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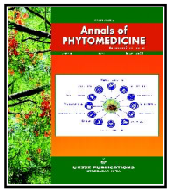

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Investigation on antifungal metabolites of Chinese caterpillar fungus *Ophiocordyceps sinensis* (Berk.) against wilt causing pathogen, *Fusarium* spp.

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Abstract

Soil-borne diseases pose a major threat to the production of both agricultural and horticultural crops. *Fusarium* genus is one of the common soil-borne fungi which causes wilt disease with typical symptoms of yellowing, stunting, and wilting and leads to even 100 per cent yield loss under severe conditions. Owing to the residual effects of fungicides and pesticides, eco-friendly approaches are the current focus of researchers all over the globe. *Ophiocordyceps sinensis* (Berk.) G.H. Sung and his team popularly called as Chinese caterpillar fungus is an entomophagous fungus that belongs to the phylum Ascomycota secretes secondary metabolites known to possess antimicrobial properties and used in medicine. In this view, the present study was formulated to tap antimicrobial metabolites from *O. sinensis* against *Fusarium* spp. The ethyl acetate and methanolic fractions of cell-free culture filtrate (18 days old) of *O. sinensis* at a concentration of 3000 ppm showed maximum inhibition of mycelial growth of *Fusarium oxysporum* f. sp. *lycopersici* (43.10 and 46.6 per cent, respectively), followed by *Fusarium oxysporum* f.sp. *cubense* (42.20 and 41.10 per cent, respectively), when tested by poisoned food technique. Similarly, the methanolic and ethyl acetate fraction of *O. sinensis* of CFC condensate tested at higher concentrations of 9000 ppm by paper disc assay, showed maximum inhibition of *F. o* f.sp *cubense* (72.20 and 70.20 per cent, respectively), followed by *F.* of. sp *lycopersici* (61.10 and 66.60 per cent, respectively). Characterization of antimicrobial metabolites of the methanolic fraction of CFC of *O. sinensis* through GC-MS analysis indicated the presence of twelve different compounds. Among these, n-decanoic acid, glycerin, and 1, 2, 3-benzenetriol was expressed with the highest peak area percentage which might be responsible for antifungal activity. Chitinase gene expression evidenced with the expression of amplicon size of 1047 bp indicates that *O. sinensis* may also play a major role as a biocontrol agent. This study paves the way for identifying the antifungal compounds of *O. sinensis* against *Fusarium* spp.

1. Introduction

Ophiocordyceps sinensis (Berk.) G.H. Sung and his team popularly called summer grass and winter worm or caterpillar fungus (Cooke, 1892), is an entomophagous fungus, which belongs to the phylum Ascomycota. The fungus parasitizes larvae of ghost moths (Lepidoptera) or Thitarodes (*Hepialus* spp) moths. The erstwhile known anamorphic state of the genus *Cordyceps, viz., Beauveria, Metarhizium,* and *Paecilomyces* have been reported as biocontrol agents against insect pests and nematodes. It is one of the most valuable medicinal fungus known by pharmaceutical industries which helps in curing various diseases and its metabolites act as antibacterial, immunomodulatory (Zhong *et al.*, 2009), immunosuppressive (Dong and Yao, 2011), anticomplementary (Zhou *et al.*, 2009), antitumor, anti-inflammatory, antioxidant, anti-diabetes, antifatigue, and antiaging. It also possesses various biologically active compounds like cordycepin (Zhou *et al.*, 2009), polysaccharide (Zhong *et al.*, 2009 and Dong and Yao, 2011), adenosine (Zhang *et al.*, 2010), ergosterol (Das *et al.*, 2010), cordymin (Shrestha *et al.*, 2012) and ethyne and ergotamine (Sangeetha *et al.*, 2015b). Although, there is little work studied in *Ophiocordyceps* spp. against plant pathogens. More fascinatingly, *Cordyceps sobolifera* has been used as a potential biocontrol agent against *Colletotrichum gleosporioides* and *Cochilobolus miyabeanus* (Imtiaj and Lee, 2007). Varughese *et al.* (2012) reported that the two antifungal depsidones cordycepsidone A and cordycepsidone B isolated from *C. dipterigena* being antagonistic to *Giberella fujikori.* Sangeetha *et al.* (2015a) reported the antimicrobial activity of culture filtrates of *O. sinensis* against *F. oxysporum* f. sp. *lycopersici* and *F. oxysporum* f. sp. *cubense*. Despite its greater potential, the fungus, *O. sinensis* has not been explored properly in agrochemical industries. In this present investigation, experiments were carried out to study the antifungal activities of biomolecules from *O. sinensis.*

