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Secondary Metabolites Approach to Study the Bio-Efficacy of *Trichoderma asperellum* Isolates in India

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A B S T R A C T

10 isolates of *Trichoderma asperellum* was used for characterization of secondary metabolites through gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS) analysis to establish valid correlation between the production of antifungal metabolites and their bio-efficacy as BCAs. The investigation revealed that the culture filtrate of *T. asperellum* isolates were showed the presence of 673 secondary metabolites at different retention time with a range of 39 (Ta-20) to 101 (Ta-12) with GC-MS. Out 673 volatile metabolites, 55 metabolites were found to be most abundant from which seven metabolites from Ta-14 and Ta-20, six metabolites from Ta-8, Ta-17 and Ta-29, five metabolites from Ta-45, Ta-15, Ta-10 and Ta-12 and remaining three metabolites from Ta-2 isolate respectively. Further, the five isolates *viz.*,Ta-2, Ta-8, Ta-10, Ta-20 and Ta-45 were used for the LC-MS and study showed the presence of nine antifungal metabolites viz., Viridin, Viridiol, Butenolides, Harzianolides, Ferulic acid, Viridiofungin A, Cyclonerodiol, Massoilactone and Gliovirin. Hence, these isolates were produced highest number of major volatile and antimicrobial compounds. Therefore, these isolates viz., Ta-45, Ta-10, Ta-20, Ta-8, and Ta-2 were considered as high potential bio-control agents against *Sclerotium rolfsii* pathogens.

Introduction

The worldwide 1.5 million fungal species were identified and among them around 10% have been discovered and described. Out of 10%, only1% fungal species has been examined for secondary metabolites based on characterization (Weber *et al*., 2007). The *Trichoderma* species has various features that could helpful for researcher's community. Amidst these diverse characteristics, which involved in production of abundant secondary metabolite compounds and some compounds are known function and rest of compounds often have vague or unidentified its functions in the organism and which are significant importance to humankind in a different field such as agricultural applications, industrial and medical. The fungus produced certain volatile compounds and these volatile

compounds are commonly used as antibiotic as well as immunosuppressant activities (Srinivasa *et al*., 2014).

Trichoderma viride is the most widely used as a fungal an atagonist not only in India and other countries also. The most of *T. Viride* isolates have been submitted in gene bank; from which India are actually known as *Trichoderma asperellum* or its cryptic species (*T. asperelloides*). Sriram *et al*., 2013, characterized *Trichoderma* spp. by morphologically and also amplified the ITS and tef1 regions using oligonucleotide bar-code. Antibiosis is a key role for antagonistic interactions amid micro-organisms and with adequate production of antibiotic (by *Trichoderma* spp.), could be utilized as biological control agents against several plant-pathogenic fungi (Weindling *et al*., 1936). Though, the role of antibiosis in bio-control needs to be intensely explored, because of huge number of *Trichoderma* species and its strains could yield large number of antibiotics as well as secondary metabolite compounds. The fungus has a potentiality to produce volatile compounds such as, ethylene, hydrogen cyanide, alcohols and ketones and non-volatile compounds like peptides; hence these compounds are effectively inhibit the mycelial growth of disease causing fungi. Therefore, the *Trichoderma* spp. has an ecological advantage in soil and the rhizosphere of cultivated crop plants as well a strees spp. (Harman *et al*., 2004; Schnurer *et al*., 1999).

The *Trichoderma* spp has produced various volatile compounds and which are physiologically active; hence, these compounds were involved in signaling transduction in the microbial kingdom. Galindo *et al*., 2004, well-described 6-pentyl-a-pyrone (6-PAP) as a volatile product of secondary metabolism and this compounds act as herbicide and antimicrobial. In addition to, Combet *et al*., 2006, was reported, eight carbon volatile compounds such as 1-octen-3-ol, 3-octanone, 3-octanol and 1-octen-3-one and these compounds are typical mushroom components and they play important role such as insect attractants, exhibit fungi-static and fungicidal effects (Chitarra *et al*., 2004; 2005; Okull *et al*., 2003).

Sclerotium rolfsii is a one of the highly destructive soil borne plant pathogen and which causes destructive diseases in more than 500 plant species. Hagan (1999) reported that, *S. rolfsii* as well as root knot nematode were caused exceedingly damages in southern USA. This fungus causes diseases in many crops viz., tomato, cucumber, brinjal, soybean, maize, groundnut, bean, watermelon, etc. this fungus causes various types of diseases viz., collar rot, sclerotium wilt, stem rot, charcoal rot, seedling blight, damping-off, foot-rot, stem blight and root-rot in various economically valued crops (Dwivedi *et al*., 2016).

The advent of molecular biology era would support in the identification of known as well as unknown secondary metabolite compounds. The Gas Chromatographic (GC)-Mass Spectrometric (MS) and Liquid Chromatographic (LC)-Mass Spectrometric (MS) methods are recent and extensively used techniques for the analysis of volatile and also antifungal compounds in biological systems (Namera *et al*., 1999; Ramos *et al*., 1999; Tarbin *et al*., 1999; Mohamed *et al*., 1999; Pichini *et al*., 1999). These methods have been involved different mechanisms or process such as extraction, separation, purification and characterization of any compounds.

