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Utilizing *Trichoderma harzianum* T(MP)-7 volatiles for effective management of stem end rot of avocado incited by *Botryosphaeria scharifii* SE(TKD)-7

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Abstract

Avocado, a vital nutritious fruit crop, faces significant threats from various pests and diseases, with *Botryosphaeria* spp. emerging as a serious postharvest pathogen affecting nursery and field conditions. This pathogen was isolated from infected avocado fruits, while Trichoderma species were isolated from the avocado rhizosphere. Evaluation of *Trichoderma* VOCs against *Botryosphaeria scharifii* SE(TKD)-7 using a paired plate assay revealed substantial inhibition of pathogen mycelial growth. Trichoderma isolates T(MP)-7 and T(PD)-2 exhibited reduced mycelial growth of 2.4 cm and 3.2 cm, respectively, compared to the control, representing percent inhibitions of 73.33% and 64.44%, respectively. Scanning Electron Microscopy (SEM) analysis demonstrated alterations in Botryosphaeria mycelium caused by *Trichoderma* VOCs, resulting in shorter and abnormal mycelium and the absence of spore production. Moreover, GC-MS analysis identified various antifungal compounds in Trichoderma VOCs, including octane, oxirane, hexanediol, oxalic acid, and pentane-1-ol, highlighting their biocontrol potential against fungal pathogens. These findings underscore the efficacy of *Trichoderma* VOCs in inhibiting pathogen growth and suggest their potential application in plant disease management strategies.

Key words: Avocado, postharvest fungal diseases, biocontrol agents, *Trichoderma* spp., microbial volatile organic compounds (mVOCs), GC-MS analysis

Introduction

Avocado (*Persea americana* Mill.) is indeed native to Mexico but has gained widespread cultivation in tropical and subtropical regions worldwide due to its nutritional value and culinary versatility. Its composition, rich in monounsaturated fatty acids, lipids, phytochemicals, minerals such as potassium, iron, and phosphorus, as well as vitamins E, B and C, makes it highly sought after in global markets. The nutritional benefits of avocado consumption, linking it to a higher-quality diet overall (Drewster *et al.*, 2013; Magwaza *et al.*, 2015). However, despite its popularity and nutritional value, the commercialization of avocado fruits faces challenges due to high occurrences of postharvest fungal diseases such as anthracnose and stem end rot (SER). These diseases can significantly impact producer losses, even as the production and export of avocados have expanded from emerging countries (Oduol *et al.*, 2013). Avocado stem-end rot is caused by a variety of fungi, including species belonging to the family *Botryosphaeriaceae* such as *B. parva*, *B. ribis*, *B. lutea*, *B. dothidea*, and *B. rhodian* (Valencia *et al.*, 2019). As the avocado fruit ripens, symptoms of stem end rot (SER) appear. It manifests as shriveling, followed by brown to black rot that begins at the fruit stem end. Internal vascular bundles may gradually turn black to brown as the rot spreads, eventually consuming the entire fruit (Pérez-Jiménez 2008; Twizeyimana *et al.*, 2013; Guarnaccia *et al.*, 2016). The application of fungicides may lead to the emergence of pesticide resistance (Yoon *et al.*, 2013) and can have negative consequences for people (Fattahi *et al.*, 2015).

Biological control agents (BCAs), offer a possible alternative strategy for managing these diseases. During postharvest storage, *Trichoderma* spp. have been frequently employed to safeguard commercially important fruits and vegetables, including tomatoes, chilies, mangoes, apples, bananas, strawberries, and others (Begum *et al.*, 2010). In the case of bananas, the postharvest crown rot disease complex, which occurs during storage at room temperature and in cold storage, is caused by *L. theobromae* and *Colletotrichum musae*. *Trichoderma viride*, *T. harzianum*, and *T. koningii* have shown antagonistic action against these pathogens (Yahia *et al.*, 2011). Additionally, it has been noted that *T. harzianum* controls banana anthracnose, preserves fruit quality after harvest, and lessens naturally occurring fruit diseases (Sangeetha *et al.*, 2009). *Trichoderma* sp. employ diverse biological control strategies, including competition for nutrients and available space, mycoparasitism, and the production of antimicrobial metabolites (Elad *et al.*, 1999; Troian *et al.*, 2014; Damodaran et al., 2025). Moreover, they induce resistance systems (Xiao-Yan *et al.*, 2003) and plant growth promotion (Howell *et al.*, 2003; Martínez-Medina *et al.*, 2014). Recently, considerable attention has been focused on microbial volatile organic compounds (mVOCs), which are released by antagonists and possess antibacterial and growth-promoting properties in many plants (Almeida *et al.*, 2023).

