GC-MS Analysis of Secondary Metabolites of Endophytic Colletotrichum Gloeosporioides Isolated from Camellia Sinensis (L) O. Kuntze

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Abstract: **- Tea plant, *Camellia sinensis* (L) O. Kuntze is a well known cash crop of Assam, North East India. Various endophytic fungi are reported to have born inside the tissue of tea plant. We isolated an endophytic fungus and it was identified as *Colletotrichum gloeosporioides* based on morphological and molecular characteristics. Endophytic fungi are very common to produce extracellular hydrolytic enzyme and secondary metabolites on the culture fluid. Quantitative estimation of hydrolytic enzyme revealed that it showed highest protease activity (10 IU/µl) than chitinase activity (2.8 IU/µl). GC-MS analysis of ethyl acetate extract revealed the presence of different secondary metabolites. Analysis revealed the presence of different metabolites and among them it had highest amount of Pentadecanal and very low amount of Oxalic acid and allyl octyl ester.**

Key words: Tea plant, Endophytic fungi, *Colletotrichum gloeosporioides,* GC-MS,

I. INTRODUCTION

Tea plant *Camellia sinensis* (L) O. Kuntze belongs to theacae family and is a major cash crop in Assam, North-East India. Occurrences of different species of endophytic fungi were reported inside the healthy tissue of tea plant[1, 2, and 3]. *Colletotrichum sp.,* which is a well known pathogen causes serious disease 'anthracnose' in some crops like cereals, coffee and legumes[4, 5,] and on tropical fruits like banana, avocado, papaya, coffee, passion fruits and others [6] are very frequent to isolate as endophytic fungus from the tissues of healthy leaves and branches of tea plant [7, 8]. Apart from tea plant, *Colletotrichum gloeosporioides* was also commonly isolated as endophytic fungus from a range of plant species[9, 10 and 11].

It is reported that outside the culture of the host tissue; endophytic fungi are known to produce a number of secondary metabolites which have anti-cancer, anti-fungal, anti-diabetic and immunosuppressant activity[12, 13]. A number of antimicrobial substances have been recognized as from different species of endophytic fungi[14-20]. Taxol, a bioactive molecule, which was isolated from endophytic fungus *taxus brevifoliai*[21] is used as anticancer drug for ovarian and breast cancer. Some endophytic fungi isolated from tea plant like the *FHM* and *Guignardia sp.* also produced metabolic products which showed strong inhibitive activity against *Candida albicans*[22]. The metabolic products of endophytic fungi strain *FHM* can inhibit the growth *of Guignardia sp., Pestalotiopsis sp., Colletotrichum sp., Bacillus subtilis., Staphylococcus aureus and Candida albicans* (Internet site: http://mt.china-papers.com/1/?p=137235).

So, we focused our studies on isolation and identification of the endophytic fungi and characterization of the metabolites produced by them in culture.

II. MATERIALS AND METHODS

A. Collection, processing and isolation of samples

Healthy tea plants were selected from tea estate of Assam and diseased free leaves were picked from healthy plants and brought to the laboratory in sterile plastic bags and processed within 12 hours after sampling to reduce the chance of contamination. Following procedure was followed for isolation of endophytic fungi[23]. Leaves (1 cm^2)

were cut and washed in running tap water. The leaves segments were treated with 96% ethanol for 30 sec and washed with sterile water for 30 sec. Later the leaves were treated with NaOCl-Distilled water (1:3) for 5min followed by 96% ethanol for 30 sec. Finally the leaves were washed with sterile water for 1-2 mins followed by drying. The processed leaves segments were then placed in petri dishes containing potato dextrose agar (PDA) medium. The petri dishes were sealed with parafilm and incubated at 28°C in the incubator. After 3-5 days of incubation, the initial growth appeared and the emerging hyphal tips were transferred to the petriplates for vitality test and to the thin layer PDA slant for sporulation of the fungi. Vitality test were carried out to verify the proper surface sterilization[24].

