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Evaluating metabolite profiling of endophytic contagious disconnects of five ethno-pharmacologically significant plants of Meghalaya

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Abstract

Crude metabolite extracts derived from the culture broths of endophytic fungi of five ethno-pharmacologically important plant species used by the ethnic tribes of the hill state of Meghalaya in North-East India were screened for the presence of toxins and secondary metabolites using agar plug paper chromatography, thin layer chromatography (TLC) and liquid chromatography-mass spectroscopy (LC-MS). The endophytic fungal isolates were found to produce a wide range of toxins, growth hormones and antibiotics upon searching in a fungal mycotoxin database. Relatedness among the fungal isolates was determined based upon their extra-cellular metabolite production pattern. The endophytic fungal isolates in the present study showed a wide range of metabolomic diversity and can be explored as potential microbial cell factories for production of a diverse range of biomolecules. Taxonomic relatedness of the endophytes based upon the snapshots of their secretomes, presented a contrasting picture in relation to their morphological identity. Rapid metabolome analysis of endophytic fungi using analytical techniques like LC-MS coupled with mass related search in fungal specific metabolite databases is bound to increase our insights about fungal chemo-taxonomy as a whole and bio-prospection of useful fungal metabolites in particular.

Keywords: Endophytic fungi, ethnic tribes, medicinal plants, agar plug paper chromatography, thin layer chromatography (TLC), liquid chromatography-mass spectroscopy (LC-MS), chemo-taxonomy, metabolome.

INTRODUCTION

Identification and ascertaining taxonomic identity of endophytic fungi using available taxonomic tools at the disposal of fungal taxonomists is often difficult as most of the endophytic fungal isolates tend to present cryptic properties (Ganley et al., 2004). In order to arrive upon the best possible taxonomic classification of the endophytes, it is often advised that the workers use a polyphasic approach to ascertain the most likely taxonomic identity of the fungal isolates (Samson et al., 2007; Perrone et al., 2008). In recent years, efforts to find a stable molecular taxonomic barcode for fungi has also led to the characterization and decoding of genes encoding for functionally conserved proteins in the fungal genome such as the tubulin, cytochrome oxidase 1 and actin genes (Samson et al., 2004; Seifert et al., 2007; Roe et al., 2010) However, molecular techniques like sequencing of rRNA regions, protein encoding genes are limited by the fact that the new sequences generated by workers worldwide have to be compared to known sequences already deposited in public sequence databases like the nucleotide database of NCBI.

Therefore, if a fungal taxonomist is to stumble upon a previously undescribed sequence from a unique endophytic fungal species or isolates, with a very low similarity percentage to any known nucleotide sequence, then the precision of the molecular approach becomes limited. This limitation is further enhanced by the fact that even isolates of the same species of fungi may show different metabolic characters under a given set of conditions (Kubicek et al., 2003). In order to eliminate these major stumbling blocks, the approach that is slowly

Table 1. The host plant of the five endophytic fungal isolates along with their ethno-medicinal usage.

Endophytic fungal isolate	Host plant	Sacred forest	Habitat	Ethno-medicinal use of the host plant
RS07PF	*Potentilla fulgens*	Nongkrem (Lum Shyllong, Laitkor)	Open grassland	Treatment of diabetes mellitus
RS07OS	*Osbeckia stellata*	Cherrapunji (Law Kyntang)	*Pinus Kesiya* forest undergrowth	Treatment of cuts and wounds and as a remedy for toothaches
RS07OC	*Osbeckia chinensis*	Cherrapunji (Law Kyntang)	*Pinus Kesiya* forest undergrowth	Used as an antitussive, expectorant and febrifuge. A decoction is used in the treatment of watery diarrhoea, dysentery and excessive sputum production in coughing.
RS07CC	*Camellia caduca*	Mawphlang (Law Lyngdoh)	Forest floor	Extracts used as an astringent, digestive, carminative and diuretic herbal medicine
RS07SK	*Schima khasiana*	Umsaw Nongkhrai (Pahampdem)	Forest floor	Used as an anthelmintic and rubefacient

gaining hold in the field of fungal taxonomy is that of a combined polyphasic approach with molecular based taxonomy coupled with secondary metabolite profiling (Frisvad et al., 2008; Stadler et al., 2010).

The current investigation was undertaken with a systems biology approach in mind and aimed at generating metabolite production profiles of five morphologically cryptic and functionally novel endophytic fungal isolates of the ethno-medicinal plants *Potentilla fulgens, Osbeckia stellata, Osbeckia chinensis, Camellia caduca* and *Schima khasiana* (Table 1) of the 'Sacred forests' of Meghalaya (India) with a view to classify them based on their metabolic profiles.

MATERIALS AND METHODS

Isolation of endophytic fungi from the medicinal plants

About 100 root and stem pieces of the selected plants were washed in tap water followed and then air dried. The plant parts were treated with sodium hypochlorite (0.5%) solution and subsequently flamed in the laminar air flow before removing the outer layers with sterile forceps and surgical blades. 2 cm long pieces of these roots and stems pieces were then placed on Petri plates containing water agar and incubated at 24°C.After incubation for 7 days, hyphal tips of developing fungi were aseptically removed and placed on potato dextrose agar (PDA) as per the procedure described by Strobel et al. (2005).

Preparation of the fungal culture broth crude extracts

1 ml each of the broth culture of the five endophytic fungal isolates that is RS07PF, RS07OS, RS07OC, RS07CC and RS07SK were aseptically transferred under laminar flow to 1.5 ml microfuge tubes and centrifuged at 13000 rpm for 5 mins at 4°C in a refrigerated ultra-centrifuge (Haraeus Biofuge). The culture free supernatant was then filtered through 0.2 micron syringe filter and transferred to new 1.5 ml microfuge tubes and kept at 4°C until used.