2. Materials and Methods

2.1 Collection of fungal cultures

Pure cultures of *Ophiocordyceps sinensis*, *Fusarium oxysporum* f. sp. *lycopersici* (Sacc.) Synder and Hansen, *Fusarium oxysporum* f. sp. *cubense*, *Fusarium ciceri*, *F. oxysporum*, *Fusarium* spp. were obtained from the culture collection facility available in the Department of Plant Pathology, TNAU, Coimbatore.

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2.2 Extraction of bioactive compounds of *O. sinensis* using different solvents

Four mycelial discs measuring 6 mm diameter each, cut from the margin of a 5 d old colony of *O. sinensis*, were inoculated to 100 ml of sterilized sabouraud dextrose agar (SDAY) broth (adjusted to pH 5.5) taken in 250 ml conical flasks. The flasks were later placed on a rotary shaker at 120 rpm and incubated at 25°C in darkness for 18 days. After incubation, the culture filtrate and the mycelial mat were separated by filtration through Whatman No. 40 filter paper. The filtrate was further centrifuged at 10,000 rpm and the mycelium free culture filtrate was extracted with organic solvents, *viz.*, methanol, ethanol, and ethyl acetate, (v/v) sequentially. The organic extracts of each solvent were evaporated separately under reduced pressures using a rotary evaporator at 40°C. The condensate of each solvent was dried and dissolved in methanol (1mg/ml) and filtered with a membrane filter (0.2 µm) and stored at 4°C for further study.

2.3 Testing of secondary metabolites extracted with different solvents

The antimicrobial activity of each solvent fraction of *O. sinensis* was evaluated by poison food technique. Crude extracts of CFC filtrate of different solvents fraction mixed separately with 1000, 2000, 3000, 6000, and 9000 ppm with 100 ml of sterilized molten PDA medium and poured into sterile petri plates and allowed to solidify. At the center of the poisoned medium, a 6 mm mycelial disc of the test pathogen was introduced and the petri plates were incubated at 28 ± 2°C. Constant observations were made and the per cent inhibition of mycelial growth of *Fusarium* f. sp. *lycopersici*, *Fusarium* f. sp. *cubense*, *F. ciceri*, and *Fusarium* spp. were recorded. The per cent inhibition was calculated by using the formula as proposed by Vincent (1927).

2.4 Ethyl acetate and the methanolic fraction of CFC filtrates of *O. sinensis* against *Fusarium* spp.

Based on the above studies, ethyl acetate and the methanolic fraction of CFC filtrate found effective at 3000 ppm were again tested at higher concentrations. The inhibitory effect of ethyl acetate and the methanolic fraction of CFC filtrate of *O. sinensis* were evaluated by the paper disc assay method. Sterilized Whatman filter paper disc was immersed in crude extracts of the CFC filtrate of ethyl acetate and methanolic fractions at the concentration of 3000, 6000, 9000 ppm and placed at one cm apart from four corners of sterilized petri plate containing PDA medium. The pathogen was placed at the center. The mycelial growth and zone of inhibition were recorded after 7 d of incubation. Based on the observations, a per cent reduction over control was calculated. Each dose was replicated three times. Sterile water used as a control was also maintained to assess the effect of bioactive molecules (Bauer *et al.*, 1966). The per cent inhibition was calculated by using the formula as proposed by Vincent (1927).

2.5 Detection of bioactive molecules through gas chromatography mass spectrometry analysis (GC-MS)

Characterization of biomolecules of CFC filtrate condensate of *O. sinensis* (Methanolic fraction dissolved in DMSO) was done by GC–MS analysis. The column Elite-5MS (100 per cent Dimethyl-polysiloxane), 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 set at 250°C and was kept constant for 36 min. The electron impact energy was 70 eV, Julet 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.

