

RESEARCH ARTICLE

Production of Volatile Organic Compounds of *Fusarium oxysporum* f. sp. *lycopersici* on Coinoculation with the Metabolites of *Chaetomium globosum*

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ABSTRACT

The present study focused on divulging the effect of 15 and 30 days old culture filtrates of *Chaetomium globosum* on the mycelial growth and toxin production of *Fusarium oxysporum* f. sp. *lycopersici*. The cell-free culture (CFC) filtrate of *C. globosum* was inoculated in the liquid medium of *F. oxysporum* f. sp. *lycopersici*. The results revealed that mycelial growth was reduced in the CFC filtrate of *C. globosum* inoculated medium compared to control (pathogen alone). The mycelial dry weight of the *F. oxysporum* f. sp. *lycopersici* was 0.864g in fifteen days old CFC filtrate followed by 30 days old CFC filtrate of *C. globosum* (1.374g) amended medium. Metabolites from the CFC filtrate and control were extracted separately using chloroform followed by ethyl acetate and the extract was subjected to GC-MS analysis. GC-MS analyses showed that methyl ester group was present in 15 days old culture filtrate condensate compared to 30 days old CFC filtrate condensate. The compounds are methyl tetradecanoate, octadecanoic acid, methyl ester, and DL-Proline, 5-oxo-, methyl ester. Thirty days old culture filtrate condensate of *C. globosum* contained more phenol groups like phenol 2,4-bis (1,1-dimethylethyl) at 17.62 RT followed by diisooctyl phthalate, 17-pentatriacontene, cholestan-3-ol, 2-methylene-, (3a,5a) and bicycle [4.1.0]heptanes,-3-cyclopropyl,-7-hydroxymethyl,trans. Metabolites of *F. o. f. sp. lycopersici* were also characterized by GC-MS, which showed very few volatile compounds like methyl tetradecanone, oleic acid, eicosyl ester, methyl stearate, and bis(2-ethylhexyl)phthalate. These compounds were not detected in co-inoculation of 15 and 30 days old CFC filtrate of *C. globosum* and *F. o. f. sp. lycopersici*. The difference in the volatile profile may be due to the effect of the metabolites of *C. globosum* on *F. o. f. sp. lycopersici*.

Keywords: Culture filtrate condensate; *Chaetomium globosum*; *Fusarium oxysporum* f. sp. *lycopersici*

INTRODUCTION

Fusarium causing wilt disease in tomato persists in soil for long period and produces survival structures such as chlamyospores (Goyal *et al.*, 1973 and Shuxian *et al.*, 1998). Complete control of *Fusarium* is very difficult with a conventional method. Even though fungicides are used for the management of many pathogens, continuous use of such chemicals are reported to induce the resurgence of existing pathogens and the emergence of new races, posing a greater threat. At this juncture, biomolecules from the secondary metabolites have multiple advantages to manage plant pathogens and induce plant growth. Microbes producing secondary metabolites are called mycotoxin, present in the growing medium as well as in the plant tissue. Secondary metabolites of several fungi are known

to possess an inhibitory effect on plant pathogenic fungi, bacteria and viruses (Janssens *et al.*, 1992; Breheret *et al.*, 1997 and Marumoto *et al.*, 1997). The involvement of secondary metabolites in plant disease and particularly in the interaction between beneficial and pathogenic fungi, has been studied by several scientists (Fravel, 1988). Every microorganism produces different nature of secondary metabolites with low molecular weight compounds. *Trichoderma* spp is known to produce several secondary metabolites that are toxic for plant pathogenic fungi. Secondary metabolite compounds from *Trichoderma* spp are pyrone 6-pentyl-2H-pyran-2-one; koniginins; viridians; harzianopyridone; azaphilones; butenolides ; hydroxyl -lactones; isocyano metabolites; diketopiperazines; gliotoxin; gliovirin and peptaibols. A pyrone 6-pentyl-2H-pyran-2-one is a metabolite purified from the

