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Isolation and Identification of Volatile Metabolites from the Biocontrol Agent *Trichoderma harzianum* through GC-MS Analysis

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Trichoderma **belongs to a group of filamentous fungi, is an effective biocontrol agent. There are several antagonistic mechanisms exercised by** ***Trichoderma*** **species such as nutrient competition, antibiotic production and mycoparasitism. Use of biocontrol agents for the management of phytopathogens is an economical, ecological and environmental friendly process. The present study involves the isolation and characterization of volatile secondary metabolites from the culture filtrate of** ***Trichoderma harzianum*** **(Th. Azad). The in vitro assay of extracted volatile metabolite of** ***Trichoderma harzianum*** **(Th. Azad) shows growth inhibition of all the tested phytopathogens (*Rhizoctonia bataticola* 24%, *Fusarium oxsporum ciceri* 29.36%). The inhibition varied from species to species. Highest growth inhibition was achieved with *Fusarium oxsporum ciceri* (29.36%). Identification and profiling of volatile metabolites produced by *T. harzinum* (Th. Azad) yielded 15 compounds with 15 sharp peaks. Out of the different compounds obtained, 1H-Purine-6-amine (2-Fluro Phenyl) methyl), is first time found to be present in *Trichoderma harzianum* (Th. Azad). This compound works as a plant growth regulator.**

Key words: *Trichoderma,* Secondary metabolites, Antagonistic, Volatiles metabolites.

Biological control is a safe, effective and ecofriendly method for the management of plant diseases. *Trichoderma* species are the most widely studied biocontrol agents (BCA). They are effective against a wide variety of soil borne pathogens such as *Fusarium*, *Rhizoctonia*, *Sclerotium rolfsi*, *Pythium* etc. *Trichoderma* sp commonly live in association with plant roots. *T. harzianum* is a well known biocontrol agents responsible for the increase in plant growth and providing resistance against phytopathogenic microorganisms (Abdullah, F., *et al*., 2005). Due to these beneficial effects, some strains of *Trichoderma* such as *T. harzianum, T. atroviride* and *T. asperellum* are used as plant protection agents for the biocontrol of plant pathogens and plant growth promotion in agriculture (Harman, G.E., *et al* 2004, Harman, G.E et 2006, Verma, M *et al* 2007). The mechanism employed by *Trichoderma* against pathogens are mycoparasitism, antibiosis, competition for food and space, induce plant resistance, secretion of cell wall degrading enzymes (chitinase, glucanase, cellulase etc) and production of volatile and non volatile secondary metabolites (Howell CR 2003). In *Trichoderma* species these volatile and non volatile secondary metabolites play a crucial role in mycoparasitic action. *Trichoderma* species are known to produce over 40 different metabolites which play an important role in mycoparasitic action (Vinale, F *et al* 2008).

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The main aim of this manuscript was to evaluate *Trichoderma harzianum* (Th. Azad) for the production of volatile metabolites and identification of the secreted volatile metabolites through GC-MS analysis.

MATERIALS AND METHODS

Cultivation of *T. harzianum*

T. harzianum strain (Th. Azad) used in this study was isolated from the student farm CSA, Kanpur and maintained on potato dextrose agar (PDA) (Himedia, USA) at 28±2°C for 5 days.

Dual culture assay

Antagonistic potential of *Trichoderma* sp was examined by dual culture technique as described by Morton and Stroube. A 5mm disc was taken from the edge of actively growing colonies of fresh fungal cultures and placed on the surface of a fresh PDA plate 4 cm apart. The plates were incubated at 28 ± 2°C for 7 days. Control plates were maintained without *Trichoderma*. The experiment was replicated thrice and percent growth inhibition was calculated by the following formula

$I = (C-T)/C \times 100,$

where C is mycelial growth in control plate, T is mycelial growth in test organisms inoculated plate and I is inhibition of mycelial growth

Evaluation of antagonistic activity through production of antifungal Volatile Metabolites