B. Identification with morphological characteristics and Molecular approaches

The isolated endophytic fungi were inoculated on thin layer PDA slant and PDA containing petriplates and allowed to grow at 28˚C in incubator for 8-10 days. Morphological studies included colony and conidial morphology of the fungal isolates and was carried out when the mycelium occupied the whole plate or the test tube.
For molecular identification, the fungal isolate was grown in test tubes containing PDB media and allowed to grow at 28˚C for 5-7 days. The mycelium was harvested and extraction of genomic DNA was carried out by using Nucleopore gDNA Fungal Bacterial Mini Kit (50) as per manufacturer's instructions.
Using primer pair ITS1 and ITS4[25], amplification of rDNA ITS was carried out in a thermo cycler (Gen Amp 9700, Applied Biosystem, US) and the conditions of PCR was as follows: 95˚C for 5min, 35 cycles of 94˚C for 45 sec, 60˚C for 30sec, 72˚C for 45 sec and final 72˚C for 5 min. The product size was approximately 575 base pair which was visible in 1.2% agarose gel under UV light. PCR product was purified using PCR purification kit (Fermentas, Lithuania) and sequencing of the PCR product was done by a Sanger's Dideoxy method on applied Biosystem 3730XL (Bioloink, New Delhi, India). The sequence was submitted to NCBI Genbank database which provided the accession number of the isolate.

C. Preparation of fungus for enzyme assay, GC-MS and FTIR analysis

The isolated endophytic fungus was grown on PDA plates for 4-5 days at 28˚C in dark. Three pieces of 6 mm plug of the isolate was cut from the margin of the plate and inoculated on erlenmeyer flasks (250 ml) containing 150 ml of PDB. The flasks were then incubated in shaking incubator (Labtech, Korea) at 120 rpm at 28˚C. The culture was harvested after 5 days, the mycelium was separated with Whatman No 1 filter paper and cell free supernatant was prepared through endotoxin free 0.2 µm PES syringe filter for enzyme assay. For GC-MS and FTIR analysis the mycelium was separated by vacuum filtration using bottle top filter to collect the culture filtrate. The filtrate was extracted with Ethyl acetate in 1:1 ratio and allowed to shake for 2 hrs in rotary shaking incubator. The filtrate was air dried in a closed chamber in a dark room.

D. Quantitative enzyme assay

The most important hydrolytic enzymes protease and chitinase was selected for quantitative estimation. For chitinolytic activity, 1 ml of culture supernatant, 0.3 ml of 1 M Na-Acetate buffer (P^H 4.7) and 0.2 ml of colloidal chitin was taken on test tube. It was incubated at 40˚Cfor 6-24 h. The mixture was centrifuged at 12,225 g for min at 6˚C and after that 0.75 ml of the supernatant was collected. There, 0.25 ml of 1% solution of dinitrosalycilic acid (DNS) in 0.7M NaOH was added and it was followed by the addition of 0.1 ml of 10 M NaOH. The mixture was heated at 100˚C for 5 min. It was cool down to room temperature and absorbance was taken at 582 nm (A_{582}).Blank was prepared same as above protocol except culture supernatant was added after addition of DNS[26].
For proteolytic activity 1ml of 1% soluble casein and 1 ml of enzyme extract was taken on a test tube. It was incubated for 1 h at 45˚C. After incubation 3ml of 5% TCA was added to stop the reaction and was kept in ice for 15 mins. The mixture was centrifuged at 5000 rpm for 30 mins at 4˚C. Finally OD was measured at 280 nm. Blank or control was prepared in the same way except 3 ml TCA was added before incubation[27].

E. FT-IR analysis

Infra Red- spectral analysis was performed using Shimadzu FT-IR 8300 instrument. KBr pellet was prepared by mixing 1mg of fungus extract with 10mg of anhydrous potassium bromide. The spectra were recorded from 500 to 4500 cm^{-1}. Thirty-two scans at a resolution of 4 $cm^{-}1$ were averaged and referenced against air.

