Journal of Biological Control, 37(4): 220-225, 2023, DOI: 10.18311/jbc/2023/34295

Research Article

Identification of bio-active compounds in indigenous *Trichoderma asperellem* against *Fusarium oxysporum* f. sp. *ciceris*: A causal agent of chickpea wilt

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ABSTRACT: Wilt caused by *Fusarium oxysporum* f. sp. *ciceris* (Padwick) is an important soil-borne disease and considered as most serious and widespread disease of chickpea throughout the world. Twenty indigenous isolates of *Trichoderma* spp. were isolated from the rhizosphere of chickpea in different geographic regions of Karnataka. Among twenty isolates, a higher concentration of volatile compounds was produced by TR-14 (72.22%) followed by TR-19 (66.67%) and TR-9 (65.93%). Further, the efficient strain that is TR-14 was identified as *Trichoderma asperellum* molecularly and was used to extract metabolites by using solvent extraction technique and subjected to GC-MS/MS analysis. The results have shown presence of twenty-nine compounds at different retention times ranging from 4.910 to 21.868 min. and the mass-to-charge (m/z) ratio from 43 to 190. Out of twenty-nine compounds, the concentration of 2-Imidazol-1-ylmethyl-pyridine 1-oxide ($C_9H_9N_3O$) was highest with the maximum area (14375440) and retention time (19.842). Apart from this compound, 7-Isopropylidene-5-methyl-2, 3-diazabicyclo (2.2.1) hept-5-ene-2,3 dicarboxylic acid, diethyl ester was present at the highest peak with a retention time of 20.021.

Keywords: Bio-active compounds, *Fusarium oxysporum* f. sp. *ciceris*, GC-MS/MS, *Trichoderma asperellum*

(Article chronicle: Received: 02-07-2023; Revised: 11-10-2023; Accepted: 14-10-2023)

INTRODUCTION

Chickpea is an annual legume crop which is one of the earliest cultivated legumes and the world's second most important pulse crop after common bean. Its production is often subjected to significant yield losses due to diseases and insect pests ranging from 50-100 per cent in tropical and 5-10 per cent in temperate regions (Van-Emden *et al*., 1988). Among the diseases caused by fungi affecting chickpea, wilt caused by *Fusarium oxysporum* f. sp. *ciceris* (Padwick) is an important soil-borne disease and is considered the most serious and widespread disease throughout the chickpea-growing areas of the world (Nene & Reddy, 1987). The fungus, *Fusarium oxysporum* f. sp. *ciceris* is a soil-borne pathogen, but few reports indicated that it is also seed-borne and facultative saprophyte. It can survive in the soil for up to six years in the absence of a susceptible host (Haware *et al*., 1978).

The biological control of plant pathogens is an eye-catching alternative to decrease the heavy dependence of modern agriculture on costly chemical fungicides (Harman *et al*., 2004; Lewis *et al*., 2001). The secondary metabolites are organic compounds that are not directly involved in the normal growth, development or reproduction of an organism and are not required for the survival of the organism. These compounds are synthesized in the stationary phase of the growth cycle of antagonists. *Trichoderma* spp. is a filamentous fungus which is an asexually reproducing ascomycete with the sexual teleomorph of genus *Hypocrea*. It produces both volatile and non-volatile metabolites that adversely affect the growth of different fungi (Moses *et al*., 1975). However, the success of any biological control depends on a clear understanding of the mechanism of antagonism, particularly the production of bioactive compounds. There are no reports on the identification of bioactive compounds and their antagonistic potential of indigenous *Trichoderma s*trains against chickpea wilt. Hence, the present investigation aimed to identify different bio-active compounds produced by indigenous *Trichoderma* strains and their antagonistic potential against the *Fusarium oxysporum* f. sp. *ciceris* in chickpeas.

MATERIALS AND METHODS

Isolation and maintenance of the pathogen (*Fusarium oxysporum* f. sp. *ciceris*)

Chickpea plants showing typical symptoms of wilt were collected from chickpea fields. The infected stem bits were inoculated on Potato Dextrose Agar (PDA) medium and the plates were incubated at 28 ± 1°C for 5 to 7 days. The pathogen was purified by using the hyphal tip technique (Rangaswami, 1972) and culture tubes were preserved in a refrigerator at 4°C. Koch's postulates were proved by using the susceptible variety Annigeri-1.

Isolation and maintenance of native *Trichoderma* strains

Twenty *Trichoderma* strains were isolated from the soil by serial dilution technique using *Trichoderma* Selective Medium (TSM) developed by Elad and Chet (1983).