To check the efficacy of volatile metabolites produced by *Trichoderma harzianum* against soil borne phytopathogens inverse plate technique given by Dennis and Webster (1971) and Goyal *et al* (1994) was used. Mycelial disc (5mm) from both *Trichoderma* and test pathogens were centrally inoculated on two separate PDA plates and allowed to incubate at 28 ± 2°C for 24 hours. After 24 hours the lid of each plate was removed and both the plates were sealed together with help of parafiilm tape and incubated at 28 ± 2°C for seven days. In this mehtod the pathogen remain directly exposed to the volatile metabolites produced by *Trichoderma*. In control plate *Trichoderma* sp. replaced with the plain media plate. After seven days the colony diameter of control as well as *Trichoderma* and pathogen inoculated plate was measured and percent growth inhibition was calculated by using the following formula

$I = (C"T)/C \times 100,$

Where C is mycelial growth in control plate, T is mycelial growth in test organism inoculated plate and I is inhibition of mycelial growth.

Volatile metabolite Extraction procedure

For the extraction of volatile compounds *Trichoderma* species inoculated into 500 ml of PDB medium and incubated 28°C for 25 days. After incubation period content of the flasks were filter through muslin clothes. The liquid phase obtained after filtrations is used for extraction. Extraction was done with Hexane (1:1). Upper phase of the solvent which contain volatile compounds was collected through the separating funnel (Dubey *et al* 2011). Solvent was removed from the collected phase and obtained residue is dissolved in acetone. This sample was then used for GC-MS analysis.

Apparatus and chromatography conditions

GC-MS analysis was done by Indian Institute of Toxicological Research, Lucknow. GC-MS analysis was done by the SHIMAZDU QP2010, an oven temperature from 50°C to 280°C at 4°C/min and held at this temperature for 5 min; inlet and interface temperatures were 250°C and 280°C, respectively. Carrier gas was He at a flow rate of 1.0 ml/min (constant flow). 0.2 ml of sample was injected under split of 20:1. EIMS: electron energy, 70 eV. Interpretation of mass spectrum GC-MS was conducted using data base of NIST, having more than 62,000 patterns. The spectrum of the known compounds was compared with the NIST library

RESULTS AND DISCUSSION

The in vitro antagonistic activity of *Trichoderma harzianum* against phytopathogens was tested by dual culture technique (Table 2). *Trichoderma harzizanum* (Th. Azad). efficiently inhibit all the tested pathogens. Antagonism of *Trichoderma* species against phytopathogens has already been studied. Dubey *et al* 2007 tested ten isolates of *T.viride*, *T.harzianum* and *T.virens* against *Fusarium oxysporum* and found that isolate of *T.viride* and *T. harzianum* shows the greatest capacity to inhibit fungal growth

Scanning electron microscopy analysis (Fig. 1 & 2) of hyphal interaction between *Trichoderma harzianum* and phytopathogens clearly indicates that *Trichoderma* coils around the phytopathogen and parasitized the phytopathogen mycelium. Elad *et al* 1983 observed the hyphal interaction between *Trichoderma* and *Sclerotium rolfsii* through SEM and showed that *Trichoderma* coils around the phytopathogen cells

Volatile metabolite produced by *T.harzianum* (Th. Azad) found to reduce the mycelial growth of both the tested phytopathogens (Table 3). In 2006 Eziashi E.I. *et al* conducted and experiment and found *Trichoderma* sp are capable of antagonize *Ceratocystis paradoxa* through the production of volatile and non volatile metabolites. Patil A *et al* 2012 Conducted an experiment with three different species of *Trichoderma* (*T. harzianum, T. flavofuscum and T. viride),* and observed that the volatile metabolites of all the selected species of *Trichoderma* showed broad spectrum inhibition of *Pythium.*