2.6 Chitinase gene expression of *O. sinensis*

The genomic DNA of *O. sinensis* was subjected for PCR amplification using a range of primer pairs, *Bbchit1-15*'CCCTTCTACCCTTGACTTGTTC-3' and *Bbchit1-2* 5'TAT CTACAAATATGTACCAAC-3' for the detection of the chitinase gene. PCR was performed in a thermocycler (Eppendorf-Master Cycler nexus gradient S-Eppendorf, A.G, Hamburg, Germany) by using the conditions included 5 min preheating step at 95°C followed by 35 cycles consisting of denaturation at 95°C for 30 sec, annealing at 60°C for 30 sec, extension at 72°C for 2 min and followed by a final extension at 72°C for 10 min.

2.7 Statistical analysis

All the experiments were performed in triplicate and the treatment means differences were evaluated with Duncan's Duncan's Multiple Range-Test (DMRT) at 5% significance (Gomez and Gomez, 1984). The package used for analysis was IRRISTAT version 92-1 developed by the International Rice Research Institute Biometrics unit, the Philippines.

3. Results

3.1 Testing the antifungal potential of *O. sinensis* against *Fusarium* spp.

3.1.1 Poisoned food technique

Bioactive compounds were extracted from the CFC (Cell-free culture) filtrate condensate of *O. sinensis* (18 d old) with different solvents, *viz.*, methanol, ethanol, ethyl acetate. The concentration of solvent fractions was adjusted to 1000 ppm, 2000 ppm, and 3000 ppm was represented in Figure 1. Among the solvents tested, the methanolic and ethyl acetate fractions of CFC filtrate condensate at 3000 ppm showed the maximum mycelial inhibition of *F. o.* f.sp. *cubense* (42.20 and 41.10 per cent, respectively), *F. o.* f.sp. *lycopersici* (43.10 and 46.60 per cent, respectively), *F. cicero* (40.00 and 42.20 per cent, respectively), *Fusarium* spp. (41.10 and 40.00 per cent, respectively) and *F. oxysporum* (35.50 and 43.30 per cent, respectively), followed by 2000 ppm, the mycelial inhibition of *F. o.* f.sp. *cubense* (40.00 and 41.11 per cent, respectively), *F. o.* f. sp. *lycopersici* (41.10 and 44.4 per cent, respectively), *F. ciceri* (40.00 and 42.20 per cent, respectively *Fusarium* spp. (44.40 and 37.70 per cent, respectively) and *F. oxysporum* (33.50 and 43.30 per cent, respectively).