Agar-plug paper chromatography of the crude extracts

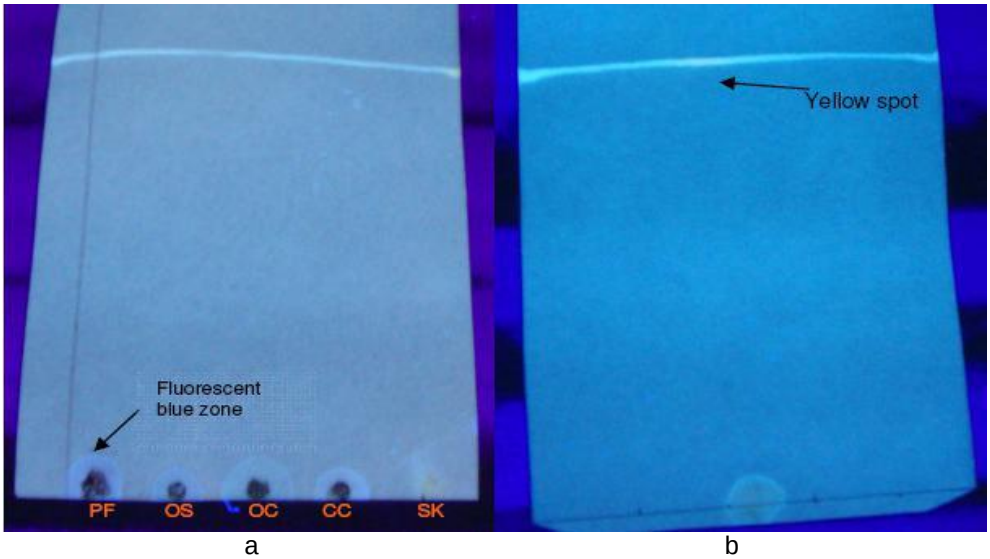
Agar-plug chromatography of the crude extracts derived from the endophytic fungal isolates was carried as per the protocol of Filtenborg et al. (1983) with some minor modifications in the form of using the filter paper (Whatman No. 1, USA) instead of the TLC plates. The chromatograms generated using chloroform: acetone: 2-propanol in the ratio 85:15:20 were visualized under UV light.

Thin layer chromatography (TLC) of the crude extracts

Thin layer chromatography was performed using silica gel 60 F_{254} (Merck) and a 9:1 mixture of HPLC grade chloroform (SD fine chem., India) and methanol (Spectrochem, India) was used to develop the TLC chromatograms for a period of about 1 h. Iodine (pre-sublimed) pellets were put into the iodine chamber and the dried TLC plates were kept in the chamber for visualization of the metabolites. Plates were also visualized under UV light.

Liquid chromatography-mass spectroscopy (LC-MS) analysis

The crude extracts obtained from the endophytic fungal culture broths were concentrated in a rotary evaporator (RE-300, Stuart, UK) at 50°C. The samples were then lyophilized in a lyophilizer (Scanvac, Denmark). 50 µg of the s ample was dissolved in 1 ml 50% acetonitrile (HPLC grade) in water (HPLC grade). The sample



a b

Figure 1. (a) Paper chromatogram visualized under UV light. Agar plugs of each of the endophytic fungal isolates were used to spot the paper before development with the solvent system. Fluorescent blue regions are observed in all the endophytic fungal species. (b) Observation of a yellow spot at the end of the solvent front in case of the endophytic fungal isolate RS07CC.

was then filtered through 0.2 um filter paper (GH polypropylene 0.2 um pore size, 47 mm diameter filter paper) and kept for sonication for 30 min to get a clear solution. The samples were then transferred to UPLC vials (LC-MS certified 12x32 mm clear presilt combo, Waters, India) and kept under refrigeration at 10°C till the LC-MS analysis. The LC-MS analysis was carried out in a Waters' ultra performance liquid chromatography (UPLC) coupled with Waters' Q-ToF Premier Mass Spectrometer.

The LC column used was ACQUITY UPLC BEH C18 RF 1.7 µm, 2.1 x 50 column. The mycotoxin database Myco_DB (Senyuva et al., 2008) was used for the tentative identification of the fungal metabolites from the mass values so obtained. A phylogenetic tree of the endophytic fungal isolates based upon their production of some major metabolites was created using numerical taxonomy software NTsys (version 2) using the Jaccard's similarity coefficient and the neighbor-joining algorithm. Presence of a particular metabolite was scored as 1 and absence scored as 0.The attempt to profile the metabolites produced by the fungal isolates was to look at the polyphasic approach to their identification. Hence, the LC-MS data (metabolite peaks and mass spectrums) were compared specifically for the mycotoxin groups and related secondary metabolites using the database generated for such metabolites developed by Senyuva et al., (2008).

RESULTS

Agar plug paper chromatography of the endophytic fungal metabolites showed presence of UV-visible components in the crude extracts of the endophytic fungal metabolites (Figure 1a). A yellow spot (both light and UV visible) near the end of the eluting solvent front was particularly evident in the case of the isolate RS07CC (Figure 1b). The bluish green colouration around the agar plug spots in the chromatographic paper indicated the possible production of aflatoxins by the fungi under investigation.

Thin layer chromatography of the endophytic fungal extracts was seen to form a blue-violet colour in the zone where they were spotted into the TLC plate after treatment with iodine vapours (Figure 2). Faint blue zones were also observed in all endophytic fungal extracts except RS07CC. The extracts were more or less static and exhibited only a negligible movement in TLC chromatogram in relation to the developing solvent front (Figure 2).

The total ion chromatogram of the extracts of five endophytic fungal isolates namely RS07PF, RS07OS, RS07OC, RS07CC and RS07SK obtained by the liquid chromatography of the acetonitrile fractions of the crude endophytic metabolite extracts showed more or less uniform peaks except for the isolate RS07CC (Figures 3 to 7). Visually, the chromatograms obtained from RS07PF, RS07OC and RS07SK are quite comparable. The chromatogram obtained from RS07OS (Figure 4) although, similar to the earlier mentioned three, show more visible peaks in comparison. The chromatogram obtained from RS07CC (Figure 6) was unique in comparison to the others. Major metabolites tentatively detected by LC-MS In the fungal metabolite extracts using the database of Senyuva et al. (2008), were compared for their similarity pattern (Table 2). Detection of the metabolites was done by manual searching of the mycotoxin database for related mass and retention times. All the endophytic fungal isolates were detected to produce aurantioclavine, austdiol, oleic acid, jasmonic acid-ethyl ester, diaportin acid and walleminone. Abscissic acid was detected in all the isolates except isolate RS07PF. RS07CC did not produce any type of

