Plant Archives Vol. 24, Special Issue (GABELS), 2024 pp.104-108 e-ISSN:2581-6063 (online), ISSN:0972-5210

Plant Archives

Journal homepage: http://www.plantarchives.org
DOI Url : https://doi.org/10.51470/PLANTARCHIVES.2024.v24.SP-GABELS.016

GC-MS PROFILING OF METABOLITES OF ENDOPHYTIC FUNGI *CURVULARIA PSEUDOROBUSTA* FROM *DISTIMAKE DISSECTUS*

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ABSTRACT

In recent days endophytic fungi are recognized as an excellent source of widely occurring natural producers of natural products, mostly secondary metabolites. The use of these fungi may benefit to current demand for novel biomolecules in medical, agriculture and pharmaceutical industries. This research emphasis on the numerous fungal endophytes that have been isolated from *Distimake dissectus* which has known to be a rich herbal source. The isolates were identified based on the morphological identification and molecular characterisation. *Curvularia pseudorobusta* was found to be the most predominant.The selected fungal isolate *Curvularia pseudorobusta* was screened for the production of secondary metabolites, followed by extraction using ethyl acetate. Ethyl acetate fraction of culture filtrate was subjected to GC-MS analysis, resulted in the identification of several compounds and some are known to have medicinal properties, such as anti-fungal and anti-bacterial effects. This analysis revealed the presence of various metabolites/bio-compounds viz., 3-Octen-2-one, 1-Undecene 9-methyl, 1-Dodecanol, Oxalic acid, allyl hexadecyl ester, 2-Undecanol, Formic acid, oct-2-yl ester etc. These findings suggest the potential bio active compounds could be used in the development of antimicrobial agents that can be used in pharmaceuticals and agrochemicals. Fungal based products offer a unique opportunity to discover novel therapeutic agents to combat various infectious agents and agricultural pathogens. This study is an important step to investigate the biodiversity of fungal endophyte and to explore novel bioactive compounds from natural resource.

Keywords: *Distimake dissectus*, *Curvularia pseudorobusta.*, Endophytic fungi, Bioactive compounds, Gas Chromatography-Mass Spectrometry (GC-MS Profiling).

Introduction

Endophytic fungi are an endosymbiotic group of microorganisms that colonize in plants and microbes that can be readily isolated from any microbial or plant growth medium. They act as reservoirs of novel bioactive secondary metabolites, such as alkaloids, phenolic acids, quinones, steroids, saponins, tannins, and terpenoids that serve as a potential candidate for antimicrobial, anti-insect, anticancer and many more properties. (Goud *et al*., 2016) These fungi are instrumental in bolstering plant growth and fortifying resistance to various environmental stressors encountered by plants. Beyond that, they also contribute to the nutritional status of plants by improving nutrient uptake, including essential elements like phosphorus, rock phosphate, and atmospheric nitrogen that may otherwise be inaccessible to the plants (Maaloum *et al*., 2020). A key mechanism through which endophytic fungi benefit plants is by producing bioactive molecules that not only stimulate growth but also trigger adaptive responses and enhance immunity (Yang *et al*., 2019). Fungal based bioactive metabolites isolated from endophytes offer a unique chance for discovering a new therapeutic drug against various maladies. (Strobel and Daisy, 2003). Endophytes are emerging as a new source of novel natural products with potential application in various fields (Urooj *et al.*, 2021).

Materials and Methods

Collection of plant material

The selected plant *Distimake dissectus* was collected from its natural habitat. The symptomless and apparently healthy plants were selected. A total of five leaves, stem and flower samples were collected separately from five plants. The samples were placed in pre-sterilized zip-lock polythene bags, stored at 4ºC and transported to the laboratory. Fresh plant materials were used for the isolation of fungal endophytes to reduce the chance of contamination. Thus, collected plant materials were subjected to surface sterilization within few hours after sampling and processed within 24 hrs of collection.