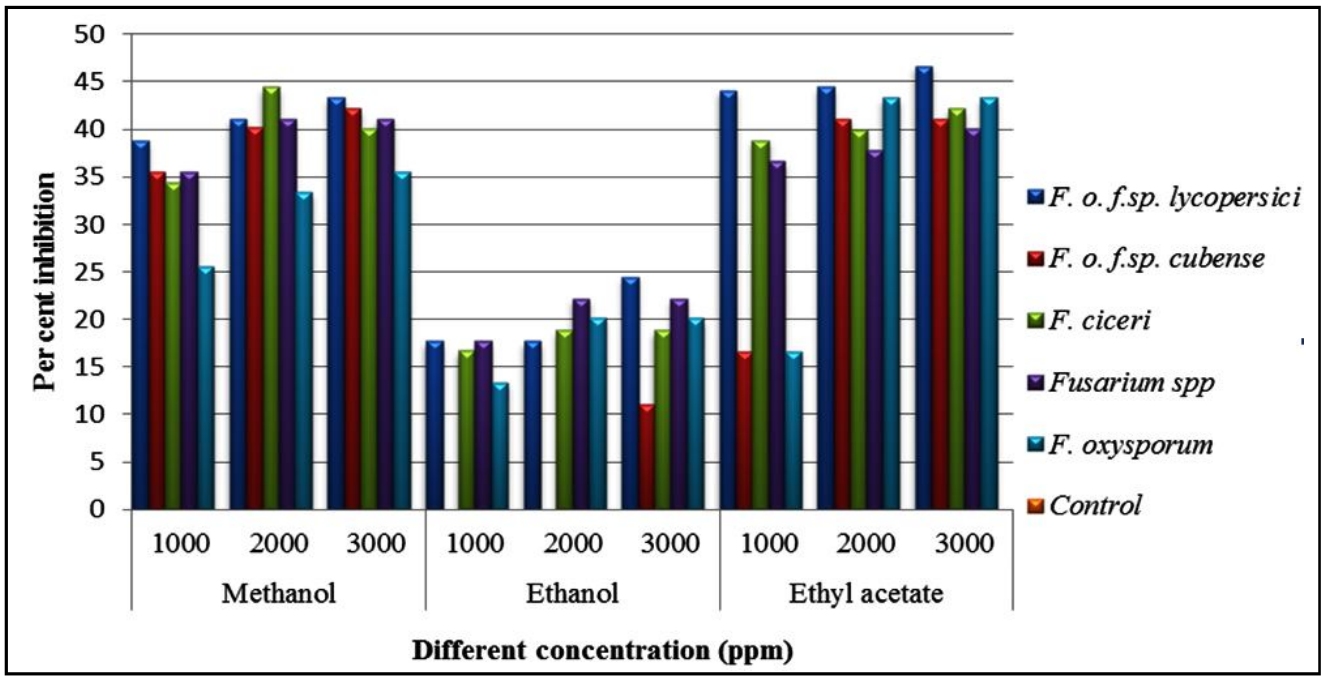

Figure 1: Effect of the different solvent fraction of *O. sinensis* against *Fusarium* spp.

In the case of methanolic and ethyl acetate fractions of CFC filtrate condensate, a concentration of 1000 ppm showed the minimum mycelial inhibition of *F. o.* f. sp. *cubense* (35.5 and 16.60 per cent, respectively), *F. o.* f. sp. *lycopersici* (38.80 and 41.10 per cent, respectively), *F. ciceri* (34.00 and 38.80 per cent, respectively), *Fusarium* spp. (35.50 and 36.60 per cent, respectively), *F. oxysporum* (16.60 and 25.50 per cent, respectively). The ethanolic fraction of culture filtrate condensate in 1000, 2000, and 3000 ppm concentration showed the lowest mycelial inhibition of *F. o.* f. sp. *cubense* (0.00, 0.00 and11.11 per cent, respectively), *F. o.* f. sp. *lycopersici*(17.70, 17.71 and 24.40 per cent, respectively), *F. ciceri* (16.60, 18.80 and 18.80 per cent, respectively), *Fusarium* spp. (17.70, 22.20 and 22.20 per cent, respectively) and *F. oxysporum* (13.30, 20.00 and 20.00 per cent, respectively). Also, further study to confirm the above assay methanolic and ethyl acetate fraction was tested against *F. o.* f. sp. *lycopersici* and *F. o.* f. sp. *cubense* at higher concentrations.

3.1.2 Paper disc assay

The methanolic and ethyl acetate fractions of *O. sinensis* CFC condensate tested by paper disc assay showed the maximum inhibition in *F. o.* f. sp. *cubense* (72.20 and 70.20 per cent, respectively) followed by *F. o.* f. sp. *lycopersici* (61.10 and 66.60 per cent, respectively) at a concentration of 9000 ppm, followed by 6000 ppm which showed the inhibition of in *F. o.* f. sp. *cubense* (61.10 and 55.50 per cent), followed by *F. o.* f. sp. *lycopersici* (53.30 and 54.40 per cent respectively). The minimum inhibition was noticed at the concentration of 3000 ppm in *F. o.* f. sp. *cubense* (42.20 and 44.40 per cent), followed by *F. o.* f. sp. *lycopersici* (35.80 and 40.00 per cent, respectively) in methanolic fraction (Table 1; Figure 2). The result of current investigation conclusively indicated non-polar solvents methanol could be effectively used for extraction of bioactive compounds from *O. sinensis* compared to other solvents.

Table 1: Testing of ethyl acetate and methanolic fractions of CFC filtrate condensate of *O. sinensis* against *Fusarium* spp.