Metabolomic approach in the present study revealed the metabolites profile to understand its bio-control, biomass degradation and human pathogenicity potentiality of the *T.*

asperellum isolates present in India. A total of 10potential isolates of *T. asperellum* were selected based on its bio-efficacy and were further characterized for secondary metabolites through GC-MS and LC-MS analysis techniques to establish valid correlation between the production of antifungal metabolites and their bio-efficacy as BCAs.

Materials and Methods

Bio-efficacy of *Trichoderma asperellum* isolates against *Sclerotium rolfsii*

10 isolates of *Trichoderma asperellum* were procured from Indian Institute of Horticultural Research (IIHR), Bengaluru (Table 1) and these potential isolates were tested for their bio-efficacy in *in-vitro*condition against *Sclerotium rolfsii* at IARI, New Delhi.

Dual culture method

The isolates (*Trichoderma*) and test fungus (*Sclerotium rolfsii*) were grown on potato dextrose agar (PDA) @ 28±20 ^{0}C for a week. The target fungus and *Trichoderma* mycelium were cut from its periphery with 5mm disc and transferred to sterilized petri plates which encompass PDA media. Each plate consists of two discs, one from *Trichoderma* and other from test pathogen and both the discs were placed 7cm away from each other. All the plate kept for incubation @ 28±20 ^{0}C and observed growth of antagonist and test fungus (after eight days). The index of antagonism as percent mycelium growth inhibition of test pathogens was calculated as per ref.

Characterization of secondary metabolites of *T. asperellum* isolates

A total of 10 isolates of *T. asperellum* were used for characterization of secondary metabolites with recent and widely used GC-MS and LC-MS techniques.

Cultivation of isolates

The potential bio-control *T. asperellum* isolates obtained from the earlier studies were grew for 5 days on PDA media at 30±2^0 C. The isolates mycelium (5mm in diameter) was inoculated in a flask containing 250 ml of potato dextrose broth (PDB). The flask mouth was plugged using cotton wool, wrapped and sealed using aluminum foil and Para film respectively. The flasks were incubated @ 30±2^0 C (12h darkness, 12h light) on rotary shaker for 21 days @ 120 rpm.

Extraction and separation of antifungal metabolites

The culture filtrate of *T. asperellum* was obtained by straining through the muslin cloth. A 225ml aliquot of ethyl acetate added into inoculums cultured in a 1000 ml Erlenmeyer flask and the flask was kept overnight to ensure that the fungal cell died. Next day, culture filtrate was filtrated using Buchner vacuum funnel and filtrated culture was collected along with ethyl acetate phase, water phase and rest of cell debris (mycelium) was thrown away.

The ethyl acetate phase and with other polar constituents were separated from the water phase (medium) with the help of Buchner vacuum separation funnel and along with the sodium sulphate salt. The water phase was evaporated using rotary evaporated shaker @ 40^0 C. immediately after evaporation; the polar constituents were collected in ethyl acetate extract. The extracted solvents were diluted in 100ml of n-hexane to remove fatty acids and other non-polar elements, and then prepared 1000ppm extracted compounds with hexane solvent (n- hexane extract). The acetonitrile layer of the culture filtrate was used to perform GC-MS and LC-MS analysis immediately or it can be stored in the deep freezer at -20^0 C.

Isolation of volatile compounds from isolates

Isolation of volatile compounds was performed (Yang *et al*., 2009) with some modifications. The SPME fibre coated with carboxan-polydimethyl siloxane-divinylbenzene (50/60µm, CAR/PDMS/DVB; Supelco, Bellefonte, PA, USA), used for the analysis, because of its high sensitivity towards aroma compounds and excellently reproducible. The 1 g each *T. asperellum* isolate was homogenized with 100 ml double distilled water using a commercial blender. The slurry was transferred to a 250 ml conical flask and 5 g of NaCl was added. Subsequently, the flask was sealed with a teflon-lined septum and the samples were kept stirred @ 37±1°C. After 20 min of equilibration between the solution and the headspace, the fibre was exposed to the headspace of sealed flask for 60 min. prior to sampling. Further, the fibre was preconditioned for 1hr @ 260°C in the GC injection port as per instructions of the manufacturer's.

Gas chromatography

Gas chromatography GC-FID analysis was carried out by a Varian-3800 gas chromatograph system with SPME sleeve adapted to injector on a VF-5 column (Varian, USA), 30 m x 0.25 mm i.d, and 0.25 µm film thicknesses. The helium gas was used as a carrier; along with flow rate of 1ml min^{-1}; injector 250 °C and detector 260°C temperatures. The column temperature for program as follows: The 40 °C for 4 min was initial oven temperature and time, subsequently it was increased 3 °C /min up to 180 °C, held for 2 min, further the temperature has increased at 5 °C/min until it reach to 230 °C and maintained constant time for 5 min. For desorption, the SPME device was introduced in the injector port for chromatographic analysis and remained in the inlet for 15 min. Initially injection mode was split-less and then, split mode (1:5) after 1.5 minutes. For the qualitative identification of volatile substances and computation of retention time and index, the following standards, ethyl acetate, propanol, isobutanol, hexanol, 1-octene-3-ol and eugenol were co-chromatographed.

