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GC-MS based profiling and quantification of bioactive secondary metabolites from *Trichoderma harzianum*

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Abstract

Trichoderma harzianum is well known for its ability to produce a variety of bioactive secondary metabolites, many of which have important antifungal properties. The purpose of the study was to identify and quantify the metabolites produced by *T. harzianum* using gas chromatography-mass spectrometry (GC-MS). The *T. harzianum* was cultivated in potato dextrose broth, and its metabolites were extracted with ethyl acetate and analysed on a Shimadzu GC-MS-QP 2010 system. The analysis revealed a complex profile of volatile and semi-volatile compounds, including 6-pentyl-2H-pyran-2-one, hexadecanoic acid, tetradecanoic acid, octadecanoic acid, and several aromatic and complex organic compounds. These metabolites are known to contribute to membrane disruption, ergosterol biosynthesis inhibition, and oxidative stress in phytopathogenic fungi. Quantitative analysis indicated that the relative abundance of these compounds, with some low-abundance metabolites showing high bioactivity potential. These findings provide valuable insights into the metabolic profile of *T. harzianum*, supporting its potential role in the development of sustainable, natural antifungal agents.

Keywords: *Trichoderma harzianum*, GC-MS, secondary metabolites

Introduction

Trichoderma species are widely recognised as potent biocontrol agents in sustainable agriculture because of their ability to antagonise a wide range of phytopathogenic fungi through a variety of mechanisms including mycoparasitism and competition (Harman *et al*., 2004) [6]. Among these species, *Trichoderma harzianum* has attracted considerable attention for its robust production of bioactive secondary metabolites that inhibit pathogenic fungi and improve the defense of plants. These secondary metabolites include volatile organic compounds (VOCs), polyketides, terpenes and various fatty acids, many of which directly affect the fungal membrane, inhibit the growth of the fungal spores and interfere with the pathogenic organisms' signalling pathways (Reino *et al.*, 2008; Mukherjee *et al.*, 2013) [7, 8].

Gas chromatography-mass spectrometry (GC-MS) is a powerful analytical tool for profiling of metabolites, enables characterization of volatile and semi-volatile compounds with antifungal properties (Siddiquee *et al.*, 2012) [1]. The metabolites identified by GC-MS from *T. harzianum* have been shown to include compounds such as 6-pentyl-2H-pyran-2-one (6-PP), which is known for its strong antifungal and plant growth-promoting activity (Chaudhary *et al.*, 2022) [11]. Fatty acids like hexadecanoic acid and octadecanoic acid, commonly found in *T. harzianum*, have been reported to alter membrane fluidity and disrupt cellular integrity of target fungal pathogens (Avis & Bélanger, 2001; Ahluwalia *et al.*, 2015) [5, 4]. Moreover, aromatic compounds and phthalate esters, such as di-n-octyl phthalate, possess documented antifungal activity by affecting ergosterol biosynthesis and inhibiting mitochondrial function in fungi (Zhang *et al.*, 2018) [9].

The biocontrol efficacy of *T. harzianum* is further enhanced by synergistic interactions among its secondary metabolites, which collectively target multiple fungal pathways, including cell wall biosynthesis, oxidative stress response, and protein synthesis (Vinale *et al.*, 2012) [3]. These compounds not only exhibit direct antifungal activity but also trigger systemic resistance in host plants, activating defense-related gene expression and enhancing tolerance to environmental stressors (Shoresh *et al.*, 2010; Singh *et al.*, 2022) [10, 11].

Given the growing concerns about resistance to fungicides and environmental sustainability, the identification and characterization of natural antifungal compounds produced by *T.*

harzianum offers a promising strategy for the development of environmentally friendly crop protection methods. This aim of this study to analyse and quantify the secondary metabolites produced by *T. harzianum* using GC-MS. By elucidating the chemical profile and concentration of these compounds, this work contributes to a better understanding of the metabolites production potential of *T. harzianum*, supports its role in sustainable crop protection.

Materials and Methods

Fungal Cultivation

Trichoderma harzianum was grown in potato dextrose broth (PDB) at 25 °C for 14 days with continuous shaking at 140 rpm in a 2 L Erlenmeyer flask containing 1 L of sterile PDB. The culture filtrate was separated from the mycelia using non-absorbent cotton.

Metabolite Extraction

The culture filtrate was subjected to a triple extraction procedure with the same volume of ethyl acetate for the purpose of isolating the secondary metabolites. The resulting water fraction was evaporated to dryness by means of a rotary evaporator (Heidolf, Germany) at a reduced pressure of 55 °C. The dry residues of the metabolites were weighed and reconstituted with ethyl acetate at GC-MS grade concentration of 1 g per ml for further analysis.