Fraction of bioactive molecules	Concentrations (ppm)	*F. o.* f. sp. *lycopersici*		*F. o.* f. sp. *cubense*	
		Growth (mm)	PI	Growth (mm)	PI
Ethyl acetate fraction	3000	58.00 (50.06)	35.80^{f} (36.75)	52.00 (46.49)	42.20^{f} (40.45)
	6000	42.00 (40.39)	53.30^{d} (46.89)	35.00 (35.57)	61.10^{c} (51.45)
	9000	35.00 (36.27)	61.10^{b} (51.41)	25.00 (30.17)	72.20^{a} (58.18)
Methanolic fraction	3000	54.00 (47.29)	40.00^{e} (39.23)	50.00 (38.52)	44.40^{e} (41.78)
	6000	41.00 (39.81)	54.40^{c} (47.29)	40.00 (38.12)	55.50^{d} (48.15)
	9000	30.00 (33.21)	66.60^{a} (54.69)	27.00 (31.98)	70.00^{b} (56.79)
Control		90.00	0.00	90.00	0.00
CD (p = 0.05)		1.49	-	1.27	-

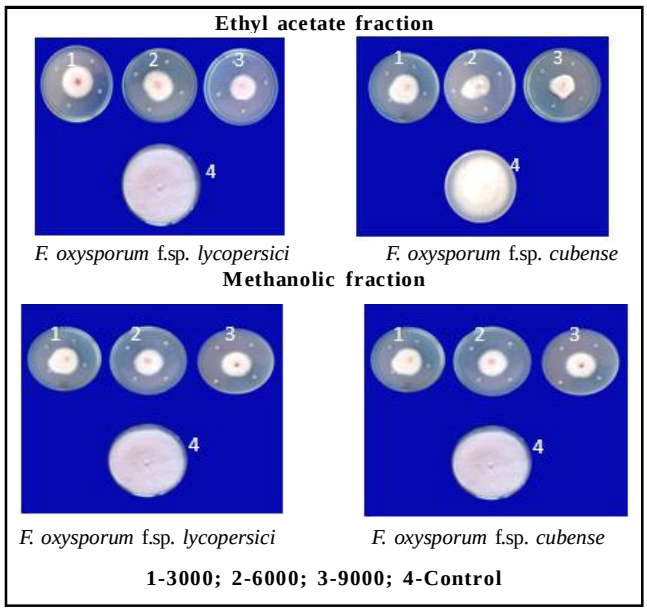

Figure 2: Effect of ethyl acetate and methanolic fractions of CFC condensate of *O. sinensis* against *Fusarium* spp.

3.3 Profiling of volatile organic compounds (VOCs) of *O. sinensis* through GC-MS

The methanolic fraction of CFC filtrate condensate was subjected to GC-MS analysis. The identity of compounds was confirmed through the NIST library 2011 and the AMDIS software program. The results showed an array of biomolecules as presented in Table 2 and Figure 2.

Twelve different compounds, *viz.*, hydroxylamine; glycerin; 4H-pyran-4-one, 2, 3-dihydro-3, 5-dihydroxy-6-methyl; dianhydromannitol; mexiletine; methyl eugenol; 1,2,3-Benzenetriol; cyclopropane, nonyl; cyclohexane-1,4,5-triol-3-one-1-carboxylic acid; 2-(allylamino)-5-ethyl-1,3,4-thiadiazole; cyclopropane, 1,2-dibutyl and n-decanoic acid have been detected. Among the 12 compounds, the highest peak area was observed with n-decanoic acid (32.95 per cent) at 24.398 RT, followed by glycerin (10.67 per cent) at 10.92 RT and 1, 2, 3-benzenetriol (5.24 per cent) at a retention time of 18.893 (Figure 3 and Table 2).

3.4 Chitinase gene expression

In this present study, the presence of chitinase producing gene was identified by the PCR analysis using gene-specific primer indicate the name of the primer *O. sinensis* contained chitinase producing gene, which was amplified at 1047 bp (Figure 3).