GC-MS techniques

The Varian-3800 gas chromatograph coupled with Varian 4000 GC-MS/MS mass selective detector was used to perform GC-MS analysis. The VF-5MS (Varian, USA), column (30 m x 0.25 mm ID with 0.25 µm film thickness) were used for separation of volatile compounds by applying the same temperature programme as mentioned in GC-FID analysis. The Mass detector was used for separation of volatile compounds and this mass detector conditions were: EI-mode at 70 eV, injector, 250 °C; ion source, 220 °C; trap, 200 °C; transfer line, 250 °C and full scan range, 50–450 amu. The helium gas (carrier gas) and a flow rate of 1 ml.min-1. 2.5 were used for the identification of components of the volatile compounds. The identified volatile compounds were compared with the mass spectra and the data system libraries (Wiley-2009 and NIST-2007).

LC-MS techniques

LC-MS parameters i.e. Ultra Performance Liquid Chromatography (UPLC) was performed on an Acquity H-Class® UPLC system (Waters Corporation, Milford, USA);equipped with a quaternary solvent manager, an auto-sampler maintained at 4°C, a waters AccQ-TagTM Ultra column (5 mm × 1.2 mm, 0.2 µm particles) with a pre-filter heated at 55°C, and which coupled with a tandem quadrupole detector. The two

different solvents were used: Solvent A: Methyl alcohol (MeOH): Water: Acetic acid (HAc) with a ratio of 80:19:1 whereas, solvent B: Methyl alcohol (MeOH) and with gradient flow (2C), A: B 0' (80: 15), 0.5'(80: 15), 10'(60:40), 10.5'(60:40), 14'(80:15), 15' (80:15).The nonlinear separation gradient was used (21). The mobile phase flow rate of 0.15 ml/min, One microliter of sample was injected in duplicate into the UPLC system.

ESI-MS/MS and UPLC-MS/MS analysis were carried out on a Xevo TQD® (Waters Corporation, Milford, USA). In this investigation the parameters used for detection was followed ref. The ESI source was operated at 135°C with a desolvatation temperature of 350°C, a 650 L/h desolvatation gas flow rate and a capillary voltage was set 3.5 kV. The extractor voltage was set 3.2 V, and the radio frequency voltage was set 3 V. The collision gas was used as Argon whereas, collision energies varied with 19 eVto 35eV. Integration and quantitation were performed using the software's were Waters Target Links-TM and Masslynx.

Results and Discussion

The aim of present investigation was to develop a metabolomic method and which can be utilized to identify potential *T. asperellum* isolate against soil-borne pathogens (*Sclerotium rolfsii*). GC-MS and LC-MS techniques were explored to identify volatile as well as antifungal compounds produced by *T. asperellum* and to develop metabolomic profiling. Isolation of volatile compounds from *T. asperellum* isolates were performed as described by ref (Yang *et al*., 2009), with slight modifications (under typical solvents). The GC –MS data was de-convoluted using the software's (Wiley-2009 and NIST-2007) and which measured with mass spectra to match the entries in the compound library.

In the present investigation, it was revealed that, the culture filtrate of the 10 isolates of *T. asperellum* showed the presence of 673secondary metabolites compound at different retention time *viz.*,Ta-2 (57), Ta-8 (68), Ta-10 (86), Ta-12 (101), Ta-14 (53), Ta-15 (73), Ta-17 (71), Ta-20 (39), Ta-29 (61) and Ta-45 (64) by GC-MS (Table 2). The volatile compounds were detected in the culture samples and which constitute members of the different compounds and with various classes such as alkanes, alcohols, ketones, pyrones (lactones), fatty acids, benzene derivatives including cyclohexane, cyclopentane, simple aromatic metabolites, terpenes, isocyano metabolites, some polyketides, butenolides and pyronesfuranes, monoterpenes, and sesquiterpenes, for which these compounds were fungal origin and which was previously reviewed by ref. (Magan *et al*., 2000). *T. asperellum* was produced high percent abundance compounds and numerous minor peaks of secondary metabolites produced by fungus. The identified metabolites and compositions of compounds were presented in table 3 and figure 1. Among the identified compounds, the most abundant compounds such as 6-Pentyl-2H-Pyran-2-One (22.04%), 2,3,5,5,8a-pentamethyl-6,7,8,8a-tetrahydro-5H-Chromen-8-ol (15.85%) from Ta-2 isolate, whereas Toluene (26.24%), 2,4, Ditert-butyl phenol (14.48%) and 6-Pentyl-2H-Pyran-2-One (27.52%) from Ta-8 isolate, 1,5, Dimethyl-6-methylene spiro (2, 4) heptanes and 2,4, Ditert-butyl phenol (17.00%) from Ta-10, 1, 5, Dimethyl-1-methylenespiro (2,4) heptanes (17.50%) and N,N-Dimethyl-1-(4-methylphenyl) ethanamine (24.11%) from Ta-12, Benzenethanol (39.06%) from Ta-14, Toluene (22.38), 1,5-Dimethyl-6-methylenespiro (2.4) and heptanes (13.03) from Ta-15. 6-Pentyl-2H-Pyran-2-One (21.81%) from Ta-17. Anethanol (19.55%) and 1-Hydroxy-2,4-di.tert butyl benzene (16.68%) from Ta-29, 1,5, Dimethyl-6-