Volatile compound production by native *Trichoderma* strains

The test for inhibitory volatile compounds production by twenty strains of *Trichoderma* was carried out by using the inverted plate technique (Dennis & Webster, 1971). The colony diameter of the pathogen was measured and compared with the control. The per cent inhibition was calculated by using the formula of Vincent (1947).

$$I = \frac{(C - T)}{C} \times 100$$

where,
I = Per cent inhibition
C = Radial growth of fungus in control
T = Radial growth of fungus in treatment

Molecular detection of efficient *Trichoderma* strain

The efficient *Trichoderma* strain was characterized based on ITS genes. The total genomic DNA of fungi was extracted by using the Cetyl Trimethylammonium Bromide (CTAB) method. ITS genes were amplified from fungal genomic DNA using fungal universal primers; ITS1-F (CTTGGTCAT-TTAGAGGAAGTAA) and ITS4-R (TCCTCCGCT-TATTGATATGC). Primer sequences were synthesized at commercial facilities (Eurofins, Bangalore, India). Sequencing was carried out by Sanger's dideoxy chain-termination method and aligned by using BLAST analysis (http://www.ncbi.nlm.nih.gov/BLAST).

Extraction of bio-active compounds from *Trichoderma asperellum*

The efficient *Trichoderma asperellum* was grown on Potato Dextrose Broth (PDB) in five-litre haffkine flat bottom flasks containing a two-litre medium at 28 ± 1°C for 21 days. The culture filtrate was obtained by straining through the muslin cloth. Compounds were extracted by solvent extraction method into ethyl acetate (EtOAc) at the ratio of 1:1 (v/v). The upper layer of solvent, which may contain antifungal compounds, was collected by separating the funnel into conical flasks. Ethyl acetate was evaporated from the collected upper phase by using a rotary evaporator at 35°C under reduced pressure. Finally, the residue obtained in the rotary evaporator was resuspended in solvent (acetone) for GC-MS/MS analysis.

Identification of bioactive compounds by GC-MS/MS analysis

GC-MS/MS parameters were performed by using Agilent 7890B GC with 7000C MS system, used for identification and quantification with an oven temperature of 75°C for 1 min and 30°C/min to 300°C for 2 min; inlet and transfer line temperature is programmed at 280°C and 290°C, respectively. The flow rate of helium gas is 1.0 ml/min. 1 µl samples were injected under a split of 3:1. The ionization mass spectroscopic analysis was done with 70eV. Interpretation of mass spectrum GC-MS/MS analysis was done by matching a list of known compound spectra with Agilent's GC-MS/MS Mass Hunter, NIST MS Library and NIST's Automated Mass Spectral Deconvolution and Identification Software.

RESULTS AND DISCUSSION

Isolation and purification of pathogen

The whitish colony with woolly abundant mycelium of fungus was observed at 7 days after incubation. Later it turned to pink. The identification of pathogens was based on mycelial and conidial characteristics through standard mycological keys (Barnett and Hunter, 1972). The fungus produced abundant macroconidia which were spindle-shaped, curved and containing three to five septa, microconidia were fusiform with rounded apexes and no septa. The chlamydospores were globose to oval, thick-walled and occurred terminally or intercalary (Nelson *et al*., 1981).

Isolation and maintenance of native *Trichoderma* strains

After the incubation, all twenty strains showed typical greenish colonies on TSM and produced characters similar to *Trichoderma* under a microscope. Similarly, Someshwar and Sitansu (2011) and Nagamani *et al*. (2015) isolated native *Trichoderma* strains from different soil samples successfully by serial dilution technique using *Trichoderma* Specific Medium and incubated for 7 days at 28 ± 1°C.

Volatile compound production by native *Trichoderma* strains

The results showed that all the strains produced a considerable amount of volatile compounds which varied among strains. A higher concentration of volatile compounds was produced by the TR-14 strain (72.22%) followed by TR-19 (66.67%) and a lower concentration by TR-18 (2.59%)

(Table 1). Nagamani *et al.* (2017) conducted a study which enables to justify that volatile metabolites produced by *T. asperellum* were found most efficacious in inhibiting the mycelial growth of *F. oxysporum* f. sp. *ciceris* by 86.70 per cent. Further, volatile compounds produced by *T. harzianum* showed a strong inhibitory effect on the mycelial growth (79.25%) of *F. oxysporum* f. sp. *ciceris* followed by the *T. viride* (64.16%) (Mohit *et al*., 2019).