Surface sterilization and isolation of fungal endophytes

The leaf, stem and flower samples were rinsed gently in running tap water to remove dust and debris. Samples were surface sterilized by sequential rinsing in 70% (v/v) ethanol for one minute, 3.5% (v/v) Sodium hypochlorite for two minutes. The samples were rinsed three times with sterile distilled water and dried on sterile blotters under laminar airflow to ensure complete drying (Schulz *et al.*, 1993). Bits of 1.0X0.1 cm size were excised with the aid of sterilized blade. Segments of leaf, stem and flower were evenly placed on water agar (WA) medium (15 g/L). The Petri dishes were sealed using Parafilm ™ and incubated at 27±2º C in a light chamber with 12 hours of light followed by 12 hours of dark cycles for 4-6 weeks. The Petri dishes were monitored periodically to check the growth of endophytic fungal colonies from the segments. The hyphal tips which grew out from the segments were transferred separately onto fresh Potato Dextrose Agar (PDA) slants with a sterile fine tipped needle under stereo binocular microscope and incubated at 27±2ºC for 10-15 days and pure cultures were maintained at 4ºC for further use.

Identification of endophytic fungi

Morphological identification was done by inoculating the endophytic fungi on PDA plates followed by seven days of incubation and observation of colony and spore morphology. The slides of each fungal endophytes were prepared by tease mount method using lactophenol cotton blue stain and observed under the light microscope with 400X magnification. The identification was based on the observation of mycelia, fruiting bodies, conidial characters according to the standard identification manuals (Domsch *et al*., 1980; Singh *et al*., 1991; Barnett and Hunter, 1998; Mathur and Kongsdal, 2003).

Molecular characterisation of fungal endophytes using ITS - PCR and electrophoresis:

Mycelial plugs from different endophytic fungi were inoculated into Potato dextrose broth and were grown in still culture at 27 ± 2 °C for 7–10 days. Genomic DNA was extracted from the freeze-dried mycelial mat using the cetyl-trim ethylammonium bromide (CTAB) method with trivial modifications (Gashgari *et al*., 2016). The concentration of DNA was measured using nanodrop spectrophotometer at 260 and 280 nm. The DNA was amplified with the PCR technique using a PCR kit. The target regions of the rDNA, ITS1, ITS2 regions and 5.8S gene was amplified using ITS1 (5'-TCCGTAGGTGAACCTG CG-3') and ITS4 (5'- TCCTCCGCTTATTGATA TGC-3') primers (White *et al*., 1990). The PCR was performed in a thermal cycler by using the following programme: 94°C for 2 min (initial denaturation), 35 cycles of 94 °C for 1 min (denaturation), 47 °C for 15 s (primer annealing), 72°C for 30 s (primer extension), followed by 10 min of final extension at 72°C. Subsequently, the amplified products were analysed with horizontal agarose gel electrophoresis through 1% agarose gel supplemented with ethidium bromide along with the 100bp DNA marker.

DNA bands on the gel is visualised under a UV light trans-illuminator and documented. The amplified PCR products were sent to Dextrose laboratory ltd, Bangalore for purification and sequencing. Sequencing similarity searches were achieved for the obtaining fungal sequences and compared with ITS sequence data from strains available from the GenBank database (National Centre for Biotechnology Information website; http://www.ncbi.nlm.nih.gov/) by using the BLAST sequence match routines.

Extraction of metabolites from fungal endophyte

Mycelia from actively growing 7-day-old endophytic pure cultures were inoculated aseptically into 500 ml of PDB contained in Erlenmeyer flasks in duplicates. The inoculated flasks were incubated at 28 ± 2°C for 21 days. The flasks were examined for any contamination. Culture broths were filtered through muslin cloth. The supernatant was transferred to a separating funnel, to which an equal volume of ethyl acetate (1:1 v/v) was added, and extracted thrice by strong agitation. The extract obtained in this way was concentrated in a Rotary flash evaporator and stored in vials for further use.

GC-MS Analysis

The ethyl acetate fungal extract was subjected to GCMS analysis to analyse various metabolites present in it. The Clarus 680 GC was used in the analysis employed a fused silica column, packed with HP-5MS (5% biphenyl 95% dimethylpolysiloxane, 30 m × 0.25 mm ID × 250μm df) and the components were separated using Helium as carrier gas at a constant flow of 2 ml/min. The injector temperature was set at 280°C during the chromatographic run. The 1μL of extract sample injected into the instrument the oven temperature was as follows: 100 °C (2 min); followed by 200 °C at the rate of 10 °C min−1; and 200 °C, where it was held for 3min and then followed by 300°C at the rate of 25°C min−1; it was held for 10.00 min.The mass detector conditions were: Inlet line temperature 250 °C; ion source temperature 230°C; and ionization mode electron impact at 70 eV, a scan time 0.2 sec and scan interval of 0.1 sec. The fragments from 40 to 600 Da. The spectrums of the components were compared with the database of spectrum of known components stored in the GC-MS NIST (2014) library.