While *Trichoderma* spp. has well-documented biocontrol capabilities, it remains unclear how microbial volatile organic compounds (mVOCs) interact with and suppress plant pathogenic fungi (Srandharan *et al.*, 2020). Consequently, the purpose of this research is to test the antifungal volatiles of *Trichoderma* sp.

and *Botryosphaeria* sp. *in vitro* and characterize these volatiles through GC-MS analysis.

Material and methods

Pathogen and antagonist: The Stem End Rot disease-causing agent, *Botryosphaeria scharifii*, was isolated from avocado fruits exhibiting characteristic development of dark, sunken lesions at the stem end of the fruit. The tissue segmentation method of pathogen isolation was employed using Potato Dextrose Agar (PDA) medium in Petri plates. A pure culture of *B. scharifii* was obtained using the single hyphal tip approach. The morphology of the isolated pathogen conidia was determined by observing them under a light microscope. The conidia appeared light brown to dark brown and double-celled, cylindrical to oval shaped conidia with striations. Molecular confirmation of the species as *B. scharifii* SE(TKD)-7 was performed using the Blast search in the National Centre for Biotechnology Information (NCBI) Database and received the Accession Number: OR196111. Ten different *Trichoderma* sp. were isolated from the native region of avocado, obtained from the Department of Plant Pathology, AC & RI, Madurai and the efficacy of their volatiles was assessed against the stem end rot pathogen.

Assessing the effectiveness of volatile organic compounds generated by *Trichoderma* spp. against *Botryosphaeria scharifii* SE(TKD)-7: The efficacy of *Trichoderma* sp. volatile organic compounds (VOCs) against *B. scharifii* was evaluated using an inverted or paired plate assay. Mycelial plugs from *Trichoderma* sp. and *B. scharifii* were placed on separate PDA plates. The plate with *B. scharifii* was then positioned above the *Trichoderma* sp. plate, secured together with parafilm, and incubated at 28±2°C for four days. Control plates contained only *B. scharifii*. Each treatment was replicated three times. After the seven-day incubation period, the reduction in growth was assessed and expressed as a percentage inhibition over control using the following formula by Vincent (1947)

Per cent Inhibition (%)= [(Dc-Dt)/ Dc] ×100

Dc is the average diameter of fungal growth (cm) in the control. Dt is the average diameter of fungal growth (cm) in the treatment.

Scanning Electron Microscope (SEM) Examination: The reduced mycelium of *Botryosphaeria* sp. was taken from a paired plate and firmly affixed onto aluminum stubs for scanning electron microscopy (SEM). To enhance visibility, the samples underwent a sputter-coating process to deposit gold particles. An ion coater was utilized to ionize the gold particles before SEM examination. The bacterial colonies were fixed for 24 hours using a 3:1 ratio of glutaraldehyde and osmium tetroxide vapors to prepare them for SEM imaging. Following fixation, a 48-hour air-drying period was observed. Subsequently, the treated samples were coated with a layer of gold via sputter-coating (Soliman *et al.*, 2016) from Madurai Kamarajar University, Madurai. The microscopic features of the samples were then analyzed using an SEM instrument of the TESCAN VEGA3 SBH model.

Preparation of crude extracts of *Trichoderma* sp. for GC-MS: The crude extracts of potent *Trichoderma* sp. were prepared by culturing a mycelial disc from *Trichoderma* isolate T(MP)-7 in 200ml potato dextrose broth at 25°C for seven days. Culture filtrates were obtained by filtration and centrifugation, followed by extraction with hexane. Volatile organic compounds (VOCs) were concentrated using a rotary evaporator and diluted before filtration through a 0.4µ bacterial filter.

