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Exploring the Plant Growth Promoting and Antifungal Potential of Endophytic and Rhizospheric fungi isolated from Litchi chinensis Sonn.: Metabolite profiling and Molecular characterization

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Abstract

The unique characteristics of Tezpur litchi varieties in terms of their size, quality, taste and relatively small seed size helped to recognize the Geographical Indication (GI) in 2014. The present study aimed to investigate the endophytic and rhizospheric fungal communities associated with Tezpur litchi (var. Bilati) for their plant growth-promoting (PGP) and biocontrol potential. Fungal isolates were evaluated for indole-3-acetic acid (IAA) production, phosphate solubilization activity (PSA), and antagonistic activity against the phytopathogen *Alternaria alternata* (MTCC 3880). The isolated fungi were found belonging to the genera *Aspergillus*, *Colletotrichum*, *Fusarium*, *Penicillium*, and *Mycelia sterilia*.

Promising results were obtained for IAA production, with the highest values recorded at 179.25 ± 3 µg ml $^{-1}$ and 143.13 ± 3 µg ml $^{-1}$ by soil isolate SF32 and endophytic isolate BE23, respectively. All isolates exhibited varying degrees of PSA, with the highest value (3.44 ± 0.04 SI) observed in endophytic isolate LE07. Antifungal activity screening revealed significant inhibition of *A. alternata* by endophytic isolate BE14 (41.9 ± 1.4 mm zone of inhibition) and soil isolate SF32 (29.4 ± 0.8 mm). Subsequent molecular identification 18S ITS rDNA sequencing confirmed BE14 and SF32 as *Penicillium citrinum* and *Aspergillus aculeatus*, respectively. This study reports the association of *P. citrinum* in litchi and identifies the potential antifungal properties. Fourier transform infrared (FTIR) analysis of the crude metabolite from *P. citrinum* revealed the presence of various functional groups, including alcohols, alkanes, and aromatic compounds. Gas chromatography-mass spectrometry (GC-MS) analysis tentatively identified four major compounds: Succinic-acid-2,4,6-trichlorophenyl-3-methylbut-3-en-1-yl-ester, 1,5-but(3-cyclopentylpropoy)-1,1,3,3,5,5-hexamethyltrisiloxane, Hexamethyl-cyclotrisiloxane and Tris(tert-butyldimethylsilyloy)arsane. These findings suggest the presence of potentially bioactive metabolites with antifungal properties in *P. citrinum*.

In conclusion, this study highlights the diverse fungal communities associated with Tezpur litchi and identifies potential candidates for promoting plant growth and managing fungal diseases through eco-friendly approaches. Further investigations are warranted to elucidate the specific mechanisms underlying the observed PGP and biocontrol activities of these promising fungal isolates.

1. Introduction

Fungi are cosmopolitan microorganisms which exist in a wide range of environments like terrestrial, freshwater, and marine ecosystems (Jones et al. 2019; Dayarathne et al. 2020; Dong et al. 2020). Some fungi may cause serious diseases in humans, plants, and animals giving rise to many health challenges (Giacomazzi et al. 2016; Hyde et al. 2018). However, many are saprophytic and playing a crucial role as decomposers of organic matter. Also, many are contributing to advances in medicine, industries, and sustainable agriculture (Abo Nouh 2019; Solomon et al. 2019).

Fungal communities associated with plants have been identified as major growth promoters (Murali et al., 2012). Rhizospheric fungi decompose organic matter and make them available to plants (Murali et al.,

2012). They are capable of solubilizing phosphates to plant available forms (Jyoti et al., 2013; Li et al., 2015). Similarly, the endophytic fungal association benefits the host plant in numerous ways, like enhancing the root and shoot biomass of the host plant by producing growth hormones (IAA)(Mehmood et al. 2018; Pandey et al. 2018; Roy et al. 2023). Studies show that endophytic fungi confer resistance to phytopathogens by secreting antibiotic compounds such as phenolic metabolites (Deka and Jha 2020; Sornakili et al. 2020). These endophytic fungi are ubiquitous to plants, and plants without endophytic fungi are very rare (Zabalgogeazcoa 2008). Endophytic fungal diversity is controlled by the plant species and its specific organs (Wearn et al. 2012). Whereas, in the plant rhizosphere, the fungal species are also influenced by the soil's physiochemical properties (Zhou et al. 2021). Available phosphorous (P), urease activity, and the organic content in cultivated crops determine the fungal community composition in the rhizosphere (Zhou et al. 2021). Moreover, climate has an important influence on soil fungal diversity (Tedersoo et al. 2014; Newsham et al. 2016).

Litchi (*Litchi chinensis* Sonn.) from the family Sapindaceae is a popular tropical fruit crop. horticulture crop of Tezpur, Assam, India. The agro-climatic conditions of Tezpur (26.6528 N, 92.7926 E)from north eastern part of India favour litchi cultivation and six litchi varieties (Bombay, Bilati, Shahi, Elaichi, Piyaji, and China) have been identified with excellent qualities (Gogoi et al. 2020). The unique varieties of litchis only specific to Tezpur have coveted Geographical Indication (GI) in 2014 (Gogoi et al. 2020). Among the six varieties, the Bilati litchi towers all by having the highest fruit weight and circumference (Gogoi et al. 2020). Recent studies on crops like coffee (*Coffea arabica*), apple (*Malus* x *domestica*) and strawberry (*Fragaria* x *ananassa*) suggest that climatic conditions, soil characteristics and microbial diversity play crucial roles in improving quality crops by inducing genetic variability and resistance (Veloso et al. 2020; Abdelfattah et al. 2021; Sangiorgio et al. 2022).

Fungal species associated to cultivated crops like rice (*Oryza sativa*), wheat (*Tritichum aestivum*), maize (*Zea mays*), soyabean (*Glycine max*) have been well studied (Abdolmaleki et al. 2023; Airin et al. 2023; Farhat et al. 2023; Orole et al. 2023; Sodhi and Saxena 2023). However, litchi crop is not well attended with regard to fungal association. Moreover, the exceptional characteristic of Tezpur litchi (var. Bilati) inspire us to study the endophytic and rhizospheric fungal communities associated with the plant and to screen for their growth-promoting potential if any.

The aim of the present investigation was to isolate and identify endophytic fungi (colonizing in leaves, roots, and barks) along with the rhizosphere from *Litchi chinensis* Sonn. (var. Bilati) of Tezpur and to screen them for the potential use in agricultural production.

2. Materials and Methods

2.1 Collection of Samples

Leaf, bark, and root samples from eight healthy (without disease symptoms) litchi (*L. chinensis* Sonn. var. Bilati) plants were collected aseptically along with the rhizospheric soil (top 0-20cm of soil layer).The

collection of both soil and plant samples were done during the harvesting season of litchi (June-July of 2022). Samples were collected from two litchi orchards of Tezpur, Assam (26.693491 N, 92.824764 E, and 26.677472 N, 92.815549 E). The collected samples were stored at 4 C and the isolation of endophytic fungi from the collected samples were done within 6h of collection time.

2.2 Isolation of Endophytic and Rhizospheric fungi from *Litchi* plants

Using a sterile knife, freshly gathered and cleaned samples were cut into 108 segments, each made up of 36 leaves, barks, and roots that were of around 2 cm long and 0.5 cm broad. The segments were immersed in 70% ethanol for 3 minutes, 4% sodium hypochlorite (NaClO) aqueous solution for 5 minutes, 70% ethanol for 1 minute, and finally 0.1% mercuric chloride ($HgCl_2$) for 3 minutes (Strobel et al. 2002). After cleaning the segments with sterile distilled water, the surface sterilization efficiency of each segment was determined using Schulz et al.(1993) imprint method. Following the segments' inoculation, three distinct media namely Czapeck-Dox-Agar, Potato-Dextrose-Agar, and Rose-Bengal Chloramphenicol Agar were added to petriplates measuring 90 × 15 mm. After inoculation, the plates were incubated at 28 ± 1°C until the segments were surrounded by a mycelium or colony. To isolate the rhizospheric fungi the media mentioned above was used after dilution (10^{-4}) with water. The plates were evenly spread with a sterile L-shaped glass rod and incubated at 28 ± 1°C for 3–5 days (Waksman 1944). Streptomycin sulphate (50 µg mL^{-1} concentration) was used in all the media to prevent bacterial contamination. All the media were procured from HiMedia Laboratories Pvt. Ltd., India. The sporulated fungi were observed under a compound microscope (LABOMED CxL MONO) and identified based on their morphology and reproductive structure using a standard fungal identification manual (Barnett and Hunter 1998).