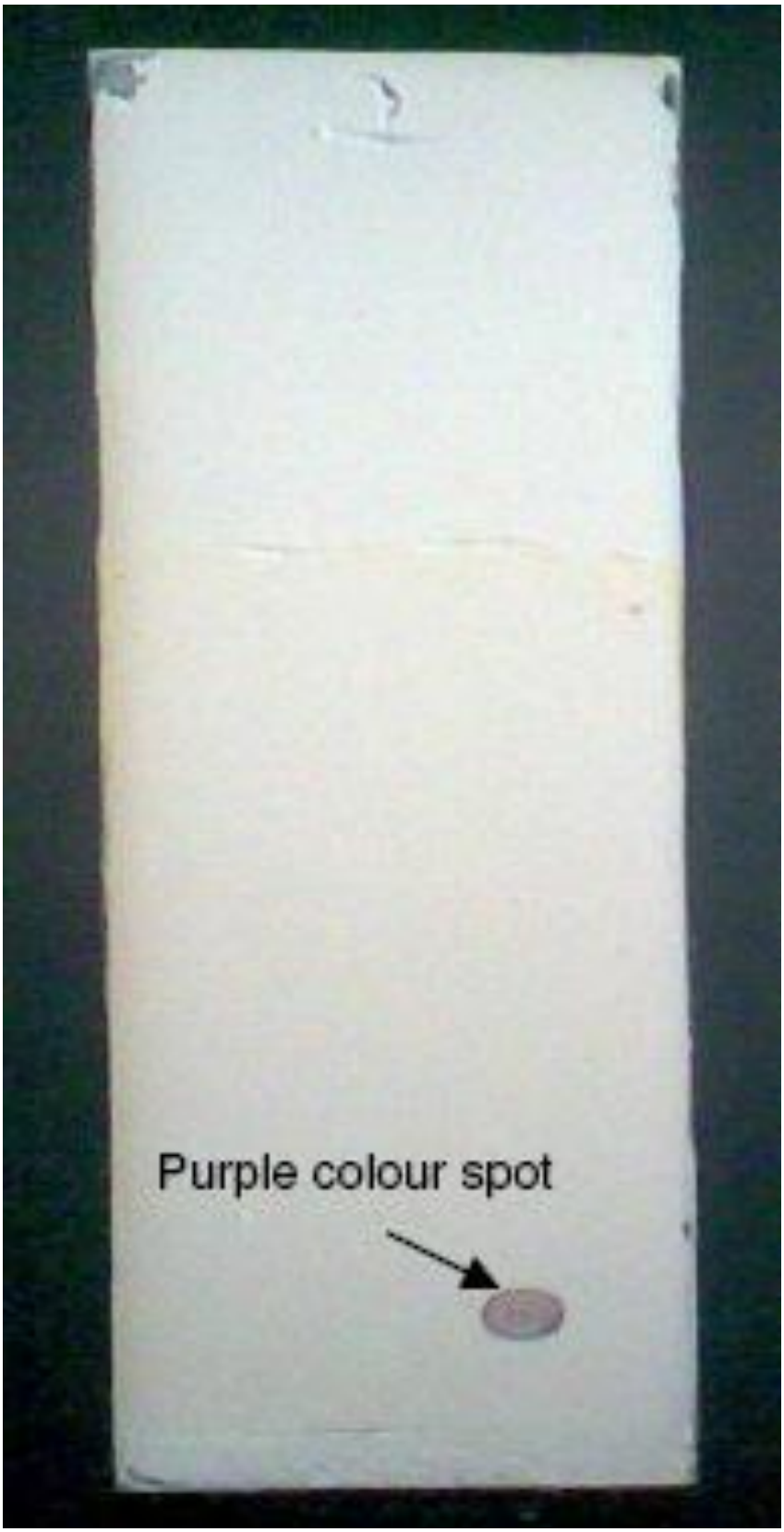


Figure 2. Thin layer chromatogram in Merck silica gel 60 F_{254} showing a characteristic purple blue spot in the area of spotting the crude endophytic fungal metabolic extracts (RS07PF) upon exposing the TLC plate to iodine vapours.