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culture filtrate of *Trichoderma* spp, responsible for coconut aroma and has shown antifungal activity against several plant pathogens (Scarselletti and Faull., 1994; Worasatit *et al.*, 1994). Likewise, *Chaetomium* spp also produces several biologically active compounds such as chaetoglobosin A, C, D, E, G, R, chaetoviridins, chetomin, chaetocin, chaetochromins, cochliodinol, mollicellin, oosporein, ergosterol, allantoin, uracil, and sterigmatocystin (Brewer *et al.*, 1968; Cole and Cox, 1981; Park *et al.*, 2005; Fogle *et al.*, 2007; Aggrawal, 2015). Biswas *et al.* (2000 and 2012) studied the characterization of antifungal metabolites from *C. globosum* and their antagonism against fungal plant pathogens. Soyong (1992) and Soyong *et al.* (2001) showed that a specific isolate of *C. cupreum* produced secondary metabolites that significantly suppressed tomato wilt caused by *F. oxysporum* f. sp. *lycopersici* and later found that this isolate of *C. cupreum* produced rotiorinols A to C and rotiorin, which also exhibited antifungal activity against *Candida albicans* (Kanokmedhakul *et al.*, 2006 and Phong *et al.*, 2016). *C. globosum* produces the antibiotic chaetoglobosin C, which suppressed the growth of many plant pathogens such as *Colletotrichum gloeosporioides*, *C. dematium*, *Fusarium oxysporum*, *Phytophthora palmivora*, *P. parasitica*, *P. cactorum*, *Pyricularia oryzae*, and *Sclerotium rolfsii* (Soyong *et al.*, 2001). Some compounds have been reported to possess significant biological activities, such as cytotoxic, enzyme inhibitory, and antibiotic (Gunatilaka, 2006; Scherlach *et al.*, 2010). The antimicrobial activity of chaetoglobosin-C, which is produced by *C. elatum* CHE01, and *C. lucknowense* CLT01 could be involved in the disease control mechanism of these antagonistic fungi against tomato wilt fungus *F. oxysporum* f. sp. *lycopersici* (Thohinung *et al.*, 2010). With this background, the current study on the reduction of secondary metabolite secretion of *F. oxysporum* f. sp. *lycopersici* in the culture medium was tested by adding beneficial microbe of *C. globosum* under *in vitro* conditions.

MATERIAL AND METHODS

Isolation and identification of *C. globosum* and *F. oxysporum* f. sp. *lycopersici*

Fusarium sp. was isolated from the infected plant tissue of the tomato. The same field bioagent of *Chaetomium* spp was also isolated and both pathogens and bioagents were identified based on the morphological and molecular level, and the molecular sequence of *C. globosum* (MN069626.1) and *F. oxysporum* f. sp. *lycopersici* (MN071397.1) were submitted into NCBI.

Collection of cell free culture (CFC) filtrates

The conical flasks containing sterilized PDA broth

were inoculated with mycelial discs of *C. globosum*, measuring 6 mm diameter (5 numbers in each flask). The flasks were placed in an incubator cum shaker maintained at 25 °C and agitated at 120 rpm. The culture filtrates were collected at 15 and 30 days after inoculation under aseptic conditions, filtered through a membrane filter (0.2 µm). The CFC filtrates were used to arrest the mycelial growth and metabolite production of *F. oxysporum* f. sp. *lycopersici*.

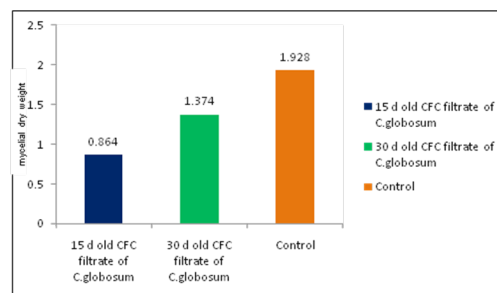


Figure 1. Effect of Cell-Free Culture (CFC) filtrate of *C. globosum* on mycelia growth of *F. oxysporum* f. sp. *lycopersici*

Bioassay of cell-free culture filtrates of *C. globosum* on inhibition of the mycelial and metabolite production of *F. oxysporum* f. sp. *lycopersici*

Cell-free culture filtrates (15 and 30 days old) of *C. globosum* were aseptically mixed with sterilized liquid medium separately in each flask at the rate of 1 mL in 100 mL broth. Then, a 5 mm mycelial disc of the test pathogen was inoculated in the same flask, placed in an incubator, and maintained at 25 °C, and agitated at 120 rpm. The ingredients of the liquid medium contained 0.5 g malt extract; one gram of peptone; one gram of KH_2PO_4 , 0.3g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; 0.3g KCL; one mL of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ solution (0.005g dissolved in one lit.) and one mL of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ solution (0.01g dissolved in one lit) per lit. The pathogen alone in the liquid medium without cell-free culture filtrates served as a control. Three replications were maintained in each treatment. Ten days after incubation, the mycelial mat and the culture filtrates were separated by filtration through Whatman No.40 filter paper. The mycelial dry weight of the pathogen was taken separately in each treatment to know the biomass production.