2.3 Colonization Frequency and Isolation Frequency

The colonization frequency (CF%) of endophytic fungi was determined following the formula of Hata and Futai (1995)

$$\mathrm{CF} = \left(\frac{\mathrm{Ncol}}{\mathrm{Nt}}\right) \times 100$$

where,

Ncol = number of leaf/bark/root segments colonized by specific fungus,

Nt = total number of leaf/bark/root segments plated

The isolation frequency (IF%) of rhizospheric fungi was calculated by using the formula given by Baweja et al. (2020):

$$\mathrm{IF} = \left(\frac{\mathrm{Ni}}{\mathrm{Nt}}\right) \times 100$$

where,

Ni = Number of unique fungal genera isolated in a plate

Nt = Total number of fungal genera isolated in a plate

2.4 PCA analysis

The dimensionality-reduction method in principle component analysis (PCA) was utilised to summarise the frequency of colonisation of endophytic and rhizospheric fungi that were isolated from soil and each type of plant tissue. Correlation matrix in the data set of colonization frequency was used to generate PCA in PAST 4.03.

2.5 Indole Acetic Acid (IAA) production

The fungal isolates were cultivated in Czapek's Dox (CD) broth at 27 ± 1 C. After inoculating in 20 ml of CD broth (with 0.1% L-tryptophan and without L-tryptophan) the mycelium of 1 cm (diameter) was incubated for 10 days using a rotatory shaker (HiMedia Laboratories Pvt. Ltd.). This followed by centrifugation of 5 ml of each cultured broth for 30 mins at 6000 rpm. The supernatant (1 ml) was then combined with one drop of orthophosphoric acid and 2 ml of Salkowski's reagent (300 ml of concentrated sulfuric acid and 15 ml of 0.5 $FeCl_3$).

Appearance of pink colour indicated production of IAA by the tested endophytes following the method by Fouda et al. (2015). To quantify the same, the optical density (OD) at 530 nm was measured using a UV spectrophotometer (Eppendorf Biospectrometer kinetic 6136FP603197). The IAA concentration was calculated using a standard IAA graph.

2.6 Phosphate Solubilization Activity (PSA)

Each isolate was inoculated on Pikovskaya's agar plates (HiMedia Laboratories Pvt. Ltd.) with a loopful of mycelium. The plates were subsequently grown at 28 ± 1 C for 5–7 days. The activity in phosphate solubilization by the isolates as indicated by the formation of a clear zone around the colony was recorded (Pandey et al. 2006). The formula given by Premono et al., 1996 used to calculate the phosphate solubilization index (SI).

$$\mathrm{solubilizationindex\,(SI)} = \frac{(\mathrm{colonyzonediameter} + \mathrm{clearingzonediameter})}{(\mathrm{colonydiameter})}$$

2.7 Antagonistic Activity

Dual culture approach was followed to evaluate the antagonistic activity of the fungal isolates against *Alternaria alternata* (MTCC 3880) (Khare et al., 2010). In PDA plates, culture discs containing each fungal isolate and *A. alternata* were positioned opposite each other. Whereas, in control plates *A. alternata* was used without *A. alternata*. and the plates were incubated at 28 ± 1°C. The following formula was used to

evaluate the Colony interactions as a percentage of inhibition of *A. alternata's* radial growth was measured using the following formula (Khare et al. 2010).

$$\mathrm{percentageofinhibition} = \frac{\mathrm{R2} - \mathrm{R1}}{\mathrm{R1}} \mathrm{X100}$$

Where,

R1 - Radius of the radial growth of the pathogen towards the opposite side in the control plate,

R2 - Radius of the radial growth of the pathogen towards the opponent antagonist in the test plate.

2.8 Statistical Analysis

Analysis of variance (ANOVA) was done for the isolates at $P \leq 0.05$ using Statistical Package for the Social Science (SPSS 26.0) for Windows. Duncan's Multiple Range Test (DMRT) test was done alongside between the isolates to indicate a significant difference among the data at 5% significance level ($P \leq 0.05$).

2.9 Fungal culture and extraction of metabolite

Fungal isolates were grown in 250 ml erlenmeyer flasks with 100 ml of potato dextrose broth and cultured at room temperature for 7 days while being shaken sporadically. The mycelial mass was then separated from the culture broth by filtering. The filtered soup was added together with an equal volume of ethyl acetate using a separating funnel. After shaking the mixer vigorously for 10 minutes, the two distinct, immiscible layers formed within 5 minutes. The secondary metabolites-containing top layer of ethyl acetate was isolated. The solvent was then evaporated in a rotary evaporator at 35–40°C while reduced pressure was applied, resulting in a concentrated extract. The extract was then dissolved in 10% DMSO and stored at 4°C for the antifungal test (Talukdar et al. 2021).

2.10 Antifungal assay

Agar cup diffusion method was used to measure the antifungal activity of the extracted metabolites. Each agar cup (6 mm diameter) held 100 µL of the crude extract had been dissolved in dimethyl sulfoxide (DMSO) and the control cup was prepared with only DMSO. For every case, three replicates were kept. After 24 hrs of incubation at 28 ± 1°C, the zones of inhibition on the plates were measured and contrasted with the control. The diameter (mm) of the inhibition zones determined the strength of the antifungal activity (Tayung et al. 2011).

2.11 Molecular Identification of potent isolates

Fungal isolates documented promising IAA and antifungal activity was used for ITS rDNA sequencing and phylogenetic studies. Following Chen et al.(2011), the selected fungi were cultivated in PDA medium, and genomic DNA was extracted using the CTAB method. ITS1 and ITS4 primers were used to amplify the fungal DNA. For the PCR amplification, a total of 25ml of aqueous solution was utilized. Reaction mixture components included 50–100 ng of isolated fungal genomic DNA, 0.5 U of Taq DNA polymerase,

1x concentration of PCR buffer (Invitrogen, Life Technologies, Brazil), 10 pmol of forward and reverse primers, 2.5 mM of $MgCl_2$, 200 mM of each of the four deoxyribonucleotide triphosphates (dNTPs). Genomic DNA was denaturated for 5 mins at 95°C to start the reaction. Subsequently, the DNA underwent 39 cycles consisting of denaturation for 30 s at 95°C, annealing for 45 s, elongation for 1 min at 72°C, and a final extension for 7 mins at the same temperature. The amplicons were resolved in 1.5% agarose gel using 0.5x tris-acetate-EDTA (TAE) buffer. The sequencing was done using an automated sequencer (ABI 3730XL) and a BigDye Terminator sequencing kit.

The sequences were added to the NCBI GenBank database the accession numbers were received. The evolutionary distances was expressed in base substitutions per site and were calculated using the maximum composite likelihood method (Tamura et al. 2004). There were 16 nucleotide sequences in this investigation. The 1st, 2nd, 3^{rd,} and noncoding codon locations were covered. For every sequence pair, all unclear places were eliminated (pairwise deletion option). In MEGA X, evolutionary studies were performed (Kumar et al. 2018). 500 ITS rDNA sequences from various related species were obtained from the GenBank database to create the phylogenic tree.