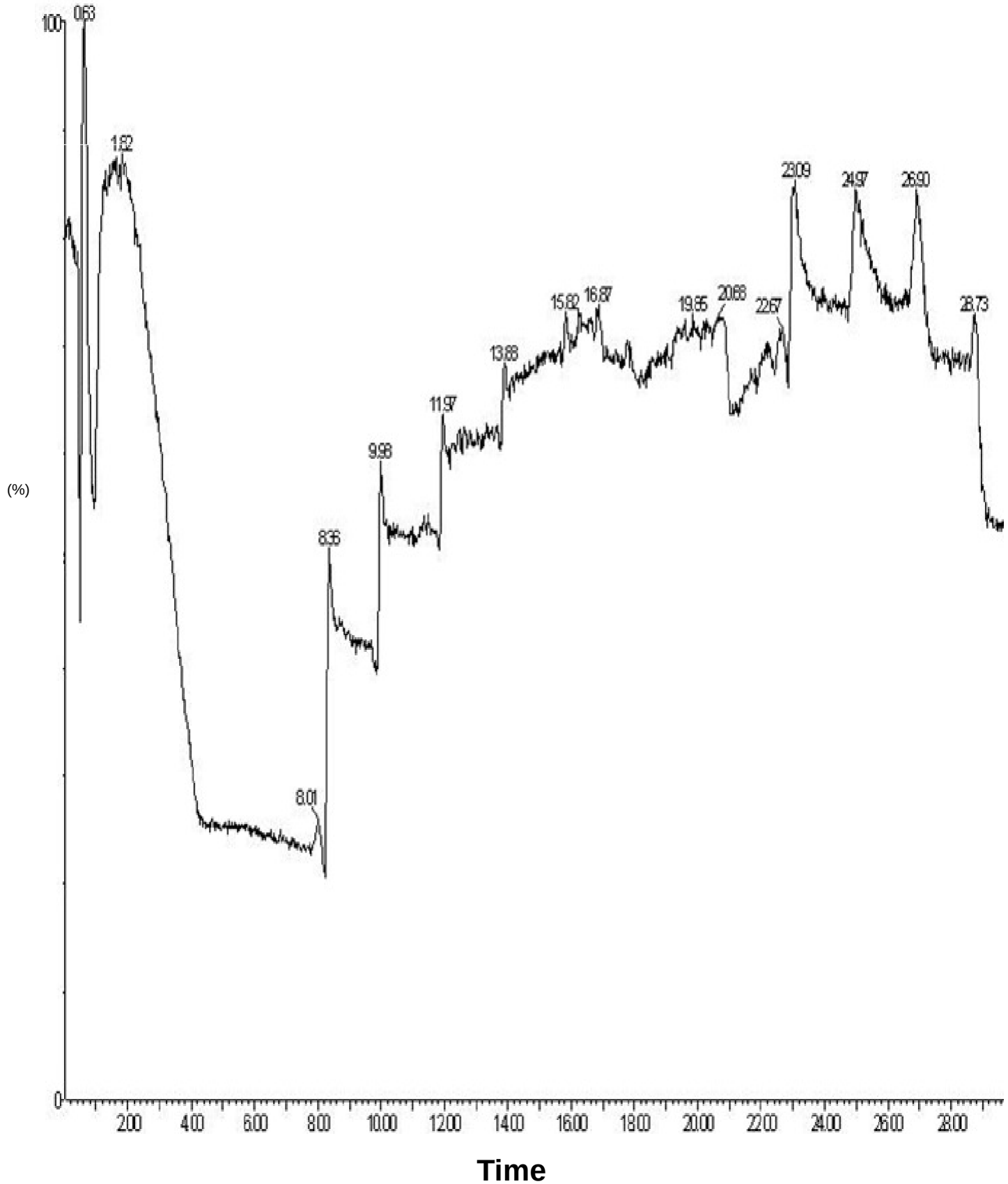


Figure 3. Chromatogram of endophytic fungal isolate RS07PF obtained by the LC-MS analysis.

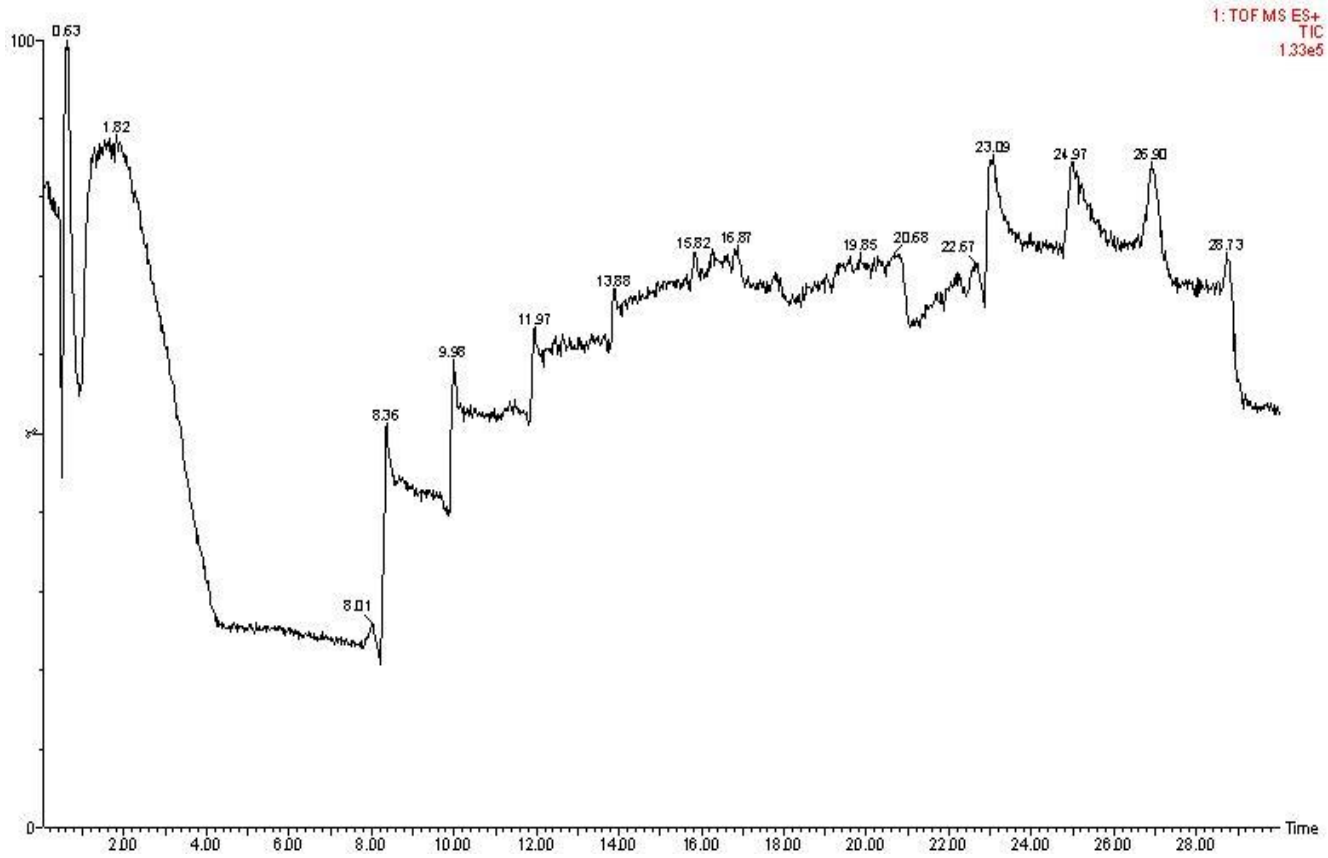


Figure 4. Chromatogram of endophytic fungal isolate RS07OS obtained by the LC-MS analysis.