F. GC-MS analysis

The crude extract of the fungus was subjected to GC-MS analysis for further purification and chemical characterization of metabolites. GC-MS analysis was performed using an Agilent 240 MS series chromatograph equipped with an Agilent ion trap mass-spectrometer[28]. Samples were separated on a column of Agilent 19091J- 433 model and the size of the column is (30mx 250µm x 0.25mm). The mobile phase was methanol water (linear gradient from 30% to 100% methanol over 30 min) at a flow rate of 1ml / min–1 at 30 325°C. The volume of the injected extract was 50µl and Helium was used as carrier gas. An ion mass spectrometer and PDA detector were used to monitor the eluted compounds. Compounds were identified by absorbance at nm over 10 to 25 min (total analysis time 35 min). Particular compounds structures were putatively identified and evaluated by comparing the molecular masses (m/z values) of the eluted compounds with literature data and standards.

III. RESULT and DISCUSSION

A. Identification of endophytic fungus

After confirmation through vitality test, the isolated endophytic fungus was identified as *Colletotrichum gloeosporioides* based on colony morphology, conidial shape and size[29]. The colony colour of the isolate was white to plae grey, which contained cylindrical conidia and the size was 14.4-3.6 µm. The growth rate of the isolate was 11mm/day and rate of sporulation was 23 X 10^4(spore/ml). Previous report also revealed that *C. gloeosporioides* was also frequently isolated as endophytic fungi from tissues of healthy leaves and branches of tea plant[7].
Only morphological characteristics can't be considered for accurate identification of *Colletotrichum* species due to lots of variation within the isolates of a species or among the species. To overcome species delineation within the genus *Colletotrichum*, analysis of the sequences of ribosomal DNA were extensively used[30, 31]. The submitted ITS sequence of our isolate (Accession no KF053197) clustered with the representative reference *Colletotrichum gloeosporioides* isolates (AB981196 and KJ676453) published in NCBI GenBank database (**Fig 1**) and confirmed our isolate as *C. gloeosporiodes*. The phylogenetic tree (**Fig 1**) was constructed using Kimura 2-parameter[32] method and the analysis involved 12 nucleotide sequences of six different species.

