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ORIGINAL ARTICLE

Antagonistic profiling of volatile and non-volatile secondary metabolites produced by *Trichoderma asperellum* towards pathogenic *Fusarium oxysporum* f. sp. *ciceri*

Bhushan Popat Birari*, Sunil Ingle

Department of Plant Pathology, Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola, India

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*Corresponding address:
bhushanbirari8@gmail.com

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Abstract

Fungi of the genus *Trichoderma* are widely marketed as biocontrol agents against plant pathogens. However, some *Trichoderma* species may produce toxic secondary metabolites, underscoring the need for comprehensive safety assessments to ensure their ecological safety. In this study, the antifungal activities of volatile secondary metabolites (VSMs) and non-volatile secondary metabolites (nVSMs) produced by six *Trichoderma asperellum* strains (TaSrBh, TaSrYa, TaSrBu, TaSrUm, TaSrGa, and TaSrCh) were evaluated against the chickpea wilt pathogen *Fusarium oxysporum* f. sp. *ciceri*. Bioassays of VSMs and nVSMs revealed that TaSrGa and TaSrYa exhibited the strongest antifungal activity. The ethyl acetate extract of the TaSrYa strain showed excellent growth inhibition (60–100%) of *F. oxysporum* f. sp. *ciceri*, prompting further investigation into its metabolic profile. To identify potential fungicidal compounds, spectrometric analyses were performed on the ethyl acetate extract of TaSrYa. The predominant compounds identified included 9-octadecenoic acid (26.32%), dehydroacetic acid (25.71%), 9-undecenal 2,10-dimethyl (11.31%), 7,10-octadecadienoic acid (8.62%), 9,12,15-octadecatrienoic acid 2,3-dihydroxypropyl ester (Z,Z,Z) (6.18%), and cis-Z-α-bisabolene epoxide (4.96%), which are likely responsible for inhibiting pathogen growth. Additionally, two-dimensional gas chromatography and spectroscopic analyses of volatiles from TaSrGa identified 67 VSMs, categorized as alcohols, alkanes, esters, acids, and terpenes. The examined *T. asperellum* strains demonstrated strong antagonistic effects against the pathogen responsible for chickpea wilt, suggesting that these biocontrol agents could be used in the formulation of natural fungicides, offering an eco-friendly alternative to synthetic fungicides.

Keywords: antifungal, *Fusarium oxysporum* f. sp. *ciceri,* non-volatile secondary metabolites, *Trichoderma asperellum,* volatile secondary metabolites

Introduction

Chickpea (*Cicer arietinum* L.) is a key legume crop cultivated extensively in the Mediterranean region and across the world (Sunkad *et al*. 2019). It ranks as the third most important pulse crop globally, following dry bean (*Phaseolus vulgaris* L.) and dry pea (*Pisum sativum* L.). Currently, chickpeas are grown as a major pulse crop in 57 countries (Merga and Haji 2019). Worldwide, 143,00000 tons of chickpeas are harvested annually from 137,00000 hectares of cultivated area (FAOSTAT 2020). Chickpea is a vital source of protein for millions of people in developing countries. In addition to its high protein content (20–22%), chickpeas are also rich in fiber and essential minerals such as phosphorus, calcium, magnesium, iron, and zinc, and contain significant amounts of β-carotene (Yegrem 2021). However, chickpea cultivation is challenging due to its vulnerability to diseases caused by soil-borne pathogenic fungi. The pathogen *Fusarium oxysporum* f. sp.

ciceri causes chickpea vascular wilt, which is one of the major biotic constraints in chickpea-grown areas. *F. oxysporum* f. sp. *ciceri* causes yield losses ranging from 10 to 100% in India (Venkataramanamma *et al.* 2022).

Carbendazim is one of the most commonly used and recommended fungicides for controlling wilt disease in legumes (Srivastava *et al.* 2011). However, the over-reliance on synthetic pesticides, often resulting from their frequent misuse, has raised significant environmental and health concerns, leading to increased global restrictions on their use (Pradhan *et al.* 2022). Research on alternative approaches to controlling fungal diseases has advanced considerably, often necessitating the implementation of multiple methods, known as integrated disease management (IDM) (Villa *et al.* 2017; Das and Pattanayak 2020). Among these methods are the use of disease-resistant cultivars, adequate water and soil management, fertilization, crop rotations, and BCAs, all aimed at maintaining or increasing agricultural productivity while reducing chemical inputs (Salim *et al.* 2017). Despite this progress, much research is still needed to expand the global application of biological control for plant pathogens, as biopesticides account for only approximately 2% of all pesticides sold worldwide (Legrand *et al.* 2017).

The use of chemical fungicides raises serious concerns about toxic residues and their potential health impacts in food products (Li *et al.* 2016). Therefore, there is an urgent need to develop and deploy biological control agents in the crop root zone as a sustainable strategy for controlling chickpea wilt caused by *F. oxysporum* f. sp. *ciceri. T. asperellum* is a well-established antagonist that controls pathogens by employing an arsenal of mechanisms, such competition, antibiosis, and mycoparasitism (Harman *et al.* 2004). Aside from inducing the defense responses in plants, *T. asperellum* also produces growth-stimulating hormones. Its bioactive secondary metabolites and secreted enzymes act synergistically against plant pathogens (Yassin *et al.* 2022; You *et al.* 2023). Many *Trichoderma* species actively colonize chickpea roots during the vegetative phase, triggering broad-range induced systemic resistance (ISR) (Mukherjee *et al.* 2012). In addition to mycoparasitism, *Trichoderma* application is linked to increases in plant biomass and vigor (Da Silva *et al.* 2023).

Mycoparasitism and antibiosis are the primary pathogen-combating mechanisms employed by *Trichoderma* species, which produce both volatile and non-volatile organic compounds (Javaid *et al.* 2023; Birari *et al.* 2024). Cell wall degradation enzymes help to destroy the first line of defense present in pathogens, while VSMs and nVSMs produce remote inhibition. Terpenes isolated from *Trichoderma* species have demonstrated a range of bioactive properties, including antimicrobial, anti-microalgal, anticancer, and cytotoxic effects (Zhang *et al.* 2021). Fungal volatile organic compounds, which are a diverse group of carbon-based substances, typically have low molecular weights, low polarity, low boiling points, and high vapor pressures (Lakhdari *et al.* 2023). These compounds are often lipophilic and encompass a variety of chemical classes, including alcohols, benzenoids, aldehydes, alkenes, acids, esters, ketones, and terpenes (Hung *et al.* 2015; Lemfack *et al.* 2018).

The goal of the current study was to assess the antagonistic potential of *T. asperellum* against *F. oxysporum* f. sp. *ciceri.* Additionally, the research aimed to characterize the antifungal profiles of VSMs and nVSMs produced by *T. asperellum* using gas chromatography-mass spectrometry (GC-MS) analysis.

Materials and Methods

Trichoderma asperellum pure culture and pathogen

The fungal antagonist strains (Table 1) used in this study were previously isolated from soil samples collected at various locations in Maharashtra, India. The strains of chickpea wilt-causing fungal pathogen *F. oxysporum* f. sp. *ciceri* (GenBank accession number ON041443) were isolated from wilted chickpea vascular tissues and obtained from the mycology and biocontrol unit laboratories of the Plant Pathology Section, Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola. Before further experimental utilization, all the strains were grown on PDA and stored in a refrigerator (at 4°C).

Table 1. *Trichoderma asperellum* strains used to control *Fusarium oxysporum* f. sp. *ciceri*

No.	Place	Location	Codes
1	Bhandara	21° 10′ 30.039″N 79° 39′ 20.966″E	TaSrBh
2	Yavatmal	20° 23′ 19.658″ N 78° 7′ 13.4652″ E	TaSrYa
3	Buldhana	20° 32′ 12.645″ N 76° 10′ 51.132″ E	TaSrBu
4	Umarkhed	19°36′5.18122″N 77°41′19.6121″E	TaSrUm
5	Gadchiroli	20° 11′ 5.5392″ N 79° 59′ 41.269″ E	TaSrGa
6	Chandrapur	19° 58′ 13.166″ N 79° 18′ 12.096″ E	TaSrCh

Antagonistic efficacy of *Trichoderma asperellum* culture filtrate

The antifungal effect of *T. asperellum* culture filtrate on the test pathogen *F. oxysporum* f. sp. *ciceri* was evaluated using the method described by Dennis and Webster (1971), with minor modifications. A 6 mm fungal disc from a six-day-old *T. asperellum* culture was inoculated into 100 ml of sterilized PDB medium and incubated at 28 ± 2°C for 14 days under continuous shaking. After incubation, the culture was filtered through Whatman No. 1 filter paper into a sterilized conical flask. The filtrate was then centrifuged at 10,000 rpm for 15 min, and the supernatant was passed through a sterile cellulose membrane Millipore filter to remove spores. The resulting filtrate, which contained secondary metabolites (SMs), was used for further testing.