methylene spiro (2,4),heptanes (16.93%), P-Propenyl phenyl methyl ether (20.31%) and 2,4-Di-tert-butyl phenol (19.77%) from Ta-45, and Epizonarene (29.71%), 2,5-Di-tert-buytlphenol (10.04%) and 2,3,5,5,8a-pentamethyl-;7,8,8,8A-tetra hydro-5H-chromen-8-ol (16.43%) from Ta-20. Only few compounds were innovative and rest of compounds was previously known. Amidst compounds, the most abundant metabolite identified in this study was 6-pentyl-alpha-pyrone (6-PP) followed by Toluene, Azulene and Anethol.

The compound, 6-PP was reported and characterized by Collins and Halim, 1972(23), and they identified as one of the key bioactive compounds of several isolates, e.g., *T. asperellum* has reviewed by (24, 25, 2). The most important volatile compound was obtained from pyrone (peak 13 from Ta-2, peak 63 from Ta-12, peak-36 from Ta-17, peak 14 from Ta-20 and peak 42 from Ta-45 respectively).This compound is oxygen heterocyclic compound and dehydroderivative showing characteristics of coconut odour and which is the peculiar characteristic to identify the *T. asperellum* (earlier *T. viride*).

This is a nontoxic flavoring agent and which was chemically synthesized for industrial purposes before its discovery as a natural product and which was involved in cellular function, plant growth regulation, plant defense response and antifungal activity (El-Hassan *et al*., 2009; Reino *et al*., 2008; Siddiquee *et al*., 2012). The metabolomic profiling was done using 21 days old culture filtrate of five potential isolates of *T. asperellum* viz., Ta-2, Ta-8, Ta-10, Ta-20 and Ta-45 were selected for further analysis with LC-MS techniques based on their bio-efficacy test using dual culture method. The study revealed that, the Ta-45 isolates showed highest percent inhibition up to 80.04% followed by Ta-10 (74.56%), Ta-20 (73.79%) and Ta-8 (70.26%). The Ta-2 isolate (58.13%) showed lowest percent inhibition among 10 isolates of *T. asperellum* and to establish valid correlation between the production of antifungal metabolites and their efficacy as BCAs (Fig.2.1 and 2.2).

Further, preliminary experiment was performed to optimization of extraction yield and LC-MS chromatographic profiling. ESI-MS/MS spectrum of Ta-2 isolate showed four prominent peaks correspondingly four compounds were tentatively identified as Butenolides ($C_4H_4O_2$) with the molecular ion peak exhibited at 243.3 m/z, Cyclonerodiol ($C_{15}H_{28}O_2$) with peak mass exhibited at 241.38 m/z, Ferulic acid ($C_{10}H_{10}O_4$) with molecular ions at 195.18 m/z and Gliovirin ($C_{20}H_{20}N_2O_8S_2$) with peak mass exhibited at 481.5 m/z.

Similarly, the spectrum of Ta-8 isolate showed 6 peaks correspondingly six compounds were tentatively identified as Ferulic acid ($C_{10}H_{10}O_4$) with molecular ions at 195.18 m/z, Harzianolides ($C_{13}H_{18}O_3$) with molecular ions at 223.28 m/z, Cyclonerodiol ($C_{15}H_{28}O_2$) with peak mass exhibited at 241.38 m/z, Viridin ($C_{20}H_{16}O_6$) with molecular ions at 353.09 m/z, Gliovirin ($C_{20}H_{20}N_2O_8S_2$) with peak mass exhibited at 481.5 m/z and Mass oil actone ($C_{10}H_{16}O_2$) with molecular ions at 169.232 m/z.

The spectrum of Ta-10 isolate showed five prominent peaks correspondingly five compounds were tentatively identified as Ferulic acid ($C_{10}H_{10}O_4$) with molecular ions at 195.18 m/z, Viridin ($C_{20}H_{16}O_6$) with molecular ions at 353.09 m/z, Viridiol ($C_{20}H_{18}O_6$) with molecular ions at 355.35 m/z, Gliovirin($C_{20}H_{20}N_2O_8S_2$) with peak mass exhibited at 481.5 m/z and Viridiofungin A ($C_{31}H_{45}NO_{10}$) with peak mass exhibited at 562.7 m/z.

