Journal of Applied and Natural Science 9 (2): 954 - 959 (2017)

JANS

Characterization of volatile secondary metabolites from *Trichoderma asperellum*

Nitish Rattan Bhardwaj[1*] and J. Kumar[2]

[1]Crop Improvement division, ICAR-Indian Grassland and Fodder Research Institute, Jhansi-284003 (Uttar Pradesh), India
[2]Department of Plant Pathology, G.B. Pant University of Agriculture and Technology, Pantnagar-263145 (Uttarakhand), India
*Corresponding author. E-mail: nitish.rattanbhardwaj@gmail.com

Received: June 6, 2016; Revised received: March 28, 2017; Accepted: May 1, 2017

Abstract: Many *Trichoderma* isolates are known to secrete several secondary metabolites with different biological activities towards plants and other microbes. The production of such compounds varies according to the strain. In the present study, volatile secondary metabolites from the culture filtrate of *Trichoderma asperellum* strain were characterized using Gas chromatography-Mass spectrometry (GC-MS). Results of GC-MS detected 43 secondary metabolites in the *T. asperellum* strain including many important volatile secondary metabolites such as 1,2-Benzenedicarboxylic acid, 2-butoxy-2-oxoethyl butyl ester (peak area-3.59%), 1,2-Benzenedicarboxylic acid dibutyl ester (peak area-2.02 %), 2H-Pyran-2-one (peak area-66.63 %), palmitic acid (peak area-2.86 %), several phenolic isomers, methyl cyclohexane etc., all reportedly having effective pesticidal activity. The results indicated that these secondary metabolites could be useful for biological control applications of *T. asperellum* strain against diverse plant pathogens.

Keywords: GC-MS, Metabolites,*Trichoderma*, Volatile

INTRODUCTION

Trichoderma spp. are present in nearly all types of soil and other diverse habitats. In relation to other fungi in soil, these are the most prevalent fungi belonging to the genus *Trichoderma* under Deuteromycotina, Hyphomycetes, Moniliales, and Moniliaceae. This genus comprises large number of fungal strains like *T. asperellum*, *Trichoderma atroviride*, *T. harzianum*, *T. hamatum*, *T. koningii*, *T. virens* and *T. viride*, widelyused as bio-control agents of plant diseases incited by fungal and oomycete pathogens and in addition these are found effective in increasing plant growth and development (Harman and Bjorkmann, 1998; Singh *et al*. 2006, Shoresh *et al*., 2010), Viterbo & Horwitz, 2010, Tucci *et al*., 2011). *Trichoderma* strains exhibit biocontrol activity against fungal phytopathogens either indirectly, by competing for nutrients and space, modifying the environmental conditions, promoting plant growth and plant defensive mechanisms and antibiosis, or directly by mechanisms such as mycoparasitism. These indirect and direct mechanisms may act synergistically and their importance in the biocontrol process depends on the *Trichoderma* strain, the antagonized fungus, the crop plant, and the environmental conditions, including nutrient availability, pH, temperature, and iron concentration (Harman *et al*., 2004). *Trichoderma* species have many characteristics that make them of significant interest to the research community. Among these characteristics is the production of natural products or secondary metabolites. These secondary metabolites of volatile or non-volatile nature, often have obscure or unknown functions that are of considerable importance to humankind in medical, industrial or agricultural applications.Secondary metabolic compounds appear as intermediate or end products of heterogenous metabolic pathways and belong to various structural classes such as mono- and sesquiterpenes, ketons, lactones, alcohols and esters compounds (Schnurer*et al*., 1999; Korpi *et al.,* 2009). These secondary metabolites from *Trichoderma* spp. are involved in different biological processes like biocontrol between microorganisms and pathogens (Howell, 2006) mediating resistance against parasites and diseases (Leitgeb*et al*., 2007; Reithner*et al*., 2005, 2007,Viterbo*et al*., 2007) or they may be produced to enhance competition between species and in order to facilitate reproductive processes (Sivasithamparam and Ghisalberti, 1998). Secondary metabolites from *Trichoderma* act against plant pathogens and can have plant growth promoting (Vinale*et al*., 2008) and resistance inducing effects on plants, thus making plants less susceptible to fungal pathogens (Harman *et al*., 2004). Volatile secondary metabolites produced by *Trichoderma* spp. include

compounds such as pyrones (Claydon*et al*., 1987), anthraquinone, butenolide (Almassi*et al*., 1991), cyclopentyl isocyanide, isonitrine-type compounds and peptaibols (Claydon *et al*., 1987; Goulard *et al*., 1995; Hlimi *et al*., 1995) which have been reported to play vital role in managing the plant pathogens like *Gaumannomyces graminis* var. *tritici* (Ghisalberti *et al*., 1990), *Rhizoctonia solani* and *Fusarium oxysporum*f.sp. *lycoersici* (Scarselletti and Faull, 1994) and *Phytohthora* (Reino*et al*., 2008). The production of volatile secondary metabolites varies between different *Trichoderma* strains and *Trichoderma* strains with effective secondary metabolites are potential candidates for the biological control of plant diseases as these could be exploited for management of plant pathogens.Therefore, it is essential to characterize the volatile secondary metabolites produced by a particular *Trichoderma* strain, so that its candidature as an efficient biocontrol strain could be proved. Keeping above things in mind, present study was conducted to characterize volatile secondary metabolites produced by *T. asperellum* strain through gas chromatography - mass spectrometry (GC-MS).