To assess the growth reduction of the test pathogen by the *T. asperellum* culture filtrate, 6 mm fungal discs of the pathogen were placed on PDA plates containing 5%, 15%, and 25% culture filtrate. The plates were incubated at 28 ± 2°C to allow the pathogen to grow fully on the control plates. Control plates, containing only the pathogen without the culture filtrate, were also prepared. The percentage inhibition of mycelial growth (PIMG) of the pathogen was recorded once the control plate was completely covered by mycelium. Three replicates were used for each treatment. The radial growth of the mycelium on the treatment plates (R2) was measured and compared to the radial growth on the control plates (R1), with the following formula used to calculate PIMG:

$$PIMG = [(R1–R2)/R1] \times 100.$$

Antagonistic efficacy of *Trichoderma asperellum* VSMs

The susceptibility of *F. oxysporum* f. sp. *ciceri* to *T. asperellum*-mediated VSMs was evaluated in vitro using a remote confrontation assay (Li *et al*. 2023). In this assay, six mm mycelial plugs of *T. asperellum* and the pathogenic *F. oxysporum* f. sp. *ciceri* were each placed at the center of separate Petri dish bottoms containing PDA. The two Petri dish bottoms were then carefully stacked, with the *T. asperellum* dish placed upside down and the *F. oxysporum* f. sp. *ciceri* dish positioned right-side up. The two Petri dishes were sealed together with Parafilm (HiMedia, India) tape to prevent the escape of any volatile compounds produced by the fungi (Daami-Remadi and Mahjoub 2001). Incubation conditions were consistent with those used in the previous method.

For the control, a similar setup was used, except the upper dish contained a six mm fungal plug of *F. oxysporum* f. sp. *ciceri*, while the lower dish contained only plain PDA medium. The experiment was monitored until the mycelium of the *Fusarium* pathogen in the control dish reached the edge of the plate. The average colony diameter (in mm) of the treated pathogen was measured and recorded to assess the growth inhibition caused by the VSMs produced by *T. asperellum*.

Antagonistic efficacy of diffusible *Trichoderma asperellum* SMs

The bilayer assay (Angel *et al*. 2016) was adapted to assess the antifungal activity of diffusible SMs produced by *T. asperellum* against *F. oxysporum* f. sp. *ciceri*. In this assay, six mm mycelial plugs of *T. asperellum* were inoculated onto a sterilized PDA medium and incubated for 8 days at 28 ± 2°C to form a fungal lawn. Afterward, a second agar layer containing benomyl (a fungicide known to inhibit *Trichoderma* growth) was poured over the lawn. The purpose of adding benomyl was to halt the growth of *T. asperellum* into the second layer of agar, ensuring that only diffusible SMs (and not physical interactions) would be responsible for the observed effects on the pathogen. The second agar layer was prepared by mixing 15 ppm benomyl with molten PDA, and 10 ml of this formulation was poured over the 8-day-old *T. asperellum* lawn to create the bilayer (Fig. 1).

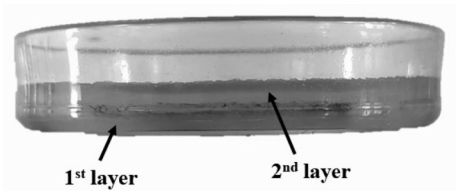

Fig. 1. Strategy to test antifungal effect of *Trichoderma asperellum* diffusible SMs

Next, a six mm plug of *F. oxysporum* f. sp. *ciceri* was placed at the center of the second agar layer. Two single-layer control treatments were also prepared for comparison. The first control consisted of 20 ml of PDA supplemented with 15 ppm benomyl in a separate Petri dish. After solidifying, a six mm plug of *F. oxysporum* f. sp. *ciceri* was placed at the center of the plate, serving as a negative control (NC). The second control consisted of a PDA dish without benomyl, with a 6 mm plug of *F. oxysporum* f. sp. *ciceri* inoculated at the center.

The growth inhibition of *F. oxysporum* f. sp. *ciceri* (FGI) in the bilayer and single-layer controls was measured 8 days after inoculation. FGI represents the percentage reduction in *F. oxysporum* f. sp. *ciceri*

growth due to the antifungal effect of the diffusible metabolites produced by *T. asperellum*. The FGI was calculated using the following formula:

$$FGI (\%) = (R1 - R2) / R1 \times 100\%,$$

where: R1 was the control without *T. asperellum,* and R2 was the treatment with *T. asperellum*.

Extraction of nVSMs from *Trichoderma asperellum*

Trichoderma asperellum nVSMs extraction was prepared as described by Youassi *et al.* (2024) with minor changes. The *T. asperellum* strains (TaSrBh, TaSrYa, TaSrBu, TaSrUm, TaSrGa, and TaSrCh) were grown on Potato Dextrose Agar (PDA) (HiMedia, India) plates at 28 ± 2°C for 7 days. Once the mycelial edges were actively growing, 6 mm plugs were cut and aseptically transferred into separate 2 l Erlenmeyer flasks containing 800 ml of sterile Potato Dextrose Broth (PDB) medium (HiMedia, India). The cultures were incubated at 28 ± 2°C for 31 days without disturbance. After incubation, the culture filtrates of each *T. asperellum* strain were processed to extract nVSMs using immiscible organic solvents (n-hexane, ethyl acetate, and dichloromethane) separately in equal volumes within a separating funnel. The organic phases of each solvent were filtered through Whatman No. 4 filter paper (HiMedia, India), dried over anhydrous sodium sulfate (HiMedia, India), and then concentrated using a rotary evaporator (Buchi) under reduced pressure to remove the solvents (Pradeep *et al.* 2024). The resulting nVSMs of each strain were recovered as respective crude extracts, *viz.* n-hexane (HeE), dichloromethane (DcE), and ethyl acetate (EaE). The crude nVSMs extracts were then diluted to a concentration of 10 mg · ml^{-1} in methanol (master nVSMs) and stored in a refrigerator until further evaluation for antifungal activity against *F. oxysporum* f. sp. *ciceri*.

Antagonistic efficacy of nVSMs extracted from *Trichoderma asperellum*

The antagonistic effect of nVSMs from individual *T. asperellum* strains (TaSrBh, TaSrYa, TaSrBu, TaSrUm, TaSrGa, and TaSrCh) at concentrations of 150, 300, 450, and 600 µg · ml^{-1} was evaluated against *F. oxysporum* f. sp. *ciceri* using the poisoned PDA technique. The respective nVSMs were dissolved in a 1% (w/v) Tween 20 solution suspended in methanol to create a final master solution of 10 mg · ml^{-1}. Appropriate volumes of each nVSMs stock solution were then mixed with molten PDA to achieve the desired final concentrations of 150, 300, 450, and 600 µg · ml^{-1}. The PDA, containing these concentrations and a negative control (NC) consisting of Tween 20 + methanol, was poured into sterile Petri dishes.

Afterward, each Petri dish was inoculated with a six mm fungal plug of *F. oxysporum* f. sp. *ciceri* at the center. The positive control was set using tebuconazole (Folicur 250 EC, 25.9% w/w, Bayer) at a concentration of 60 ppm. The experiment was replicated, and incubation conditions, along with the measurement of percent inhibition, followed the same protocol as in the previous methods.

A three-factor completely randomized design (6 × 3 × 4) was used to structure the experimental trial. The factors included six *T. asperellum* strains, three types of organic solvent extracts (HeE, DcE, and EaE), and four concentrations (150, 300, 450, and 600 µg · ml^{-1}).

Gas chromatography high resolution mass spectrometry (GCHRMS)

The GCHRMS analysis was conducted with modifications to the methods described by Angel *et al.* (2016) and Yassin *et al.* (2021). The analysis was conducted at the Sophisticated Analytical Instrument Facility (SAIF) of the Indian Institute of Technology (IIT) Bombay.

The GCHRMS scans of TaSrYa ethyl acetate extract were performed using a Gas Chromatography Mass Spectrometer (Model: AccuTof JMS-T100GCV, JEOL Ltd., Tokyo, Japan), equipped with an EI/CI ion source, a Time of Flight (TOF) analyzer, and a mass range of 10–2000 amu with a resolution of 6000. The GC system (Agilent 7890) consisted of an automatic liquid sampler, FID detector, and headspace injector, all of which were interfaced with the mass spectrometer.

A capillary column (Rxi-5 ms, Restek Corporation, Bellefonte, Pennsylvania, USA) was used for separation, with a length of 60 m, a diameter of 0.25 mm, and a film thickness of 0.25 µm. The EI system was employed for GCHRMS scans, with an ionization energy of 70 eV. Helium was used as the carrier gas, with a flow rate of 1 ml · min^{-1}, and a splitless injection of 0.5 µl was performed.

