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GC-MS analysis of culture filtrates of *Trichoderma harzianum* and their efficacy of biocontrol measures of plant pathogens

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Abstract

Trichoderma species is a filamentous soil borne fungus known to be an effective bio control agent against plant pathogens. The chemical compositions of culture filtrates of *Trichoderma harzianum* were investigated using Perkin-Elmer Gas Chromatography–Mass Spectrometry, while the mass spectra of the compounds found in the extract was matched with the National Institute of Standards and Technology (NIST) library. Gas Chromatography - Mass Spectrometry analysis from *T. harzianum* showed five retention times 1.91, 4.28, 4.52, 26.42 and 30.21 min. There, action time 1.91 corresponds to 3,6,9,12,15,18-hexaoxa-1-triacontanolacid; 4.28corresponds to N- (tau-benzyl-N(1)-(tert-butoxycarbonyl) histidyl) serine methyl ester acid; 4.52 corresponds to didecyl phthalate acid; 4.22 corresponds to methoxy glutamic acid methyl ester; 4.32 corresponds to triacontanoic acid.

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Introduction

Biological fungicides may act to suppress the population of the pathogenic organisms through competition. Stimulated plant growth may allow plants to quickly outgrow any pathogen effects or damage the pathogen by means of toxins produced (Cook, 2000; Gilreath, 2002). Bio control agents are derived from natural materials such as animals, plants, bacteria, fungi and other living organisms. Fungus exhibiting myco parasitic behaviour eliminates the threat of synthetic fungicides. The goal, the biological control methods can be effectively used along with other methods of disease control. The soil mycoflora has the potentiality to secrete antibiotic substances. The soil fungi include both 'soil in habitants' and 'soil invaders. The interactions between these two groups of organisms are the determinant factors of the competitive survival of the pathogens in soil which include antagonism and symbiosis. Fungal antagonism is usually characterized as the mechanisms that protect the prior colonists of the substrates from colonization by other organisms (Garrett, 1956; 1970).

The bio control mechanisms of soil borne antagonistic fungus were also established (Haran *et al.*, 1996; Zhihe *et al.*, 1998; Panneerselvam and Saravanamuthu, 1994; 1996). Antagonistic interactions and cell free culture filtrate have been used to demonstrate the role of antibiotics in biological control (Naik and Sen, 1992; Panneerselvam and Saravanamuthu, 1994). *Trichoderma* species is a filamentous soil borne fungus known to be an effective bio control agent against plant pathogens. Weindling and Emerson (1936) stated that they could excrete extra cellular compound called gliotoxin. Since then antibiotics and extracellular enzymes are isolated and characterized. *Trichoderma harzianum* is known for its mycoparasitic and antagonistic mechanism for the control of fungal disease. *Trichoderma harzianum* lone or incombination with other *Trichoderma* species can be used in the biological control of several plant diseases (Papavizas, 1985; Chet, 1987; Samuels, 1996). Although *Trichoderma* sp. is ubiquitous, the type of fungi in the soil can affect growth, proliferation and effectiveness as biocontrol agent.

The possible mechanism of antagonism employed by *Trichoderma* sp. are realized so far including competitions, antibiosis by producing non-volatile, volatile antibiotics and exploitation (Harman and Hadar, 1983). The application of *Trichoderma* species can control a large number of foliar leaves disease caused by soil borne fungi i.e., *Fusarium* sp., *R. solani*, *Pythium* sp., *S. sclerotium, S. rolfsii* in vegetable fields, fruits and other crops (Ngo *et al.*, 2006). Aside from having few adverse effects and promising therapeutic applications, bio-efficient natural compounds are a promising source of new antioxidants and antibacterial agents (Wassima *et al.*, 2023). Some strains of *T. longibrachiatum* and *T. orientale* are found to be toxic to humans, especially in immunocompromised patients (Afrasa *et al.*, 2022). The antagonistic activity of *Trichoderma* sp. against different plant pathogens occurs through different mechanisms of action, including antibiosis, mycoparasitism and competition for nutrients and space (Yassin *et al.*, 2021). The rate of decomposition process increases when there is the inoculation of Trichoderma spp. in an agricultural waste substrate such as empty fruit bunches (EFB), palm oil mill effluent (POME) and crop residues (Zin and Badaliuddin, 2020). In *Trichoderma*, such essential, highly adaptive functional traits combine to form a coherently evolved group of fungi ideally suited to restore a diseased plant environment, thus capable of promoting eco-friendly, sustainable agricultural applications (Laszlo *et al.*, 2024). The aim of this study to determine the organic compounds present in the *Trichoderma harzianum* with the aid of GC-MS technique, which may provide an insight in its use in biocontrol.