Analysing the crude extracts of *Trichoderma* sp. using GCMS: The crude hexane extracts from *Trichoderma* sp. were analysed using a Shimadzu GC-MS QP-2020 system. This system included a mass spectrometer with a silica capillary column and an auto-sampler gas chromatograph. GC-MS detection utilized a 70-eV electron ionization device. The temperature program for the oven ranged from 80 °C to 300 °C, with a helium carrier gas flow rate of 1.5 mL/min. The injection volume was 1 µL, and ion source and injector temperatures were set at 230 °C and 250 °C, respectively. Mass spectra were acquired within a fragment selection range of 50-550 amu at an energy of 70 eV. The GC procedure typically lasted 35 minutes. Compound identification was based on a comparison with NIST libraries (Hiller *et al.*, 2009).

Statistical analysis: The mean differences between treatments were statistically assessed following the instructions outlined by Gomez and Gomez (1984). Analysis of Variance (ANOVA) was conducted, followed by Duncan's Multiple Range Test, with a significance level set at 5 %.

Results and discussion

Isolation of pathogen: The pathogen *Botryosphaeria* sp. was isolated from infected avocado fruits using the tissue segmentation method as per Saeed *et al.* (2017) and Al-Jabri *et al.* (2017). Infected twigs were cut into 0.2-0.5 cm pieces, surface sterilized with 1% sodium hypochlorite for 2 min, rinsed three times with sterilized distilled water, and dried with sterilized tissue paper for pathogen isolation. *Trichoderma* species were isolated from the avocado rhizosphere and confirmed based on their phenotypic characteristics. Likewise, *Trichoderma* isolates from the shallot rhizosphere were cultured on Potato Dextrose Agar (PDA) and identified through both morphological and molecular methods (Gezgin *et al.*, 2023; Susila *et al.*, 2023).

Microbial volatile organic compounds of *Trichoderma* sp. against the *Botryosphaeria scharifii* SE(TKD)-7 : In a paired dish technique, two isolates of *Trichoderma* sp., namely T(MP)-7 and T(PD)-2, were evaluated for their ability to inhibit the mycelial growth of a pathogenic organism. The results indicated that T(MP)-7 exhibited the least mycelial growth, measuring 2.4 cm, and subsequently inhibited the mycelial growth of the pathogen by 73.33 % compared to the control. On the other hand, T(PD)-2 displayed a slightly higher mycelial growth of 3.2 cm but still showed a significant reduction of 64.44 % in comparison to the control (Fig. 1 and 2). Likewise, in their study, Sridharan *et al.* (2020) discovered that *T. longibrachiatum* EF5 emitted VOCs that hindered the growth of both *S. rolfsii* (reduced by 57%) and *Macrophomina phaseolina* (reduced by 35%) in experiments using inverted plates. Moreover, in a tripartite assay conducted with a partition plate, *T. longibrachiatum* EF5 released VOCs that suppressed the growth of *S. rolfsii* and *M. phaseolina* by 68% and 33%, respectively, in comparison to controls. Wanjiku *et al.* (2021) assessed the effectiveness of *Trichoderma* spp. (*T. atroviride*, *T. virens*, *T. asperellum*, and *T. harzianum*) against avocado stem-end rot fungal pathogens (*Lasiodiplodia*

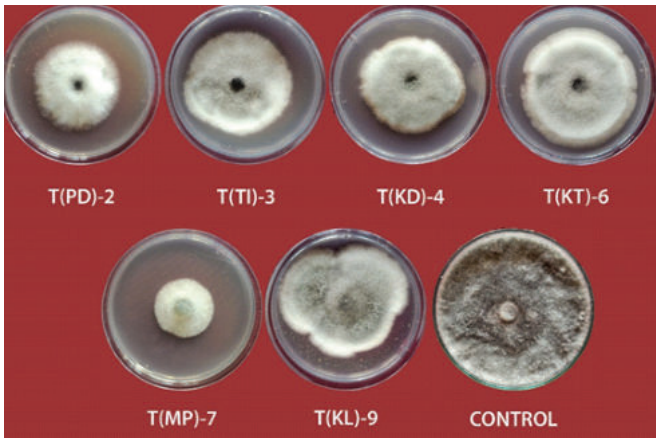

Fig. 1. Antifungal efficacy of volatile organic compounds produced by *Trichoderma* spp. against *Botryosphaeria scharifii* SE(TKD)-7