The oven temperature program was as follows: it started at 80°C, then increased by 8°C · min^{-1} to 200°C, followed by an 8°C · min^{-1} ramp to 275°C. The temperature was then increased by 5°C · min^{-1} to 280°C and held isothermally at 280°C for 5 minutes. Mass spectra were acquired at 70 eV, with a scan interval of 0.5 s and a mass range from 50 to 500 m/z. The total run time for the analysis was 40 min. The NIST (National Institute of Standards and Technology) library was used to match each compound's mass fragmentation spectra.

Headspace-solid phase microextraction – two-dimensional gas chromatography time of flight mass spectrometry (HS-SPME-GCGC--TOF-MS)

The results of the remote confrontation assay indicate that TaSrGa is a valuable source of antifungal VSMs that inhibit the growth of *F. oxysporum* f. sp. *ciceri*. To identify these VSMs, TaSrGa was subjected to two-dimensional gas chromatography-mass spectrometry.

Solid-phase microextraction (SPME) is a modern technique used to capture volatile compounds from samples, offering superior extraction efficiency compared to traditional methods for volatile sampling. This solvent-free technique ensures direct concentration of analytes from the headspace of a sample (Taibi *et al*. 2024). For sample preparation, a sterile 20 ml glass chromatography vial (2 cm diameter) was filled with PDA medium and prepared as a slant. After solidification, a TaSrGa mycelial disc was inoculated under aseptic conditions. The inoculated vial was sealed with a PTFE (polytetrafluoroethylene) septum, secured with an aluminium lid, and incubated in a BOD incubator for 14 days (Ruangwong *et al*. 2021).

To capture the VSMs from the headspace of the vial, an SPME fiber (65 µm Polydimethylsiloxane/divinylbenzene) was inserted into the vial through the septum. The sample was incubated at 45°C for 30 min, then shaken at 250 rpm to facilitate the absorption of the volatile compounds. Afterward, the volatiles were desorbed by injecting the fiber into the GC port HS-SPME required the injector to be operated in a splitless mode to ensure complete desorption of the volatile organic compounds into the capillary column. Separation of the analytes was achieved using two capillary columns, Rxi-5MS (30 m) and Rxi-17silMS (2 m). The GC system was coupled with a time-of--flight mass spectrometer (TOF-MS).

Based on the available literature, the oven temperature was programmed to increase from 45°C (initial temperature) to 230°C (final temperature) at a rate of 8°C · min^{-1}. The acquired chromatograms were processed and analyzed using ChromaTof software and the NIST database.

Statistical data interpretation

Data from the experiments were analyzed using General Linear Models (GLM) and mean comparison procedures with the Statistical Package for the Social Sciences (SPSS, Version 29.0.2, 2022, IBM). Post hoc tests were conducted to perform multiple comparisons between groups.

Results

Antagonistic efficacy of *Trichoderma asperellum* culture filtrate

The cell-free (CF) supernatants of *T. asperellum* strains (TaSrBh, TaSrYa, TaSrBu, TaSrUm, TaSrGa, and TaSrCh) were tested for their antifungal activity against the pathogenic agent *F. oxysporum* f. sp. *ciceri* at concentrations of 5%, 15%, and 25%. As shown in Table 2, all tested concentrations of culture filtrate exhibited varying degrees of inhibition in the mycelial growth of *F. oxysporum* f. sp. *ciceri*.

The culture filtrate of TaSrYa significantly inhibited the mycelial extension of *F. oxysporum* f. sp. *ciceri*, with inhibition rates of 21.44 ± 1.38%, 38.25 ± 1.27%, and 56.93 ± 0.70% at 5%, 15%, and 25% concentrations, respectively. TaSrGa was the second most effective isolate, showing 52.06 ± 1.10% mycelial inhibition at the 25% concentration. Overall, the antifungal activity of all *T. asperellum* culture filtrate increased with higher

Table 2. Antagonistic efficacy of *Trichoderma asperellum* culture filtrate against *Fusarium oxysporum* f. sp. *ciceri*

Culture filtrate concentrations	Strains	Mycelial growth [mm]	Mycelial inhibition [%]
5%	TaSrBh	80.30	9.76 ± 1.36 l
	TaSrYa	70.22	21.44 ± 1.38 h
	TaSrBu	76.88	14.50 ± 0.70 j
	TaSrUm	80.10	10.94 ± 0.58 kl
	TaSrGa	74.21	17.23 ± 0.74 i
	TaSrCh	78.20	13.06 ± 1.10 jk
	Control	90.00	00.00 ± 00.00 m
15%	TaSrBh	73.89	17.98 ± 0.68 i
	TaSrYa	55.64	38.25 ± 1.27 c
	TaSrBu	66.49	26.22 ± 0.69 ef
	TaSrUm	69.13	23.42 ± 1.14 gh
	TaSrGa	60.22	34.21 ± 0.89 d
	TaSrCh	68.00	24.46 ± 1.28 fg
	Control	90.00	00.00 ± 00.00 m
25%	TaSrBh	66.00	26.88 ± 1.39 e
	TaSrYa	38.99	56.93 ± 0.70 a
	TaSrBu	56.44	37.83 ± 0.56 c
	TaSrUm	60.20	32.70 ± 1.57 d
	TaSrGa	42.70	52.06 ± 1.10 b
	TaSrCh	55.42	38.61 ± 1.14 c
	Control	90.00	00.00 ± 00.00 m

Duncan's post hoc test interpreted data showing the same lowercase letters next to mean ± standard deviation in the column, suggesting that mean values are not significantly different from each other ($p \leq 0.05$)

concentrations. TaSrYa and TaSrGa exhibited greater mycelial inhibition at all concentrations than the other strains (TaSrBh, TaSrBu, TaSrUm, and TaSrCh).

Antifungal effect of *Trichoderma asperellum* VSMs

The antifungal efficacy of the VSMs produced by *T. asperellum* strains was evaluated using the remote confrontation assay. All the strains demonstrated significant mycelial inhibition of *F. oxysporum* f. sp. *ciceri*. Among the strains, TaSrGa exhibited the highest growth inhibition at 52.57 ± 1.19%. The next most effective isolate was TaSrYa, with 44.80 ± 1.69% inhibition, followed by TaSrBu at 28.75 ± 2.26%. The analysis of variance revealed a non-significant difference ($p < 0.001$) in the percent mycelial inhibition of strain TaSrBh (23.55 ± 2.59), TaSrUm (25.75 ± 1.43), and TaSrCh (24.28 ± 1.43) relative to each other. *F. oxysporum* f. sp. *ciceri* was most sensitive to strains TaSrGa and TaSrYa.

The observed inhibition of mycelial growth is likely due to the volatile antifungal compounds released by *T. asperellum* (Fig. 2). The results of the remote confrontation assay clearly demonstrated the ability of *T. asperellum* to inhibit the mycelial growth of *F. oxysporum* f. sp. *ciceri* (23.55% to 52.57%) without direct contact. This technique effectively highlighted the inhibitory effects of *T. asperellum* VSMs on *F. oxysporum* f. sp. *ciceri*, even when acting remotely (Fig. 3).

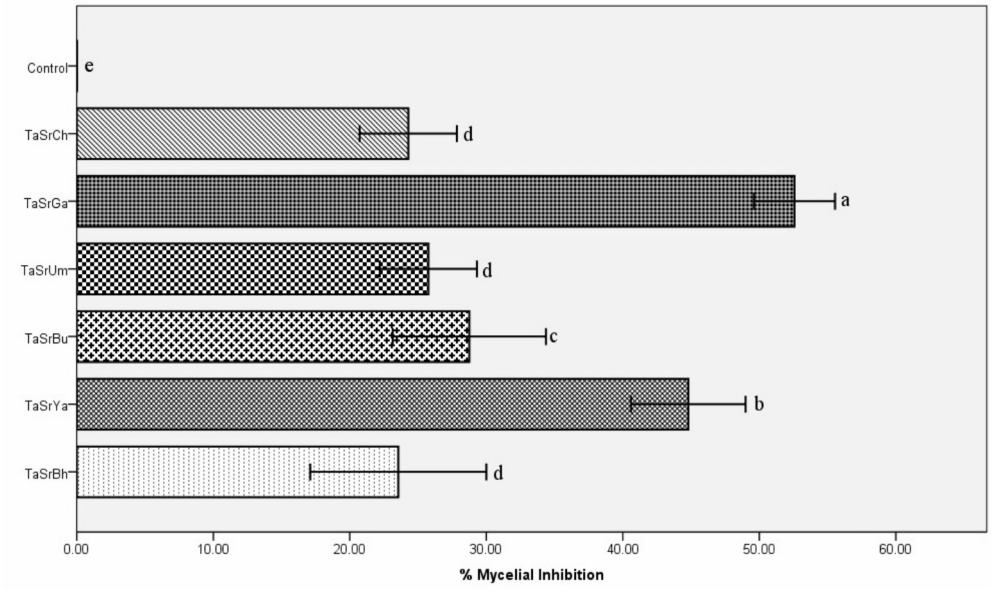

Fig. 2. Bar graph showing the antagonistic effect of VSMs produced by *Trichoderma asperellum.* Same lowercase letters over bars indicate that mean values are not significantly different from each other ($p \leq 0.05$), according to Duncan's post hoc test