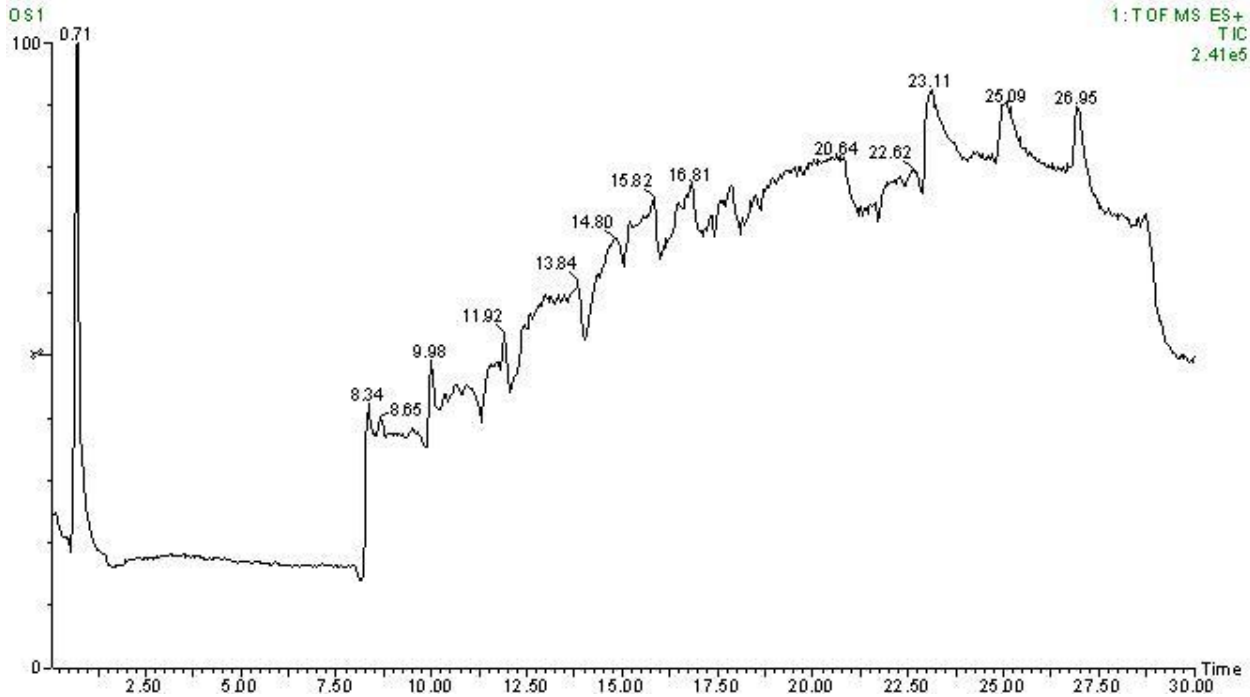


Figure 5. Chromatogram of endophytic fungal isolate RS07OC obtained by the LC-MS analysis .

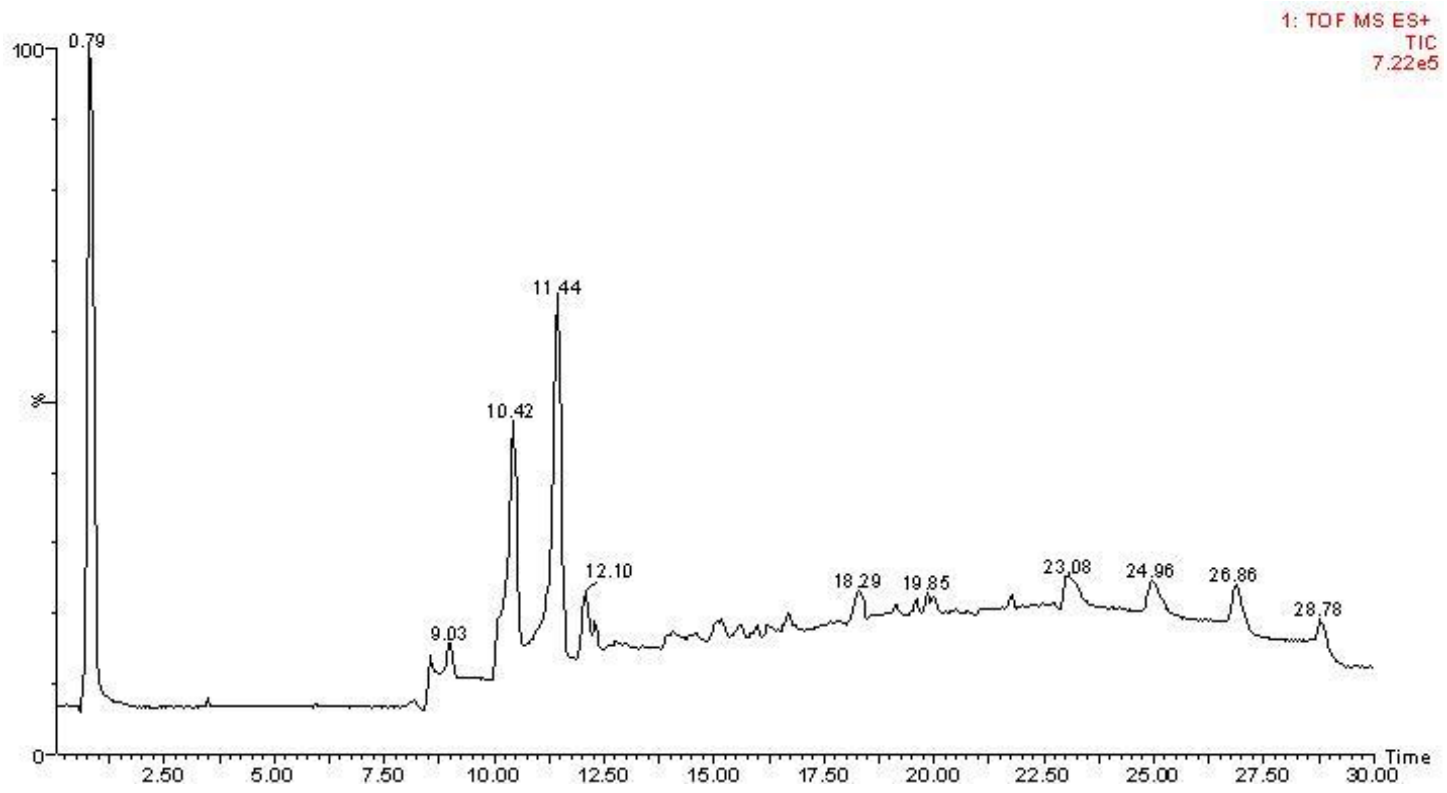


Figure 6. Chromatogram of endophytic fungal isolate RS07CC obtained by the LC-MS analysis.