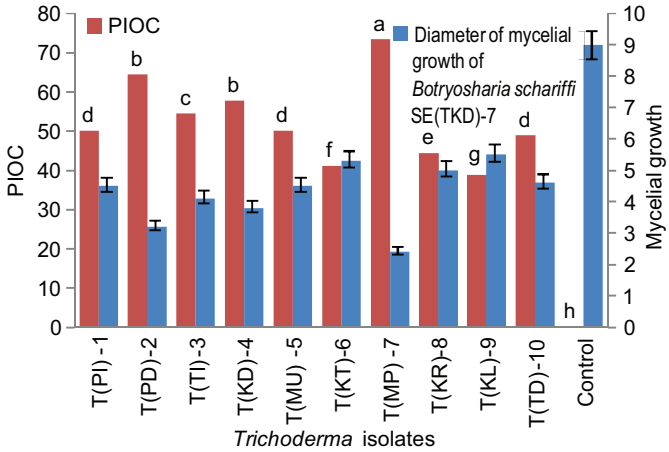

Fig. 2. Efficacy of volatile organic compounds produced by *Trichoderma* spp. against *Botryosphaeria scharifii* SE(TKD)-7 by paired plate

theobromae, *Neofusicoccum parvum*, *Nectria pseudotrichia*, and *Fusarium* solani) *in vitro*. *T. atroviride* showed the highest inhibition of mycelial growth against *N. parvum* (48%), *N. pseudotrichia* (55%), and *F. solani* (32.95%), while *T. harzianum* was most effective against *L. theobromae*. *T. virens* displayed the highest inhibition against *N. pseudotrichia* (45%), while *T. asperellum* was the least effective against all pathogens.

From these studies, different *Trichoderma* strains exhibited varying antifungal efficacy against different pathogens, indicating that the production of volatile organic compounds (VOCs) by *Trichoderma* may serve as a potential mode of action against pathogens (Yamunarani *et al.*, 2019).

Assessing *Trichoderma* sp. VOCs impact on *Botryosphaeria scharifii* SE(TKD)-7 via Scanning Electron Microscopy: In the paired plate approach, *Trichoderma* isolates T (MP)-7 showed the least mycelial growth of *Botryosphaeria* sp. with modified cultural features. Scanning electron microscope analysis revealed alterations in *Botryosphaeria* mycelium caused by *Trichoderma* VOCs, resulting in shorter and abnormal mycelium and absence of spore production. This contrasts with unaffected mycelium and spore production in the control plate (Fig. 3). Similarly, SEM observations from You *et al.* (2022) reported that *B. cinerea* hyphae treated with T-51 VOCs appeared crumpled and withered compared to untreated hyphae. Notably, *F. oxysporum* showed no significant differences between treatment and control groups. Chen *et al.* (2016) discovered that VOCs from *T. koningiopsis* YIM PH30002 inhibited *Epicoccum nigrum*, containing β-phellandrene, cyclohexene, and cycloocta-2,4-dien-1-ol. These compounds likely play an antifungal role and induce changes in mycelial structure

GC-MS analysis of mVOCs of *T. harzianum* T(MP)-7: GC-MS analysis revealed that *Trichoderma* sp. produce various antifungal compounds. The crude metabolites of *Trichoderma* sp. T(MP)-7 were analyzed using GC/MS, identifying prominent antifungal compounds such as Octane, 4-methyl, Oxirane, 2,2-dimethyl-3-propyl, 3,4-Hexanediol, 2,5-dimethyl, Oxalic acid, 3-n-Propyl-5-methylhexan-2-one, 2-Heptanone, 4,6-dimethyl, Dodecane, 2-cyclohexyl, 2-Penten-1-ol, 2-methyl, 3-n-Propyl-5-methylhexan-2-one, 3-Methyl-2-butenoic acid, hexadecyl ester, Dodecyl acrylate, Octadecane, 1-chloro, and 1-Chloroeicosane (Fig. 4, Table 1).

According to Tomah *et al.* (2020), a variety of bioactive substances produced by *Trichoderma citrinoviride*, including quinolone, heptadecane, heneicosane, 6-pentyl-2H-pyran-2, phenol, 2-(6-hydrazino-3pyridazinyl), 17-methoxy-4-methyl-