Table.1 Details of the *T.asperellum* isolates used for present study

Strain No.	Source	Place	Optimum temperature for growth on PDA	Incubation time	Subculture period	A brief description or distinctive features of the microorganism
Ta-2	Tamoto, rhizosphere	Devanahalli, Bengaluru	25 to 30ºC	5-7 days	Once in 3 months	Conidiophores on PDA media gives typically comprising a fertile central axis or the central axis 100-150 µm long and flexuous, with lateral branches paired or not and typically arising at an angle at or near 90° with respect to its supporting branch, sometimes lateral branches at widely-spaced intervals when near the tip of the conidiophore and arising at closer intervals when more distant from the tip; phialides arising singly from the main axis or in whorls of 2-3 at the tips of lateral branches or at the tip of the conidiophore. The central axis (1.7-)2.2-3.2(-4.5) µm wide. Conidia dark green, sub-globose, on CMD, (3.0)3.5-4.5(-5.0) x (2.7-)3.2-4.0(-4.8) µm, L/W = (0.8-)1.0-1.2(-1.5), conspicuously tuberculate. Ref: http://nt.ars-grin.gov/taxadescriptions/keys/
Ta-8	Cauliflower, rhizosphere	Bangalore (Hoskote)				
Ta-10	Rose, Green house	Bangalore (Hoskote)				
Ta-12	Sugarcane, rhizosphere	Devanahalli, Bengaluru				
Ta-14	Plantation crops	Bangalore (Hoskote)				
Ta-15	Plantation crops	Bangalore(Hoskote)				
Ta-17	Plantation crops	Bangalore(Hoskote)				
Ta-20	Maize,rhizosphere	Sollapur				
Ta-29	Field	Iskon				
Ta-45	Cumin	Ajmer				

Table.2 List of total number of Volatile metabolites produced from the *T.asperellum* isolates

Sl. No.	Isolates	Volatile compounds
1	Ta-2	57
2	Ta-8	68
3	Ta-10	86
4	Ta-12	101
5	Ta-14	53
6	Ta-15	73
7	Ta-17	71
8	Ta-20	39
9	Ta-29	61
10	Ta-45	64
	Total	**673**

Table.3 The most abundant volatile metabolites identified from the *T.asperellum* isolates using GC-MS

Sl. No.	Isolates	Peak No.	RT	Chemical Name	Chemical Structure	MW g/mol	Abundance (%)
1.	Ta-2	13	34.30	6-Pentyl-2H-pyran-2-one	$C_{10}H_{14}O_2$	166	22.04
		24	41.64	2,3,5,5,8a-Pentamethyl-6,7,8,8a-tetrahydro-5H-chromen-8-ol	$C_{14}H_{22}O_2$	222	15.85
		47	51.84	3,4,4-trimethyl-2-Hexenoic acid	$C_9H_{14}O_3$	170	09.10
2.	Ta-8	45	34.04	6-pentyl-2H-Pyran-2-one,	$C_{10}H_{14}O_2$	166	27.52
		20	18.79	Toluene	C_7H_8	92	26.24
		49	35.84	2,4-Di-tert-butylphenol	$C_{14}H_{22}O$	206	14.48
		57	41.30	(3E)-4-(3-Hydroxy-2,6,6-trimethyl-1-cyclohexen-1-yl)-3-penten-2-one	$C_{14}H_{22}O_2$	222	03.61
		43	33.35	Chamigren	$C_{15}H_{24}$	204	02.41