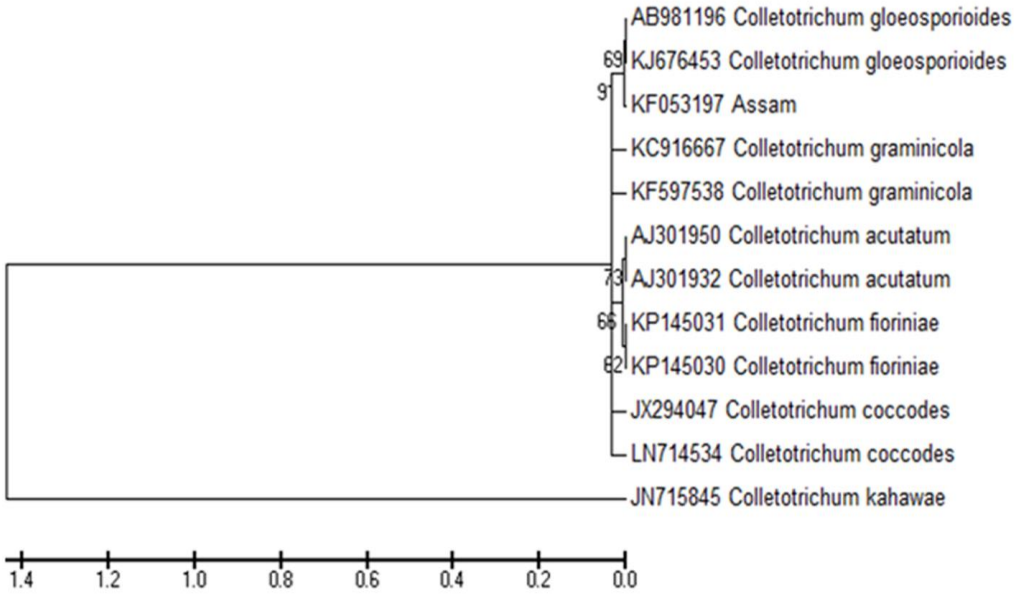


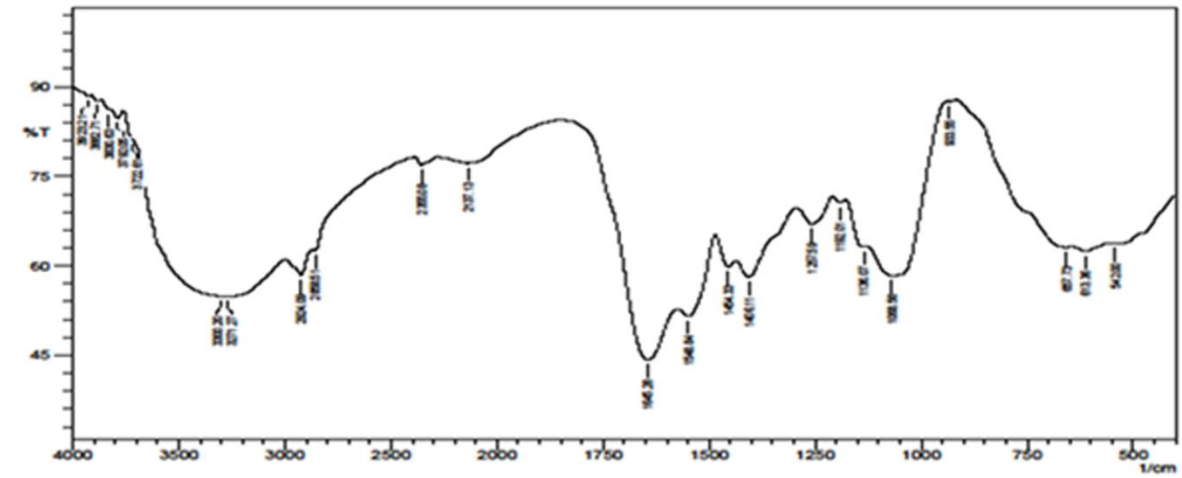
Fig 1: The evolutionary history was inferred using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method and are in the units of the number of base substitutions per site. The analysis involved 12 nucleotide sequences. Evolutionary analyses were conducted in MEGA5.

B. Qualitative and quantitative assay of hydrolytic enzymes

5 days old cell free filtrate of the fungus showed good chitinase activity (2.8 IU/µl) and protease activity (10 IU/µl). The hydrolytic enzyme like chitinase and protease which are also produced in culture filtrate, play a major role to inhibit the pathogens along with the secondary metabolites. The chitin degrading enzyme, chitinase and its role in biological control and plant defence mechanism is known[33, 34, 35]. Fungal protease also plays a significant role in mycoparasitism in pathogen control mechanism[36].

C. Fourier Transform Infrared Spectroscopy (FTIR) analysis

Crude extract of endophytic fungus *C. gloeosporioides* was applied for FTIR analysis and it was predicted that the wave number 3300.20 and 3271.27 may be C-H stretch of aromatics/ =C-H stretch of alkenes or O-H stretch of carboxylic acids or –C=H-H: C-H stretch of alkenes terminals; the wave number 2924.09 and 2858.51 C-H stretch of alkanes; the wave number 2355.08 and 2137.13 may be predicted as CΞN stretch of Nitriles or CΞC- stretch of alkynes. The wave number 1645.28 and 1548.84 may be –C=C- stretch of alkenes/N-H bond of 1° amines; the wave number 1454.33 and 1406.11 may be predicted as C-H bond of alkanes and C-H nock of alkanes. The peak with intensity 12.57.59, 1192.01 and 1136.07 may be C-N stretch of aromatic amines or C-O stretch of alcohols, carboxylic acids, esters or ethers or C-H wag (-CH_2X) alkyl halides. The wave number 933.55 is the stretch of O-H bond of carboxylic acid. The wave number 657.73, 613.36 and 542.00 may be the stretch of –CΞC-H:C-H bond of alkynes or C-Br stretch of alkyl halides (Fig 2).