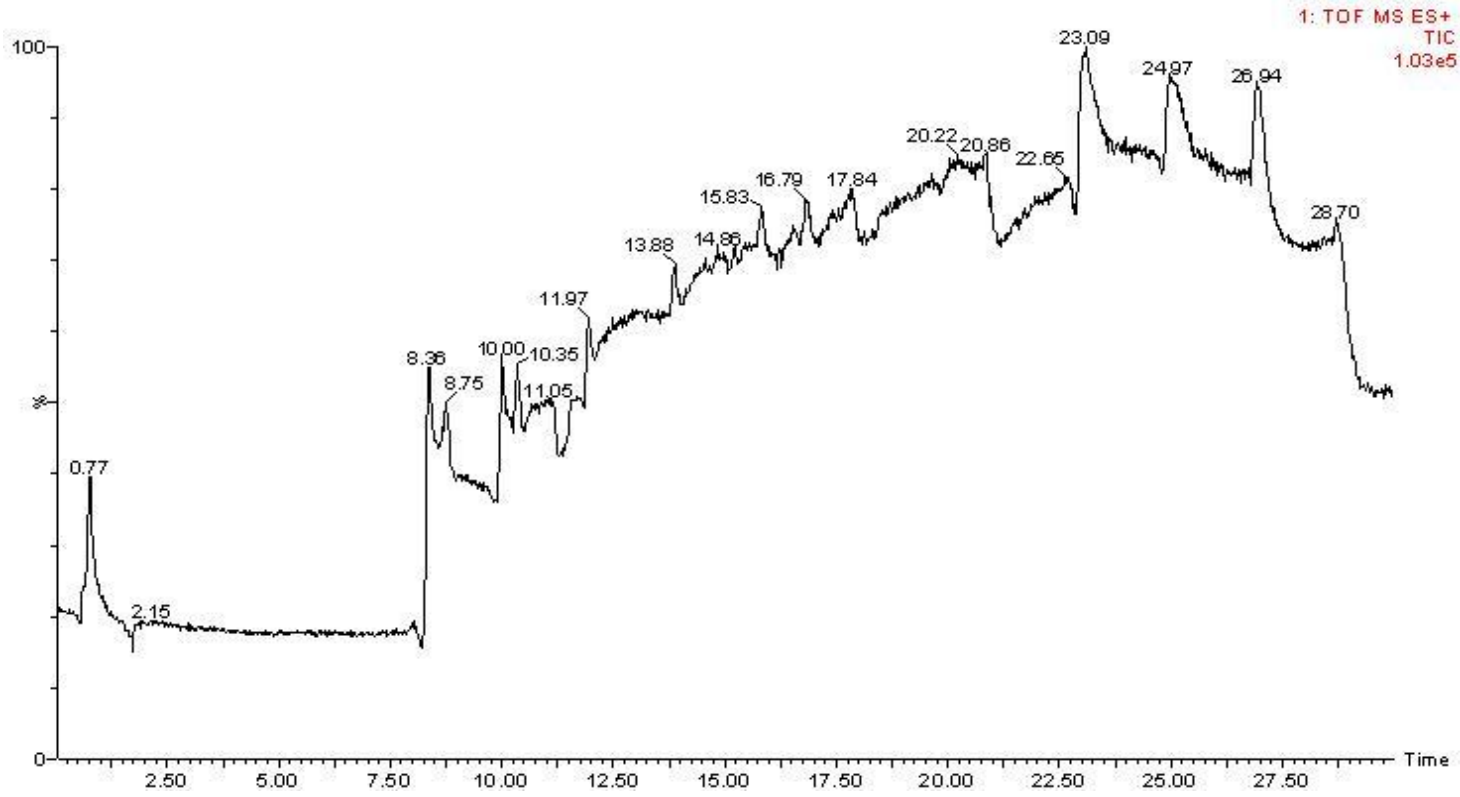


Figure 7. Chromatogram of endophytic fungal isolate RS07SK obtained by the LC-MS analysis. .

aflatoxin whereas the other four isolates showed the presence of one or the other form of aflatoxin that is aflatoxicol I, aflatoxin B2, aflatoxin G2, aflatoxin G2a and aflatoxin M1 (Table 2). This result is in agreement with the one observed in agar plug paper chromatography and TLC of the fungal isolates. Only RS07CC was shown to produce Antibiotic Y whereas all the isolates produced one or the other form of antimycins.

Shikimic acid was detected in the crude extract of the endophytic fungal isolate RS07SK (Table 2). Fusicoccin, an important antimicrobial agent was detected in the culture extracts of RS07PF, RS07OS and RS07SK (Figure 8). Caffeine was detected in the crude extracts of the isolates RS07PF, RS07OSand RS07OC. Indolacetic acid was detected in the isolates RS07PF and RS07OC. The crude extracts of the endophytes isolated from *P. fulgens* and *O. stellata*, have shown DNA damage protective activity in one of our previous studies (Bhagobaty and Joshi, 2008). Gibberellic acid was also detected in the extracts of the fungal isolates RS07OS and RS07OC (Table 2). Cytochalasins (Type B, H, and J) was not detected in the crude extract of isolate RS07OC and RS07SK, while one or the other was detected in the rest of the isolates. Riboflavin was detected in the case of RS07OS, RS07OC and RS07SK while tryptophan was detected to be present only in the crude extracts of RS07OS and RS07OC (Table 2). Many other metabolites relevant for ascertaining the toxicity of the endophytic fungal species or their metabolic diversity were shown to be produced by the isolates (Table 2).

DISCUSSION

The presence of a visible yellow coloured spot in the chromatogram (Figure 1b) may be due to the presence of griseofulvin or related metabolites (Belofsky et al., 1998). The technique of agar plug TLC is reported to be effective in generating metabolite profiles of fungi (Frisvad et al., 1989). Therefore, prior to more accurate TLC screening of the metabolites, the agar plug technique provided us with an idea of the metabolite production by the endophytic fungi. The production of growth promoting substances or hormones are very crucial in the successful establishment of a symbiotic life cycle within the host plant tissues. Uptake of genes from competing plant pathogens or the plant host itself during the evolutionary course of the symbiosis have generated a much wider range of metabolic diversity in case of fungal endophytes (Schardl et al., 1991; Strobel, 2002). This case is evident when we take the example of ochratoxins, trichoverrin, trichoverrol and verruculotoxin (Table 2). A single endophytic fungal isolate like RS07OS, which was identified to be *Syncephalastrum racemosum,* was shown to produce a combination of these metabolites.

Thus, it is suggested that the production or absence of a particular metabolite in the crude extracts of fungal fermentation broths as such, must not be used as specific biochemical markers to ascertain the exact taxonomic identity of the endophytic isolates. We thus decided to take a more holistic view and tried to analyze the relatedness of the five endophytic fungal species based upon their total metabolic profiles so obtained, using the LC-MS data and the available database. The metabolite production profiles generated for each of the endophytic fungal isolates was used to create a matrix based on the presence (1) or absence (0) of a particular metabolite. This matrix was fed into the numerical taxonomy software NTSys to generate a phylogenetic tree showing the relatedness of the endophytic fungal isolates based on their metabolite production profiles (Figure 9). Isolate RS07PF and RS07SK are shown to be closely related to each other with respect to their metabolite production profiles.