Result and Discussion

Isolation and identification of fungal endophytes

The isolates were isolated and cultured**.** The isolates were identified using morphological and microscopic characteristics with the support of molecular analysis. Molecular characterization of isolated endophytic fungi with Gen Bank accession numbers is depicted in table 1.

Table 1 : Molecular characterization of isolated endophytic fungi from *Distimake dissectus* with Gen Bank accession numbers

Sl. No.	Organism	Sequence ID
1	*Aspergillus tubingensis*	XR_004775241.1
2	*Fusarium verticillioides*	XM_018898866.1
3	*Colletotrichum spaethianum*	XR_007414293.1
4	*Trichoderma rifaii*	OM515093.1
5	*Alternaria solani*	OM522508.1
6	*Nigrosporamusae*	KY019455.1
7	*Penicillium digitatum*	NW_014574583.1
8	*Mucor janssenii*	MH870818.1
9	*Rhizopus koreanus*	NR_164543.1
10	*Curvularia pseudorobusta*	MH857148.1

The discovery of various endophytic fungi species within *Distimake dissectus*, including *Aspergillus tubingensis*, *Fusarium verticillioides*, *Colletotrichum spaethianum*, *Cladosporium herbaroides*, *Trichoderma rifaii*, *Alternaria solani*, *Nigrosporamusae*, *Penicillium digitatum*, *Mucor janssen*, *Rhizopus koreanus* and *Curvularia pseudorobusta* underscores the potential richness and diversity of endophytic fungi in this ecological niche. This diversity opens up new avenues for exploring the unique characteristics and potential applications of these endophytic fungi and their metabolites. As the study of endophytic fungi and their secondary metabolites holds great promise for various scientific and industrial applications, In this present research work, *Curvularia pseudorobusta* was selected for further evaluation as this was most predominant.

The ethyl acetate extract of *Curvularia pseudorobusta* was characterized and identified by GC-MS analysis. The interpretation on mass spectrum GCMS was conducted using the database of National institute standard and technology (NIST). The spectrum of the unknown component was compared with the spectrum of the known components stored in the NIST library. The active principles with their molecular formula, molecular weight, exact mass and NIST no are represented in Table 2 and Fig. 1.

Table 2 : Biochemical compounds identified in ethyl acetate extract of *Curvularia pseudorobusta*

Sl No.	Chemical name	Molecular formula	Molecular weight	Id#	Exact mass	NIST#:
1	d-Proline,N-methoxycarbonyl-, pentyl ester	$C_{12}H_{21}NO_4$	243	112166	243.1470585	320788
2	d-Proline, N-methoxycarbonyl-, isohexyl ester	$C_{13}H_{23}NO_4$	257	112521	257.162708	320789
3	d-Proline, N-methoxycarbonyl-, hexyl ester	$C_{13}H_{23}NO_4$	257	112522	257.162708	320790
4	d-Proline, N-methoxycarbonyl-, dodecyl ester	$C_{19}H_{35}NO_4$	341	112526	341.256609	320795
5	5-Methyl-1-heptanol	$C_8H_{18}O$	130	20744	130.135765	113701
6	(S)-(+)-5-Methyl-1-heptanol	$C_8H_{18}O$	130	20767	130.135765	237035
7	1-Hexene, 3,5-dimethyl	C_8H_{16}	112	2742	112.1252007	113470
8	1-Decene, 8-methyl	$C_{11}H_{22}$	154	37298	154.172151	6117
9	Isoxazole, trimethyl	C_6H_9NO	111	88350	111.0684137	1575
10	3-Octen-2-one	$C_8H_{14}O$	126	4888	126.104465	46426
11	3-[N-Aziridyl] butyraldehyde hydrazone	$C_6H_{13}N_3$	127	88358	127.110947	257031
12	Thiophene-2-carboxylic acid, 2,4,6-trichlorophenyl ester	$C_{11}H_5C_{13}O_2S$	306	88437	305.907583	325731
13	2-Propenoic acid, 2-methyl-, 2-propenyl ester	$C_7H_{10}O_2$	126	8398	126.0680795	229264
14	Acetic acid, trifluoro-, 3,7-dimethyloctyl ester	$C_{12}H_{21}F_3O_2$	254	7301	254.149364	58167
15	1-Undecene, 9-methyl	$C_{12}H_{24}$	168	37297	168.1878	61825
16	1-Hexene, 3,5-dimethyl	C_8H_{16}	112	2742	112.1252007	113470