Materials and methods

Isolates of fungi

Totally 48 species were isolated from paddy field soil, Thanjavur Dt., Tamil Nadu. About ten species were selected to screen for their antifungal activity against plant pathogenic fungi.

Antibiotic interactions assay

A preliminary screening was conducted against *Bipolaris oryzae* with all the fungi isolated from the soil. Based on this, *Trichoderma harzianum* species were selected for further antagonistic assay.

Culture filtrate method (Skidmore and Dickinson, 1976)

Agar blocks of equal size (5 mm dia) was cut from the actively growing margin of the individual species of soil fungi namely *Trichoderma harzianum* inoculated separately into the 250ml conical flasks containing 100ml sterile potato dextrose broth. The flasks were incubated at 25 ± 2°C for 15 days. After incubation, the cultures were filtered through Whatman No.1 filter paper and seitz filter (G5). The filtrates were transferred in to conical flasks and stored at 4°C for further use. The culture filtrates were added separately to the cooled PDA medium to give the concentration of 5, 10, 15 and 20%. The amended media was dispersed separately in to Petri dishes and allowed to solidify. After solidification, 5 mm agar block was cut from the actively growing margin of the test fungus and inoculated at the centre of each plate. The plates were incubated at 25 ± 2°C for five days. The radial growth was measured periodically at 24 hrs interval and the mean growth rate was calculated. Control was also maintained.

Percentage of inhibition growth = {(Growth in control – Growth in treatment)/ (Growth in control)} ×100

The culture filtrate technique, *T. harzianum* inhibited the growth of the pathogen to the maximum extent. Hence, *T. harzianum* was taken for further antimicrobial compound separation.

GC-MS analysis of the culture filtrate

Extraction of antifungal compound (Liu et al., 2007)

The fungi which showed promising activity against the pathogen was cultured in liquid Potato Dextrose Agar medium at 25°C in darkness for three weeks. After incubation, the *Trichoderma harzianum* culture was filtered twice through Whatman No.1 filter paper and Seitz filter (G.5). *T. harzianum* 100 ml of culture filtrate, 10 ml of ethyl acetate was added in a separation funnel (250 ml), shaken well for 3 min. and the solvent and aqueous layer were separated. The ethyl acetate layer of the culture filtrate was used for further analysis**.**

Gas chromatography-mass spectrometry (GC-MS)

Volatile components were identified by GC-MS using a column Elite-1 (100% Dimethyl poly siloxane), 30 x 0.25 mm x 1µm df equipped with GC clarus 500 Perkin Elmer. The turbo mass-gold-perkin-Elmer detector was used. The carrier gas flow rate was 1 ml per min, split 10:1, and injected volumes were 2 µl. The column temperature was maintained initially at 110˚C for 2 min (hold) followed by increases up to 200˚C at the rate of 10˚C per min (no hold), up to 280˚C at the rate of 5˚C for 9 min (hold). The injector temperature was 250˚C and this temperature was held constant for 36min.The electron impact energy was 70eVJ ulet, line temperature was set at 200˚C and the source temperature was set at 200˚C. Electron impact (EI) mass scan (m/z) was recorded in the 45-450 a MU range. Using computer searches on the NIST Ver.2.1 MS data library and comparing the spectrum obtained through GC-MS the compounds present in the crude sample were identified.

Results

Culture filtrate analysis of T. harzianum

The maximum percentage of inhibition on the growth of the potato dextrose agar medium amended with 20 % of the culture filtrate of *Trichoderma harzianum* (77.41%). In culture filtrate technique, *Trichoderma harzianum* inhibited the growth of the pathogen to the maximum extent. Hence, *T. harzianum* was taken for further antifungal compounds separation, purification and molecular characterization.

GC-MS analysis of culture filtrates of T. harzianum

Gas chromatography – mass spectrometry analysis of ethyl acetate extract of *T. harzianum* showed five retention times 1.91, 4.28, 4.52, 26.42 and 30.21 min. The reaction time 1.91 corresponds to 3,6,9,12,15,18-hexaoxa-1-triacontanol acid; 4.28

corresponds to N-(tau-benzyl-N(1)-(tert-butoxycarbonyl) histidyl) serine methyl ester acid;4.52 corresponds to didecylphthalate acid;4.22corresponds to methoxy glutamic acid methyl ester ; 4.32 corresponds totriacontanoic acid (Table 1; Fig. 1 and 2).