MATERIALS AND METHODS

T. asperellum strain was obtained from culture repository of biocontrol lab, Department of Plant Pathology, G.B.P.U.A.& T., Pantnagar. Liquid culture of *T. asperellum* strain was analyzed for the presence of secondary metabolites by using chromatographic analysis followed by Mass spectrometry for the identification of separated components. Procedure followed for extraction of secondary metabolites was adopted from Siddiquee *et al*. (2012) with few modifications. Briefly, *T. asperellum* strain was grown on potato dextrose broth (PDB) at 25±1°C for 25 days. Culture filtrate was extracted by straining through muslin cloth. Metabolites were extracted by solvent extraction method into hexane in the ratio of 1: 1 (v/v). Solvent (hexane) was evaporated from the solution using rotary evaporatorwith a rotor speed of 120 rpm at 40^0C until the residues were visible. Obtained residues obtained were re-suspended in solvent (acetone) for further characterization by GC-MS. GS-MS analysis was performed in GCMS-QP2010 Plus ultra. The column temperature settings were programmed to begin with 80^0C for 2 minutes, followed by an increase at a rate of 10^0C/min. till 250^0C followed by final injection temperature of 280^0C. The linear velocity of carrier gas was 40.5 cm/sec. Samples were injected by splitless mode with sampling time of 1 minute. The ionization for MS detection was performed with ion source temperature of 230^0C and interface temperature of 270^0C. Starting time for acquisition after injection was 5 min. and end time was 44.49 min. The detected compounds were identified by matching the electron impact spectra against the National institute of standards and technology (NIST) library.

RESULTS AND DISCUSSION

Volatile secondary metabolites have been attributed to play a key role in the mycoparasitism of *Trichoderma*

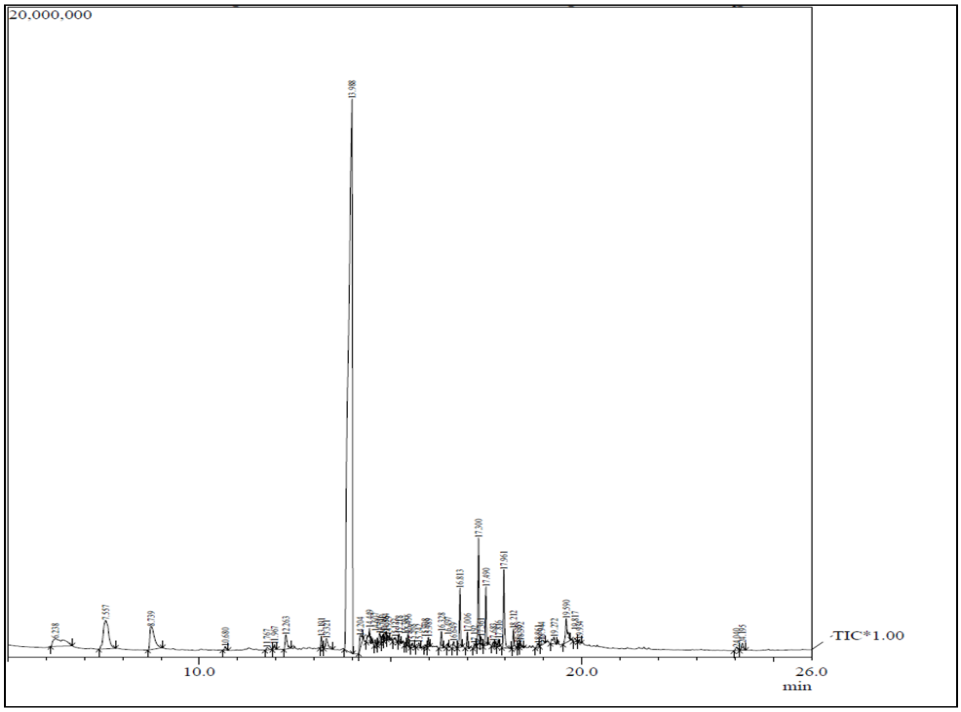

Fig. 1. *Chromatogram showing major secondary metabolites produced by T. asperellum strain obtained through Gas chromatography-mass spectrometry*

Table 1. Major secondary metabolites produced by *T. asperellum* strain along with their characteristics.