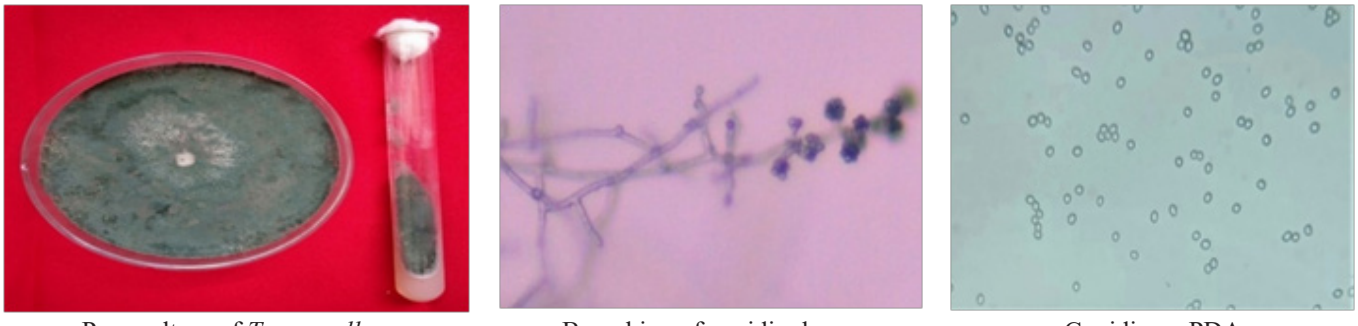
Molecular detection of efficient *Trichoderma* strain

In molecular detection, the ITS primers produced an amplified product size of 550-650 bp. Further, the ITS sequence was BLAST searched and results showed that isolate TR-14 belonged to *Trichoderma asperellum* Samuels, Lieckf. and Nirenberg, with accession number MW063489. The colonies of *T. asperellum* on PDA were initially white but later became greenish. Morphological characteristics of *T. asperellum* involve thickened, pigmented, darkened and branched hyphae. Conidiophores were branched, walled thickened and darkened. Phialides were produced solitary or in groups, terminal phialides were 5-6 in number. Conidia were globose to subglobose to rarely elongated, smooth walled thickened and darkened (Figure 1).

Table 1. Effect of volatile compounds produced by native *Trichoderma* spp. isolates on mycelial inhibition of *F. oxysporum* f. sp. *ciceris*

Sl. No	Isolate code	Colony growth* (mm)	Per cent mycelial inhibition*
1	TR-1	70.00	22.22 (28.12)
2	TR-2	60.00	33.33 (35.26)
3	TR-3	35.00	61.11 (51.41)
4	TR-4	82.00	8.89 (17.28)
5	TR-5	40.00	55.56 (48.18)
6	TR-6	41.67	53.70 (47.12)
7	TR-7	50.00	44.44 (41.80)
8	TR-8	50.70	43.70 (41.38)
9	TR-9	30.67	65.93 (54.28)
10	TR-10	85.00	5.56 (13.63)
11	TR-11	65.00	27.78 (31.80)
12	TR-12	31.00	65.56 (54.06)
13	TR-13	43.33	51.85 (46.06)
14	TR-14	25.00	72.22 (58.19)
15	TR-15	75.00	16.67 (24.09)
16	TR-16	40.00	55.56 (48.18)
17	TR-17	40.67	54.81 (47.76)
18	TR-18	87.67	2.59 (9.26)
19	TR-19	30.00	66.67 (54.73)
20	TR-20	85.67	4.81 (12.67)
21	Control	90.00	0.00 (0.00)
S. Em. ±		-	**1.02**
CD at 1%		-	**3.91**

*Mean of three replications
Figures in the parenthesis are arcsine transformed values.



Pure culture of *T. asperellum* | Branching of conidiophores | Conidia on PDA

Figure 1. Cultural and morphological characteristics of *T. asperellum.*

Extraction and identification of bio-active compounds in *Trichoderma asperellum*

The GC-MS/MS analysis revealed that the extract showed the presence of 29 compounds at different retention times ranging from 4.910 to 21.868 min. The mass-to-charge (m/z) ratio of compounds ranged from 43 to 190 (Table 2). The chromatogram of compounds showed different peaks (Figure 2). The compound 2-Imidazol-1-ylmethyl-pyridine 1-oxide ($C_9H_9N_3O$) was found with the maximum area (14375440) and retention time (19.842) as compared to other compounds detected. The structure of the compound is shown in Figure 3. Another compound namely 7-Isopropylidene-5-methyl-2, 3-diazabicyclo (2.2.1) hept-5-ene-2,3 dicarboxylic acid, diethyl ester was present at its highest peak with a retention time of 20.021 and the structure of the compound is shown in Figure 4. Similarly, the characterization of volatile metabolites from the culture filtrates of the *T. asperellum* strain using GC-MS was carried out by Nitish and Kumar (2017). Further, they also showed that GC-MS analysis detected 43 secondary metabolites in the *T. asperellum* strain including many important volatile metabolites reportedly having effective pesticidal activity. Twenty-eight compounds in the fermentation liquor of *T. asperellum* GDFS1009 *via* GC-MS analysis were also reported (Qiong *et al*., 2017). The culture filtrate of *T. asperellum* isolates showed the presence of 673 secondary metabolites at different retention times with a range of 39 (Ta-20) to 101 (Ta-12) with GC-MS (Srinivasa *et al*., 2017).