Table 1. Characteristic features of antifungal compounds

#	Molecular formula	Molecular weight	RT time	Name of the compounds
1	$C_{24}H_{50}O_7$	450.7	1.91	3,6,9,12,15,18-hexaoxa-1-triacontanol
2	$C_{22}H_{30}N_4O_6$	446.5	4.28	N-(tau-benzyl-N(1)-(tert- Butoxycarbonyl) histidyl) serine methyl ester
3	$C_{28}H_{46}O_4$	446.7	4.52	Didecylphthalate
4	$C_{21}H_{28}N_2O_9$	452.5	26.40	Methoxy glutamic acid methyl ester
5	$C_{30}H_{60}O_2$	452.8	30.21	Tria contanoic acid

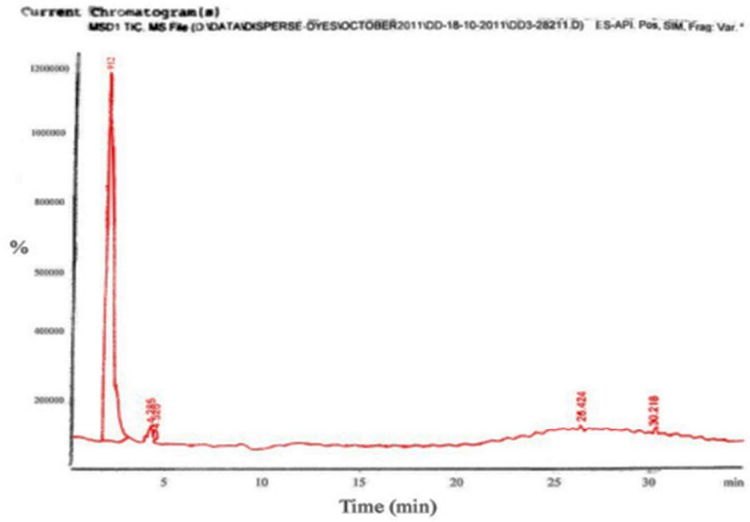

Fig. 1. Characteristic features of antifungal compounds isolated from *T. harzianum* by GC-MS

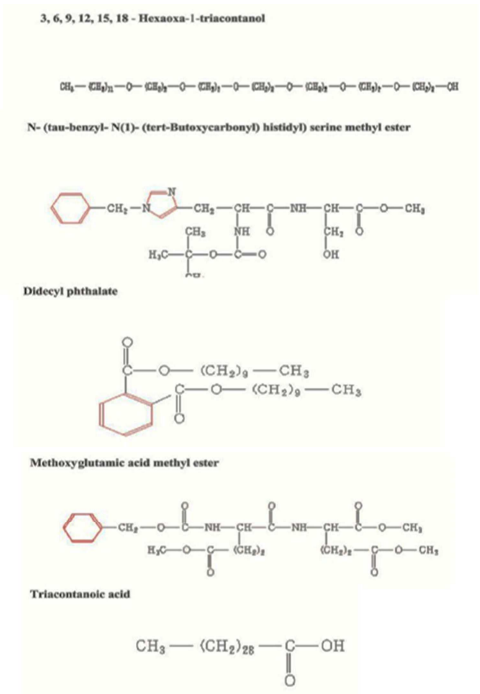

Fig. 2. Analysis of chemical structures of bioactive compounds from *T. harzianum* by GC-MS method

Discussion

In the present investigation, Gas chromatography mass spectrum analysis of ethyl acetate culture filtrate of *T. harzianum* was subjected to GC-MS analysis to find out the components produced by the fungus, it yielded five prominent peaks with retention time of 1.91,4.28,4.52,26.42 and 30.21 min which indicated the presence of five compounds such as 3,6,9,12,15,18-hexaoxa-1-triacontanol acid; N-(tau-benzyl-N(1)- (tert-butoxycarbonyl) histidyl) serine methyl ester acid; di decylphthalate acid ; methoxy glutamic acid methyl ester; triacontanoic acid. The production of volatile and non- volatile substances of antagonistic fungi in liquid cultures, which are inhibitory to spore germination and mycelial growth of pathogenic fungi, has been reported by several workers (Mukherjee and Tripathi, 2000; Saikia and Gandhi, 2002; Prasad and Rangeshwaran, 2001).