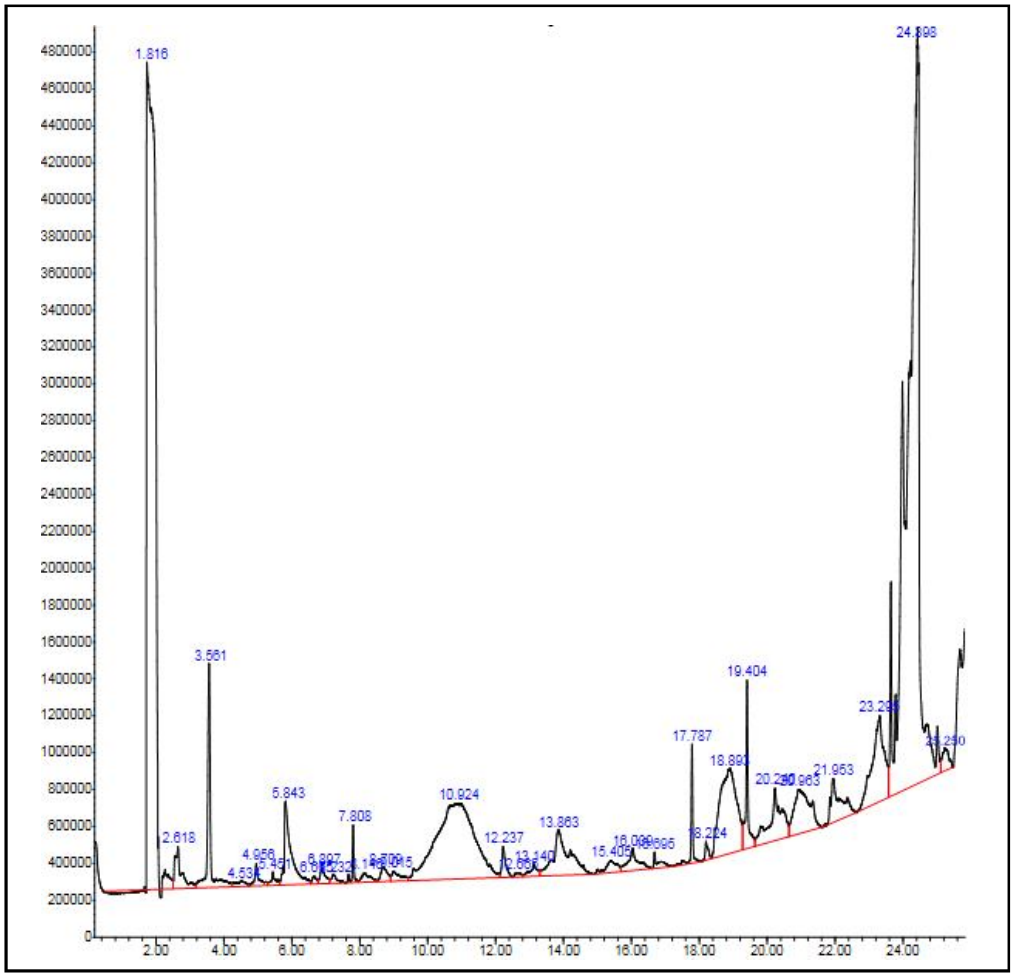

Figure 3: Chromatogram of methanolic extract of CFC filtrate condensate of *O. sinensis*.