17	2,6-Dimethyl-6-trifluoroacetoxyoctane	$C_{12}H_{21}F_3O_2$	254	34734	254.149364	215969
18	1-Dodecanol	$C_{12}H_{26}O$	186	2092	186.198365	63858
19	Thiocyanic acid, 4-oxotricyclo[3.3.1.1(3,7)]dec-2-yl ester, (1α,2α,3β,5α,7β)	$C_{11}H_{13}NOS$	207	103536	207.071785	37585
20	p- Acetoacetanisidide	$C_{11}H_{13}NO_3$	207	85311	207.089543	340958
21	6-Butanamide, N-(2-methoxyphenyl)-3-oxo	$C_{11}H_{13}NO_3$	207	17145	207.089543	75376
22	Oxalic acid, allyl hexadecyl ester	$C_{21}H_{38}O_4$	354	2467	354.27701	309244
23	Oxalic acid, allyl dodecyl ester	$C_{17}H_{30}O_4$	298	2464	298.214409	309240
24	2-Undecanol	$C_{11}H_{24}O$	172	3963	172.182715	114102
25	2-Butenedioic acid (E)-, bis(2-ethylhexyl) ester	$C_{20}H_{36}O_4$	340	37727	340.26136	339147
26	Fumaric acid, 2-ethylbutyl 2-ethylhexyl ester	$C_{18}H_{32}O_4$	312	53946	312.230059	405634
27	2-Butenedioic acid (E)-, bis(2-ethylhexyl) ester	$C_{20}H_{36}O_4$	340	17715	340.26136	232972
28	Fumaric acid, 2-ethylhexyl 8-chlorooctyl ester	$C_{20}H_{35}ClO_4$	374	37722	374.222387	405578
29	Bis(2-ethylhexyl) maleate	$C_{20}H_{36}O_4$	340	6250	340.26136	232973
30	Fumaric acid, 2-methylpentyl 2-ethylhexyl ester	$C_{18}H_{32}O_4$	312	89509	312.230059	405648
31	Fumaric acid, 2-ethylhexyl isobutyl ester	$C_{16}H_{28}O_4$	284	37456	284.19876	339137
32	Carbonic acid, bis(2-ethylhexyl) ester	$C_{17}H_{34}O_3$	286	25201	286.250795	383146
33	Formic acid, oct-2-yl ester	$C_9H_{18}O_2$	158	37282	158.13068	368945
34	Fumaric acid, 2-ethylhexyl 1,1,1-trifluoroprop-2-yl ester	$C_{15}H_{23}F_3O_4$	324	37434	324.154844	405569