The effect of volatile metabolites on the mycelial growth of phtyopathogenic fungi is evident from the Figure 3. The volatile metabolites produced by *T.harzinum* inhibit the mycelial growth of all the test pathogens with different rates *Fusarium oxsporum Ciceri* (29.36%) and *Rhizoctonia bataticola* (24%).These findings are in close resemblance to those of Ayoubi *et al* 2012. Siddique *et al* 2009 showed the inhibitory properties of both volatile and non volatile metabolites produced by *T.harzianum* against *Ganoderma boninense.*

In *Trichoderma* there are many volatile metabolites which have been reported to play an important role in mycoparasitic activity. Sivasithamsaran and Ghiberti in 1998 showed that *Trichoderma* species are able to produce more than 40 metabolites which help in mycoparasitic action. GC-MS analysis of partially purified crude extract yields 15 compounds with sharp peaks (Fig. 4, Table 4). The peak with retention time 13.30 corresponds to Benzene acetic acid, ethyl ester, 17.83 corresponds to Phenol 2,4 Bis (1,1-dimethylethyl ether), 22.37 corresponds to Veridiflorol, 22.80 corresponds to Benzyl Benzoate, 25.91 corresponds to 1,2-benzene dicarboxylic acid, butyl 8- metylnoyl ester, 26.10 corresponds to Hexadecanoic acid, 32.03 corresponds to Hexadecanoic acid, bis(2-ethylhexy)ester, 33.18 corresponds to Retenoic Acid, 33.46 Corresponds to 1,2 Benzene dicarboxylic acid, bis(2-etylhexyl)

Table 1. Identification of *Trichoderma harzianum*

S. No	Strain code	Source	ITCC No	Fungus Identified	Crop	GPS Location
1	*Th azad*	CSA Univ. Farm	6796/12	*T.harzianum*	Chickpea	Latitude: 26.4912 ºN Longitude: 80.3070ºE

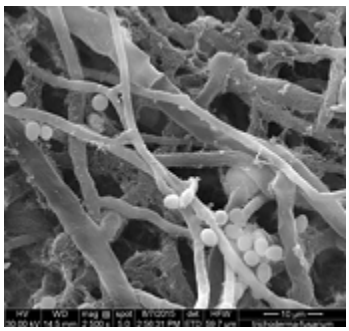
Table 2. In vitro antagonistic potential of *Trichoderma* isolates against *phytopahogens* through dual culture

Trichoderma species	Growth of *Foc* at 72h (mm)		Growth of *R.b.* at 72h (mm)	
	Mycelial growth	% inhibition in mycelial growth	Mycelial growth	% inhibition in mycelial growth
T. harzianum	13.5	55.0	37.5	31.8
CD@5%	4.2		3.3	

Table 3. Effect of volatile metabolites produced by *Trichoderma harzianum* on the mycelial growth of Phytopathogens

Pathogen	Growth inhibitionby *T. harzianum*
Fusarium oxsporum Ciceri	29.36%
Rhizoctonia bataticola	24%

ester, 34.76 corresponds to 1H-Purine-6-amine (2-Fluro Phenyl) methyl), 35.87 corresponds to 2,2,4-trimethyl-3(3,8,12,16-tetramethyl-hepta deca-3,7,11,15,=-tetraenyl cyclo hexanol, 37.17 corresponds to 6h,8H-Benzo(10,11) Chrysenol (1,12-cd)pyran-6,8-dione, 37.33 corresponds to 2-(((4-(Dietylamino)phenyl) metylidine amino) Benzoic acid, 38.53 corresponds to Dimetyl(4-(2phenyl-1,9b-hydro-5-oxa-3,3a-diazacyclopenta(a)naphthalene-4y-phenyl)amine and 43.82 corresponds to 1-(1,5-dimethylHexyl)-3A,6,6,12A-TetramethylDodecahydro-1H-Cyclopenta(A)Cyclopropa(E)Phenatherene-7,11-Dione.