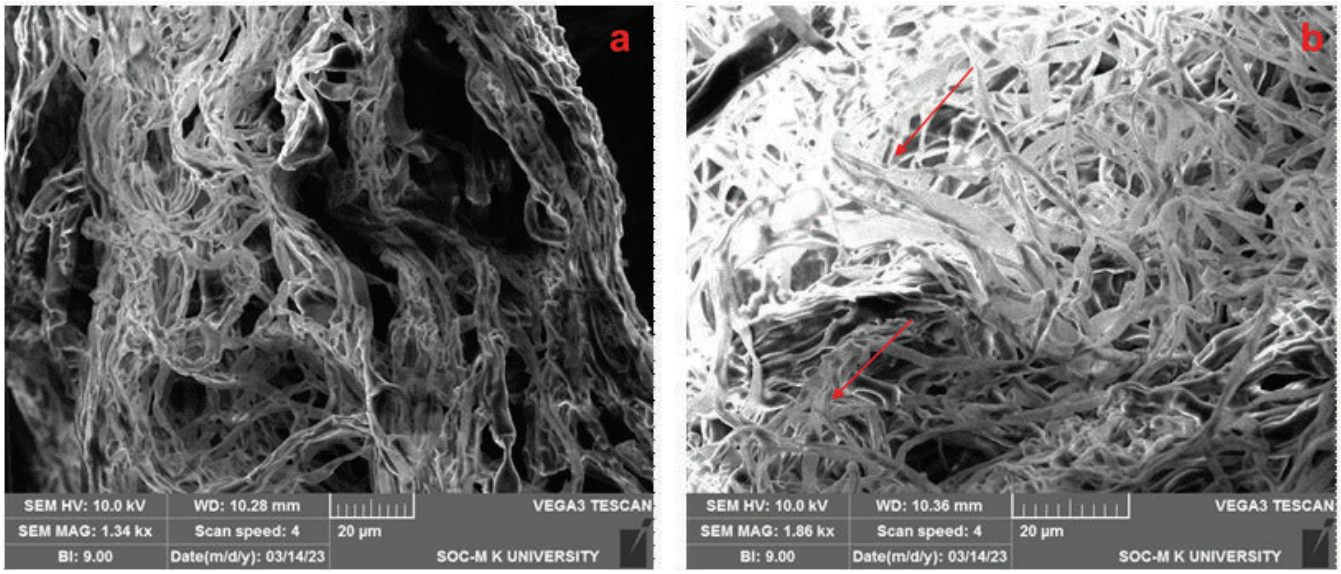

Fig. 3. Effect of VOC of by *Trichoderma* spp. against *Botryosphaeria scharifii* SE(TKD)-7 observed by Scanning Electron Microscope: (a) Scanning electron micrographs showing the hyphal morphology of *Botryosphaeria scharifii* untreated healthy hyphae; (b) shows the shriveled hyphae of *B. scharifii* SE(TKD)-7 treated by *Trichoderma harzianum* T(MP)-7 VOCs.

Table 1. Bioactive compounds and their peak area %, retention time, molecular weight, molecular formula of promising *Trichoderma* sp.