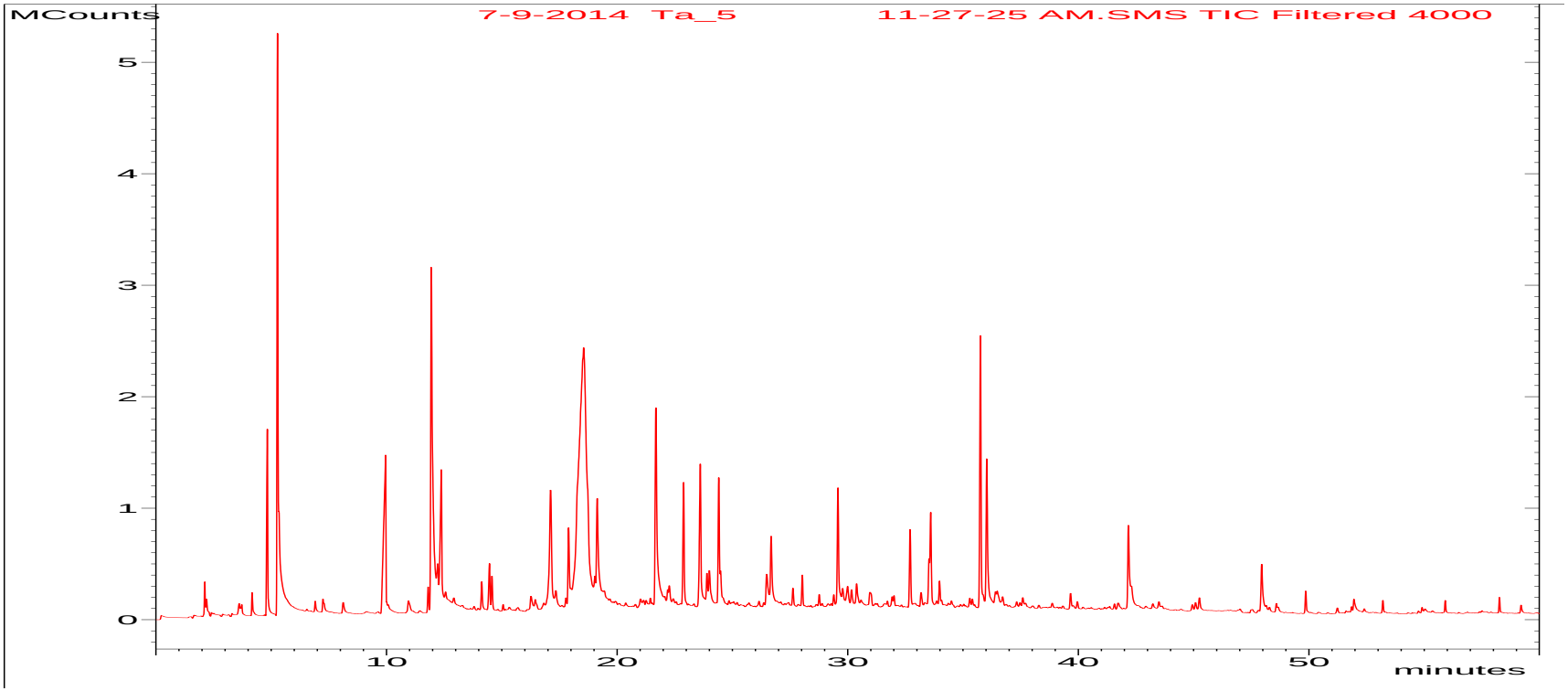
		26	21.68	Azulene	$C_{10}H_8$	128	01.79
3.	Ta-10	17	14.22	1,5-Dimethyl-6-methylenespiro(2.4)heptane	$C_{10}H_{16}$	136	19.49
		68	35.83	2,4-Di-tert-butylphenol	$C_{14}H_{22}O$	206	17.00
		47	26.56	Anethole	$C_{10}H_{12}O$	148	13.89
		48	27.45	2-Methyl-1-indanone	$C_{10}H_{10}O$	146	05.91
		61	32.72	1,4-Epoxy-1,2,3,4-tetrahydronaphthalene	$C_{10}H_{10}O$	146	02.01
4.	Ta-12	40	26.74	N,N-Dimethyl-1-(4-methylphenyl) ethanamine	$C_{17}H_{22}$	291.81	24.11
		11	14.33	1,5-Dimethyl-6-methylenespiro(2.4)heptane	$C_{10}H_{16}$	136	17.50
		63	34.06	6-Pentyl-2H-pyran-2-one	$C_{10}H_{14}O_2$	166	13.01
		89	41.34	(3E)-4-(3-Hydroxy-2,6,6-trimethyl-1-cyclohexen-1-yl)-3-penten-2-one	$C_{14}H_{22}O_2$	222	02.54
		68	35.12	1H-Benzocycloheptene	$C_{15}H_{24}$	204	02.23
5.	Ta-14	16	18.55	Benzeneethanol	$C_8H_{10}O$	122	39.06
		6	09.96	1-(4-Methoxyphenyl)-1-methoxypropane	$C_{11}H_{16}O_2$	180	08.73
		43	35.75	2,4-Bis(1,1-dimethylethyl)phenol	$C_{14}H_{22}O$	206	08.28
		7	12.37	1-Propylcyclohexanol	$C_9H_{18}O$	142	06.45
		24	21.68	Azulene	$C_{10}H_8$	128	05.33
		27	24.40	4-pentyl-Benzoyl chloride	$C_{12}H_{15}ClO$	210	03.72
		38	33.59	2,5-Cyclohexadiene-1,4-dione	$C_{14}H_{20}O_2$	220	03.10
6.	Ta-15	14	18.85	Toluene	C_7H_8	92	22.38
		6	14.19	1,5-Dimethyl-6-methylenespiro(2.4)heptane	$C_{10}H_{16}$	136	13.03
		47	35.80	2,4-Di-tert-butylphenol	$C_{14}H_{22}O$	206	10.35
		26	26.51	Anethole	$C_{10}H_{12}O$	148	08.17
		59	41.35	2,3,5,5,8a-Pentamethyl-6,7,8,8a-tetrahydro-5H-chromen-8-ol	$C_{14}H_{22}O_2$	222	07.61
7.	Ta-17	36	33.92	6-pentyl-2H-Pyran-2-one,	$C_{10}H_{14}O_2$	166	21.81
		56	41.33	2,3,5,5,8a-Pentamethyl-6,7,8,8a-tetrahydro-5H-chromen-8-ol	$C_{14}H_{22}O_2$	222	12.58
		20	21.68	Azulene	$C_{10}H_8$	128	08.04
		51	40.00	Eudesma-3,7(11)-diene	$C_{15}H_{24}$	204	08.27

