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European Journal of Experimental Biology, 2012, 2 (3):539-542

ISSN: 2248 –9215
CODEN (USA): EJEBAU

Profiling metabolites in different day cultures of a root endophyte, *Frankia* Brunchorst from *Casuarina equisetifolia* L. using GC-MS-MS

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ABSTRACT

The GC MS-MS investigation was carried out to identify the biosignalling compounds in Frankia that make the Frankia to enhance nodulation in Casuarina equisetifolia. Mass spectrum investigation of Frankia culture resulted in identification of biosignalling compounds viz., Isoterpinolene eluted at retention time (RT) 12.468, 2, 4, Phenol-bis (1, 1-dimethyl ethyl) at Rt 18.126 and 1-Dotriocantanol at Rt 19.105, reported to have free radical scavenging activity. The triterpenes Viminalol, β-Amyrine, betulin identified in Frankia were found to be involved in the protection of oxygen sensitive nitrogenase from oxidation.

Key words: *Frankia*, Biosignalling, *Casuarina equisetifolia*, Terpenoids, Hopanoids.

INTRODUCTION

Frankia are nitrogen-fixing actinomycete symbionts that cause the formation of perennial nodules on the roots of a botanically diverse group of bushes and small trees belonging to eight families, 25 genera, and well over 200 species [10].*Casuarina equisetifolia* an ecologically and economically important tropical coastal trees nodulated by Frankia. An additional mechanism operates when nodules are mature and functional; root infections as well as growth of nodules are regulated by the N content of the shoot [14]. *Frankia* strains can use a variety of organic and inorganic source of nitrogen for growth, including amino acids, urea, nitrate, ammonia and N_2. It has been suggested that chemotaxis to bioactive compounds is important for the establishment of root-associated growth of Frankia. Several aromatic compounds are important for signaling in gene expression between plants and some bacteria, and play a major role in the communication between plants and beneficial rhizosphere bacteria. Nitrogen fixation is utilized by numerous prokaryotes, including bacteria, actinobacteria, andcertain types of anaerobic bacteria. Microorganisms that fix nitrogen are called diazotrophs.Some higher plants, and some animals (termites), have formed associations (symbioses) with diazotrophs. Nitrogen fixation also occurs as a result of non-biological processes [12]. N_2-fixing microbes are utilized for formulating biofertilizers for legumes as well as nonlegumes for crop production in many countries [1-4] as a source of secondary metabolites of potential interest and play an important role in the regulation of plant communities in terms of growth and resistant to their herbivores.The present study aims at delineate the biosignalling compounds in Frankia for nodulation capacity in *Casuarina* species by using GC-MS-MS analysis.

MATERIALS AND METHODS

Isolation of Frankia

Nodules of *C. equisetifolia* were collected from plantation at Panampalli, Kerala (INDIA) lies between (76 ° 44' E latitude, 10° 47' N longitude). From the nodules the Frankia was isolated using Diem&Dommergues [6] method.

Frankia culture

Frankia strain was grown in static BAP minimal medium supplemented with sodium pyruvate as carbon source [11], and maintained at 28 ºC for 4 weeks. The homogenized cell suspension was lyophilized and used for the present study.

Sample preparation

Total Lipid Extraction

Different days *Frankia* culture were extracted according to ($CHCl_3/CH_3OH/H_2O$, 2:2:1·8, by vol., 38 ml) Bligh&Dyer method [3]. A total lipid extracted from the sample has evaporated to dryness. The residue was dissolved in hexane, filtered through solid phase extraction column.

GC-MS –MS Conditions

GC–MS-MS analysis of the extracts was conducted on a Varian 4000 GC system (split less injector, 315°C) linked to a MS/ MS detector (interface temperature 300°C; source temperature 210°C; electron energy 70 eV). A VF-5ms (30 m, 0.25 mm thickness) was used with helium as the carrier gas. The oven temperature was programmed at 60°C (held for 1 min), from 60 to 100°C at 10°C/min and from 100 to 300°C at 5°C/min (held for 35 min). The mass spectrometer was operated in full scan mode (m/z 50–900; 0.2 s).