2.12 Characterization of crude metabolite

FTIR analysis

Few drops of the extracted metabolite were placed on a KBr aperture plate, which was then positioned underneath another aperture plate to prevent any trapped gas bubbles. There are no lines added to the spectra since the KBr plates are translucent to infrared light. The analysis was done in Perkin Elmer Frontier IR 101880. The obtained spectra were then compared and evaluated for the shifting of functional peaks (Pervez et al. 2015).

GC– MS analysis: HPLC grade methanol was used to prepare the sample solution for GC–MS analysis of the crude metabolite following Huang et al. (2007). The GC conditions were as follows: capillary column of length 60 m (Perkin Elmer Elite-5 MS) with internal diameter 0.25 mm composed of 5% diphenyl 95% dimethyl polysiloxane (low bleed). Helium was used as carrier gas at a rate of 1 ml/min. The inlet temperature was set at 280°C and oven temperature was initially programmed at 60°C for 5 min, then increased to 180°C at a rate of 10°C with a 5 min holding time with the final temperature at 300°C at a rate of 20°C ending with a 5 min. The ion source temperature was maintained at 200°C at total run time of 39 min. GC-MS was analyzed using electron impact ionization at 70 eV. Diluted sample of 1.0 µl was injected in a splitless mode. The quantification and identification of the compounds were accomplished using a total ion chromatogram (TIC). Library searches for the peaks were carried out using the S/W Turbomass NIST library. The structure of the compounds was retrieved from PubChem database of the National Library of Medicine (https://pubchem.ncbi.nlm.nih.gov/).

3 Results

3.1 Endophytic and Rhizospheric fungal isolates

Altogether 89 and 38 fungal isolates were obtained from healthy plant tissues and rhizospheric soil respectively. By comparing colony and spore morphologies with standard identification manual (Barnett and Hunter 1998) it was found that the isolates were comprised of fungal genera *Aspergillus, Colletotrichum, Fusarium, Penicillium,* and non-sporulating mycelia sterilia. The colonization/isolation frequencies of endophytic and rhizospheric fungi is presented in Fig. 1

3.2 Principle Component Analysis (PCA):

The PCA showed that the genus *Penicillium* is dominant in leaves of litchi plants, whereas Mycelia sterilia is dominant over barks. Similarly, *Fusarium* was dominant on root tissues and *Aspergillus* was dominant in rhizospheric soil. In Fig 2 the PCA biplots is shown with active variables and active observations.

3.3 IAA production and phosphate solubilization

Out of 127 isolates tested for the production of IAA; 19 endophytic and 9 rhizospheric fungi demonstrated notable production of IAA. It has been observed numerously that there is a positive correlation between the production of IAA, phosphate solubilization and disease resistance in fungi (Bader et al. 2020; Napitupulu et al. 2021). Consequently, the phosphate solubilization activity of these (19+9=28) isolates was evaluated. With 143.136±3.94 μgml^{-1}, the endophytic isolate BE23 showed the highest IAA production, followed by LE20 (118.058±1.98 μgml^{-1}). Whereas, rhizospheric fungal isolate SF32 demonstrated an exceptionally elevated production of IAA, with a concentration of 179.25±3.75 μgml^{-1}. Statistical analysis showed the 28 isolates were grouped in 17 groups which showed significant difference across groups by $p \leq 0.05$ in production of IAA in presence of tryptophan. However, 9 groups were formed among the isolates showing production of IAA showing significant difference. Thus, indicating that production of IAA is independent in presence of tryptophan among the isolates.

Phosphate solubilization was found to be positive in 12 isolates. The documented PSA values of 8 endophytic isolates were between 1.8 and 3.4 SI. Isolate LE07 showed the highest phosphate solubilization of 3.442±0.04SI and 4 rhizospheric fungal isolates exhibited PSA values between SI 1.9 and 3.1. Among them, isolate SF11 displayed the highest PSA, measuring SI 3.16±0.13. Table 1 displays the IAA production and PSA for each of the 28 isolates. Statistically, it was observed that the isolates had 7 groups having significant difference in PSA with $p \leq 0.05$.

3.4 Antagonistic activity

Strong inhibition of *A. alternata* was noted by the tested endophytic fungi (>55%). With 79±0.50% inhibition, the endophytic isolate LE24 showed the highest level of suppression, followed by BE14 with 78.5±0.61%. Similarly, rhizospheric isolate SF04 displayed the highest inhibition, at 80.8±0.50 followed by isolate SF32 with inhibition, at 80.5±0.54%. Table 2 shows the fungal isolates and their respective inhibition ability against *A. alternata.* Fig 3: displays the photo-plates of dual culture assay of LE24, BE14, SF32, and SF04 along with their graphical representations. Statistics showed the isolates had 8 related groups having significant difference across them with $p \leq 0.05$.

3.5 Antifungal Assay

The result from antifungal assay with crude metabolites of 4 promising isolates (LE24, BE14, SF32, and SF04) showed a zone of inhibition > 25mm. However, the isolate BE14 revealed exceptionally high antifungal activity with the zone of inhibition of 41.9±1.46 mm. Table 3 lists the results of antifungal activity of the 4 potent fungal isolates. Fig 4: displays the photoplates showing the zone of inhibitions.

3.6 Molecular identifications of the potent isolates

The isolates BE14 and SF32 were found to have the best antifungal activity and IAA production respectively. Formerly, BE14 morphologically identified as *Pencillium* sp. and SF32 as *Aspergillus* sp. were sequenced for 18s rDNA for ITS1 and ITS4 for their molecular identifications. The sequences were submitted to GenBank and the accession number was obtained as PP203028 and PP196577 respectively. The evolutionary history was done using Maximum Parsimony Method. The BLAST analysis revealed that BE14 (*Penicillium* sp.) had the closest homology with *Penicillium citrinum* and SF32 (*Aspergillus* sp.) with *Aspergillus aculeatus* (Table 4). 9 (*Penicillium*) and 8 (*Aspergillus*) sequences with the complete ITS rDNA region (18S rDNA-ITS1-5.8S-ITS2-rDNA) were taken into consideration for phylogenetic analysis after the sequences underwent filter search. The generated tree revealed that the isolate BE14 clustered with *Penicillium citrinum* and isolate SF32 clustered with *Aspergillus aculeatus* (Fig 5).

3.7 Characterization of crude metabolite

FTIR analysis of the crude metabolite of *P. citrinum* showed bands at 3438 cm^{-1}, 2914 cm^{-1}, 1417 cm^{-1}, 1638 cm^{-1}, 1310 cm^{-1}, 1044 cm^{-1}, 776 cm^{-1} and 698 cm^{-1}. The bands were compared with standard ranges of functional groups given by (Lingegowda et al. 2012). Band at 3438 cm^{-1} corresponds to the stretching vibrations of alcohol, whereas, the band at 2914 cm^{-1} correspond to the stretching vibrations of alkanes. Similarly, the bands at 1417 cm^{-1} and 1638 cm^{-1} correspond to unsaturated aliphatic and Nitro compounds, bands at 1310 cm^{-1} to alkenes, bands at 1044 cm^{-1} to carbonyl and ether, bands at 776 cm^{-1} to nitrile, acyl, methoxy, and aromatic C-H groups and band at 698 cm^{-1} to alkyl halides. The FTIR chromatogram is shown in Fig 6 and the bands corresponding to each functional group are listed in Table 5

GC-MS analysis of *P. citrinum* metabolites extracted with ethyl acetate showed two major peaks viz. 35.560 min and 36.360 min having peak area of 13.012% and 9.892% respectively. The GC-MS chromatogram is shown in Fig 7. The probable volatile compounds for the first RT peak 35.560min were Succinic acid, 2,4,6-trichlorophenyl 3-methylbut-3-en-1-yl ester and 1,5-bis(3-Cyclopentylpropoxy)-1,1,3,3,5,5-hexamethyltrisiloxane. For the second peak, 36.360 min the two major volatile compounds were found to be Tris(tert butyldimethylsilyloxy)arsane and

Hexamethylcyclotrisiloxane. The chemical formula, structure, etc. of these probable major compounds of ethyl acetate extracted crude metabolites have been presented in Table 6.