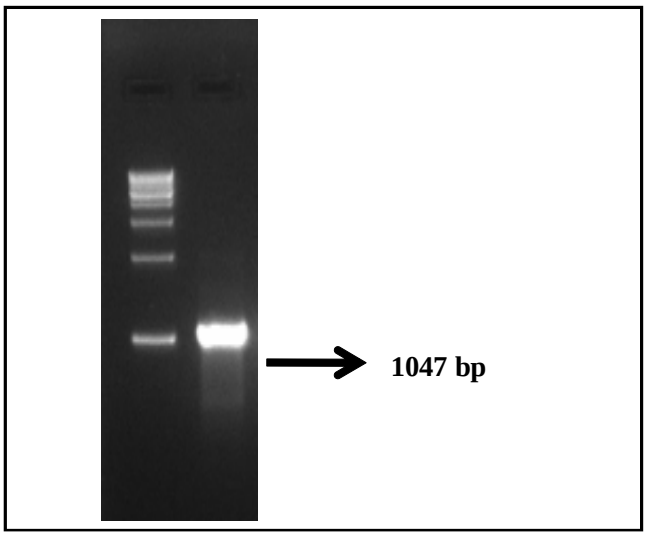

Figure 4: PCR amplification of Chitinase gene expression of *O. sinensis.*

4. Discussion

Bioactive compounds were found to be diversely distributed in the cell-free culture filtrate of *O. sinensis*. Among the different solvents used for extraction and recovery of bioactive compounds, ethyl acetate and methanolic fractions of CFC condensate had exhibited considerable antifungal activity (Kumar *et al*., 2013). In the present study, the methanolic extract of cell-free culture filtrate of *O. sinensis* produced diverse groups of potential biologically active compounds against *Fusarium* spp. It is observed that *Cordyceps* sp. had inhibited the mycelial growth of *R. solani* followed by *A. brassicae*, *F. oxysporum*, *Sclerotium rolfsii*, and *Sclerotium sclerotiorum* (Pandey, 2012). The ethanolic extract of non-edible macrofungi *Stereum ostrea* had contained antifungal properties against *Colletotrichum gloeosporioides*, *C. miyabeanus*, and *Botrytis cinerea* Imtiaj and Lee, (2007). Similarly, in our study, the methanolic and ethyl acetate fractions of cell-free culture filtrate of *O. sinensis* effectively inhibits the mycelial growth of *F. oxysporum* f.sp. *lycopersici* followed, by *F. oxysporum* f.sp *cubense* which was found to be significantly different from other *Furasium* spp at the concentration of 3000 ppm. The paper disc assay demonstrated that the CFC condensate of *O. sinensis* dissolved in ethyl acetate and methanolic fraction showed that the maximum inhibition was observed in *F. o*. f. sp *cubense* (72.2 per cent and 70.02 per cent), followed by *F. o*. f. sp *lycopersici* (61.10 and 66.60 per cent respectively), at a concentration of 9000 ppm which was significantly different from the other concentrations tested. Ahamad, (2012**)** reported that the crude ethyl acetate extract of *B. bassiana* had exhibited moderate antifungal activity at concentrations between 1200 and 1600 µg/ml against *A. tenuis, F. avenaceum, and F. graminearum* by paper disc assay. Chen and Huang, (2010) also tried the paper disc agar diffusion method to test the effect of 27 culture filtrates from 14 species of edible mushroom fungi tested against, *viz*., *Colletotrichum higgisianum*, *Phytophthora capsici*, *Fusarium oxysporum* f.sp. *lactuca*, *Alternaria brassicola*, *Rhizoctonia solani*, and *Pythium aphanidermatum* and found that the CFC of *Agaricus bisporus* slightly suppressed the mycelial growth of *Alternaria brassicola* (37.3%) and weakly inhibited the mycelial growth of *Colletotrichum higginsianum*, *Fusarium oxysporum* f.sp *lactuca,* and *Phytophthora capsici* when compared to others. Furthermore, in our investigation, GC-MS was employed to identify the antifungal compounds.

Table 2: GC-MS analysis of the methanolic fraction of CFC filtrate condensate* (18 d old) of *O. sinensis*

RT*	Compound	Molecular formula	Molecular weight (g/mol)	Mode of action	Reference
10.92	Glycerin	$C_3H_8O_3$	92.09	Defense response	Li *et al*. (2016)
12.23	4H-Pyran-4-one	$C_6H_8O_4$	144.0	Antimicrobial activity	Dahpour *et al*. (2012)
13.86	Dianhydromannitol	$C_6H_{10}O_4$	146.0	Antimicrobial	Rajeswari *et al*. (2013)
15.40	Mexiletine	$C_{11}H_{17}NO$	179.0	Antiarrhythmic agent	Labbe *et al*. (2003)
17.78	Methyleugenol	$C_{11}H_{14}O_2$	178.0	Antifungal agent	Choi *et al*. (2010)
18.89	1,2,3-Benzenetriol	$C_6H_6O_3$	126.0	Fungicidal and insecticidal properties	Euginamala and Jeyaraj, (2014)
19.40	Cyclopropane, nonyl	C_3H_6	42.08	Biopesticidal properties	Praveen Kumar *et al*. (2010)
20.96	Cyclohexan-1,4,5-triol-3-one-1-carboxylic acid	$C_7H_{10}O_6$	190.15	Pharmacheutical applications	Praveen kumar *et al*. (2010)
21.95	2-(Allylamino)-5-ethyl-1,3,4-thiadiazole	$C_4H_7N_3S$	129.19	Anti-microbial and anti-inflammatory	Popiolek *et al*. (2013)
24.39	n-Decanoic acid	$C_{10}H_{20}O_2$	172.0	Nematicidal and pesticidal properties	Praveen Kumar *et al*. (2010)