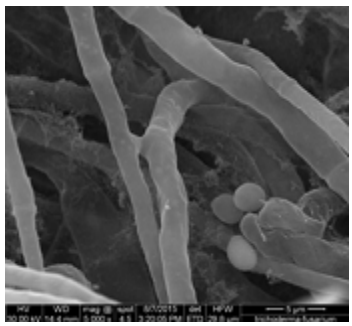
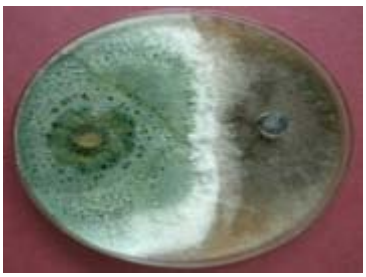
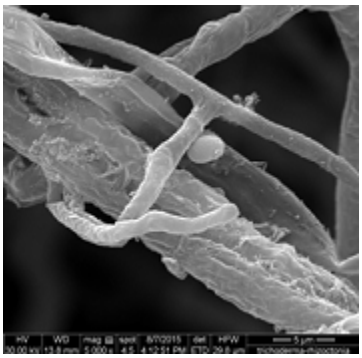


Fig. 1(a). Zone of interaction between *T. harzianum* and *Fusarium oxysporum* f.sp. *ciceri* (Dual culture)
1(b). Scanning electron micrograph on mycoparasitism of the *F. oxysporum ciceri* hyphae by the hyphae of *T. harzianum* with pincer shaped structure moving longitudinally and parallel to the hyphae of the pathogen
1(c). *T. harzianum* hyphal tip, hooks and pincer shape formed against *F. oxysporum ciceri* causing hyphal depression
**Th* = *Trichoderma harzianum* = T
**Foc*= *Fusarium oxysporum* f.sp. *ciceri* = F





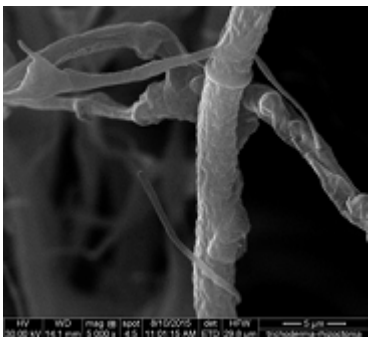


Fig. 2(a). Zone of interaction between *T. harzianum* and *Rhizoctonia bataticola* (Dual culture)
2(b). Coiling of hyphae and hyphal tip of *T. harzianum* attached and penetrating the hyphae of *R. bataticola*
2(c). Coiling of hyphae and hyphal growth depression by *T. harzianum*
**Th* = *Trichoderma harzianum* = T
**Rb* = *Rhizoctonia bataticola* = R

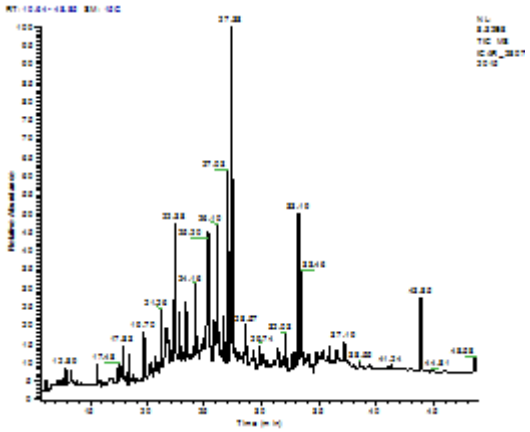


Fig. 4. GC-Mass Spectrum of the culture filtrate of *T. harzianum*

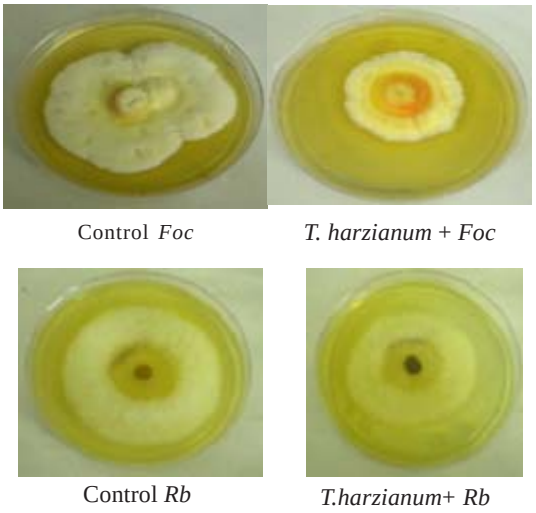


Fig. 3. Effect of *T.harzianum* volatile metabolites on the growth of Phytopathogens (*Foc*, *Rb*)