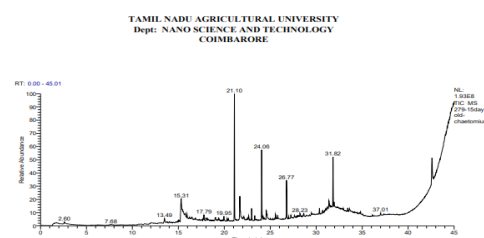


Figure 2. Total ion chromatogram of fifteen-day old Cell-Free Culture (CFC) filtrate of *C. globosum*

Extraction of Toxin

After separating the mycelial mat, the ten days old cell free culture filtrates of *F. o. f. sp. lycopersici* were taken, which were grown separately in a liquid medium containing one percent of 15 and 30 days old cell free culture filtrates of *C. globosum*. The pathogen alone was grown in a liquid medium as a control used for toxin extraction. Then the filtrate was further centrifuged at 10,000 rpm for 15 min. The cell-free culture filtrate was extracted with organic solvents like chloroform and ethyl acetate. Liquid-liquid extraction was carried out using chloroform, and the compound-containing layer was collected separately. Subsequently, the remaining aqueous layer was mixed equally with ethyl acetate. Then, the compound containing layer was separated. This was repeated three times with an equal volume of solvents. The two organic solvent layers were pooled together and it was evaporated under reduced pressure using a rotary evaporator to obtain the compound residues. The condensate of the solvent layer was dried and dissolved in methanol (1mg/mL), filtered through a membrane filter (0.2 µm), and stored at 4°C for GC-MS analysis. The compounds were also extracted from the 15 and 30th day old culture filtrate of *C. globosum* and liquid medium alone without fungus by using a method as described above. The extracted compounds were further characterized by GC-MS for compound identification.

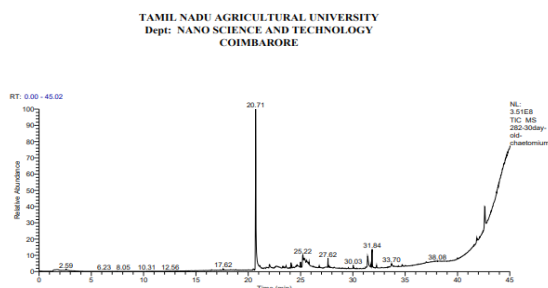


Figure 3. Total ion chromatogram of thirty days old Cell-Free Culture (CFC) of *C. globosum*

Characterization of toxin through GC-MS

Characterization of CFC filtrate condensate of *F. oxysporum f. sp. lycopersici* was done by GC-MS analysis. The column Elite-5MS (100 per cent Dimethyl poly siloxane), 30 x 0.25 mm x 0.25 µm df equipped with GC Clarus 500 Perkin Elmer and turbo mass - gold - Perkin - Elmer detector was used. The carrier gas (helium) flow rate was one mL per min, split 10:1 and injected volumes were two µL. The column temperature was maintained initially at 110°C at the rate of 10°C/min followed by increasing up to 280°C at the rate of 5°C/min and hold time 9 min. The injector temperature was 250°C and this temperature was held constant for

36 min. The electron impact energy was 70 e V, the Juliet line temperature was set at 2000°C and the source temperature was set at 200°C. Electron impact (EI) mass scan (*m/z*) was recorded in the 45-450 aMU range. Using computer searches on the NIST Ver. 2011 MS data library and comparing the spectrum obtained through GC-MS the compounds present in the sample were identified.