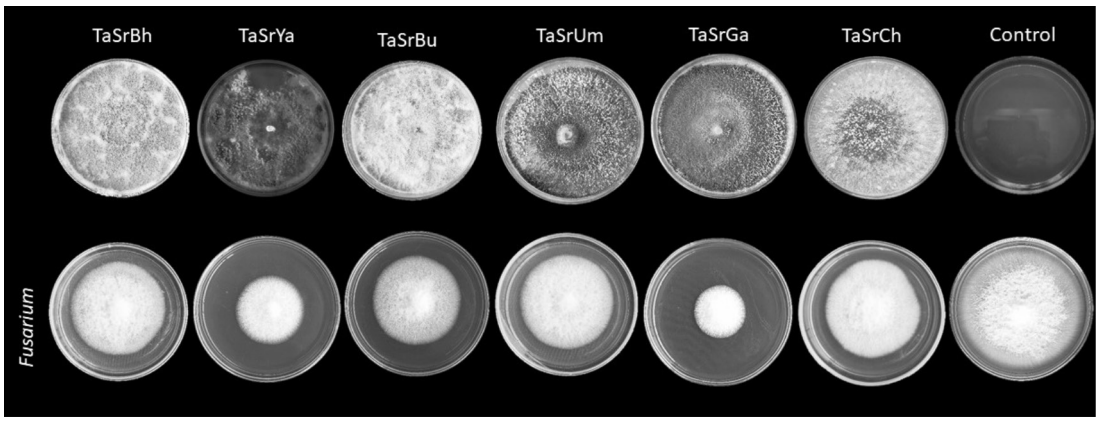

Fig. 3. Representative plates of the antagonistic remote confrontation assay

Antifungal effect of *Trichoderma asperellum* diffusible SMs

The effects of diffusible metabolites produced by *T. asperellum* were assessed using the bilayer technique. Significant mycelial extension was observed on all control plates of *F. oxysporum* f. sp. *ciceri*, including the negative control (NC) plate, which was treated with 15 ppm of benomyl. In contrast, when *T. asperellum* was present, a reduction in mycelial growth of *F. oxysporum* f. sp. *ciceri* was observed, indicating that the diffusible metabolites secreted by *T. asperellum* effectively inhibited radial mycelial growth (Table 3). The color change to brown observed in the PDA plates containing benomyl and *T. asperellum* may be attributed to the secretion of these diffusible metabolites (Fig. 4).

Mycelial growth of *F. oxysporum* f. sp. *ciceri* was measured on the 7th day to facilitate comparison across the test plates. The highest mycelial growth inhibition was observed in the plates containing benomyl + TaSrGa, which inhibited 76.17 ± 1.49% of *F. oxysporum* f. sp. *ciceri* growth. The second most effective isolate was TaSrYa, with a 72.72 ± 1.66% reduction in growth. No significant difference was found among TaSrBh, TaSrUm, and TaSrCh in terms of their inhibitory effect on *F. oxysporum* f. sp. *ciceri*. The NC plates exhibited only 58.20 ± 1.01% mycelial inhibition, which was attributed solely to the fungistatic effect of benomyl. All *T. asperellum* strains showed significantly higher mycelial growth inhibition compared to both the control treatments, suggesting that the production of diffusible metabolites by *T. asperellum* in the medium played a major role in suppressing *F. oxysporum* f. sp. *ciceri* growth.

Table 3. Antifungal efficacy of *Trichoderma asperellum* diffusible SMs against *Fusarium oxysporum* f. sp. *ciceri*

Tr. No.	Strains	Mycelial growth [mm]	Mycelial inhibition [%]
1	TaSrBh	29.44	67.28 ± 1.66 cd
2	TaSrYa	24.55	72.72 ± 1.66 b
3	TaSrBu	29.88	66.78 ± 1.82 d
4	TaSrUm	28.35	68.49 ± 1.41 cd
5	TaSrGa	21.43	76.17 ± 1.49 a
6	TaSrCh	27.46	69.48 ± 1.16 c
7	NC (with Benomyl)	37.61	58.20 ± 1.01 e
8	control	90.00	0.00 ± 0.00 f

Duncan's post hoc test interpreted data showing the same lowercase letters next to mean ± standard deviation in the column, suggesting that mean values are not significantly different from each other ($p \leq 0.05$)

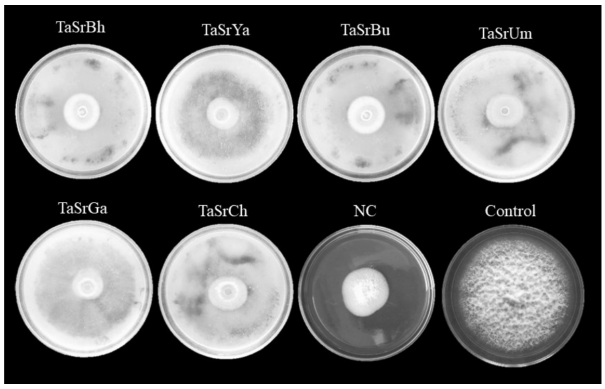

Fig. 4. Antagonistic effect of *Trichoderma asperellum* diffusible SMs against *Fusarium oxysporum* f. sp. *ciceri*

Antagonistic effect of nVSMs from *Trichoderma asperellum*

Analysis of variance of growth inhibition data showed significant interactions among all sources of variation, including the triple interaction of the factorial design: six *T. asperellum* strains (TaSrBh, TaSrYa, TaSrBu, TaSrUm, TaSrGa, and TaSrCh) × three organic extracts (HeE, DcE, and EaE) × four concentrations (150, 300, 450, and 600 μg · ml^{-1}), used in the growth inhibition assay. Growth inhibition of *F. oxysporum* f. sp. *ciceri* responded similarly to the combination of the three factors analysed, and the independent factors significantly influenced the inhibition of growth of *F. oxysporum* f. sp. *ciceri* (Table 4). In simple main effects, TaSrYa was the most significant strain, EaE was the most significant extract, and 600 μg · ml^{-1} was the most significant concentration.

In the case of *T. asperellum* strains × solvent extracts interaction effects, EaE of TaSrYa showed maximum mycelial inhibition, with the highest estimated marginal mean values. In contrast, for *T. asperellum* strains × concentrations, and solvent extracts × concentrations interactions, maximum inhibition values were observed for TaSrYa at 600 μg · ml^{-1} and EaE at 600 μg · ml^{-1} respectively.

The majority of extracts inhibited the growth of *F. oxysporum* f. sp. *ciceri*. The EaE extract of TaSrYa exhibited 27.32–84.19% inhibition within all the concentrations examined (Table 5). The HeE and DcE extracts of TaSrYa exhibited growth inhibitions of 21.50–69.33% and 20.84–77.70%, respectively. The EaE extract from all the *T. asperellum* strains showed higher inhibition values than the HeE and DcE extract toward *F. oxysporum* f. sp. *ciceri,* except for DcE extract of TaSrBh, HeE and DcE extract of TaSrUm, and HeE extract of TaSrCh. This suggests that ethyl acetate was the most efficient solvent for extracting antifungal SMs from *T. asperellum* strains. The pathogenic strain *F. oxysporum* f. sp. *ciceri* showed decreased sensitivity

Table 4. Analysis of variance on effects of *Trichoderma asperellum* strains, solvent extracts and concentration on the growth inhibition of *Fusarium oxysporum* f. sp. *ciceri*

Source	Type III sum of squares	df	Mean square	F	Sig.	Partial Eta squared
Corrected Model	79858.261[a]	71	1124.764	251.773	0.000	0.992
Intercept	431044.059	1	431044.059	96487.160	0.000	0.999
T. asperellum	4178.260	5	835.652	187.057	0.000	0.867
Solvent extract	3112.596	2	1556.298	348.370	0.000	0.829
Concentration	69381.409	3	23127.136	5176.899	0.000	0.991
T. asperellum × Solvent extract	634.609	10	63.461	14.205	0.000	0.497
T. asperellum × Concentration	1268.984	15	84.599	18.937	0.000	0.664
Solvent extract × Concentration	309.779	6	51.630	11.557	0.000	0.325
T. asperellum × Solvent extract × Concentration	972.624	30	32.421	7.257	0.000	0.602
Error	643.302	144	4.467			
Total	511545.622	216				
Corrected Total	80501.563	215				

[a]R Squared = .992 (Adjusted R Squared = 0.988), df – degrees of freedom

Table 5. Antagonistic efficacy of nVSMs extracted from *Trichoderma asperellum* against *Fusarium oxysporum* f. sp. *ciceri*