		16	17.39	1-Methylcyclooctanol	$C_9H_{18}O$	142	04.64
		35	33.60	2,5-Cyclohexadiene-1,4-dione, 2	$C_{14}H_{20}O_2$	220	03.54
8.	Ta-20	28	40.30	Epizonarene	$C_{15}H_{24}$	204	29.71
		31	41.55	.2,3,5,5,8a-Pentamethyl-6,7,8,8a-tetrahydro-5H-chromen-8-ol	$C_{14}H_{22}O_2$	222	16.43
		17	36.14	2,5-Di-tert-butylphenol	$C_{14}H_{22}O$	206	10.04
		29	41.09	octahydro-2,2,4,7a-tetramethyl-1,3a-Ethano(1H)inden-4-ol	$C_{15}H_{26}O$	222	05.17
		25	39.38	2-Naphthalenemethanol	$C_{15}H_{26}O$	222	04.16
		33	42.15	(1,5,5-Trimethyl-2-methylenebicyclo(4.1.0)hept-7-yl)methanol	$C_{12}H_{20}O$	180	04.01
		14	34.99	6-pentyl-2H-Pyran-2-one,	$C_{10}H_{14}O_2$	166	03.09
9.	Ta-29	19	26.63	Anethole	$C_{10}H_{12}O$	148	19.55
		33	36.08	1-Hydroxy-2,4-di-tert-butylbenzene	$C_{14}H_{22}O$	204	16.68
		48	41.46	5H-Benzo(b)pyran-8-ol	$C_{14}H_{22}O_2$	222	07.98
		49	42.14	Cubenol	$C_{15}H_{26}O$	222	05.56
		34	36.80	1H,4H-3a,8a-Methanoazulen-1-one, hexahydro-, (3aS)-	$C_{11}H_{16}O$	164	04.16
		4	14.17	1,5-Dimethyl-6-methylenespiro(2.4)heptane	$C_{10}H_{16}$	136	03.74
10.	Ta-45	30	26.60	p-Propenylphenyl methyl ether	$C_{10}H_{12}O$	148	20.31
		49	35.84	2,4-Di-tert-butylphenol	$C_{14}H_{22}O$	206	19.77
		10	14.23	1,5-Dimethyl-6-methylenespiro(2.4)heptane	$C_{10}H_{16}$	136	16.93
		62	41.33	2,3,5,5,8a-Pentamethyl-6,7,8,8a-tetrahydro-5H-chromen-8-ol	$C_{14}H_{22}O_2$	222	05.63
		42	33.83	6-pentyl-Pyran-2-one	$C_{10}H_{14}O_2$	166	03.98

Table.4 List of antifungal compounds identified from the *T.asperellum* isolates using LC-MS

Chemical compound/Derivatives	MW	Relative Abundance %(TIC) Total Ion Current					Antibiotic activity	References	Biological functions
		Ta-2	Ta-8	Ta-10	Ta-20	Ta-45			
Viridin (Furanosteroid)	352.09	0	259	262	378	0	Antibiotic	(32,33)	Inhibition of Fungal spore germination,Fungistatic, Anticancer
Viridiol (Steroid)	354.35	0	0	297	0	0	Antifungal	(34, 35)	Herbicidal property Antiaging
Butenolides (Trichothecene)	242.30	155	0	0	234	0	Antifungal	(36)	Insecticidaland Anti-bacterial activity
Harzianolides (Diterpenes)	222.28	0	281	0	148	0	Antifungal	(37, 26)	Plant growth regulator
Ferulic acid (Phenypropanoids)	194.18	162	966	395	111	166	Fungicide	(38, 39)	Antimutagenic, Anti-microbial antioxidant
Viridiofungin A (Alkylcitrate)	561.70	0	0	139	0	0	Antibiotic	(40, 41, 42)	Fungitoxic, Antibacterial Inhibition of Ergosterol synthesis and Serine palmitotyltransferase enzyme
Cyclonerodiol oxide (Sesquiterpenes)	240.38	110	182	0	243	0	Antifungal	(43, 44, 45, 46)	Plant growth regulator Antitumor
Gliovirin (Alkaloides)	480.06	1.28e3	300	1.92e3	201	1.12e3	Antibiotic Antiviral	(47, 48)	Immune suppressive activity, Mycoparasitic activity
Massoilactone (Pentaketides)	168.23	0	1.24e3	0	612	0	Antifungal	(49)	Plant growth regulator