S. No	RT	Name of the compound	Synonyms	Molecular formula	MW	Functional group	Peak area (%)
1	3.27	Octane, 4-methyl-	4-Methyloctane 1,3-Dimethylheptane	C_9H_{20}	128	Hydrocarbons	8.12
2	4.00	Oxirane, 2,2-dimethyl-3-propyl-	Hexane, 2,3-epoxy-2-methyl- 2,2-Dimethyl-3-propyloxirane	$C_7H_{14}O$	114	Ethers	7.44
3	4.30	3,4-Hexanediol, 2,5-dimethyl-	2,5-Dimethyl-3,4-hexanediol	$C_8H_{18}O_2$	146	Alcohols	9.24
4	5.15	Oxalic acid, cyclohexyl nonyl este	no synonyms.	$C_{17}H_{30}O_4$	298	Esters	11.22
5	6.18	3-n-Propyl-5-methylhexan-2-one	5-Methyl-3-propyl-2-hexanone	$C_{10}H_{20}O$	156	Ketones	4.19
6	6.67	2-Heptanone, 4,6-dimethyl-	4,6-Dimethyl-2-heptanone	$C_9H_{18}O$	142	Ketones	1.77
7	7.75	cis-2,3-Epoxyoctane	2-Methyl-3-pentyloxirane, (Z)-	$C_8H_{16}O$	128	Ethers	1.49
8	8.58	Octane, 4,5-diethyl-	4,5-Diethyloctane	$C_{12}H_{26}$	170	Hydrocarbons	1.37
9	8.95	Hexane, 2,3,5-trimethyl-	2,3,5-Trimethylhexane	C_9H_{20}	128	Hydrocarbons	6.57
10	9.18	Nonane, 4-ethyl-5-methyl-	4-Ethyl-5-methylnonane	$C_{12}H_{26}$	170	Hydrocarbons	7.49
11	9.79	Decane, 5,6-dimethyl-	5,6-Dimethyldecane	$C_{12}H_{26}$	170	Hydrocarbons	3.00
12	10.70	Cyclohexane, (1,2-dimethylbutyl)-	(1,2-Dimethylbutyl)cyclohexane	$C_{12}H_{24}$	168	Hydrocarbons	6.01
13	11.33	Cyclohexane, decyl	Decane, 1-cyclohexyl- Decylcyclohexane 1-Cyclohexyldecane	$C_{16}H_{32}$	224	Hydrocarbons	4.46
14	11.56	Oxirane, tetradecyl-	Hexadecane, 1,2-epoxy- Hexadecylene oxide 1,2-Epoxyhexadecane	$C_{16}H_{32}O$	240	Ethers	2.89
15	12.69	Dodecane, 2-cyclohexyl-	(1-Methylundecyl)cyclohexane 2-Cyclohexyl-dodecane	$C_{18}H_{36}$	252	Hydrocarbons	0.79
16	13.26	2-Penten-1-ol, 2-methyl-	2-Methyl-2-penten-1-ol 2-Methyl-2-pentene-1-ol	$C_6H_{12}O$	100	Alcohols	1.08
17	13.94	3-n-Propyl-5-methylhexan-2-one	5-Methyl-3-propyl-2-hexanone	$C_{10}H_{20}O$	156	Ketones	3.68
18	14.56	3-Isopropyl-5-methylhexan-2-one	3-Isopropyl-5-methyl-2-hexanone	$C_{10}H_{20}O$	154	Ketones	3.94
19	15.83	3-Methyl-2-butenoic acid, hexadecyl ester	Hexadecyl 3-methyl-2-butenoate	$C_{21}H_{40}O_2$	324	Esters	1.29
20	16.28	3-Methyl-2-butenoic acid, tridecyl ester	Tridecyl 3-methyl-2-butenoate	$C_{18}H_{34}O_2$	282	Esters	4.17
21	17.73	2-Undecanone, 6,10-dimethyl-	Hexahydropseudoionone Pseudoionone, hexahydro-	$C_{13}H_{26}O$	198	Ketones	0.42
22	28.81	2-Naphthalenemethanol, 1,2,3,4,4a,5,6,7-octahydro-α,α,4a,8-tetramethyl-, (2R-cis)-	γ-Eudesmol γ-Eudesmole	$C_{15}H_{26}O$	222	Alcohols	0.86
23	29.08	(3R,3aR,7R,8aS)-3,8,8-Trimethyl-6-methyleneoctahydro-1H-3a,7-methanoazulene	1H-3a,7-Methanoazulene, octahydro-3,8,8-trimethyl-6-methylene-, (3R,3aR,7R,8aS)-	$C_{15}H_{24}$	204	Hydrocarbons	1.1
24	31.35	Dodecyl acrylate	n-Lauryl acrylate 2-Propenoic acid, dodecyl ester	$C_{15}H_{28}O_2$	240	Esters	0.57
25	32.24	Octadecane, 1-chloro-	n-Octadecyl chloride Octadecyl chloride	$C_{18}H_{37}Cl$	288	Chlorine-containing	1.44
26	33.11	(1aR,4S,4aR,7R,7aS,7bS)-1,1,4,7-Tetramethyldecahydro-1H-cyclopropa[e] azulen-4-ol	Epiglobulol epi-Globulol	$C_{15}H_{26}O$	222	Alcohols	3.22
27	37.35	Octadecane, 1-chloro-	n-Octadecyl chloride Octadecyl chloride	$C_{18}H_{37}Cl$	288	Chlorine-containing	1.01
28	39.52	1-Chloroeicosane	Eicosane, 1-chloro- 1-Chloroicosane	$C_{20}H_{41}Cl$	316	Chlorine-containing	1.18

d-homo-18-norandrosta one, eicosane, nonadecane, benzene, propianoic acid, dibutyl phthalate, and hexadecane, are all shown to have potent antifungal activity against fungal pathogens, resulting in a growth inhibition of 77.8%. Furthermore, it has been demonstrated that the bioactive volatile dodecane, which is generated by *Trichoderma* sp., greatly inhibits the growth of mycelial organisms, especially when it comes to *Aspergillus niger* and some plant-pathogenic bacteria (Pucot *et al.*, 2021).