Table 2. List of bio-active compounds identified in acetone extract of *T. asperellum* in GC-MS/MS analysis

Sl. No.	Formula	Chemical compound	RT (min)	m/z ratio	Area
1	$C_2H_4O_2$	Acetic acid	4.910	60	6572969
2	$C_3H_6N_2O_2$	Propanediamide	5.297	59	180354
3	CH_4N_6	1H-Tetrazole-1,5-diamine	5.651	43	859362
4	$C_{16}H_{16}O_2$	2-Methylbenzyl p-toluate	6.637	104	315968
5	$C_{18}H_{18}O_4$	Oxalic acid, di(2-phenylethyl) ester	7.884	104	183578
6	$C_{15}H_{17}N$	N-Benzyl-2-phenethylamine	8.812	120	201109
7	$C_9H_{13}N$	3-tert-Butylpyridine	9.588	92	317907
8	$C_3H_6N_4$	1H-Pyrazole-3,4-diamine	10.289	98	202965
9	$C_7H_6F_2$	2,4-Diflorotoluene	10.336	127	1568533
10	$C_8H_9NO_2$	Methyl anthranilate	10.496	119	863152
11	$C_{11}H_{11}NO$	Quinolin-2-ol, 6-methoxy-4-methyl-	11.453	174	3767965
12	$C_8H_{15}N$	2-Butyl- delta. 1-pyrroline	11.844	83	314000
13	$C_{14}H_{23}N$	2,5-di-tert-Butylaniline	12.511	190	3062176
14	$C_{10}H_{11}N_3$	dl-7-Azatryptophan	13.673	131	826859
15	$C_7H_9N_3$	Trimethylpyrazole-4-carbonitrile	14.294	134	291855
16	$C_8H_{11}N_3O$	Pyrimidin-4-amine, S-acetyl-2,6-dimethyl	14.303	150	836455
17	$C_{12}H_{22}$	3,7-Decadiene, 2,9-dimethyl	14.867	55	844988
18	$C_{21}H_{39}N_3$	1,3,5-Triazine, 1,3,5-tricyclohexylhexahydro	14.879	55	741359
19	$C_{14}H_{23}N$	2,5-di-tert-Butylaniline	15.056	190	3661348
20	$C_{10}H_{10}N_2$	Pyrazole, 5-(4-methoxyphenyl)	15.571	174	456519
21	$C_7H_6N_2$	Benzonitrile, 4-amino	16.293	92	1064942
22	$C_8H_{11}BN_2$	1H-1,3,2-Benzodiazaborole, 2-ethyl-2,3-dihydro	17.426	118	1220605
23	$C_{14}H_9N_7O$	2,3-Pyrazinedicarbonitrile, 5-amino-6-((2,3-dihydro-3-hydroxy-2-oxo-1H-indol-3-yl)amino)-	18.160	92	738765
24	$C_{16}H_{33}NO$	Hexanamide, N-propyl-N-heptyl-	19.004	72	1439963
25	$C_9H_9N_3O$	2-Imidazol-1-ylmethyl-pyridine 1-oxide	19.842	93	14375440
26	$C_{15}H_{22}N_2$	7-Isopropylidene-5-methyl-2,3-diazabicyclo(2.2.1)hept-5-ene-2,3-dicarboxylic acid, diethyl ester	20.021	59	12382103
27	$C_{11}H_{17}NO$	9-Azabicyclo(4.2.1)non-2-ene, 2-acetyl-9-methyl-	20.589	83	6921906
28	$C_{11}H_{19}N$	7-Methyl-decahydro-7-aza-cyclopenta(a)pentalene	21.128	136	2121954
29	$C_{18}H_{37}NO$	Octadecanamide	21.868	59	931600