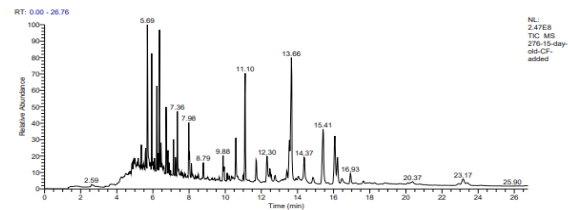


Figure 4. Co inoculation of 15 day old cell-free culture (CFC) filtrate of *C. globosum* and *F.oxysporum f.sp. lycopersici*

RESULTS AND DISCUSSION

Growth of *Fusarium oxysporum f. sp. lycopersici* in metabolite amended medium

F. oxysporum f. sp. lycopersici caused wilt disease in tomatoes leading to yield losses reducing production and productivity of tomato (Hussain *et al.*, 2016). The result of the current study is revealed that the fungus, *F. oxysporum f. sp. lycopersici* was able to grow in fifteen and thirty days old CFC filtrate of *C. globosum* amended medium at one per cent concentration. Nevertheless, the mycelia growth of *F. oxysporum f. sp. lycopersici* was less in these two treatments when compared with control.

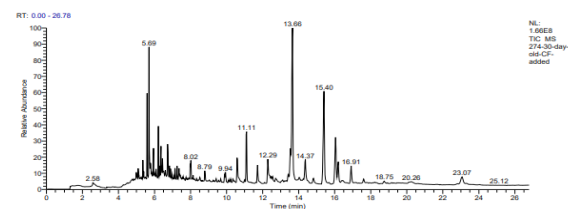


Figure 5. Co inoculation of 30th day old cell free culture (CFC) filtrate of *C. globosum* and *F. oxysporum f. sp. lycopersici*

The mycelial dry weight of the *F. oxysporum f. sp. lycopersici* was recorded only 0.864g in fifteen days old CFC filtrate followed by thirty days old CFC filtrate of *C. globosum* (1.374g) amended medium (Figure 1). Rashmi *et al.* (2011) reported that antagonistic fungal mycelial growth was suppressed when the other fungus was grown in a toxin amended medium.

Detection of compounds present in the metabolite from the CFC filtrate of *C. globosum* (Ch2) through GC-MS

Culture filtrates of 15 and 30 days - old cultures of *C. globosum* were subjected to GC-MS analysis. The results of GC-MS analysis of ethyl acetate

fraction of fifteen days old CFC filtrate condensate of *C. globosum* (Ch2) was found to have the presence of nine different compounds viz., DL-Proline, 5-oxo-, methyl ester; Cyclopropanebutanoic acid, 2-[[2-[[2-[(2-pentylcyclopropyl) methyl] cyclopropyl]methyl] cyclopropyl] methyl]-, methyl ester; Hexadecanoic acid, methyl ester; Methyl tetradecanoate; Octadecanoic acid, methyl

ester; Docosanoic acid, 1,2,3-propanetriyl ester; Diisooctyl phthalate; Tetradecanoic acid and 1-Monolinoleoylglycerol trimethylsilyl ether (Table1 and Figure 2). Soyong (1992) and Soyong *et al.* (2001) also reported that an isolate of *Chaetomium cupreum* producing metabolites significantly suppressed the mycelial growth of *F. oxysporum* f. sp. *lycopersici* causing wilt disease of tomato.

Table 1. GC-MS analysis of chloroform and ethyl acetate fraction of 15th days old cell-free culture filtrate condensate of *C. globosum* (Chg2)

RT	Compound	Molecular formula	MW (g/mol)	Peak area (per cent)	Probability
15.31	DL-Proline, 5-oxo-, methyl ester	C ₆ H ₉ NO ₃	143.14	13.52	42.41
17.79	Cyclopropanebutanoic acid, 2-[[2-[[2-[(2-pentylcyclopropyl)methyl]cyclopropyl]methyl]cyclopropyl]methyl]-, methyl ester	C ₂₅ H ₃₄ O ₂	374.6	0.80	8.12
24.06	Hexadecanoic acid, methyl ester	C ₁₇ H ₃₄ O ₂	270.45	5.96	75.73
21.10	Methyl tetradecanoate	C ₁₅ H ₃₀ O ₂	242.40	9.51	72.51
26.77	Octadecanoic acid, methyl ester	C ₁₉ H ₃₈ O ₂	298.50	3.13	51.92
28.23	Docosanoic acid, 1,2,3-propanetriyl ester	C ₆₉ H ₁₃₄ O ₆	1059.7	1.27	13.27
31.82	Diisooctyl phthalate	C ₂₄ H ₃₈ O ₄	390.60	11.94	21.35
21.71	Tetradecanoic acid	C ₁₄ H ₂₈ O ₂	229.36	5.58	49.07
37.01	1-Monolinoleoylglycerol trimethylsilyl ether	C ₂₇ H ₅₄ O ₄ Si ₂	500.9	0.51	35.71