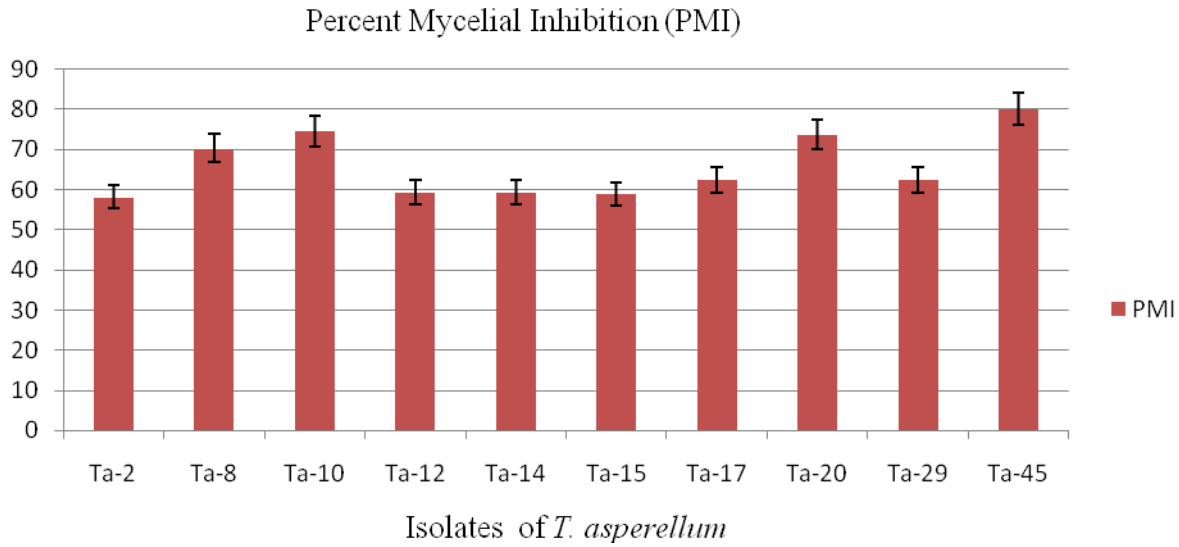
Fig.1 GC-MS spectrums of the culture filtrate of Ta-14 isolates



Ta-14 isolates showed seven secondary metabolites

Fig.2.1 Bio-efficacy of *T. asperellum* isolates effective against *S. rolfsii* (Plates)

Fig.2.2 Bioefficacy of *T. asperellum* isolates effective against *S. rolfsii*



Grand Mean= 65.94, **SEm**=0.65, **CD at 1%**=2.63, **CD at 5%**=1.92 and **CV**=1.71

Fig.4 Chromatogram of total ion current & antifungal compounds of Ta-45 isolate by LC-MS

Ta-45 isolate

The spectrum of Ta-20 isolate showed seven prominent peaks correspondingly seven compounds were tentatively identified as Massoilactone ($C_{10}H_{16}O_2$) with molecular ions at 169.232 m/z, Ferulic acid ($C_{10}H_{10}O_4$) with molecular ions at 195.18 m/z, Harzianolides ($C_{13}H_{18}O_3$) with molecular ions at 223.28 m/z, Cyclonerodiol ($C_{15}H_{28}O_2$) with peak mass exhibited at 241.38 m/z, Butenolides ($C_4H_4O_2$) with the molecular ion peak exhibited at 243.3 m/z, Viridin ($C_{20}H_{16}O_6$) with molecular ions at 353.09 m/z and Gliovirin ($C_{20}H_{20}N_2O_8S_2$) with peak mass exhibited at 481.5 m/z (Table 4 and Fig. 3).

The LC-ESI-MS negative-ion chromatogram of *T. asperellum* isolates shows the positions of significantly different metabolites. The antifungal compounds produced by the *T. asperellum* are attributed compounds for the bioactivity and have a function as bio-control agent, which may contribute to the mitigation of the unnecessary use of chemical pesticides, easily biodegradable in the soils and reduce the environmental pollution.

Among 10 isolates of *T. asperellum*, only Ta-20, Ta-10, Ta-8 and Ta-2 isolates were produced highest number of major antimicrobial compounds. Therefore, these isolates can be considered as high potential bio-control agents against *Sclerotium rolfsii* pathogens. This finding was agreements with the studies of Srinivasa and Prameela Devi, 2014; Siddiquee *et al*., 2012. From this investigation 09 major antimicrobial compounds were analyzed and this study envisages the importance of reports given by (Sivasithamparam *et al*., 1998; Vinale *et al*., 2006).

In the present study, secondary metabolites were successfully separated and identified from *T. asperellum* isolates through GC-MS and LC-MS method. Among 10 isolates, Ta-20 and Ta-10 were the highest producers of secondary metabolites and which encompasses antibiotics and found to be highly significant compared to rest of isolates.

In conclusion, *Trichoderma* species is well known for decades, and the present investigation has been confirmed that the fungus has ability to produce abundant secondary metabolites and these metabolites were quantified in same studies with the help of recent advent techniques known as GC-MS and LCMS approach. Metabolomics is a powerful tool in system biology which allows us to gain insight into the identification of unknown and known secondary metabolites in potential isolates of *T. asperellum* which is used as most predominant and promising BCA in India for the management of soil-borne pathogens (*Sclerotium rolfsii*). With the help of this approach 673 secondary metabolites were identified with GC-MS. Out of 673 metabolites, 55 metabolite compounds were found to be most abundant in all the isolates. Further, isolates viz., Ta-45, Ta-10, Ta-20, Ta-8, and Ta-2 with LCMS approach showed highest production of antifungal secondary metabolites. Therefore, these isolates can be used as high potential bio-control agents against soil borne pathogens (*Sclerotium rolfsii*). Combination of GC-MS and LC-MS approaches would help us in identifying high potential bio-control agents against soil borne pathogens in a greater extent which could have a great potential for future application of metabolites.

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