GC-MS Analysis

The extracted metabolites were analyzed using a Shimadzu GC-MS-QP 2010 system equipped with an Rtx-1 MS column coupled to a triple-axis mass spectrometer. A 1 µL sample was injected, with helium (>99.99% purity) serving as the carrier gas at a head pressure of 10 psi and a flow rate of 0.75 mL/min. The oven temperature was programmed as follows: an initial temperature of 40 °C held for 2 minutes, ramped at 4 °C/min to 120 °C, followed by a 5 °C/min increase to 200 °C (held for 5 minutes), and a final ramp of 10 °C/min to 300 °C, giving a total run time of 51 minutes. Mass spectrometry parameters included an ion source temperature of 250 °C, electron ionization at 60 eV, full scan mode over a mass range of 50-550 AMU, a transfer line temperature of 280 °C, and an electron multiplier voltage of 1222 V. Compounds identification was performed by comparing mass spectra to the National Institute of Standards and Technology (NIST) Mass Spectral Library (Shimadzu Corporation, Kyoto, Japan) with a spectral match factor ≥90%.

Quantification and Data Analysis

Th peak areas of the identified metabolites were determined by Shimadzu LabSolutions and normalized to the internal standard (D4-methanol) in order to exclude instrumental variations. The relative abundance was expressed as a percentage of the total maximum area. The experiment was conducted in three replicates.

Results and Discussion

The GC-MS analysis of *Trichoderma harzianum* identified metabolites with a high retention time that have antifungal activity and are important contributors to the biocontrol efficacy against phytopathogenic fungi (Table 1.).

Table 1: Metabolites Identified by GC-MS from *T. harzianum*

Sr. No.	Metabolites	Retention Time (min)
1	6-Pentyl-2H-pyran-2-one	20.44
2	Hexadecanoic acid	20.654
3	Tetradecanoic acid	17.76
4	Octadecanoic acid	22.37
5	Diethyl benzene-1,2-dicarboxylate	15.85
6	2,4-Bis(1,1-dimethylethyl)phenol	13.72
7	2-(2-Hydroxypropan-2-yl)-2,3-dihydrofuro[2,3-h]chromen-4-one	16.45
8	1-Monolinoleoylglycerol	23.12
9	2-(7-Hydroxyheptyl)-3,5,6-trimethylcyclohex-2-en-1-one	18.63

The gas chromatography-mass spectrometry (GC-MS) identified metabolites with retention time from Trichoderma harzianum which shows antifungal activity against phytopathogenic fungi. The metabolites include 6-pentyl-2H-pyran-2-one (20.44) (Siddiquee *et al.*, 2012) [1], which inhibits fungal membrane integrity and β-glucan synthase. Hexadecanoic acid was detected at RT (20.654) altering membrane fluidity and inhibiting spore germination (Ahluwalia *et al*., 2015) [4]. Other fatty acids, tetradecanoic acid and octadecanoic acid at RT (17.76, 22.37) respectively (Lakhdari *et al.*, 2023) [2], disrupt fungal membranes, causing leakage and growth inhibition (Avis & Bélanger, 2001) [5]. Diethyl benzene-1,2-dicarboxylate (15.85) and 2,4-bis(1,1-dimethylethyl)phenol (13.72) (Lakhdari *et al.*, 2023) [2] disrupt ergosterol biosynthesis and membrane function. Complex metabolites, 2-(2-hydroxypropan-2-yl)-2,3-dihydrofuro[2,3-h]chromen-4-one (16.45) 1-monolinoleoylglycerol (23.12) and 2-(7-hydroxyheptyl)-3,5,6-trimethylcyclohex-2-en-1-one (18.63) (Lakhdari *et al.*, 2023) [2], act synergistically, targeting enzyme inhibition and lipid peroxidation (Vinale *et al.*, 2006) [3]. The antifungal mechanisms involve membrane disruption by fatty acids, which increase permeability (Avis & Bélanger, 2001) [5], and inhibition of ergosterol and cell wall synthesis by 6-pentyl-2H-pyran-2-one. Volatile alcohols cause oxidative stress, while phenolics like 2,4-bis(1,1-dimethylethyl)phenol are similar to azole antifungal agents. Organic acids induce antagonistic microenvironments and systemic resistance and complex metabolites may target multiple pathways (Vinale *et al.*, 2006) [3]. The low peak area of 6-pentyl-2H-pyran-2-one suggests high potency despite low abundance (Siddiquee *et al.*, 2012) [1].

Conclusion

The GC-MS analysis of metabolites produced by *Trichoderma harzianum* revealed a diverse array of bioactive compounds with significant antifungal properties. Key metabolites such as 6-pentyl-2H-pyran-2-one, hexadecanoic acid, and octadecanoic acid play crucial roles in disrupting fungal cell membranes, inhibiting ergosterol biosynthesis, and impairing spore germination. Additionally, complex compounds like 2-(2-hydroxypropan-2-yl)-2,3-dihydrofuro[2,3-h]chromen-4-one and 1-monolinoleoylglycerol exhibit synergistic effects targeting lipid peroxidation and enzyme inhibition. The identification of volatile phenolics and organic acids further highlights the multifaceted antifungal mechanisms, including oxidative stress induction and systemic resistance. The phenolic compounds such as 2,4-bis(1,1-dimethylethyl) phenol further supports the multi-targeted antifungal strategy of *T. harzianum*. These findings highlight the potential of *T. harzianum* as an effective biocontrol agent against phytopathogenic fungi through synergistic action of its metabolites, paving the way for sustainable and eco-friendly crop protection strategies.

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