Strains		Mycelial growth [mm]				Mycelial inhibition [%]			
		Concentrations							
	Extracts	C1[a]	C2[b]	C3[c]	C4[d]	C1[a]	C2[b]	C3[c]	C4[d]
TaSrBh	HeE	75.99	63.11	48.88	37.51	15.55 ± 1.33 f	29.87 ± 2.40 fg	45.80 ± 3.10 gh	58.31 ± 1.66 g
	DcE	80.28	64.99	39.66	31.62	10.79 ± 1.18 g	27.78 ± 2.06 g	55.92 ± 2.05 c	64.86 ± 2.22 ef
	EaE	75.10	50.66	40.22	28.22	16.54 ± 1.49 ef	43.70 ± 2.06 c	55.30 ± 3.35 c	68.64 ± 1.49 cd
TaSrYa	HeE	70.44	48.58	34.80	27.33	21.50 ± 2.58 bcd	46.01 ± 2.45 c	61.32 ± 2.24 b	69.33 ± 1.37 c
	DcE	71.23	49.92	32.92	20.06	20.84 ± 2.59 cd	44.52 ± 2.70 c	63.41 ± 2.04 b	77.70 ± 2.08 b
	EaE	65.40	38.81	25.11	14.22	27.32 ± 2.54 a	56.86 ± 2.76 a	72.09 ± 0.93 a	84.19 ± 2.25 a
TaSrBu	HeE	69.21	59.95	42.25	34.10	23.06 ± 1.49 bc	33.38 ± 2.04 e	53.04 ± 1.48 cde	62.10 ± 2.23 f
	DcE	74.55	58.95	47.88	32.81	17.16 ± 2.60 ef	34.49 ± 1.83 e	46.78 ± 1.70 g	63.53 ± 1.59 ef
	EaE	67.97	43.92	32.58	27.28	24.46 ± 1.94 ab	51.19 ± 3.14 b	63.79 ± 0.89 b	69.67 ± 1.38 c
TaSrUm	HeE	80.93	60.17	41.14	27.99	10.07 ± 2.03 g	33.13 ± 3.36 ef	54.28 ± 2.10 cd	68.88 ± 1.11 cd
	DcE	79.98	60.58	43.92	28.22	11.12 ± 3.06 g	32.67 ± 2.81 ef	51.19 ± 2.04 def	68.64 ± 1.49 cd
	EaE	70.17	55.44	35.12	30.17	22.02 ± 1.16 bcd	38.39 ± 2.67 d	60.96 ± 2.11 b	66.46 ± 2.71 cde
TaSrGa	HeE	70.81	61.66	50.99	37.92	21.31 ± 1.58 bcd	31.48 ± 2.66 ef	43.33 ± 2.66 hi	57.85 ± 2.10 g
	DcE	72.62	57.77	43.66	30.80	19.30 ± 1.78 de	33.58 ± 1.18 e	51.48 ± 2.66 de	65.84 ± 1.72 de
	EaE	65.22	51.11	33.80	22.58	27.53 ± 2.04 a	43.18 ± 2.34 c	62.44 ± 1.15 b	74.90 ± 1.03 b
TaSrCh	HeE	72.06	61.77	46.77	32.02	19.92 ± 1.69 cde	31.35 ± 3.36 ef	48.02 ± 2.67 fg	64.41 ± 1.57 ef
	DcE	72.95	61.00	53.00	37.62	18.93 ± 1.41 def	32.22 ± 2.93 ef	41.07 ± 0.06 i	58.19 ± 2.64 g
	EaE	70.07	58.77	44.80	32.29	22.14 ± 1.11 bcd	34.68 ± 0.77 e	50.21 ± 1.15 ef	64.11 ± 1.04 ef
NC		90.00	90.00	90.00	90.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
PC		0.00	0.00	0.00	0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00

In the column, data indicate mean values ± standard deviation, n = 3, three-way factorial ANOVA followed by Duncan's post hoc test, same lowercase letters next to means suggest that mean values are not significantly different from each other ($p \leq 0.05$); [a]150 μg · ml^{-1}, [b]300 μg · ml^{-1}, [c]450 μg · ml^{-1}, [d]600 μg · ml^{-1}; NC – negative control, PC – positive control

to HeE and DcE extracts. Overall, the organic solvent extracts from strain TaSrYa showed the highest mycelial inhibition of the *F. oxysporum* f. sp. *ciceri*. In the negative control (only methanol), zero percent growth inhibition was observed. While in positive control (tebuconazole) 100% percent growth inhibition was recorded.

In the *T. asperellum* × solvents extracts × concentrations interaction, maximum mycelial inhibition of *F. oxysporum* f. sp. *ciceri* was observed in EaE of

TaSrYa and TaSrGa at 150 μg · ml^{-1} concentration with mean values of 27.32% and 27.53% respectively, which were at par with each other. In contrast, 300, 450, and 600 μg · ml^{-1} concentrations represented significant inhibition in EaE of TaSrYa, with mean values of 56.86%, 72.09%, and 84.19%, respectively.

GCHRMS

In vitro, the assay of nVSMs extracted from TaSrYa culture filtrate in ethyl acetate showed strong antifungal efficacy against *F. oxysporum* f. sp. *ciceri*. Accordingly, GCHRMS analysis was carried out to characterize the metabolic profile of nVSMs obtained from the ethyl acetate extract (EaE) of TaSrYa. The GCHRMS chromatogram (Fig. 5) showed fifteen peaks of compounds, each with its respective retention time. These compounds were identified with a matching probability of greater than 70% using the NIST library. The compounds detected in the SMs extracted from TaSrYa culture filtrate in ethyl acetate belong to the following compound classes: alkanes, alkenes, pyrones, aldehydes, sesquiterpenes, and fatty acids (Table 6).

The most dominant compounds identified in this study were 9-octadecenoic acid at 23.19 min with a 26.32% peak area, followed by dehydroacetic acid at 12.20 min with a 25.71% peak area, and 9-undecenal, 2,10-dimethyl, an aldehyde at 19.29 min with an 11.31% peak area. Figure 6 shows the structures of the major bioactive compounds.

HS-SPME-GCGC-TOF-MS

The experimental findings suggest that the VSMs emitted by the TaSrGa strain effectively inhibited the growth of *F. oxysporum* f. sp. *ciceri*, demonstrating their antifungal potential. Solid-phase microextraction (SPME) was used to capture the VSMs emitted by the TaSrGa strain, which were subsequently analyzed by GC-GC-TOF-MS. The preliminary screening of these VSMs, responsible for inhibiting *F. oxysporum* f. sp. *ciceri*, is shown in Table 7 and Figure 7.

A total of 67 compounds were tentatively identified with greater than 80% similarity using the NIST library, as listed in Table 7. These compounds belong to several classes, including alcohols, alkanes, esters, acids, and terpenes. The most abundant volatile compound identified was phenylethyl alcohol (PEA), which appeared at 882 seconds with a peak area of 3.42%, followed by 1,3-Cyclohexadiene, 5-(1,5-dimethyl-4-hexenyl)--2-methyl- (alpha-zingiberene) (sesquiterpene) at 1342 seconds (2.74% peak area), α-bisabolene at