Solid phase micro extraction (SPME) coupled to gas chromatography-mass spectrometry (GC-MS) and which can be used for the profiling of microbial volatile organic compounds (MVOCs) in the headspace (HS) of cultures of filamentous fungi. The developed method was successfully applied to cultures of the bio control fungus *Trichoderma viride*. In total, 25 volatile organic compounds were identified by applying strict criteria. The microbial volatile organic compounds were assigned to the compound classes of alcohols, ketones, alkanes, furanes, pyrones (mainly the bioactive 6-pentyl-alpha-pyrone), mono- and sesquiterpenes studied by Stoppacher *et al.* (2010). Yan *et al.* (2010) evaluated the fermentation fluid of *Trichoderma* sp., a fungus isolated from the rhizosphere soil of *Populus euphratica* Oliv., was analyzed by Gas chromatography-mass spectrometry (GC-MS) to get the basic information about the composition of the fungus. A total of forty-two substances were isolated from the fermentation fluid and thirty-nine were identified mainly including acids, nitrogen compounds, esters, hydrocarbons, alcohols, etc. The peak areas were normalized to calculate the relative content of each component.

Senthil kumar *et al.* (2011) studied the Gas chromatography mass spectrum analysis of aceto nitrile extract of the filtrate of *T. harzianum* revealed the presence of six compounds represents six major peaks. The peaks correspond with diethyl phthalate, tetradecanoic acid 9,12-octa decadienoic acid (z,z), oleic acid,1,2-benzene di oxylic acid, diiso octyl ester and squalene. Siddiquee *et al.* (2012) analysis of bio control fungus. *Trichoderma harzianum* strain FA1132 by using gas chromatography- mass spectrometry. More than 278 volatile compounds such as normal saturated hydro carbons (C7-C30), cyclohexane, cyclopentane, fattyacids, alcohols, esters, sulfur- containing compounds,simple pyrane and benzene derivatives have been identified. This proved that *T. harzianum* is capable of producing many volatile organic compounds that are produced by many other fungal species. This compounds either individually or in combination with other compounds. In present investigation suggested that meth oxyglutamic acid methyl ester and triacontanoic acid along with other compounds would have suppressed the growth of *B. oryzae*. The antifungal activity of 1, 2- benzene di carboxylic acid and diiso octyl ester have already reported by Ushadevi (2008) from them arine isolates of *P. lividum* and *T. lingorum*. Senthilkumar *et al.* (2011) reported the 1, 2-dicarboxylic acid and diisooctyl ester along with other compounds would have suppressed the growth of *F. oxysporum*. Yan *et al.* (2010) evaluated the thirty-nine substances were identified, by GC-MS study. They mainly include acids, nitrogen compounds, esters, hydrocarbons, alcohols, etc. Xin *et al.* (2005) isolated a new compound -2, 4-dihydroxy-1-butyl (4-hydroxy) benzoate and a known compound fructigenines A1 from the fungus *Penicillium auratiogriseum* which was isolated from sponge Mycale plumose.

Gas chromatography mass spectrum were analysis of ethyl acetate extract of the filtrate of *T. harzianum* revealed the presence of five compounds represents five major peaks. The peaks correspond with 3, 6, 9, 12, 15, 18-hexaoxa-1-triacontanol, N- (tau- benzyl-N(1) - (tert-butoxycarbonyl) histidyl) serine methyl

ester, didecyl phthalate, methoxyglutamic acid methyl ester and tria contanoic acid which are important biocontrol agents of *T. harzianum*. The bioactive compounds have been determined with help of Gas chromatography mass spectrum analysis from *T. harzianum*. *Trichoderma harzianum* was high potent inhibitory activity. Thus, the present study revealed the scientific validation and proof for the usage of *T. harzianum* as best eco-friendly and cost effective alternate fungicide to control all the pathogens instead of using the hazardous chemicals.

Conclusion

It has been determine with help of Gas chromatography mass spectrum analysis and provide the valuable information for the selection of drug target from *Trichoderma harzianum*. The present study, two antifungal compounds are triacontanoic acid, methoxyglutamic acid methyl ester was isolated from the *Trichoderma harzianum,* they are highly interact with photolyase of *Bipolaris oryzae* and high potent inhibitory activity, the study revealed the scientific validation and proof for the usage of *Trichoderma harzianum* as best eco-friendly and cost effective alternate fungicide to control all the fungal pathogens instead of using the hazardous chemicals.

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