Sr. No.	Molecular formula	Molecular weight	Peak area (%)	Retention time	Name of compound
1.	C_7H_9N	107	2.69	6.238	p-Aminotoluene
2.	$C_7H_7NO_2$	137	4.66	7.557	1-Methyl-2-Nitrobenzene
3.	C_9H_7N	129	3.28	8.739	Isoquinoline
4.	$C_{14}H_{30}$	198	0.17	10.680	Tetradecane
5.	$C_9H_{18}FO_2P$	208	0.17	11.767	Cyclooctylmethylphosphonofluoridoate
6.	$C_{17}H_{36}$	240	0.24	11.967	Heptadecane
7.	$C_{10}H_{14}O_2$	166.2	66.63	55.962	2H-Pyran-2-one
8.	$C_{14}H_{24}O$	208	0.27	14.607	6,8,9-Trimethyl-4-propyl-3-oxabicyclo [3.3.1] non-6-ene
9.	$C_{11}H_{16}O$	164	0.56	14.716	p-tert-Amylphenol
10.	$C_{15}H_{24}O$	220	0.27	14.791	4-Nonylphenol
11.	$C_{18}H_{30}O$	262	0.32	14.864	4-Dodecylphenol
12.	$C_{12}H_{18}O_2$	194	0.16	14.929	1,3-Cyclohexadiene-1-carboxylic acid, 2,6,6-trimethyl-, ethyl ester
13.	$C_{15}H_{24}O$	220	0.34	15.123	o-Cresol, 4-(1,1,3,3-tetramethylbutyl)-
14.	$C_{14}H_{22}O$	206	0.33	15.218	4-(1,1,3,3-Tetramethylbutyl)Phenol
15.	$C_{15}H_{16}N_4O_2$	284	0.09	15.383	1H-1,2,3-Triazolo[4,5-c]quinoline-1-hexanoic acid
16.	$C_{20}H_{42}$	282	0.34	15.456	n-Eicosane
17.	$C_{16}H_{34}$	226	0.19	15.568	5-Ethyl-5-propylundecane
18.	$C_{16}H_{19}NO_5$	305	0.25	15.742	N-(1-naphthyl)-1-deoxy-1-amino-beta-d-idopyranose
19.	$C_{18}H_{38}$	254	0.11	15.908	7,9-Dimethylhexadecane
20.	$C_{17}H_{36}O_2Si$	300	0.31	15.989	Tetradecanoic acid, trimethylsilyl ester
21.	$C_{16}H_{22}O_4$	278	0.66	16.328	1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester
22.	$C_{23}H_{48}$	324	0.13	16.497	N-Tricosane
23.	$C_{12}H_{13}N$	171	0.31	16.649	8-Propylquinoline
24.	$C_{16}H_{22}O_4$	278	2.02	16.813	1,2-Benzenedicarboxylic acid, dibutyl ester
25.	$C_{20}H_{30}O_4$	334	0.53	17.006	1,2-Benzenedicarboxylic acid, butyl octyl ester
26.	C22H46	310	0.13	17.192	Docosane
27.	$C_{18}H_{24}O_6$	336	3.59	17.300	1,2-Benzenedicarboxylic acid, 2-butoxy-2-oxoethyl butyl ester
28.	$C_{20}H_{30}O_4$	334	0.21	17.361	Butyl 2-ethylhexyl phthalate
29.	$C_{22}H_{34}O_4$	362	1.90	17.490	1,2-Benzenedicarboxylic acid, butyl 8-methylnonyl ester
30.	$C_{18}H_{26}O_4$	306	0.19	17.683	Diamyl phthalate
31.	$C_{20}H_{30}O_4$	334	0.21	17.816	Butyl 2-ethylhexyl phthalate
32.	$C_{19}H_{40}O_2Si$	328	2.86	17.961	Trimethylsilyl palmitate

Contd.

Table 1. *Contd.*

33.	$C_{19}H_{28}O_4$	320	0.74	18.212	Phthalic acid, 5-methylhex-2-yl butyl ester
34.	$C_{20}H_{30}O_4$	334	0.12	18.335	Di-hexylphthalate
35.	$C_{22}H_{34}O_4$	362	0.24	18.392	1,2-Benzenedicarboxylic acid, diheptyl ester
36.	$C_{19}H_{28}O_4$	320	0.19	18.861	Phthalic acid, 5-methylhex-2-yl butyl ester
37.	$C_{18}H_{34}O_2$	282	0.40	18.944	cis-Vaccenic acid
38.	$C_{20}H_{40}O_2$	312	0.51	19.271	Hexadecanoic acid, 1,1-dimethylethyl ester
39.	$C_{21}H_{42}O_2Si$	354	1.57	19.590	trans-9-Octadecenoic acid, trimethylsilyl ester
40.	$C_{27}H_{56}O_2Si$	440	0.19	19.817	Trimethylsilyltetracosanoate
41.	$C_{14}H_{26}O$	210	0.11	19.934	7-Tetradecenal
42.	$C_{23}H_{32}O$	324	0.22	24.040	2-[4-methyl-6-(2,6,6-trimethylcyclohex-1-enyl)hexa-1,3,5-trienyl] cyclohex-1-en-1-carboxaldehyde
43.	$C_{24}H_{38}O_4$	390	0.41	24.195	Di-n-octyl phthalate