1, 2-benzenedicarboxylic acid discovered in this study is well known for its antimicrobial activity. In 2008, Ushadevi showed the antimicrobial activity of 1, 2-benzenedicarboxylic acid isolated from *P. lividum* and *T. lignorum*. *Burkolderia capacia*, a marine bacteria produced 1,2 benzene dicarboxylic acid which has been found to posses antibacterial activity against *Aeromonas hydrophila*, *Edwardsiella tarda*, and *Vibrio ordalii* [Gohar Y. M., 2010]. Vinale *et al* 2009 demonstrated the in vitro production and inhibitory activities of secondary metabolites produced by *T.harzianum* strain T22 and T39 against several phytopathogens.

1H-Purine-6-amine (2-Fluro Phenyl) methyl) detected in *T. harzianum* culture filtrate, is a synonym of Kinetin. Kinetin is a type of cytokinin, a class of plant hormone that promotes

Table 3. Volatile Compounds Identified from *T. harzianum*

S. No.	Retention Time	Possible compound as identified using NIST Library
1	13.30	Benzene acetic acid, ethyl ester
2	17.83	Phenol 2,4 Bis (1,1-dimethylethyl ether)
3	22.37	Veridiflorol
4	22.80	Benzyl Benzoate
5	25.91	1,2-benzene dicarboxylic acid, butyl 8- metylnoyl ester
6	26.10	Hexadecanoic acid
7	32.03	Hexadecanoic acid, bis(2-ethylhexy)ester
8	33.18	Retenoic Acid
9	33.46	1,2 Benzene dicarboxylic acid, bis(2-etylhexyl) ester
10	34.76	1H-Purine-6-amine (2-Fluro Phenyl) methyl)
11	35.87	2,2,4-trimethyl-3(3,8,12,16-tetramethyl-hepta deca-3,7,11,15,=-tetraenyl cyclo hexanol
12	37.17	6h,8H-Benzo(10,11) Chrysenol(1,12-cd)pyran-6,8-dione
13	37.33	2-(((4-(Dietylamino)phenyl)metylidine amino) Benzoic acid,
14	38.53	Dimetyl(4-(2phenyl-1,9b-hydro-5-oxa-3,3a-diazacyclopenta(a)naphthalene-4y-phenyl)amine
15	43.82	1-(1,5-dimethylHexyl)-3A,6,6,12A-TetramethylDodecahydro-1H-Cyclopenta(A)Cyclopropa(E)Phenatherene-7,11-Dione.

cell division. Cytokines is a group of plant regulators that promotes cell division and leaf expansion and retarded the rate of leaf aging. Cytokines are applied in growing crops, young trees and ornamental plants to increase fruit size yield blossoms and branching. Thus, it acts as plant growth regulator. To our knowledge this is the first report which shows the presence of this compound in *Trichoderma* species.

CONCLUSION

The secondary metabolites play an important role in mycoparasitic action. These metabolites kill/degrade the hyphae of target pathogen and help in the penetration of *Trichoderma* species.This paper describes the procedure of isolation and characterization of the volatile metabolites from the culture filtrate of *Trichoderma harzianum* (Th. Azad). 1, 2-benzenedicarboxylic acid with retention time 33.46 reported in this study is well known for its antimicrobial activity. First time 1H-Purine-6-amine (2-Fluro Phenyl) methyl), which act as plant growth regulator has been discovered in the tested samples. From the above study it is clear that this *Trichoderma* isolate (Th. Azad) has both phytopathogen control as well as plant growth promotion ability. This makes this isolate a potent and effective biocontrol agent for application in crop field.

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