On the other hand, endophytic fungal isolates obtained from two different species of the same plant genera *Osbeckia* that is RS07OS and RS07OC, are grouped close to each other based on their similar exo-metabolomic fingerprints (Figure 9). These two isolates based upon the morphological and molecular study (Bhagobaty and Joshi, 2011) to ascertain their taxonomic identity were found to belong to two different groups of filamentous fungi. Isolate RS07OS was tentatively identified as *S. racemosum* (Zygomycetes) and isolate RS07OC was found to possess characteristics of Ascomycetes. The grouping generated by the numerical taxonomy software based upon the metabolite production pattern of these two isolates, reaffirms our hypothesis that the production of secondary metabolites by the fungal endophytes in general may be primarily guided by the metabolic machinery of the host plants irrespective of the fungal species involved in the symbiosis. Endophytic fungal isolate RS07CC isolated from *C. caduca* endemic to Meghalaya (India), was shown to be unique and distantly related to the other four isolates with regards to its secretome or metabolic profile (Figure 9). Taxonomic grouping of fungal species based on their metabolic profiles under a same set of *in vivo* conditions such as the one in the present study, can thus help us in our efforts to understand species relatedness or symbiosis specific gene expression patterns in endophytic fungi. The five endophytic fungal isolates which were tentatively screened for metabolites using LC-MS and the available database revealed the isolates as potential microbial cell factories for production of a wide range of biomolecules. Most of them were shown to produce mycotoxins in addition to growth promoters / plant hormones. This finding is of relevance, since it leads us to believe that majority of fungi establishing an endophytic relation inside the host plant tissues may be latent pathogens, which have somehow managed to find a perfect balance between their symbiotic and pathogenic roles in the host plant. Detection of new metabolites in fungi exhibiting

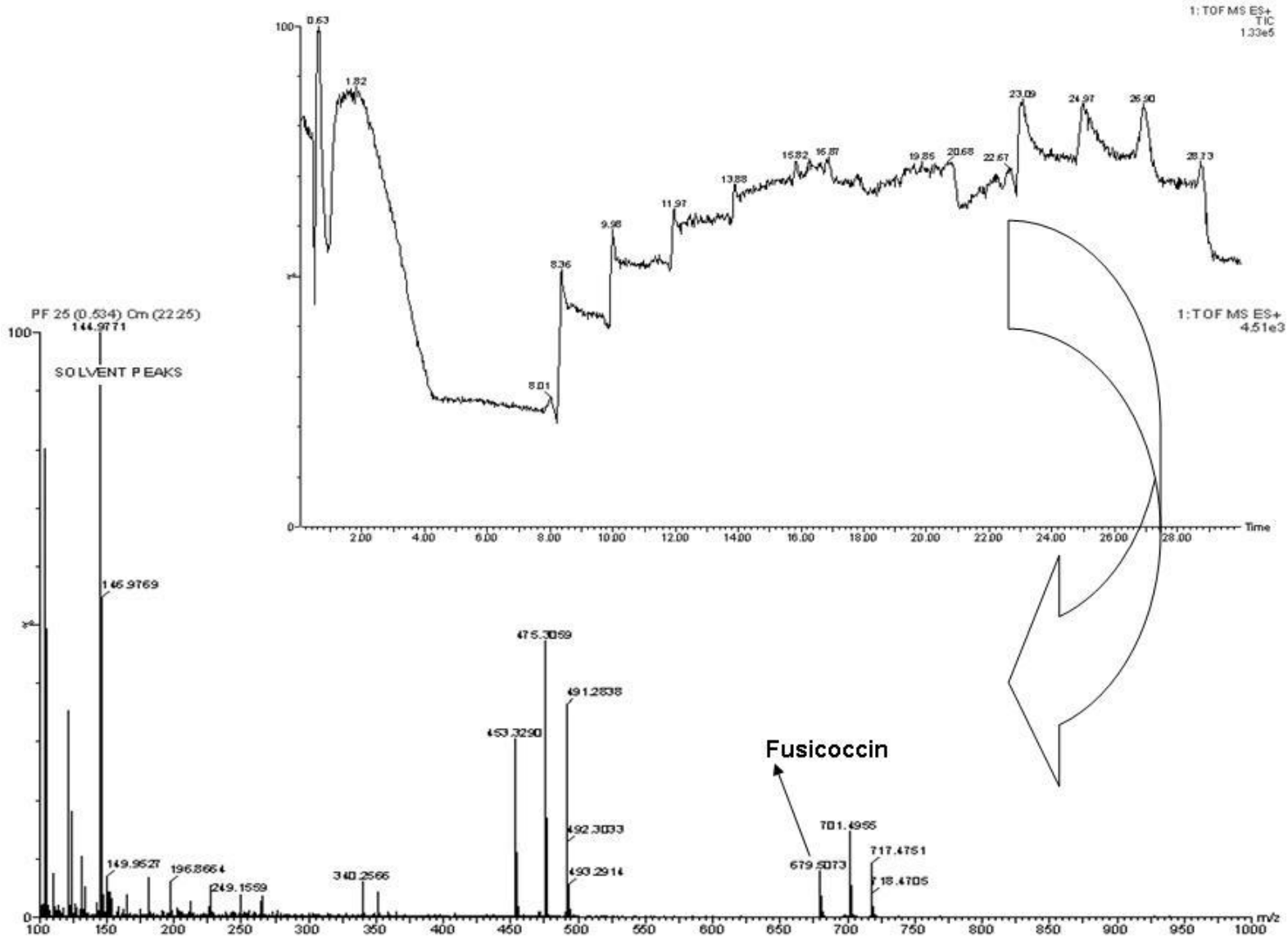


Figure 8. Peak with a retention time of 22 min in the chromatogram of endophytic fungal isolate RS07PF was analyzed using mass spectrometer. Fusicoccin was detected in the mass spec analysis upon comparison of the mass value and adducts in the mycotoxin database.

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Table 2. Metabolite production profiles of the five endophytic fungal isolates generated by the LC-MS as compared to available database of Senyuva et al. (2008).