4. Discussions

The isolated endophytic and rhizospheric fungi from Tezpur litchi (var. Bilati) belongs to class Ascomycota. Out of the recorded five major fungal genera (*Aspergillus*, *Colletotrichum, Fusarium, Penicillium* and mycelia sterilia); *Aspergillus* is pathogenic to plants (*Zea, Arachis* and *Gossypium*) (Klich 2007). However, they can be symptomless endophytes as well (Mathur et al. 2023). Similarly, *Colletotrichum, Fusarium* and *Penicillium* were reported as phytopathogens (Ma et al. 2013; Louw and Korsten 2014; Liu et al. 2016) and endophytes (Ai et al. 2023; Kumar and Prasher 2023; Roy et al. 2023). These fungal genera have been widely reported in various crops. For example, *Aspergillus niger* was reported to promote growth in various *Vigna* varieties (Mathur et al. 2023). Similarly, *Colletotrichum gloesporioides* and *Aspergillus fumigatus* isolated from the rhizosphere of *Dillenia indica* L were found to have IAA-producing and phosphate solubilizing abilities (Kumar and Prasher 2023). In addition, endophytic *Penicillium oxalicum* CX-1 was found to promote plant growth and suppress pythopathogenic fungi *Phytopthora cactorum* (Ai et al. 2023). Furthermore, seed-borne endophytic *Fusarium* sp. from indigenous rice varieties of N.E India showed promising plant growth-promoting traits (Roy et al. 2023). The fungal genera from different tissue types and rhizospheric soil of litchi varied significantly (as observed from PCA analysis) with regard to colonization behavior. *Penicillium* sp. was found to be the dominating genera in the leaves of litchi plants. In barks, Mycelia steria colonized the most. In roots, *Fusarium* occurred more frequently. In addition, *Aspergillus* was the dominant genera in rhizospheric soil. Thus, the documented diversity of endophytic and rhizospheric fungi are organ and soil-specific. The observed organ specificity of plant-associated fungi is supported by the findings of Dix, (2012) and Wearn et al. (2012).

We have screened the fungal isolates for their plant growth promotion abilities and observed positive results by rhizospheric isolate SF32 for IAA production is identified as *Aspergillus aculeatus* from molecular characterization.The potential of *Aspergillus* sp. for plant growth promotion was documented earlier (Kumar and Prasher 2023; Mathur et al. 2023). Thus, presence of such potent isolate in the litchi rhizosphere signifies the direct benefits to the litchi plants from their soil microbiota. In addition, the promising ability in phosphate solubilization of the isolates is self-explanatory for their help in nutrient acquisition by these fungi to the litchi plants. Similar findings were documented by earlier researchers which revealed multiple growth-promoting potential of plant-associated fungi (Baron and Rigobelo 2022; Roy et al. 2023).

Our results from antagonistic activity of the screened fungal isolates against a prominent litchi phytopathogen *Alternaria alternata* showed significant positive result. This verifies the tolerance ability of the fungal flora associated with Tezpur litchi plants towards potential phytopathogens. Results from antifungal assay using crude fungal metabolites against the same pathogen verifies the same. Highest zone of inhibition documented by the metabolite of isolate BE14 (a bark endophyte) was identified as

Penicillium citrinum on molecular identification. Antimicrobial properties of *Penicillium citrinum* have been documented earlier that was isolated from *Azadirachta indica* (Kumari et al. 2021), *Stephania kwangsiensis* (Luo et al. 2019) and *Ocimum tenuiflorum* (Lai et al. 2013). However, association of *Penicillium citrinum* in litchi plants is documented for the first time in this present study. Presence of functional groups such as alcohol, alkanes, unsaturated aliphatic, nitro, carboxyl, ether, nitrile, acyl, methoxy, aromatic C-H, and alkyl halides in metabolites of *P. citrinum* (from FTIR and GC-MS study) predicted the association of Succinic-acid-2,4,6-trichlorophenyl-3-methylbut-3-en-1-yl-ester, 1,5-but(3-cyclopentylpropoy)-1,1,3,3,5,5-hexamethyltrisiloxane, Hexamethyl-cyclotrisiloxane and Tris(tert-butyldimethylsilyloy)arsane in the metabolites. Prominent antimicrobial properties of these compounds isolated from endophytic fungi have been observed earlier researchers against pathogens like *Staphylococcus aureas, Pseudomonas flourescens, Escherichia coli, Streptococcus pneumoniae, Shigella* sp., *Streptomyces avermitilis* and *Candida albicans* (Rayar et al. 2015; Huang et al. 2022; Muthukrishnan et al. 2022; Zhang et al. 2023). Thus, the documented bioactive compounds in the crude metabolite of *P. citrinum* proves its observed potential for antifungal acitivity against *A. alternata.*

5. Conclusion

This study investigated the diversity and growth characteristics of endophytic and rhizospheric fungi associated with Tezpur litchi (var. Bilati). Our findings revealed that these fungi belong primarily to the Ascomycota class and exhibit significant variation depending on the plant tissue and surrounding soil. While some genera, like *Aspergillus*, can act as both pathogens and beneficial endophytes. Whereas, others like *Penicillium* and *Fusarium* reported as plant pathogens elsewhere, were also found as endophytes in litchi. Interestingly, our isolates displayed promising plant growth-promoting abilities, including IAA production and phosphate solubilization, suggesting their potential benefits to litchi cultivation. Furthermore, the identified fungal isolates exhibited antagonistic activity against the litchi pathogen, *Alternaria alternata*. This highlights the potential of these fungi for biocontrol applications. Notably, the metabolite of a bark endophyte identified as *Penicillium citrinum* displayed the strongest antifungal activity. This is the first report of *P. citrinum* association with litchi, and further studies are warranted to elucidate the specific bioactive compounds responsible for its antifungal properties.

Overall, this study contributes valuable insights into the diverse fungal communities associated with Tezpur litchi and identifies potential candidates for promoting plant growth and managing litchi diseases through eco-friendly approaches.

Declarations

Fundings

No funding was received from any organization to carry out this work.

Declaration of Conflict of Interest

The authors declared no conflict of interest for this manuscript.

Author Contribution

AR: Conceptualization, writing of the original draft, design of the figures.AH: Writing reviewing the draft, Statistical AnalysisAK: Reviewing and editing, SupervisionNG: Supervision, writing review and editing, Critical revision of the article

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Tables

Table 1: Plant growth-promoting traits of endophytic and rhizospheric fungi isolated from *Litchi chinensis* Sonn.(var.Bilati) of Tezpur, Assam.