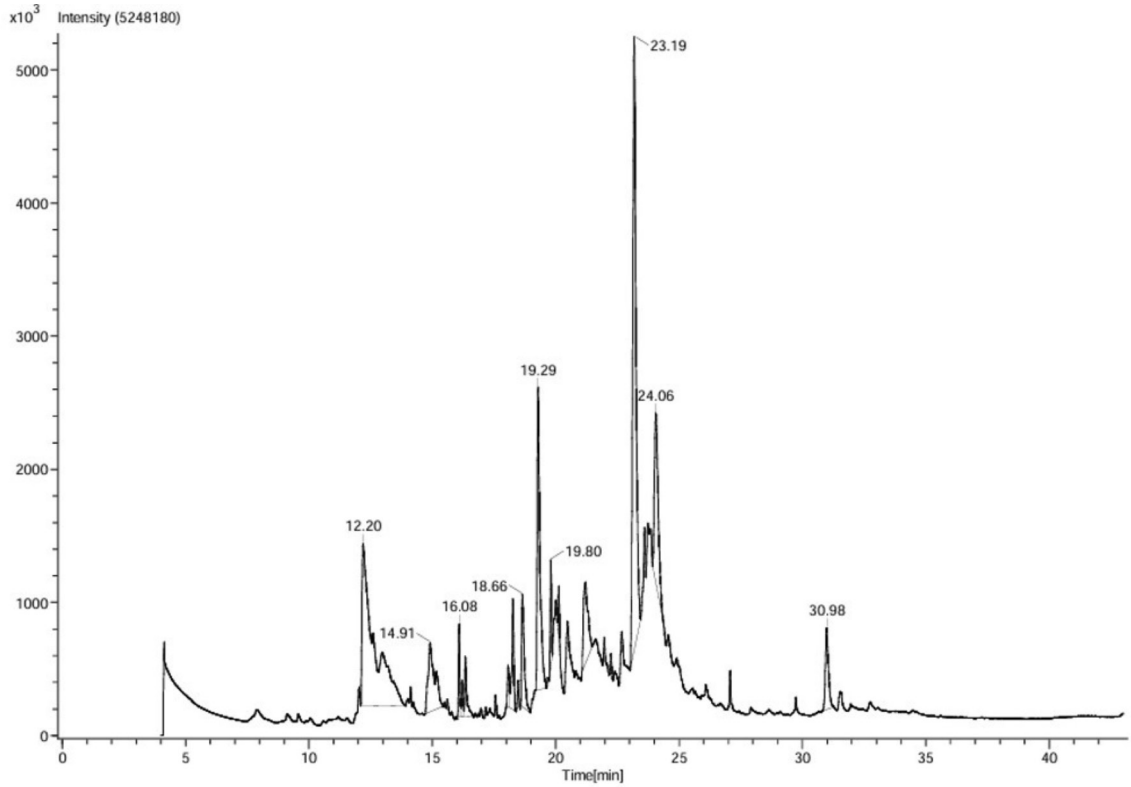

Fig. 5. GCHRMS chromatogram of nVSMs collected from TaSrYa ethyl acetate extract

Table 6. GCHRMS profiling of nVSMs derived from TaSrYa in ethyl acetate

Peak No.	RT	Compound	Molecular formula	MW	Peak area [%]	Class	References
1	12.20	Dehydroacetic acid	$C_8H_8O_4$	168	25.71	pyrone	Kumari *et al*. (2024)
2	14.91	9,12,15-Octadeca-trienoic acid, 2,3-di-hydroxypropyl ester, (Z,Z,Z)-	$C_{21}H_{36}O_4$	352	6.18	fatty acid ester	Javaid *et al*. (2023)
3	16.08	1-Hexadecene	$C_{16}H_{32}$	224	1.56	alkene	Shanmugaraj *et al*. (2023)
4	16.21	1-Chloroheptaco-sane	$C_{27}H_{55}Cl$	414	0.71	alkyl halide	Siddiquee *et al*. (2012)
5	16.34	Eicosane, 1-iodo	$C_{20}H_{41}OI$	408	1.74	alkane	Abdenaceur *et al*. (2024)
6	18.08	9-Hexadecenoic acid	$C_{16}H_{30}O_2$	254	0.39	fatty acid	Moussa *et al*. (2023)
7	18.27	Octadecane	$C_{18}H_{38}$	254	2.73	alkane	Liu *et al*. (2024)
8	18.66	Nerolidol	$C_{15}H_{26}O$	222	3.91	sesquiter-pene	Choez-Guaranda *et al*. (2023)
9	19.29	9-Undecenal, 2,10-dimethyl-	$C_{13}H_{24}O$	196	11.31	aldehyde	Chakarwarti *et al*. (2024)
10	19.80	17-Pentatriacon-tene	$C_{35}H_{70}$	490	1.61	alkene	Begum *et al*. (2016)
11	20.13	9,12-Octadeca-dienoic acid (Z,Z) methyl ester	$C_{19}H_{30}O_2$	290	0.81	fatty acid	Shaker *et al*. (2022)
12	21.21	cis-Z-α-Bisabolene epoxide	$C_{15}H_{24}O$	220	4.96	terpene	Awadalla *et al*. (2024)
13	23.19	9-Octadecenoic acid	$C_{18}H_{34}O_2$	282	26.32	fatty acid	Moussa *et al*. (2023)
14	24.06	7,10-Octadecadi-enoic acid	$C_{18}H_{32}O_2$	280	8.62	fatty acid	Lakhdari *et al*. (2023)
15	30.98	1-Heptatriacotanol	$C_{37}H_{76}O$	536	3.35	alcohols	Awad *et al*. (2018)

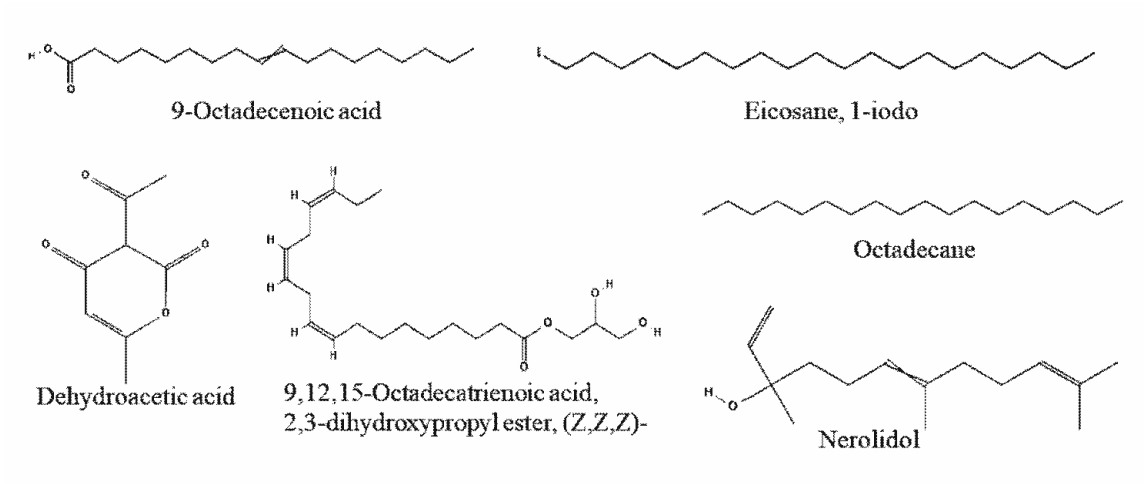

Fig. 6. Chemical structures of major bioactive compounds derived from TaSrYa ethyl acetate extract