RESULTS AND DISCUSSION

The chemical composition of the Frankia cultures was found to be a mixture of mono- and sesqui-terpenoids, and fatty acids (Table 1). Over 40 compounds were detected from different day culture fractions of Frankia using MS-MS (Fig. 1). A total of 13, 23 and 14 components were identified in 15, 25 & 30th day cultures respectively. The GC-MS-MS analysis indicates that there are variations in the presence of signaling molecules in different days of Frankia culture. Hexa decanoic acid, Phthalic acid and its ester derivatives are present in 25th and 30th day culture. A wide variety of bioactive compounds were produced by cyanobacteria [13] and a thermo tolerant bacterium produces a bioactive compound such as biocatalysts [9]. The terpine derivatives (Hopanoids) Viminalol and β-Amyrine are present in 15th day cultures which are essential for nodulation and nitrogen fixation in *C. equisetifolia.* [2] reported that the Frankia are known to contain a large percentage of hopanoids, the main lipid component of the vesicles, which are essentially produced under nitrogen-fixing conditions. Hopanoids thicken and stabilize the walls which help to keep oxygen away from the nitrogenase, [2] the hopanoids themselves play a more specific and molecular role in the oxygen protection mechanism of nitrogenous [8]. Phenol,2,4-bis(1,1-dimethylethyl)- is the only compound identified in both 15th & 25th day culture but lacking in 30th day culture, where as all other compounds identified are unique and present either any one of the culture. The bioactive compounds isoterpinolene, 2, 4, Phenol-bis (1, 1-dimethyl ethyl) and 1-Dotriocantanol were eluted at Rt 12.468, 18.126 19.105 and respectively. [5-14] reported that the compound found to have free radical scavenging activity. Antioxidants are the compounds with free radicals scavenging activity and capable of protecting the cells from free radical mediate oxidative stress. The antioxidant compounds can be derived from natural sources such as plants[7].Since oxygen inactivate nitrogenase activity, the plant produces those compounds having high affinity to oxygen originated around the root prevent it to reach the nitrogenous complex. In the present study, it is found that the Frankia has produced a complex of bioactive compounds, such as hoponoids and terpenoids at different stages during its growth period. When Frankia retained at 15th day from the date of inoculation, which are reported to produce vesicles in roots of *C. equisetifolia*. However the compounds such as isoterpinolene, 2, 4, Phenol-bis (1, 1-dimethyl ethyl) and 1-Dotriocantanol are produced in later stages of Frankia growth period tend to restrict the oxygen disturbances in the nitrogenous activity and thereby enhancing nodulation and nitrogen fixation in *C. equisetifolia.* The present study is a preliminary observation and is an eye opening to study in details about the nodulation capacity of *Frankia* in *C.equisetifolia.* Further screening of enhancing ability of those molecules in the field nursery seedling condition is under investigation.