Fungal Isolates		Plant Growth Promoting Traits		
		IAA (μgml^{-1})		Phosphate solubilization Index
		TRP (+)	TRP (-)	
Endophytic Fungi	LE03	17.08±0.13 *q*	14.88±0.50 *e,f*	2.368±0.12 *d*
	LE07	17.34±0.30 *q*	21.98±0.32 *c*	**3.442±0.04 *a***
	LE12	17.6±0.52 *q*	12.024±0.86 *h*	2.41±0.12 *d*
	LE20	**118.058±1.98** *d*	31.058±0.56 *a*	2.308±0.15 *d*
	LE24	20.806±1.61 *p*	19.734±0.83 *d*	0 *g*
	BE08	30.01±1.07 *n*	20.216±0.69 *c,d*	0 *g*
	BE09	21.018±1.60 *p*	9.164±0.81 *j*	0 *g*
	BE14	40.23±1.33 *k*	24.78±0.86 *b*	2.422±0.04 *d*
	BE23	**143.136±3.94** *b*	14.384±0.51 *e,f*	0 *g*
	BE27	33.96±1.21 *l,m*	19.1824±0.27 *e*	0 *g*
	BE30	24.904±0.87 *o*	13.692±0.76 *e,f*	0 *g*
	BE32	47.326±2.07 *j*	24.002±1.45 *e*	0 *g*
	BE34	16.586±0.78 *q*	21.292±1.02 *e*	**2.422±0.08 *d***
	RE04	26.13±1.00 *o*	12.83±0.41 *e,f.g*	2.072±0.14 *e*
	RE06	32.384±1.45 *m*	18.756±0.47 *d*	0 *g*
	RE11	35.772±1.15 *l*	14.688±0.46 *e,f*	0 *g*
	RE16	23.126±1.73 *o,p*	12.71±0.26 *e,f,g*	1.882±0.13 *f*
	RE25	16.932±1.53 *q*	10.816±0.69 *i,h*	0 *g*
	RE29	38.71±0.68 *k*	11.368±0.68 *h*	0 *g*
Rhizospheric Fungi	SF04	94.88±1.73 *f*	16.446±89 *e*	0 *g*
	SF07	28.21±0.68 *n*	10.982±95 *i,h*	2.068±0.19 *f*
	SF11	50.36±1.24 *h*	11.508±86 *h*	**3.16±0.13 *b***
	SF18	95.61±1.76 *e*	14.72±46 *e,f*	1.918±0.12 *f*
	SF24	25.27±1.76 *h*	11.8±64 *h*	0 *g*
	SF26	69.52±1.05 *g*	14.494±45 *e,f*	**2.572±0.12 *c***
	SF27	**126.58±3.25** *c*	30.178±86 *a*	0 *g*

	SF32	**179.25±3.75** *a*	12.538±76 *f,g*	0 *g*
	SF35	47.62±1.25 *i*	13.022±77 *e,f,g*	0 *g*

Different lowercase letters above each column indicate significant differences between isolates at 5% level of significance (P≤ 0.05) according to Duncan's Multiple Range Test (DMRT), "±"= Standard Deviation

Table 2: Antagonist activity of the endophytic and rhizospheric fungi against *Alternaria alternata*

against *A.alternata* (MTCC 3880)

Fungal Isolates		Inhibition of *A.alternata* (%)
Endophytic fungi	LE03	71.2±0.97 *e,f*
	LE07	69.7±2.41 *g*
	LE12	76.34±0.80 *c*
	LE20	63.9±1.29 *i*
	LE24	**79±0.50 *b***
	BE08	61.4±0.54 *j*
	BE09	74.1±1.08 *d,e*
	BE14	**78.5±0.61 *b***
	BE23	55.5±1.22 *k*
	BE27	76.3±1.52 *c*
	BE30	70.5±0.61 *f,g*
	BE32	66.5±1.17 *h*
	BE34	75.6±0.65 *c,d*
	RE04	72.4±0.96 *e,f*
	RE06	64.7±1.20 *i*
	RE11	61±10 *j*
	RE16	74.9±1.08 *c,d*
	RE25	56.4±1.08 *k*
	RE29	73±1.06 *d,e*
Rhizospheric fungi	SF04	**80.8±0.50 *a***
	SF07	56.5±0.83 *k*
	SF11	74.1±1.019 *d,e*
	SF18	74.6±0.66 *d*
	SF24	38.9±1.46 *l*
	SF26	72.1±0.96 *e,f*
	SF27	56.1±1.067 *k*
	SF32	**80.5±0.54 *a***
	SF35	73.5±1.37 *d,e*

Different lowercase letters above each column indicate significant differences between isolates at 5% level of significance (P≤ 0.05) according to Duncan's Multiple Range Test (DMRT), "±"= Standard Deviation.

Table 3: Antifungal activity of potential fungal isolates

Potent fungal isolates	Zone of inhibition (mm)
BE14	**41.9±1.46**
LE24	29.7±0.97
SF32	29.4±0.80
SF04	25.5±0.70

"±"= Standard Deviation

Table 4: Molecular identification of potent fungal isolates.

Fungal isolates	Morphological identification	Accession number	Molecular Identification	Accession number	Similarity percentage
BE14	*Penicillium* sp.	PP203028	*Penicillium citrinum*	JX192960	100%
SF32	*Aspergillus* sp.	PP196577	*Aspergillus aculeatus*	OP237368	100%

Table 5: Functional groups found in the crude metabolite of *P. citrinum* corresponding to FTIR peaks.

Wavenumbers (cm^{-1})	Functional group (bands)	Peaks found in the crude metabolite of *P. citrinum*
3200–3700	O-H stretching (Aliphatic)	3438
2700–3000	C-H stretching	2914
1780–1710	C=O stretching	-
1750–1630	Ketone, Ester, Amide C=O Stretching	-
1650-1400	Unsaturated aliphatics, -NH, Nitro compounds	1417, 1638
1400-900	C=C	1310
1340-1250	C-N	-
1000–1200	C=O/C-O-C	1044
750–870	C-N/R-O-C/R-O-CH3 stretching, aromatic C-H	776
500-730	Halogen compounds	698

Table 6 is available in the Supplementary Files section.

Figures

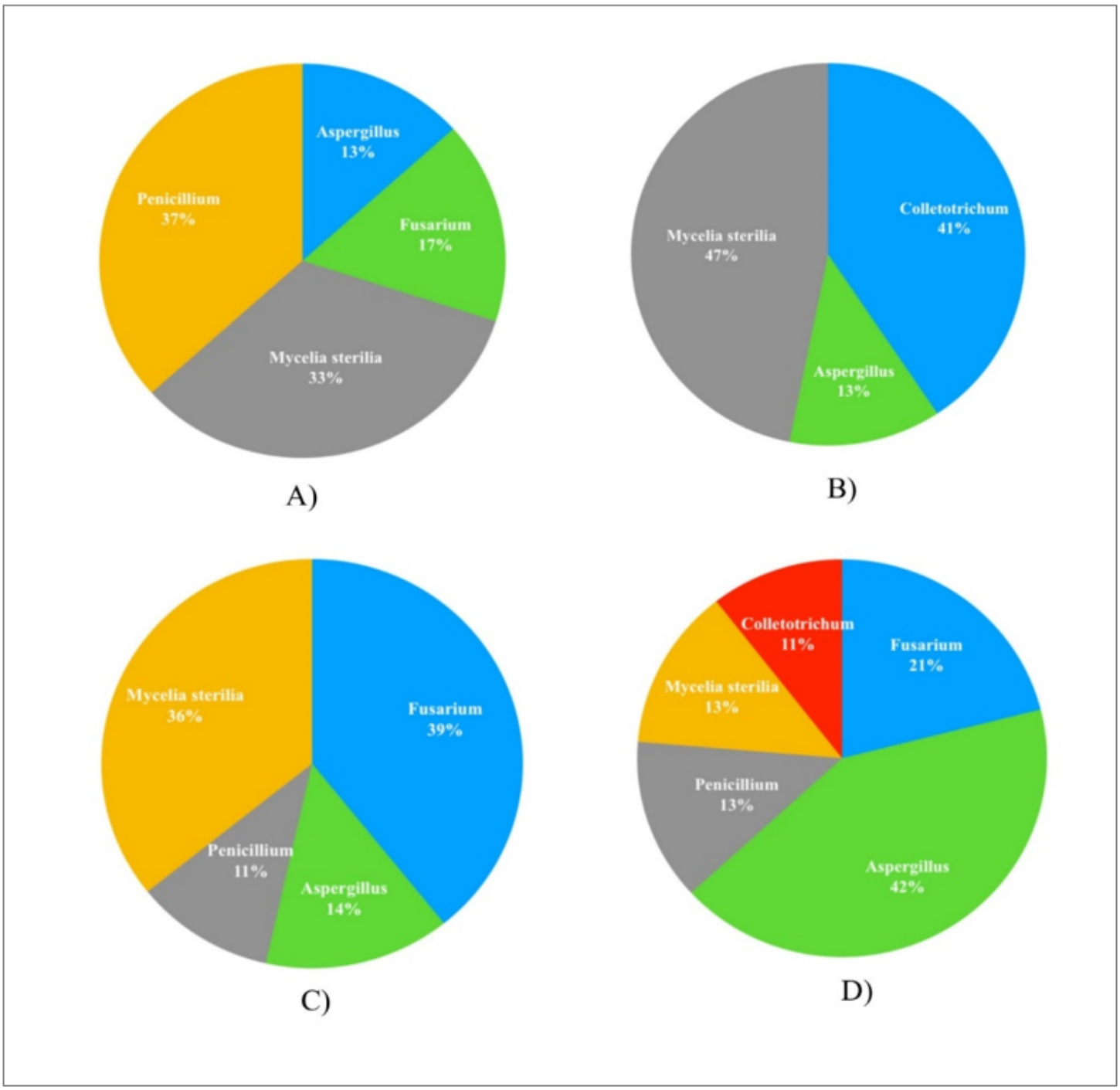


Figure 1

Colonization/Isolation frequencies of endophytic/rhizospheric fungi A) Leaves B) Barks C) Roots D) Rhizospheric soil.