	Peak	Intensity	Corr. Intensity	Base (H)	Base (L)	Area	Corr. Area
1	542	63.7239	0.7719	555.5	399.26	27.7074	1.2662
2	613.36	62.4103	0.976	642.3	557.43	17.0241	0.2837
3	657.73	63.1014	1.3003	914.26	644.22	37.1142	2.863
4	933.55	87.5648	0.064	937.4	916.19	1.2115	0.0048
5	1068.56	58.397	12.508	1128.36	939.33	33.2297	9.1596
6	1136.07	63.1498	0.9883	1178.51	1130.29	8.7178	0.3198
7	1192.01	70.5961	0.7364	1209.37	1180.44	4.3066	0.0685
8	1257.59	66.9928	3.5067	1294.24	1211.3	13.5832	1.061
9	1406.11	58.0419	4.5814	1435.04	1296.16	27.7285	1.8547
10	1454.33	59.8907	2.486	1485.19	1436.97	10.1887	0.4684
11	1548.84	51.5567	4.8676	1575.84	1487.12	22.7898	2.2449
12	1645.28	44.219	16.2474	1651.66	1577.77	55.3676	10.0897
13	2137.13	77.1947	3.1012	2279.86	1853.59	42.4814	4.1207
14	2355.08	76.858	1.4722	2385.95	2281.79	11.4806	0.4106
15	2858.51	62.5476	0.5064	2870.08	2387.87	66.1508	0.0546
16	2924.09	58.4131	3.6039	2997.38	2872.01	27.5585	1.4326
17	3271.27	54.803	0.2686	3282.84	2999.31	69.1515	1.8462
18	3300.2	54.8082	0.9476	3716.83	3284.77	92.7686	16.6027
19	3722.61	81.082	0.4974	3761.19	3718.76	3.4225	0.0953
20	3792.05	84.7391	1.3322	3822.91	3763.12	4.095	0.2
21	3830.63	86.1376	0.301	3869.2	3824.84	2.7049	0.0271
22	3862.71	87.6483	0.3959	3909.71	3871.13	2.1551	0.048
23	3923.21	88.4565	0.3089	4000.36	3911.64	4.4533	0.0691

Fig 2: FTIR result for ethyl acetate extract of *Colletotrichum gloeosporioides*

D. GC-MS analysis of the fungus

Crude ethyl acetate extract of *C. gloeosporioides* isolate was used for GC-MS analysis. The chromatogram predicted the presence of seven compounds and identified based on peak area, retention time, molecular weight and molecular formula. Database of National Institute standard and Technology (NIST) was used for interpretation of the mass spectrum of GC-MS which has more than 62,000 patterns. The mass spectrum of the unknown component was compared with the spectrum of the known components stored in the NIST library. The name, molecular weight and structure of the components of the test materials were ascertained. Pentadecanal ($C_{12}H_{30}O$) with peak area 18.71 and retention time was 39.23; 5, 7-Octadien-2-one, 3-acetyl-($C_{10}H_{14}O_2$) with retention time 28.78 and peak area was13.49, Ethanone, 1-(3-butyloxiranyl) - which molecular formula is $C_8H_{14}O_2$ along with retention time and peak area is 27.06 and 10.87 respectively. Hexadecanal ($C_{16}H_{32}O$) which retention time was 32.82 and had 4.6 peak areas. 1-Butanol, 4-butoxy- ($C_8H_{18}O_2$) had 30.25 retention time and peak area was 4.2. Very low amount constituents of the crude extract of *C.gloeosporioides* were Oxalic acid, allyl octyl ester ($C_{13}H_{22}O_4$) had 1.75 peak areas and the retention time was 19.52; Sulfurous acid, pentadecyl 2-pentyl ester ($C_{20}H_{42}O_3S$) had 1.51 peak areas and the retention time was 35.83 (Fig 3). The identification of these metabolites revealed that endophytic *C.gloeosporioides* has the capacity to produce bioactive compounds. GC-MS analysis of crude ethyl acetate extract of endophytic *C. gloeosporioides* isolated from *Lannea corammendalica* revealed the major compounds as 9-octadecenamide, hexadecanamide, Diethyl pythalate,2-methyl-3-methyl-3-hexene, 3-ethyl-2,4-dimethyi-pentane and exhibited antimicrobial activity[37]. Again *C. gloeosporioide*, the endophytic fungi which was isolated from medicinal plant *Phlogacanthus thyrsiflorus* produced volatile compounds from crude extract and was analyzed by GC-MS analysis. The main constituents were identified as Phenol, 2,4-bis (1,1-dimethylethyl), 1-Hexadecene, 1-Hexadecanol, Hexadecanoic acid, octadecanoic acid methyl ester and 1-Nonadecene[38].