Table 7. Composition of VSMs emitted by TaSrGa

No.	VSMs	R. T. (s)	Formula	Similarity [%]	Area [%]	Class	References
1	Carbamic acid, monoammonium salt	89, 1.290	$CH_6N_2O_2$	97.5	0.21	carbamate	Al-Rahbi *et al*. (2023)
2	l-Alanine ethylamide	89, 1.880	$C_5H_{12}N_2O$	82.2	0.04	alpha-amino acid	–
3	1-Propanol	125, 4.890	C_3H_8O	81.5	0.02	alcohol	Speckbacher *et al*. (2020)
4	Ethyl Acetate	133, 4.940	$C_4H_8O_2$	83.2	0.04	ester	Panchalingam *et al*. (2022)
5	Methylal	143, 4.670	$C_3H_8O_2$	82.8	0.07	ether	Aleksic *et al*. (2018)
6	Hydrazinecarboxamide	167, 1.310	CH_5N_3O	86.8	0.04	carboxylic acid	Javaid *et al*. (2019)
7	Acetic acid	209, 4.660	$C_2H_4O_2$	92.5	0.90	carboxylic acid	Jayakumar *et al*. (2021)
8	1-Butanol, 3-methyl-	233, 2.630	$C_5H_{12}O$	80.1	0.02	alcohol	Kong *et al*. (2020)
9	1,2-Ethanediol	275, 2.970	$C_2H_6O_2$	81.9	0.14	alcohol	Adelusi *et al*. (2022)
10	2,3-Butanediol	383, 3.080	$C_4H_{10}O_2$	91.8	0.16	alcohol	Shandeep *et al*. (2024) Shanmugaraj *et al*. (2023)
11	2-Furanmethanol, tetrahydro-5-methyl-	687, 2.470	$C_6H_{12}O_2$	83.8	0.12	alcohol	Nagaraj *et al*. (2023)
12	2-Hexanone	688, 2.810	$C_6H_{12}O$	83.8	0.09	ketone	Lee *et al*. (2016)
13	á-Phellandrene	755, 2.590	$C_{10}H_{16}$	86.9	2.22	terpenoid	Zhang *et al*. (2017)
14	Indane	761, 3.310	C_9H_{10}	86.0	0.06	hydrocarbon	Tabarestani *et al*. (2016)
15	Benzene, 1-ethyl-4-methoxy-	881, 3.210	$C_9H_{12}O$	82.7	0.09	ether	Griffin *et al*. (2010)
16	Phenylethyl Alcohol	882, 3.830	$C_8H_{10}O$	92.2	3.42	alcohol	Ruiz-Cisneros *et al*. (2024) Chan *et al*. (2023)
17	2H-Pyran-2-one, 6-pentyl-	883, 1.678	$C_{10}H_{14}O_2$	90.1	2.34	pyranone	Philip *et al*. (2024)
18	Benzene, 1-ethenyl-4-methoxy-	935, 3.500	$C_9H_{10}O$	80.9	0.04	ether	Griffin *et al*. (2010)
19	Decane	941, 2.140	$C_{10}H_{22}$	85.5	0.21	alkane	Safara *et al*. (2022)
20	Oxalic acid, allyl octyl ester	942, 2.560	$C_{13}H_{22}O_4$	82.3	0.03	ester	Rabha *et al*. (2015)
21	2-Nonanol	971, 2.420	C_9H_2O	80.5	0.19	alcohol	Chen *et al*. (2016)
22	Naphthalene	977, 3.900	$C_{10}H_8$	95.0	1.02	hydrocarbon	Jayakumar *et al*. (2021)
23	Tridecane	995, 1.970	$C_{13}H_{28}$	87.9	0.20	alkane	Da silva *et al*. (2023) Safara *et al*. (2022)
24	1H-Indene, 1-ethyl-2,3-dihydro-	989, 3.040	$C_{11}H_{14}$	82.7	0.06	hydrocarbon	Siddiquee *et al*. (2012)
25	1-Decanol	1037, 2.530	$C_{10}H_{22}O$	87.6	1.48	alcohol	Khan *et al*. (2018)
26	Undecane	1091, 1.900	$C_{11}H_{24}$	88.5	1.16	alkane	Da Silva *et al*. (2023) Gajera *et al*. (2020)
27	Menthyl acetate	1109, 2.680	$C_{12}H_{22}O_2$	80.5	0.15	terpenoid	Saharkhiz *et al*. (2012)
28	2-ethyl-1-hexanol	1112, 3.456	$C_8H_{18}O$	92.1	0.78	alcohol	–
29	Hexadecane	1121, 1.990	$C_{16}H_{34}$	83.1	2.05	alkane	Da silva *et al*. (2023)
30	6,7-Dimethyl-1,2,3,5,8,8a-hexahydro-naphthalene	1127, 2.890	$C_{12}H_{18}$	80.9	0.26	sesquiterpenoid	Wang *et al*. (2013)
31	Pentanoic acid, 3-methyl-, ethyl ester	1155, 2.420	$C_8H_{16}O_2$	85.9	0.22	fatty acid ester	–
32	1-Octanol	1163, 2.640	$C_8H_{18}O$	86.0	0.32	alcohol	Qin *et al*. (2022)
33	Azulene	1187, 2.440	$C_{10}H_8$	85.2	1.70	hydrocarbon	Inayati *et al*. (2019)
34	1, 1, 5-Trimethyl-1, 2-dihydronaphthalene	1187, 3.240	$C_{13}H_{16}$	83.8	0.08	hydrocarbon	–
35	Benzene, 4-ethenyl-1,2-dimethoxy-	1193, 3.930	$C_{10}H_{12}O_2$	81.0	0.70	dimethoxybenzene	Mulatu *et al*. (2022)
36	Dehydroacetic Acid	1192, 4.500	$C_8H_8O_4$	83.1	2.03	pyrone	Shavkiev *et al*. (2022)
37	1-Iodo-2-methylnonane	1235, 2.010	$C_{10}H_{21}I$	80.9	0.06	alkanes	–
38	Butanedioic acid	1239, 3.110	$C_4H_6O_4$	80.2	1.70	carboxylic acids	Abdenaceur *et al*. (2024)

Table 7. Composition of VSMs emitted by TaSrGa – continuation

No.	VSMs	R. T. (s)	Formula	Similarity [%]	Area [%]	Class	References
39	Octadecanoic acid	1242, 2.234	$C_{18}H_{36}O_2$	91.2	0.91	fatty acids	Abdenaceur *et al*. (2024)
40	Naphthalene, 1,7-dimethyl-	1247, 3.670	$C_{12}H_{12}$	89.9	0.36	hydrocarbons	–
41	Naphthalene, 1,8-dimethyl-	1265, 3.800	$C_{12}H_{12}$	88.9	0.56	hydrocarbons	–
42	Naphthalene, 1-ethyl-	1283, 3.870	$C_{12}H_{12}$	87.6	0.13	hydrocarbons	–
43	á-Farnesene	1295, 2.460	$C_{15}H_{24}$	81.8	1.74	sesquiterpene	Lee *et al*. (2016)
44	á-Myrcene	1313, 2.500	$C_{10}H_{16}$	88.9	0.93	terpenes	Loulier *et al*. (2020)
45	1-Dodecanol	1319, 2.500	$C_{12}H_{26}O$	87.0	1.55	alcohols	Da Rocha *et al*. (2020) Shavkiev *et al*. (2022) Oviya *et al*. (2022)
46	Hexadecanol	1320, 3.800	$C_{16}H_{34}O$	81.4	0.51	alcohols	Jayakumar *et al*. (2021)
47	β-farnesene	1325, 2.570	$C_{15}H_{24}$	86.8	0.06	sesquiterpene	Yassin *et al*. (2022)
48	Isoledene	1325, 2.710	$C_{15}H_{24}$	87.8	1.02	sesquiterpenoid	Inayati *et al*. (2019)
49	1,3-Cyclohexadiene, 5-(1,5-dimethyl--4-hexenyl)-2-methyl-, [S-(R*,S*)]-(alpha-Zingiberene)	1342, 2.600	$C_{15}H_{24}$	93.2	2.74	sesquiterpene	Shareef *et al*. (2016) Wiraswati *et al*. (2023)
50	Diphenylmethane	1343, 3.830	$C_{13}H_{12}$	85.5	0.05	hydrocarbons	Lu *et al*. (2021)
51	Germacrene D	1349, 2.740	$C_{15}H_{24}$	81.6	0.33	sesquiterpenoid	Philip *et al*. (2024) Van Zijll de jong *et al*. (2023)
52	á-Bisabolene	1355, 2.580	$C_{15}H_{24}$	85.2	2.55	sesquiterpene	Bai *et al*. (2023) Loulier *et al*. (2020)
53	Cyclohexane, 1-ethenyl-1-methyl--2,4-bis(1-methylethenyl)-	1355, 2.850	$C_{15}H_{24}$	88.6	0.33	hydrocarbons	Wang *et al*. (2011)
54	Naphthalene, 2-(1-methylethyl)-	1354, 3.590	$C_{13}H_{14}$	82.8	0.04	hydrocarbons	Al-Otibi *et al*. (2023)
55	1-Isopropenylnaphthalene	1360, 4.060	$C_{13}H_{12}$	92.1	0.07	hydrocarbons	–
56	Decane, 2,3,5,8-tetramethyl-	1378, 1.970	$C_{14}H_{30}$	87.4	0.50	alkanes	Habib *et al*. (2022) Dini *et al*. (2021)
57	Fluorene	1438, 4.330	$C_{13}H_{10}$	90.8	1.26	hydrocarbons	Chen *et al*. (2016)
58	1,1′-Biphenyl, 4-methyl-	1462, 4.280	$C_{13}H_{12}$	83.1	0.25	biphenyls	–
59	Dodecane, 1-iodo-	1582, 2.010	$C_{12}H_{25}I$	81.2	0.36	alkanes	Wiraswati *et al*. (2023)
60	Phenanthrene	1624, 4.970	$C_{14}H_{10}$	89.6	0.35	hydrocarbons	Tabarestani *et al*. (2016)
61	Dodecane, 2-methyl-	1636, 2.070	$C_{13}H_{28}$	88.5	0.07	alkanes	–
62	1-Tetradecanamine, N,N-dimethyl-	1732, 2.210	$C_{16}H_{35}N$	87.5	0.53	amines	El-Sayed *et al*. (2022)
63	1,2-Benzenedicarboxylic acid, butyl 2-ethylhexyl ester	1768, 3.770	$C_{20}H_{30}O_4$	82.6	0.55	esters	Abboud *et al*. (2023)
64	n-Hexadecanoic acid	1774, 2.600	$C_{16}H_{32}O_2$	81.4	0.33	fatty acids	Philip *et al*. (2024)
65	Tetradecane, 1-iodo-	1888, 2.120	$C_{14}H_{29}I$	82.4	0.39	alkanes	–
66	1,3-Dioxolane, 2-heptyl-	1906, 1.780	$C_{10}H_{20}O_2$	85.4	0.57	aldehydes	–
67	Sulfurous acid, 2-ethylhexyl hexyl ester	1966, 2.300	$C_{14}H_{30}O_3S$	85.3	0.70	esters	Alsarraf *et al*. (2024)

1355 seconds (2.55% peak area), 2H-Pyran-2-one, 6-pentyl at 883 seconds (2.34% peak area), and α-Phellandrene at 755 seconds (2.22% peak area). The structures of these major antifungal compounds, including the dominant PEA, are shown in Figure 8.