The results indicated that the presence of twelve compounds and its antifungal nature of the compounds were analyzed. Among these, n-decanoic acids, glycerin, and 1, 2, 3-benzenetriol were expressed with the highest peak area per cent which might be responsible for antifungal

activity n-decanoic acid belongs to the class medium-chain fatty acid has possessed nematicidal and pesticidal properties (Praveen Kumar *et al.,* 2010). Popiolek *et al*. (2013) illustrated that 2-(allylamino)-5-ethyl-1, 3, 4-thiadiazole had possessed a wide range of biological activities including antimicrobial and anti-inflammatory activities. Glycerin is an organo-oxygen compound reported with antibacterial, antioxidant, hypo-cholesterolemic activity and also has a defense response against plant pathogens (Li *et al*., 2016). Another compound, namely; 1, 2, 3-benzenetriol and its derivatives 1, 3, 5-benzenetriol pyrogallol compound are aromatic alcoholic compounds known for fungicidal, insecticidal, antioxidant, and antiseptic activities (Euginamala and Jeyaraj, 2014). Terpenoid and steroid molecules such as cyclopropane, 1, 2-dibutyl, cyclohexan-1, 4, 5-triol-3-one-1-carboxylic acid, and cyclopropane, nonyl has several pharmaceutical and biopesticidal applications (Praveen Kumar *et al.*, 2010). Other compounds like methylene chloride, oxime, methoxy-phenyl, mexiletine, 4H-pyran-4-one, 2, 3-dihydro-3, 5-dihydroxy-6-methyl detected from *O. sinensis* preparations have been reported to possess antimicrobial, biopesticidal, and pharmaceutical properties (Dahpour *et al*., 2012; Janani and Singaravadivel, 2014).

Fungal chitinases have been exploited in the development of bio-control strategies against insects, fungi, or nematodes (Seidl, 2008; Leger and Wang 2010). Fungi are known to possess various chitin degrading enzymes with an average of 10-20 different chitinases; where chitinases play diverse biological roles, such as cell-wall remodeling, nutrient scavenging, and aggressive functions (Seidl, 2008; Xu *et al*., 2011; Hartl *et al*., 2012 and Hamid *et al*., 2013). Over expression of *Bbchit1* was able to increase the ability of *Beauveria* (*Cordyceps*) *bassiana* to digest insect cuticle, resulting in increased virulence against insects (Murad *et al*., 2007). Similarly, in our study, *O. sinensis* is known to produce chitinase genes which might be responsible for defense response against phytopathogens. Chantasingh *et al*. (2011) reported that *O. unilateralis* express chitinase (*Ouchi*) full-length gene (1311 bp) encodes 436 amino acids with the first 20 amino acids as a putative signal peptide. The chitinase expression also enhanced fungal toxicity to another organism. The mature *Ouchi* gene was sub-cloned into an *Agrobacterium* and then transformed into *B. bassiana*. The expression of chitinase *Ifu-chit2 in vivo* suggests that the chitinase may play a role in the early stage of pathogenesis (Meng *et al.,* 2015). With this background, it is concluded that the chitinase produced by *O. sinensis* may also play a major role in hydrolyses of chitin in insects for further colonization and production of fruiting bodies.

5. Conclusion

The present study revealed that the methanolic and ethyl acetate fraction of cell-free condensate of *O. sinensis* could inhibit the mycelial growth of *Fusarium* spp. Further, characterization of the methanolic extracts of CFC through GC-MS concealed the presence of more antifungal metabolites. Interestingly, the presence of chitinase-producing genes in *O. sinensis* offers scope for use as bio-control agents. The results obtained from this study will certainly paves way for the exploration of bioactive compounds for the development of ecofriendly fungicidal formulations from *Ophiocordyceps* spp against plant pathogens.

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Conflict of interest

The authors declare that there are no conflicts of interest relevant to this article.

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