RT-Retention time, MW - Molecular weight

GC-MS analysis of ethyl acetate fraction of thirty days old CFC filtrate condensate of *C. globosum* (Chg2) indicated the presence of six major volatile compounds. The compounds are namely, hexadecanoic acid, methyl ester; phenol, 2,4-bis(1,1-dimethylethyl); bicyclo[4.1.0]heptane,-3-cyclopropyl,-7-hydroxymethyl, trans; cholestan-3-ol, 2-methylene; 17-Pentatriacontene and Diisooctyl phthalate (Table 2 and Figure 3). Biswas *et al.* (2000 and 2012) studied the identification and characterization of antifungal compounds like

chaetoglobosin and chaetomin produced by *C. globosum*. These two compounds significantly suppressed the radial growth of the plant pathogens such as *Bipolaris sorokiniana*, *Macrophomina phaseolina*, *Rhizoctonia solani* and *Phytophthora ultimum*. They also reported that chaetoglobosin at the rate of 1000ppm and 500ppm mixed medium showed only 13mm and 17mm mycelial growth of *Bipolaris sorokiniana* compared with control (69mm).

Table 2. GC-MS analysis of chloroform and ethyl acetate fraction of 30 days old culture filtrate condensate of *C. globosum* (Chg2)

RT	Compound	Molecular formula	MW (g/mol)	Peak area (per cent)	Probability
24.06	Hexadecanoic acid, methyl ester	C ₁₇ H ₃₄ O ₂	270.45	2.53	75.73
17.62	Phenol, 2,4-bis(1,1-dimethylethyl)	C ₁₇ H ₃₀ OSi	278.5	0.63	46.18
20.71	Bicyclo[4.1.0]heptane,-3-cyclopropyl,-7-hydroxymethyl, trans	C ₁₁ H ₁₈ O	166.26	31.51	5.07
22.04	Cholestan-3-ol, 2-methylene-, (3á,5â)	C ₂₇ H ₄₈ O	388.7	13.89	8.48
30.03	17-Pentatriacontene	C ₃₅ H ₇₀	490.9	0.47	21.78
31.84	Diisooctyl phthalate	C ₂₄ H ₃₈ O ₄	390.60	3.46	23.20

Identification of volatile compounds from *F. oxysporum* f. sp. *lycopersici* (alone) and CFC filtrate of *C. globosum* amended medium

The volatile compound emission was higher in fifteen days old CFC filtrate when compared to thirty days old CFC filtrate. Among these two, two similar compounds were observed namely hexadecanoic acid, methyl ester, and diisooctyl phthalate. Hexadecanoic acid and methyl ester are having high relative content and probability. Earlier, Praveen Kumar *et al.* (2010) elucidated that, hexadecanoic acid (deconic acid derivatives) possess nematocidal and pesticidal properties.

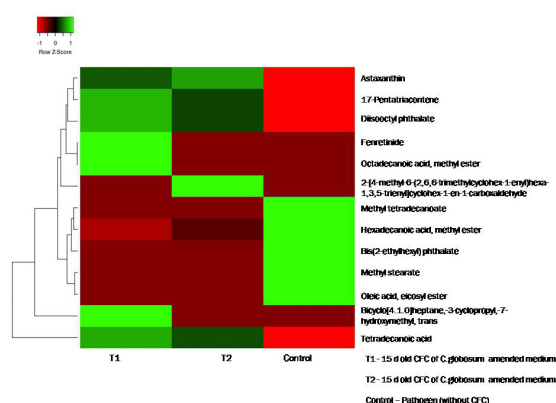


Figure 7. Heat map for *F. oxysporum* f. sp. *lycopersici* producing volatile organic compound at different conditions