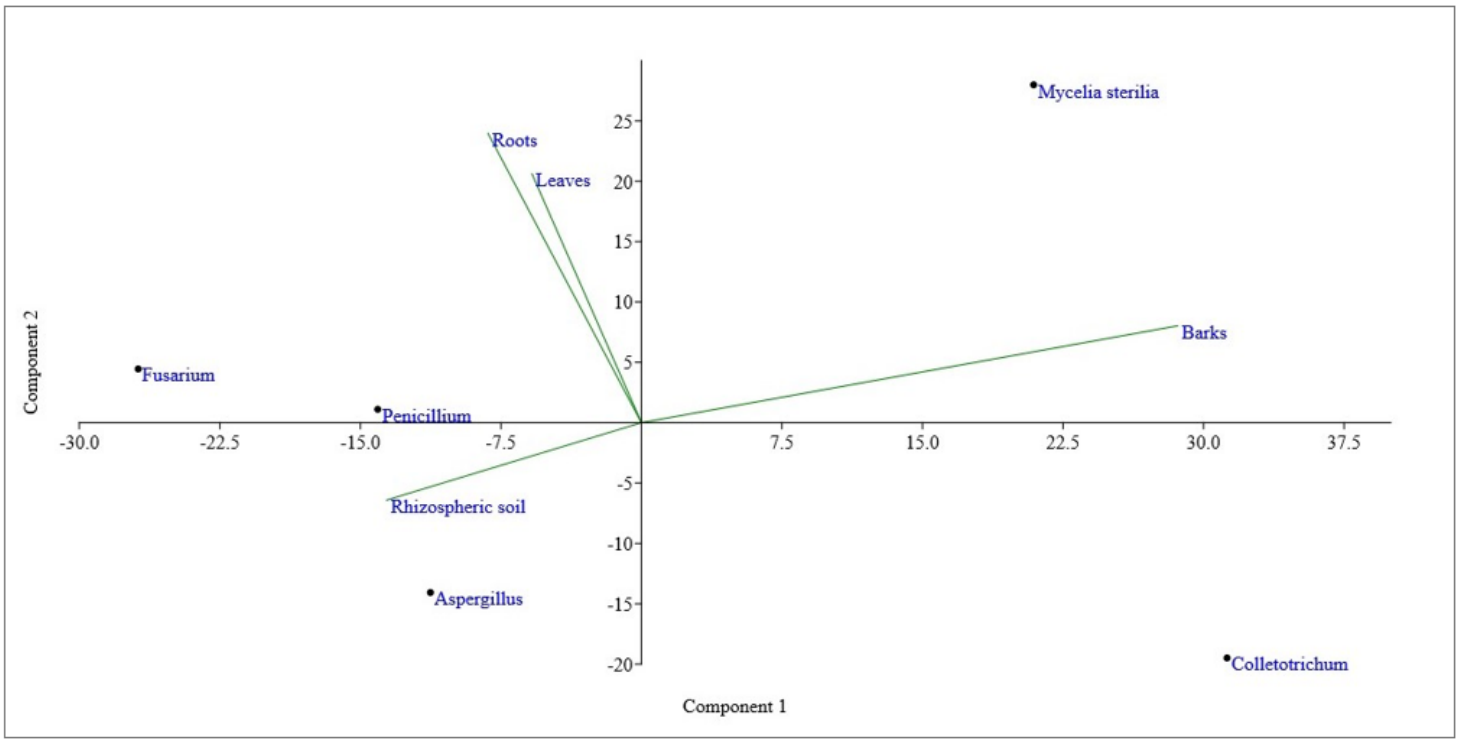


Figure 2

PCA biplots of different plant tissues and rhizospheric soil from litchi showing the colonization of different fungal genera.

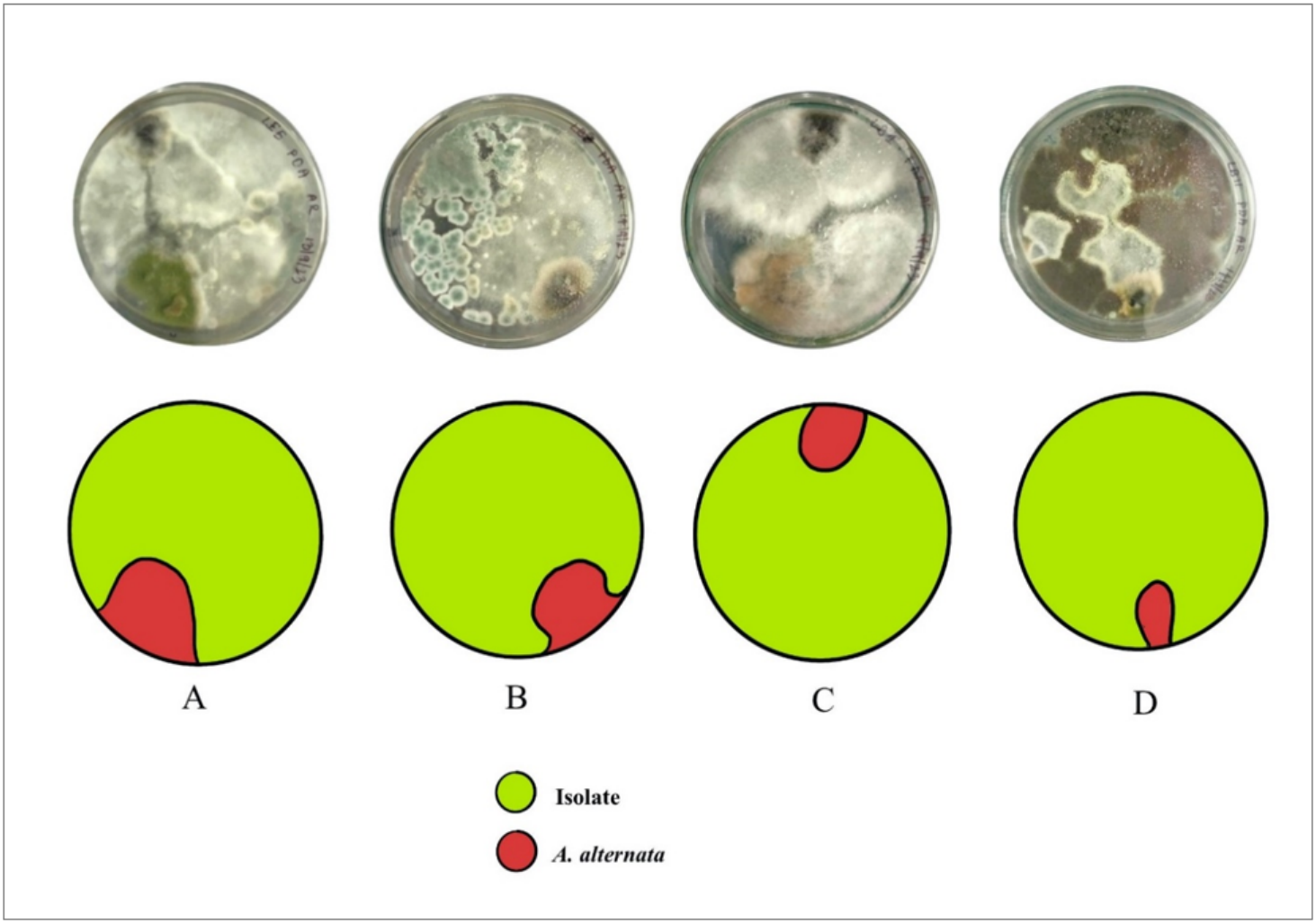


Figure 3

Graphical representation of Antagonistic activity documented by fungal isolates against *A. alternata* A= LE24; B= BE14; C= SF04; D= SF32