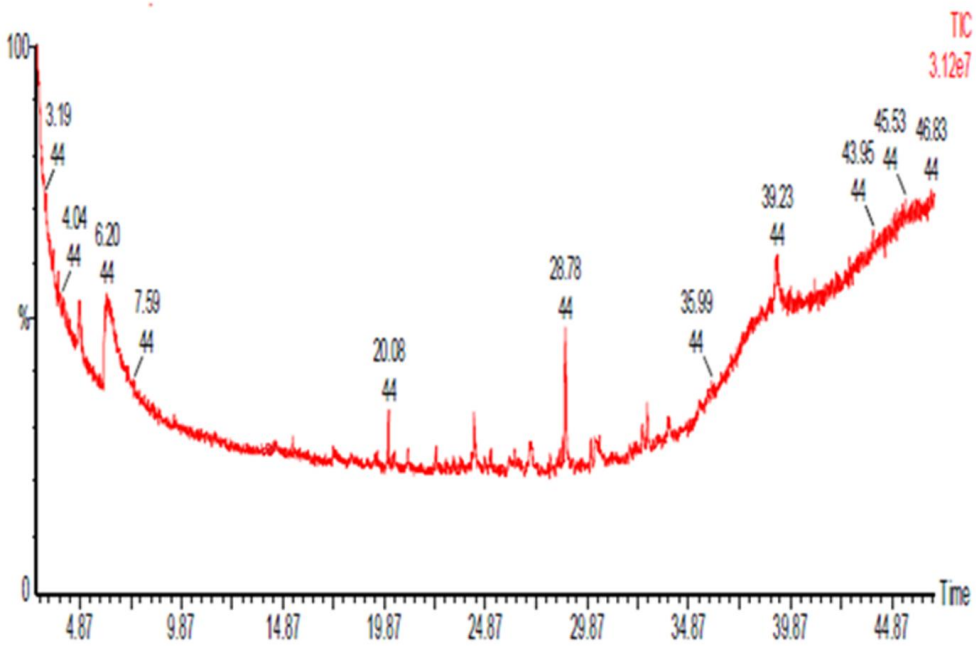


Fig 3: Chromatogram of GC-MS analysis of crude extract of *C. gloeosporioides*

From the above findings it may be concluded that *C. gloeosporioides* produces necessary secondary metabolites or enzymes on its culture filtrate. Research on *C. gloeosporioides* revealed that it has the capacity to produce secondary metabolites having antimicrobial activity. The metabolite colletotric acid produced by endophytic *C. gloeosporioides* from *Artemisia mongolica* which displayed antimicrobial activity against bacteria as well as fungus *H. sativum*[39] . The compound Nectriapyrone isolated from endophytic *C. gloeosporioides* and *G. cingulata* had strong antimicrobial activity against *S. aureus*, *E. coli*, *C. albicans*, *T. cruzi*, *L. tarentllae* and *Human T leukemia cell*[40]. Crude EtOAc extract of endophytic *C. gloeosporioides* showed strong antifungal activity against the phytopathogenic fungi *C. cladosporioides* and *C. sphaerospermum*[41]. However further research is necessary to explore the secondary metabolites of *C. gloeosporioides* which have different biological activities.

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