph 3), and the components that correspond to the peaks are listed in a table along with their structure, molecular weight, peak area percentage concentration, retention duration, and Cas value. The chromatogram of Trichoderma predicts the presence of 5-Isobutylnonane, 4,6- Dimethyldodecane, 2,6,10-Trimethyldodecane, 3,7-Dimethyldecane, 4,5-Dimethylnonane, 2,6,11-Trimethyldodecane, 5,6-Dimethyl-nonane, 4-Methylundecane, 3,3-Dimethylhexane, 2,3,6,7-Tetramethyloctane, Methyl butanoate, N-Isopentylacetamide, 3-Methylhexan-1-amine. One fungus that demonstrated encouraging results was chosen for analysis out of the six endophytes that were isolated from Phlogacanthus thyrsiflorus Nees. It is recognised that endophytic fungus found in medicinal plants can create bioactive chemicals that can be employed in pharmaceutical applications. (Zhang HW *et al*., 2006).



Graph 2. GC-MS analysis of *Trichoderma*



Graph 3. GC-MS analysis of *Fusarium proliferum*

Conclusion

The study provides a comprehensive understanding of plant pathology, emphasizing the categorization of plant diseases, importance of disease detection, and molecular identification techniques for characterizing fungal infections. The taxonomy of *Pongamia pinnata* and Asclepias syriaca plants is clarified by the isolation and genetic identification of endophytic fungi from these plants. The discovery of numerous bioactive chemicals through the qualitative screening of the fungi's ethyl acetate extracts for phytoconstituents helped to clarify their possible functions and impacts. Moreover, the study underscores the significance of bioinformatics in processing and interpreting biological data, thereby enhancing our understanding of genetic relationships and phytochemical profiles. In conclusion, the synthesis of experimental and computational approaches significantly advances our knowledge of plant pathology and contributes to the ongoing efforts in understanding and managing plant diseases.

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