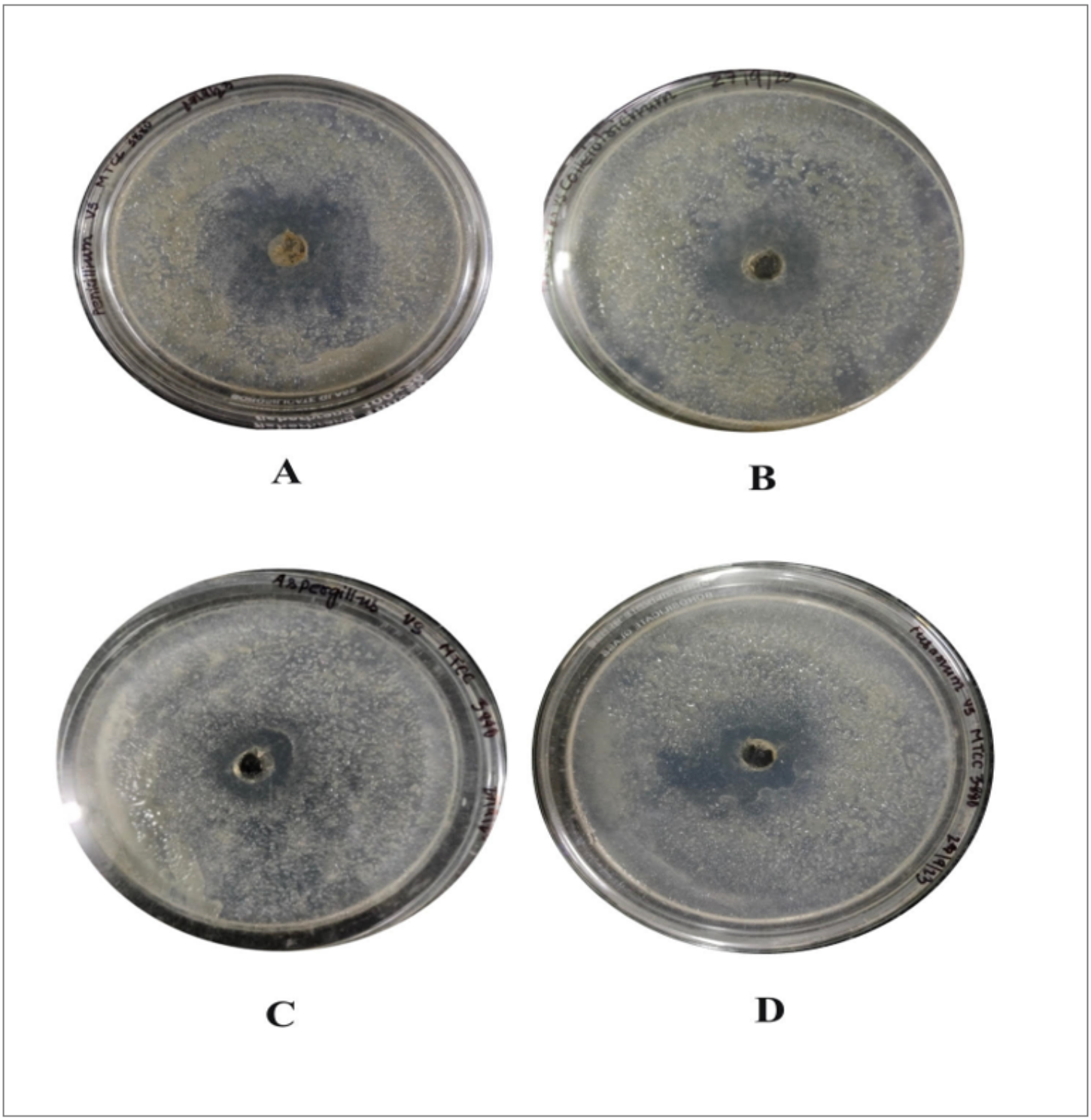


Figure 4

Agar cup diffusion plates showing zone of inhibition of *A. alternata* by the crude metabolites of potential fungal isolates A= BE14; B= LE24; C= SF32; D= SF04