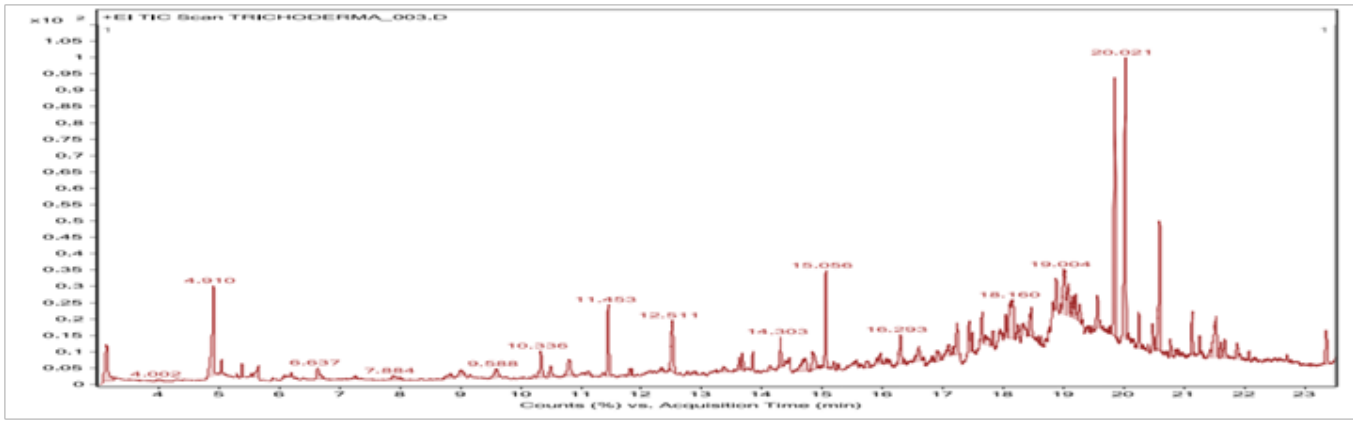


Figure 2. Chromatogram of compounds of *T. asperellum* extract obtained from GC-MS/MS analysis.

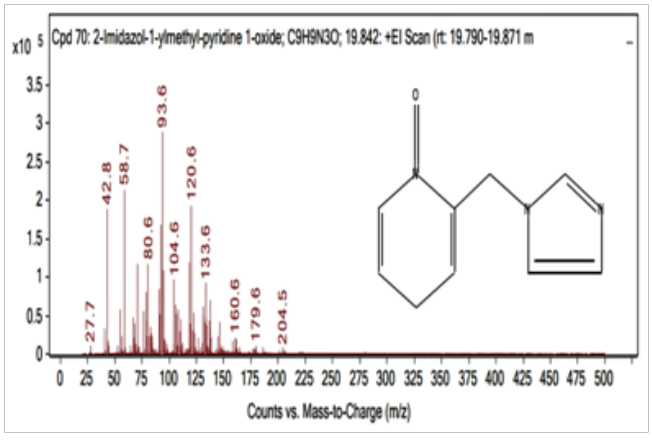


Figure 3. Mass fragmentation and structure of 2-Imidazol-1-ylmethyl-pyridine 1-oxide of *T. asperellum*.

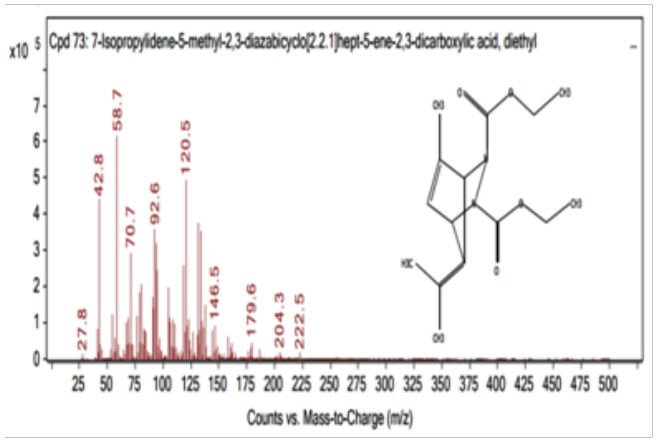


Figure 4. Mass fragmentation and structure of 7-Isopropylidene-5-methyl-2,3 diazabicyclo(2.2.1)hept-5-ene-2,3-dicar boxylic acid, diethyl ester of *T. asperellum*.

CONCLUSIONS

The findings unplug the perspective of deployment of *Trichoderma* spp. with their potentiality for inhibition of pathogens by volatile metabolites, as a sustainable approach. The present investigation reports that chickpea rhizosphere harbor diverse species of *Trichoderma* having antagonistic potential against the pathogen *F. oxysporum* f. sp. *ciceris. Trichoderma asperellum* has more potential and could able to inhibit the pathogen by producing twenty-nine various bio-active compounds.

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