Table 1. GC MS MS Profile of Frankia Culture

Retention time	Name of the compound	15th day	25th day	30th day	Peak Area	Molecular weight	Molecular formula
3.172	Silanediol dimethyl	-	-		3.172	92	$C_2H_8O_2SI$
5.241	Oxime-methoxy-phenyl	-		-	3.235	151	$C_8H_9NO_2$
5.548	3,4-dimethoxy-1-pentene	-		-	6.775	130	$C_7H_{14}O_2$
11.535	Benzaldehyde,2,4-dimethyl	-		-	4.977	134	$C_9H_{10}O$
12.067	2-Propanoic acid ethyl ester		-	-	2.736	100	$C_{10}H_{20}$
12.468	Isoterpinolene	-		-	7.547	136	$C_{10}H_{16}$
13.220	1,2,3,5 tetra methyl cyclohexane (1R,2C,3C)		-	-	7.512	140	$C_{10}H_{20}$
13.425	1,2,3,5 tetra methyl cyclohexane (1R,2C,3C)		-	-	1.037	-	-
13.619	1,2,3,5 tetra methyl cyclohexane (1R,2C,3C)		-	-	6.884	-	-
13.677	Benzene acetic acid, α-methoxy methyl ester	-		-	4.017	180	$C_{10}H_{12}O_3$
16.945	Cyclohepta siloxane, tetradecamethyl	-		-	4.679	518	$C_{14}H_{42}O_7S$
17.161	2,5-cyclohexadiene-1,4-dione-2,6-bis(1,1-dimethylethyl)-CAS	-		-	2.014	220	$C_{14}H_{20}O_2$
18.126	Phenol,2,4-bis(1,1-dimethylethyl)-CAS		-	-	3.467	206	$C_{14}H_{22}O$
18.138	Phenol,2,4-bis(1,1-dimethylethyl)-CAS	-		-	1.430	-	-
18.138	3-[(1-methyl-1H-pynl-2-yl) methylene]-2,4-(3H,5H)-furandione	-	-		2.046	191	$C_{10}H_9NO_3$
18.246	1,2,3,5 tetra methyl cyclohexane (1R,2C,3C)		-	-	3.472	-	-
18.472	1,2,3,5 tetra methyl cyclohexane (1R,2C,3C)		-	-	4.257	-	-
18.647	1-Dotriacontanol		-	-	7.045	466	$C_{32}H_{66}O$
18.879	Isotrideconol		-	-	5.953	140	$C_{13}H_{28}O$
18.979	2,6-di-(t-butyl-4-hydroxy-4-methyl)-2,5-cyclohexadiene-1-one		-	-	3.718	296	$C_{21}H_{44}$
19.105	1-Dotriacontanol		-		3.547	-	-
19.139	(1SR,4SR,5RS)-4-methyl-3-oxabicycol(3,2,0) hepta -2-ol	-			2.191/ 1.505	128	$C_7H_{12}O_2$
20.027	3-n-Hexyltriolane,s,s-dioxide		-	-	3.610	204	$C_{10}H_{20}O_2S$
20.920	Cis-4b, 5,9b,10-tetrahydro-4b,8,9b-trimethylindenol[1,2-b] indole	-	-		1.219	249	$C_{18}H_{19}N$
22.938	Phenol-2-(1-phenyl ethyl)	-		-	2.896	198	$C_{14}H_{14}O$
23.230	2,2-di-O-methylimbricaric acid	-	-		1.048	235	✓
23.231	Methylhexo-8-cyano-4-4-dimethyl-3-oxo-ol	-		-	1.692	235	$C_{13}H_{14}NO_3$
24.339	3-n-Hexyltriolane,s,s-dioxide		-	-	3.649	204	$C_{10}H_{30}O_2S$
24.727	1-Amino-3,4-dihydro-(3,7) dimethyl-2-naphthalene carbonitrile	-		-	1.471	198	$C_{13}H_{14}N_2$
25.768	Phthalic acid, pentyltridex-2-yn-1-yl-ester	-		-	8.529	414	$C_{26}H_{38}O_4$
25.770	(3R,4S)-3-(2-Notro-4-methoxy phenyl)-4-(4-hydroxy phenyl) hexane	-	-		5.714	329	$C_{19}H_{23}NO4$
26.451	2,3,5,8-tetrahydroxy-6-methyl anphthol-1-4-puinone	-		-	6.983	236	$C_{11}H_8O6$
26.685	7,9-di-tert-butyl-1-oxaspiron (4,5) deca-6,9-diene-2,8-dione	-	-		3.882	276	$C_{17}H_{24}O3$
26.999	Hexa decanoic acid, methyl ester(CAS)	-	-		1.018	270	$C_{17}H_{34}O2$
27.121	Benzene propanoic acid, 3-5-bis-(1,1-dimethyl ethyl) hydroxyl methyl ester	-	-		2.212	292	$C_{18}H_{28}O3$
327.641	Phthalic acid, butyl-2-pentyl ester	-	-		5.754	292	$C_{17}H_{24}O4$
27.645	Phthalic acid, pentyltridex-2-yn-1-yl-ester	-		-	2.086	414	$C_{26}H_{38}O4$
27.708	Hexa decanoic acid	-	-		4.020	256	$C_{16}H_{32}O_2$
28.293	Hexa decane -1,2-diol		-	-	3.071	258	$C_{16}H34O2$
28.622	Phenol-2-[(4-hydroxy phenylmethyl-)]	-		-	6.271	200	$C_{13}H12O2$
29.399	Quinolphos		-	-	4.840	298	$C_{12}H15N2O$
29.866	Phenol-(4,4-methylene bis-)CAS	-		-	6.718	200	$C_{13}H12O2$
30.264	2,3, Dihydroxy-propyl elaidate	-		-	5.053	356	$C_{21}H40O4$
35.534	Phenol-2,4-bis-(1-phenlyethyl)-	-		-	3.306	302	$C_{22}H22O$
35.803	Phenol-2,4-bis-(1-phenlyethyl)-	-		-	3.136	-	-
36.690	Phenol-2,4-bis-(1-phenlyethyl)-	-		-	5.455	-	--
37.114	Hexa decanoic acid, 2-hydroxyl-1-(hydroxyl methyl) ethyl ester	-	-		1.209	330	C19H38O4
37.383	1,2benzenedicarboxylic acid		-	-	1.585	330	C20H26O4
37.411	2-beta-(3'-oxobutyl-alpha 3,3-trimethyl-7-oxabicyclo (2,2,1) heptane	-	-		2.804	210	C13H22O2
37.418	2,Beta-(3-Oxobutyl-1-α-3,3-trime)	-		-	4.408	210	C13H43NO
40.996	13-Dococenamide,(2)	-		-	1.002	337	C22H43 N0
41.377	1,6,10,14,18,22-tetracosa hexane-3-ol-2		-	-	7.851	426	C30H50O
43.111	β-Amyrin				5.161	4.26	C30H50O
44.172	Viminalol		-	-	1.437	4.26	C30H50O