Metabolites	Endophytic fungal isolates				
	RS07PF	RSO7OS	RS07OC	RS07CC	RS07SK
Abscisic acid	—	√	√	√	√
Aflatoxicol I	√	—	√	—	—
Aflatoxin B2	√	—	√	—	—
Aflatoxin B2a	—	√	√	—	—
Aflatoxin G2	—	√	√	—	—
Aflatoxin G2a	—	√	√	—	√
Aflatoxin M1	—	√	√	—	—
Antibiotic Y	—	—	—	√	—
Antimycin A2	—	—	√	—	—
Antimycin A3	—	√	√	√	—
Antimycin A5	√	√	—	—	√
Ascorbic acid	—	—	—	√	—
Aurantioclavine	√	√	√	√	√
Austdiol	√	√	√	√	√
a-Zearalenol	—	√	√	—	—
Bezophenone	√	√	√	—	√
b-Zearalanol	—	√	√	—	—
Caffeine	√	√	√	—	—
Cytochalasin B	—	√	—	√	—
Cytochalasin H	√	√	—	√	—
Cytochalasin J	√	—	—	—	—
Diaportin acid	√	√	√	√	√
Dihydrojasmonic acid	√	√	—	—	√
Dihydroxysterigmatocystin	√	—	—	√	√
Fusicoccin	√	√	—	√	—
Gibberellic acid	—	√	√	—	—
Helminthosporin	—	—	√	—	—
Indolacetic acid	√	—	√	—	—
Jasmonic acid	√	—	—	√	√
Jasmonic acid-ethyl ester	√	√	√	√	√
Methoxysterigmatocystin	—	—	√	—	—
Ochratoxin a	—	√	√		√
Ochratoxin A-ethyl ester	—	√	√	√	
Ochratoxin A-methyl ester	—	√	—	—	√
Ochratoxin a-methyl ester	—	—	√	√	√
Ochratoxin B	—	—	√	—	—
Ochratoxin B-ethyl ester	—	√	√	√	
Oleic acid	√	√	√	√	√
Penicillic acid	√	—	—	—	—
Riboflavin	—	√	√	—	√
Rubratoxin A	—	√	√	√	—
Rubratoxin B	—	—	—	√	—
Shikimic acid	—	—	—	—	√
Sorbicillin	√	√		—	
Stipitatic acid	√	√	√	—	√
Trichodermin	—	—	—	√	—
Trichoverrin A	—	—	—	√	—
Trichoverrin B	—	—	—	√	—
Trichoverrol A	—	√	—	—	√

Table 2. Contd.

Trichoverrol B	—	√	—	—	√
Tryptophan	—	√	√	—	—
Verrucarol	√		—	—	—
Verrucine A	—	√	—	—	√
Verrucine B	—	√	—	—	√
Verrucofortine	—	√	√	—	—
Verrucosine	—	√	√	—	—
Verruculotoxin	—	√	√	—	—
Walleminone	√	√	√	√	√
w-Hydroxypachybasin	√	√	√		√
Zearalenone	—	√	—	√	—

√ represents detection of metabolites; — represents absence of metabolites

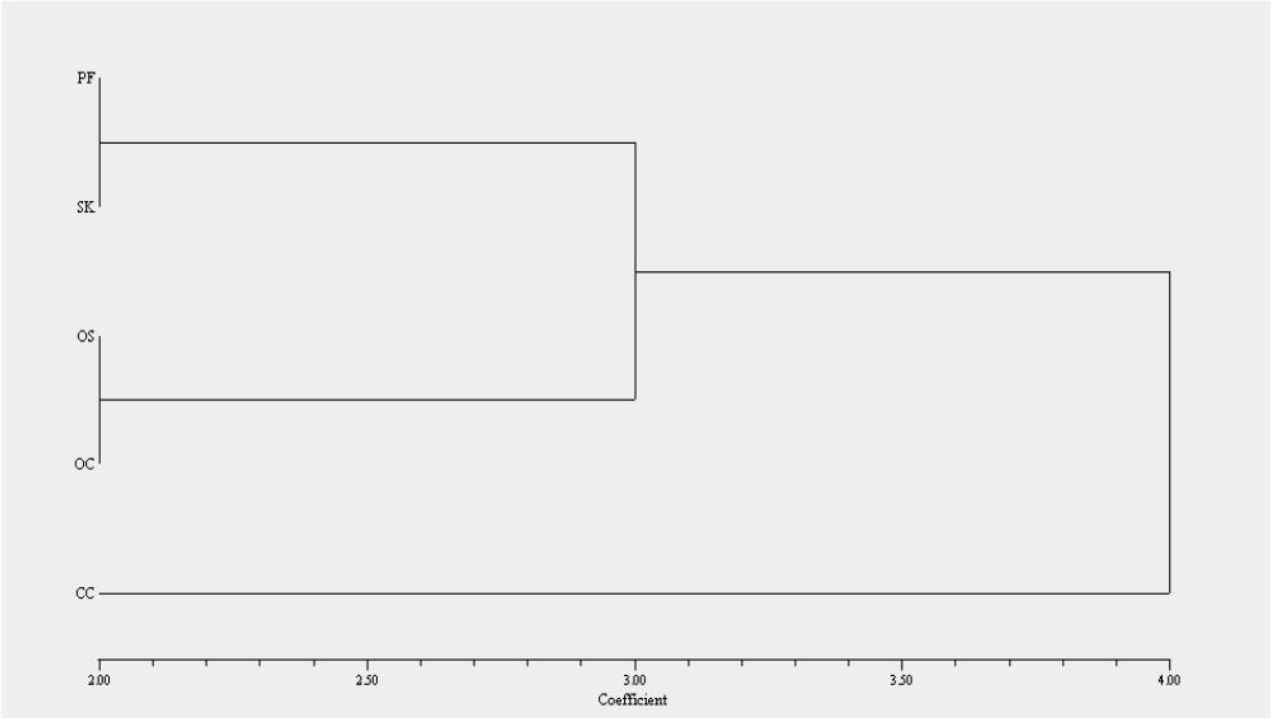

Figure 9. Dendogram of relatedness generated by NTSYSpc Version 2.0 from the metabolite production profiles of the five endophytic fungal isolates. The Neighbor-Joining algorithm coupled with the Jaccard's similarity coefficient was used for the tree generation purposes.

such diverse range of metabolite production is thus a foreseeable possibility.

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