Rao *et al.* (2022) found that multiple volatile organic compounds (VOCs) were detected through SPME-GC-MS analysis of *T. atroviride* LZ42. Among these compounds, 6-pentyl-2H-pyran-2-one (6-PP) was tentatively identified as the main component. It was found that 6-PP significantly influenced the major root orientation of tomato seedlings, although it did not notably affect the length of primary roots. Furthermore, among the pure VOCs investigated, 6-PP exhibited the strongest inhibitory

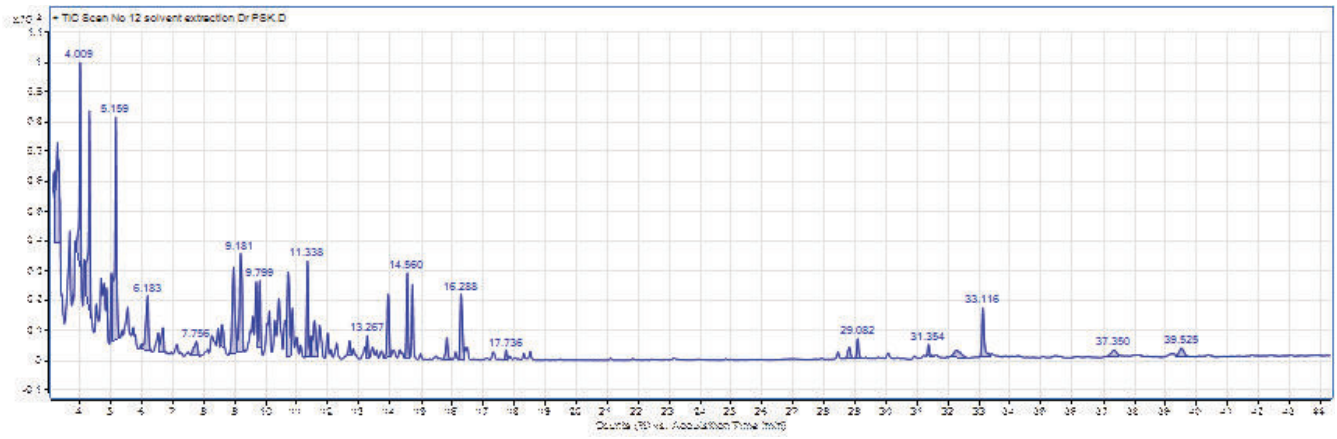

Fig. 4. GC-MS analysis of antimicrobial volatile produced by promising of *Trichoderma* sp.

effect on *F. oxysporum*. Its inhibitory potency was quantified with a 50% effective concentration (EC50) of 5.76 µL mL^{-1} headspace. This suggests that 6-PP has significant potential as a biocontrol agent against *F. oxysporum*. You *et al.* (2022) found that, growth and weight of *Arabidopsis thaliana* seedlings were significantly enhanced by *T. koningiopsis* T-51 VOCs. Analysis revealed that the VOCs of T-51 comprised twenty-four potential compounds, primarily esters, alkanes, and alkenes. This indicates that *T. koningiopsis* T-51 has the capability to employ biological control mechanisms, effectively suppressing plant diseases while promoting plant growth.

In this study, it's crucial for *Trichoderma* spp. to possess diverse biocontrol mechanisms for effective plant disease control and growth promotion in various situations. The volatile organic compounds (VOCs) produced by *Trichoderma* sp. demonstrated potent growth inhibition of plant pathogenic fungi, highlighting the significance of VOC-mediated biocontrol in enhancing plant health and combating fungal diseases.

The volatile organic compounds (VOCs) produced by *Trichoderma harzianum* T(MP)-7 demonstrated the significant role of volatiles in inhibiting plant pathogenic fungi, highlighting their importance in enhancing plant health and combating fungal diseases. In the future, further research and development efforts may focus on isolating and optimizing these signature antifungal mVOCs for commercial applications, offering sustainable solutions for disease management in Agri and horticultural ecosystems.

Competing interests: The authors declare there are no competing interests.

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