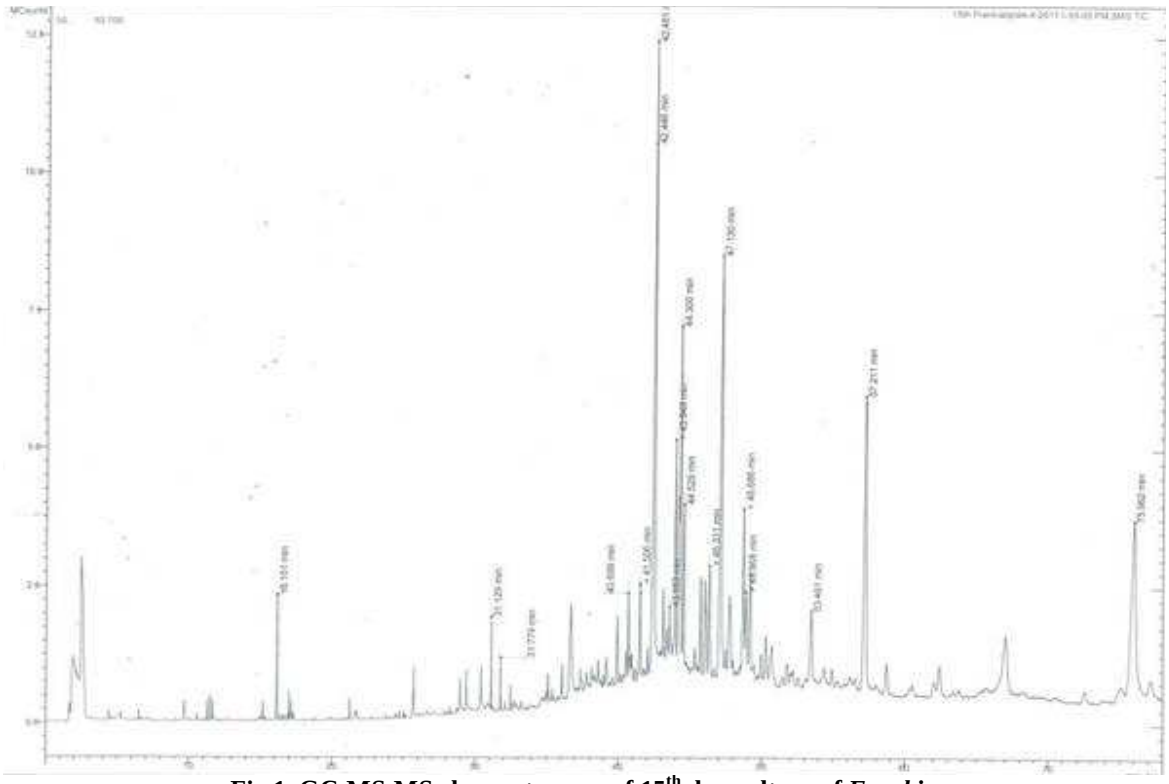

Fig.1. GC-MS-MS chromatogram of 15th day culture of *Frankia*

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