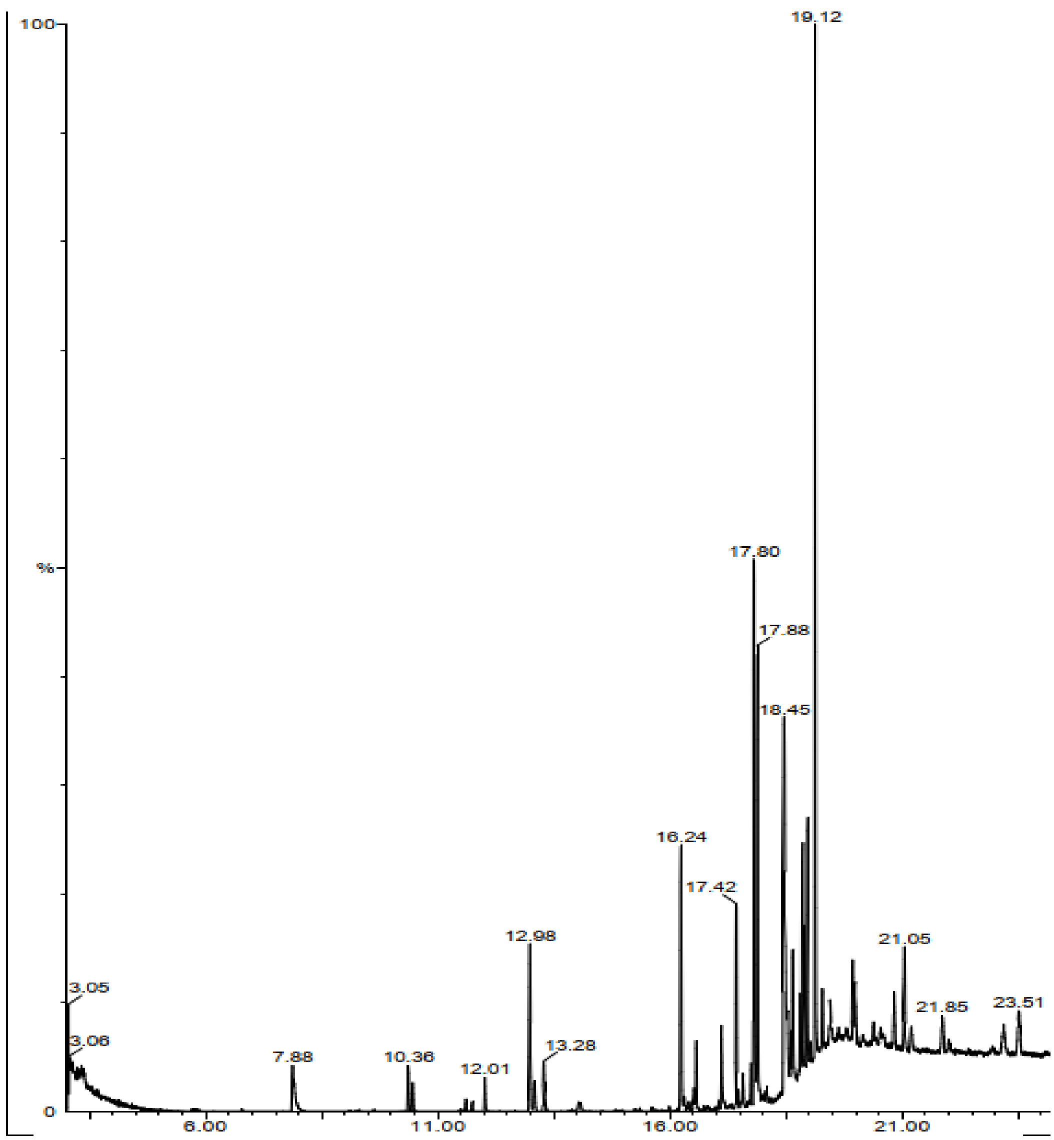

Fig. 1 : GC-MS chromatogram of ethyl acetate extract of *Curvularia pseudorobusta*

The study of endophytic fungi and their secondary metabolites holds great promise for various scientific and industrial applications. In this context, the GC-MS profiling of metabolites from *Curvularia pseudorobusta* from *Distimake dissectus* has provided provided valuable insights into the chemical composition of these secondary metabolites. Additionally, the identification and quantification of specific metabolites through GC-MS profiling has contributed to a better understanding of their bioactivity and therapeutic value. This comprehensive analysis serves as a foundation for further research and the development of medicinal and industrial applications using the secondary metabolites of *Curvularia pseudorobusta*.

In conclusion, the research conducted on endophytic fungal isolate has revealed promising findings regarding the bioactive compounds it produces. The diverse range of bioactive compounds identified in these isolates demonstrates their potential for various applications in medicine, agriculture, and industry.Moreover, exploring the ecological interactions between endophytic fungi and their host plants can provide valuable insights into the factors influencing bioactive compound production. Overall, this research emphasizes the importance of endophytic fungi in harnessing natural products for the benefit of human health and sustainable development.

Future Scope

This study can be exploited commercially to increase the synthesis of medicinal drugs.

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