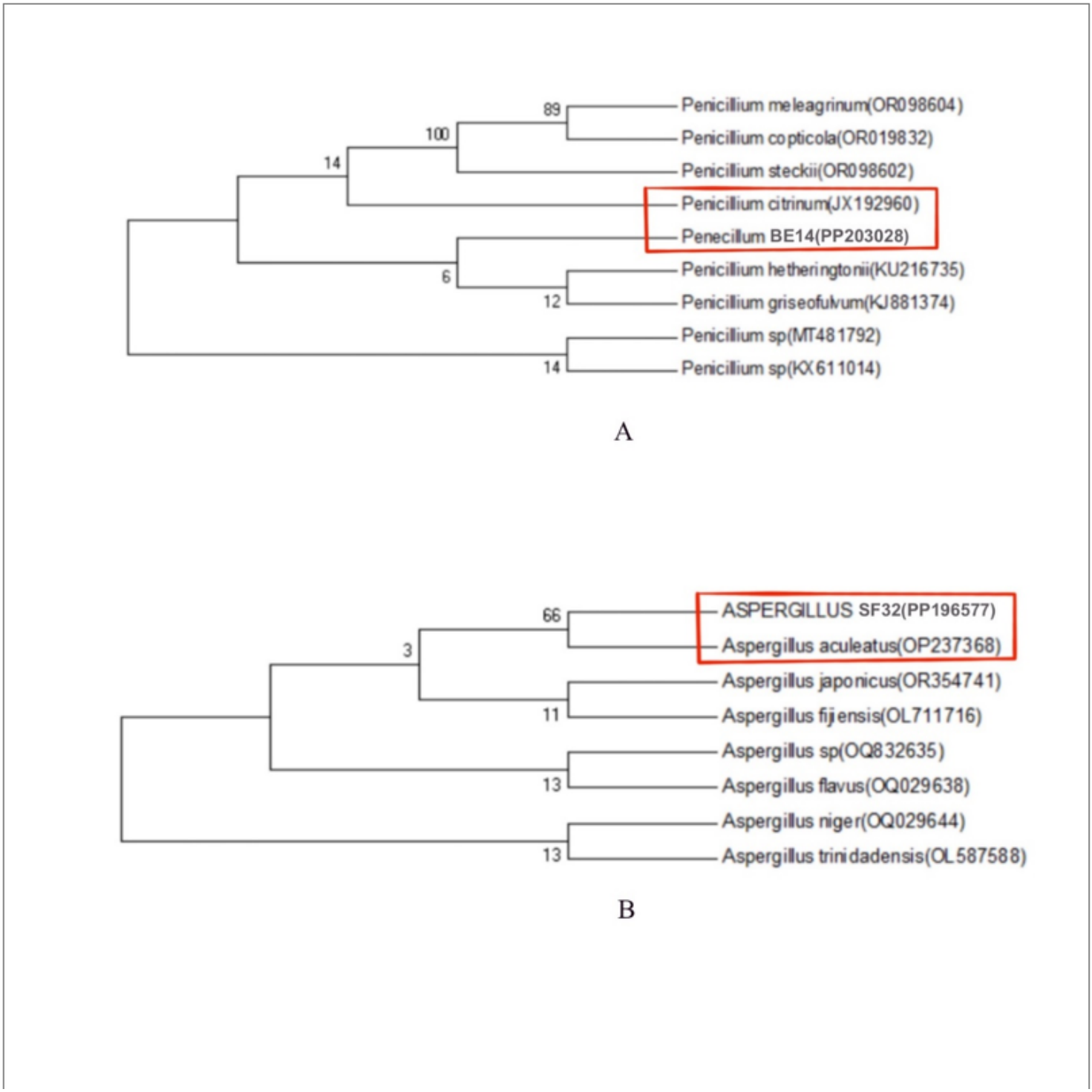


Figure 5

Phylogenetic trees based on ITS rDNA sequences and produced using the Maximum Parsimony (MP) approach demonstrate how the potent isolates are grouped within their own clades A) *Penicillium* sp. (BE14) B) *Aspergillus* sp. (SF32).

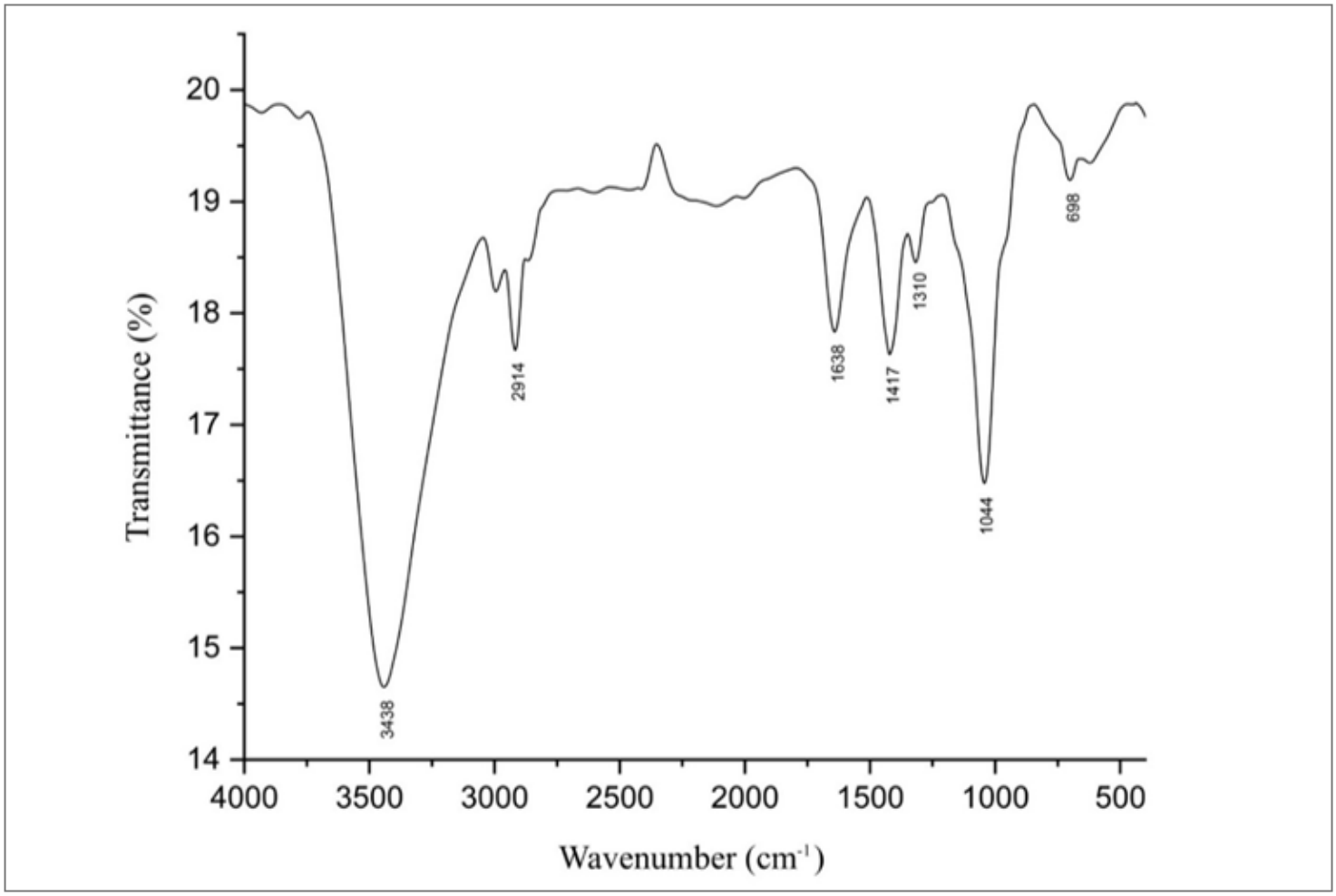


Figure 6

FTIR chromatogram of ethyl acetate extract of crude metabolite produced by *P. citrinum.*

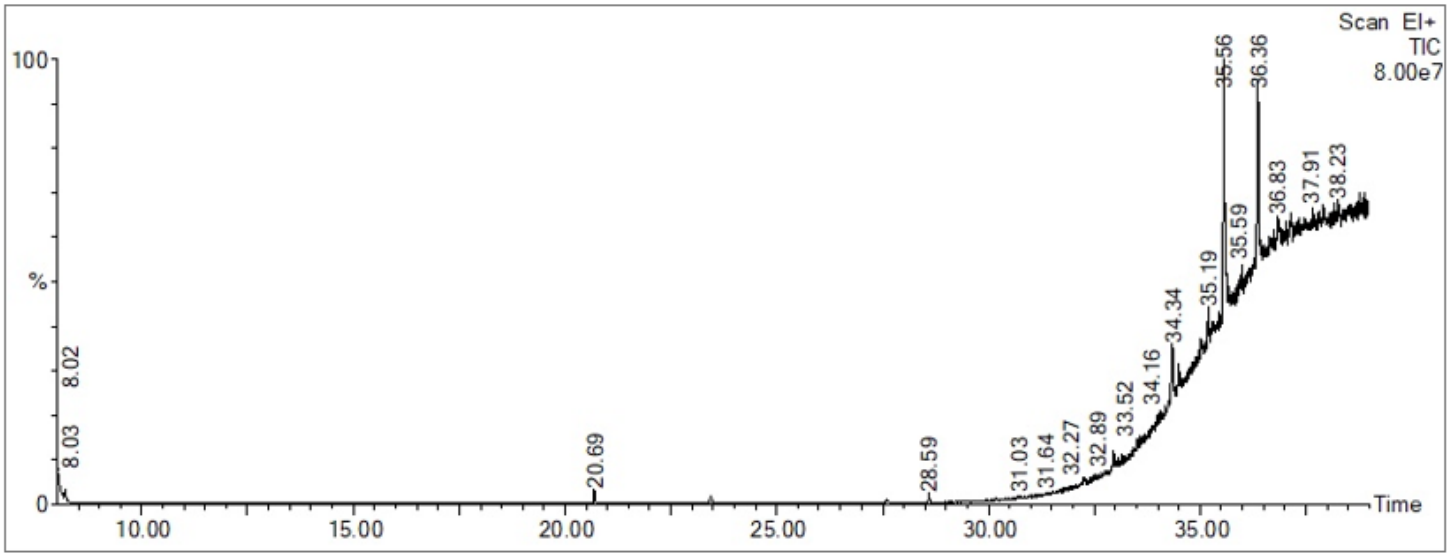


Figure 7

GC-MS chromatogram of the ethyl acetate extracted crude metabolite of *P. citrinum.*

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Table6.docx
- CompoundStructuresforTable6.docx