This compound also had antioxidant and hypocholesterolemic potential. In addition, the authors also illustrated the presence of anti-

androgenic flavor compounds, haemolytic and 5-alpha reductase inhibitors in deconic acid derivatives. Volatile metabolites produced by fungi and bacteria have been extensively profiled and reviewed by many scientists (Kai *et al.*, 2009; Kwon *et al.*, 2010 and Sangeetha *et al.*, 2018). Soil microbes even bacteria, fungi, yeast, and other beneficial and pathogenic organisms produce volatile organic compounds that have been described to promote plant growth, induce systemic resistance and inhibit bacterial and fungal growth (Ryu *et al.*, 2003; Fernando *et al.*, 2005; Farag *et al.*, 2006; Schulz *et al.*, 2010; Yuan *et al.*, 2017 and Sangeetha *et al.*, 2018). *F. oxysporum* f. sp. *lycopersici* producing volatile organic compounds under normal medium are hexadecanoic acid, methyl ester; methyl tetradecanoate; oleic acid, eicosyl ester; methyl stearate, and bis(2-ethylhexyl) phthalate. Similarly, volatile organic compounds present under fifteen days old cell-free culture filtrate amended medium are octadecanoic acid, methyl ester; bicyclo[4.1.0] heptane, -3-cyclopropyl, -7-hydroxymethyl, trans; 17-Pentatriacontene; diisooctyl phthalate; tetradecanoic acid; fenretinide and astaxanthin (Table 3 and Figure 4). Additionally, two compounds were detected namely, hexadecanoic acid, methyl ester; 2-[4-methyl-6-(2,6,6-trimethylcyclohex-1-enyl)hexa-1,3,5-trienyl]cyclohex-1-en-1-carboxaldehyde. However, the three compounds were not found (octadecanoic acid, methyl ester; bicyclo[4.1.0] heptane, -3-cyclopropyl, -7-hydroxymethyl, trans; fenretinide) in thirty days old culture filtrate amended medium (Table 3 and Figure 5). Pathogen under normal medium was compared with CFC filtrate of

Table 3. *F. oxysporum* f. sp. *lycopersici* volatile pattern studied through GC-MS

RT	Name of the Compounds	Peak area of pathogen grown on 15 th d old CFC filtrate of <i>C. globosum</i> (per cent)	Peak area of pathogen grown on 30 th d old CFC filtrate of <i>C. globosum</i> (per cent)	Peak area of pathogen (without metabolite) (per cent)
24.06	Hexadecanoic acid, methyl ester	-	0.68	3.38
21.1	Methyl tetradecanoate	-	-	4.16
26.77	Octadecanoic acid, methyl ester	3.6	-	-
20.71	Bicyclo[4.1.0]heptane,-3-cyclopropyl,-7-hydroxymethyl, trans	12.52	-	-
30.03	17-Pentatriacontene	2.68	1.92	-
31.84	Diisooctyl phthalate	4.94	3.55	-
5.69	Tetradecanoic acid	15.31	12.11	-
12.30	Fenretinide	3.67	-	-
	Oleic acid, eicosyl ester	-	-	1.17
12.29	2-[4-methyl-6-(2,6,6-trimethylcyclohex-1-enyl)hexa-1,3,5-trienyl]cyclohex-1-en-1-carboxaldehyde	-	4.15	-
14.37	Astaxanthin	2.46	3.00	-
26.77	Methyl stearate	-	-	1.94
31.84	Bis(2-ethylhexyl) phthalate	-	-	5.32

C. globosum amended medium (treatment). Four compounds were produced by *F. oxysporum* f. sp. *lycopersici*, namely, methyl tetradecanoate; oleic acid, eicosyl ester; methyl stearate, and bis(2-ethylhexyl) phthalate (Table 3 and Figure 6). Cluster heat map analysis expressed the area percentage of compounds in the column and the treatment details represent in the row (Figure 7). These compounds are having antifungal and anti-bacterial activity (Rowsh anul Habib and Rezaul Karim, 2009; Abubakar and Majinda, 2016). *F. oxysporum* f. sp. *lycopersici* also produces antimicrobial compounds for their survivability in toxin amended medium.

CONCLUSION

The current investigation revealed the reduction of mycelial growth and toxin production of *F. oxysporum* f. sp. *lycopersici* under different days old cell-free culture (CFC) filtrate of *Chaetomium globosum* amended medium. The mycelial dry weight of *F. oxysporum* f. sp. *lycopersici* was recorded to be only fifty per cent in fifteen days old cell-free culture filtrate amended medium. The volatile organic compounds were characterized through GC-MS. Fatty acid, phenol, and terpenoids compounds were present in cell-free culture filtrate of *C. globosum* and this might be responsible for the reduction of mycelial dry weight. However, the additional compounds from the culture filtrate of *F. oxysporum* f. sp. *lycopersici* have antimicrobial nature for their self-survivability. Further study is needed to test compound compatibility and explore a mode of action against plant pathogens.

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