and its interaction with plant system (Vey *et al*.,2001). Results of the present study revealed that *T. asperellum* strain produce many important secondary metabolic compounds. A total of 43 volatile compounds were detected from culture filtrate of *T. asperellum* strain which were further characterized after matching the electron impact spectra against NIST library. Major compounds identified were 1-Methyl-2-Nitrobenzene, 2H-Pyran-2-one, Isoquinoline, 1,2-Benzenedicarboxylic acid, dibutyl ester, 1,2-Benzenedicarboxylic acid, 2-butoxy-2-oxoethyl butyl ester, 1,2-Benzenedicarboxylic acid, butyl 8-methylnonyl ester, Trimethylsilyl palmitate, trans-9-Octadecenoic acid, trimethylsilyl ester, p-Aminotoluene, several phenol isomers (p-tert-Amylphenol, 4-Nonylphenol, 4-Dodecylphenol, 4-(1,1,3,3-Tetramethylbutyl) Phenol), n-Eicosane, 8-Propylquinoline, 5-Ethyl-5-propylundecane, Tetradecane, Heptadecane, Cyclooctyl methyl phosphono-fluoridoateetc (Table 1, Fig. 1). In this study, major volatile secondary metabolic compound identified was 2H-Pyran-2-one, that has been reported to helpful in mycotoxin detoxification (Cooney *et al*., 2001), antifungal (Scarselletti and Faull, 1994, Tarus*et al*., 2003) and was also found to have some role in plant growth promotion activity as reported in wheat and tomato (Vinale *et al*., 2008) suggesting that this *T. asperellum* have the ability to restrict pathogen growth as well as to have a profound effect on the growth parameters of plant system. In addition to 2H-Pyran-2-one, important volatile compound like diethyl phthalate, 1,2-benzenedioxylic acid esters, tetradecanoic acid were also identified from culture filtrate of *T. asperellum* strain. These compounds were reported to be responsible for enhanced biocontrol activity of *T. harzianum* against *Fusarium oxysporum* (Senthilkumar *et al*., 2011**).** Siddiquee *et al*. (2012) identified more than 278 volatile compounds (with spectral match factor at least 90%) such as normal saturated hydrocarbons (C7–C30), cyclohexane, cyclopentane, fatty acids, alcohols, esters, sulfur-containing compounds, simple pyrane and benzene derivatives from liquid cultures of *T. harzianum* using GC-MS by use of three different capillary columns**.** Many of these volatile compounds as reported by Siddiquee *et al*. (2012) were also found in liquid culture filtrate of *T. asperellum* strain. Isoquinoline, a volatile compound with antiprotozoal activity (Osorio*et al*., 2008) was also detected in culture filtrate of this strain. This compound was earlier reported from *penicillium pucillum* (Tajick Ghanbari *et al*., 2014). Dubey *et al*. (2011) characterized volatile secondary metabolites from *Trichoderma* sps. through GC-MS/MS and identified certain compounds like 3-methyl-heptadecanol, methyl cyclohexane, 6-nonylene alcohol, methyl-cyclopentane, 2-methyl heptadecanol, N-methyl pyrollidine, dermadin, ketotriol, koningin-A,

palmitic acid, 3-(2'-hydroxypropyl)-4-(hexa-2'-4-dineyl)-2-(5H)-furanone and 3-(propenone)-4-(hexa-2'-4'-dineyl)-2-(5H)-furanone and attributed the antifungal activity of tested *Trichoderma* spp. to these compounds. In the present study, some of these volatile compounds as reported by Dubey *et al*. (2011) were identified from culture filtrate of *T. asperellum* strain.

Conclusion

From the study, it can be concluded that *T. asperellum* strain harbours many important volatile secondary metabolites that have been reported to perform diverse functions ranging from anti-pathogenic to plant growth promotion. Thus, this *T. asperellum* strain could be further exploited for management of plant pathogens as well as to have a positive effect on the plant growth for attaining higher yield.

ACKNOWLEDGEMENTS

Senior author thanks Department of Science and Technology for financial assistance in the form of INSPIRE-fellowship (No. DST/INSPIRE Fellowship/2014/IF140216).

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