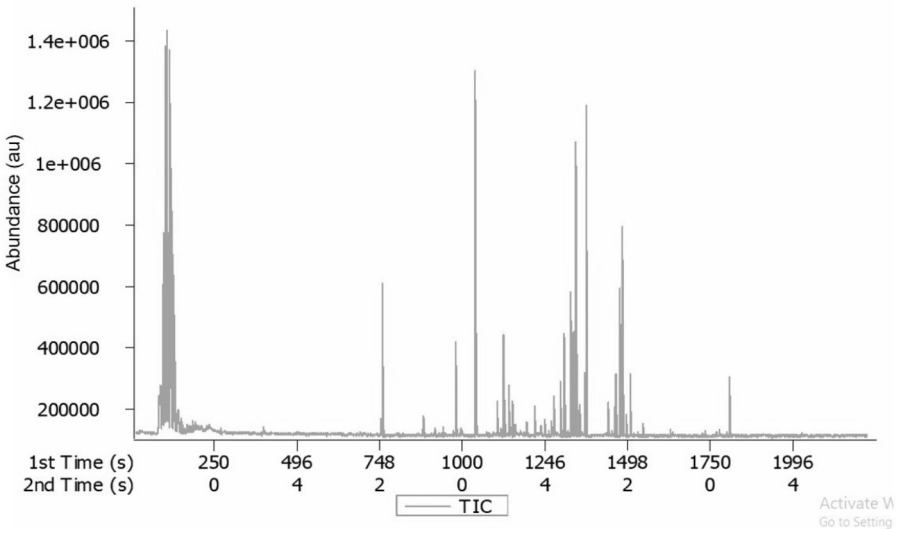

Fig. 7. Two-dimensional gas chromatographic spectrum of VSMs emitted by TaSrGa

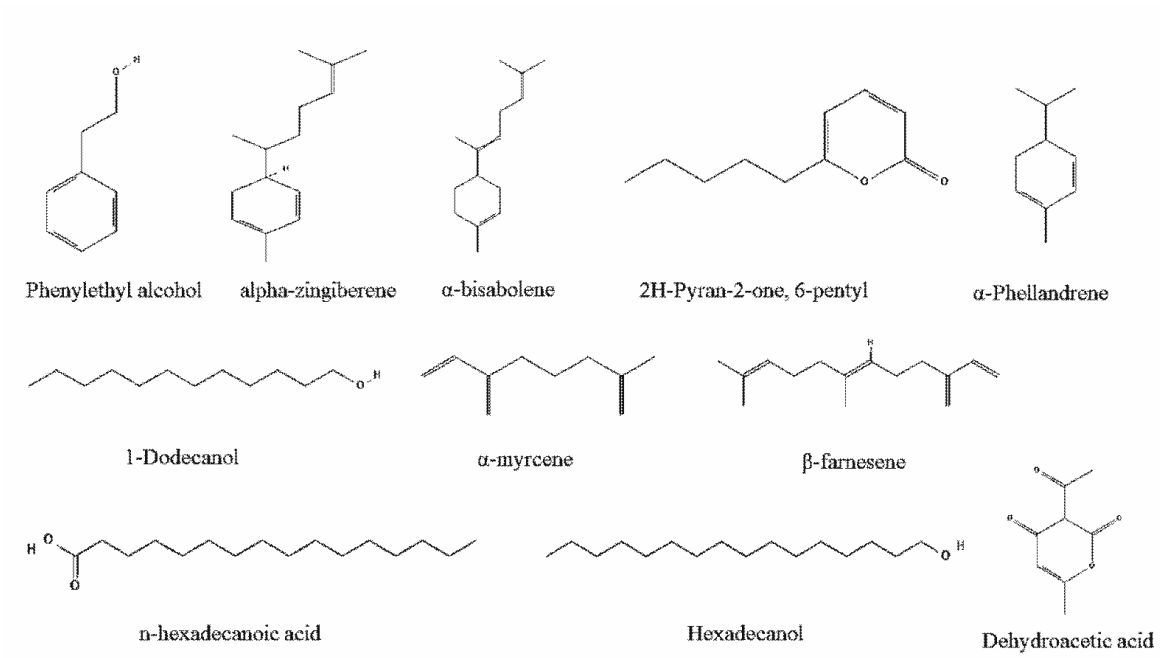

Fig. 8. Chemical structures of major bioactive TaSrGa VSMs

Discussion

Chemical fungicides are an effective method for managing the pathogen *F. oxysporum* f. sp. *ciceri*. However, their use may not always be ideal for controlling soil-borne fungal pathogens. Due to the environmental and health risks associated with chemical treatments, dependence on fungicides for managing soil pathogens is decreasing (Kumari *et al.* 2024). In contrast, biocontrol agents provide a safer and more sustainable alternative to harmful chemicals (Sehim *et al.* 2023). Among these agents, *Trichoderma* species are considered some of the most effective biological control agents, as they reduce pathogen populations through mechanisms such as competition, mycoparasitism, antibiosis, and the activation of systemic resistance (You *et al.* 2023). Specifically, *T. asperellum* has proven to be one of the most potent strains for suppressing *F. oxysporum* f. sp. *ciceri* when used as a biological inoculant.

This research began by evaluating the antagonistic efficacy of *T. asperellum* strain culture filtrates, their VSMs, and nVSMs against *F. oxysporum* f. sp. *ciceri*, the causative agent of chickpea wilt. The in vitro antagonistic results and metabolic characterization provide a foundation for further research aimed at commercializing *T. asperellum* strains for combating agricultural pathogens.

The culture filtrate of *T. asperellum* strains was assessed for its antagonistic efficacy. The culture filtrate of TaSrYa exhibited stronger inhibition against *F. oxysporum* f. sp. *ciceri* compared to the control and other strains. These results suggest that the TaSrYa strains produces more potent fungicidal bioactive metabolites than other strains. These findings are consistent with those of Meena *et al*. (2017), who reported the antifungal efficacy of *Trichoderma* spp. culture filtrates against *Alternaria alternata*, *Alternaria brassicae*, *Alternaria solani*, and *Fusarium solani*. Yassin *et al*. (2021) demonstrated the successful in vitro management of *Fusarium proliferatum* using culture filtrates of *Trichoderma viride* and *Trichoderma harzianum*. Similarly, culture filtrates of T51 and 51-31 showed inhibitory activity against soil-borne pathogens (You *et al*. 2023). The cell-free culture filtrate of *Trichoderma longibrachiatum* AD-1 suppressed the mycelial growth of *F. solani* (Abdelmoteleb *et al*. 2023). Yogalakshmi *et al*. (2021) demonstrated that *Trichoderma atroviride* culture filtrate exhibited antagonistic activity against *F. oxysporum*, achieving 77.77% mycelial inhibition. Additionally, a 30% cell-free filtrate of *T. asperellum* demonstrated 59.39% inhibition of *F. oxysporum* (Sehim *et al*. 2023).

The remote confrontation assay demonstrated the antimycotic properties of *T. asperellum* strains through the use of an array of VSMs, which resulted in 23.55% to 52.57% mycelial inhibition against *F. oxysporum* f. sp. *ciceri*. The reduction in mycelial growth of *F. oxysporum* f. sp. *ciceri* indicates that VSMs have potential as eco-friendly agents for remote management of this pathogen. Our findings align with those of You *et al*. (2022), who reported the antagonistic behavior of *Trichoderma* isolates, with the *T-51* strain exhibiting strong inhibition (43.68%) of *F. oxysporum* mycelial growth through the production of VOCs in a double-dish experiment. Additionally, mVOCs secreted by *T. longibrachiatum* inhibited the growth of *Sclerotium rolfsii* and *Macrophomina phaseolina* (Sridharan *et al*. 2020). Intana *et al*. (2021) evaluated the fungicidal effects of VSMs released by *T. asperellum* T76-14, which demonstrated 62.50% fungal inhibition of *Fusarium incarnatum*. VOCs produced by *Trichoderma harzianum* negatively affected the growth of the phytopathogenic *Diplodia* spp. (Hlaiem *et al*. 2023). The growth inhibition of phytopathogens by *Trichoderma* volatiles has been confirmed by several researchers (Phoka *et al*. 2020; Ruangwong *et al*. 2021; Rao *et al*. 2022; Shanmugaraj *et al*. 2023; Liu *et al*. 2024; Patil *et al*. 2024).

In this study, the TaSrYa strain was found to produce diffusible metabolites that significantly inhibited the growth of *F. oxysporum* f. sp. *ciceri*. Benomyl was incorporated into the agar to inhibit *Trichoderma* mycelial growth while allowing the production of diffusible metabolites. Benomyl is a commercially available fungicide that works by disrupting spindle microtubule formation, ultimately halting mitosis and inhibiting fungal growth. This allowed *F. oxysporum* f. sp. *ciceri* to grow at the minimum concentration of Benomyl while preventing *Trichoderma* from spreading into the second layer of agar. Although Benomyl reduced pathogen growth on control plates, the incorporation of *T. asperellum* strains further suppressed the mycelial growth, demonstrating the secretion of diffusible metabolites. The bilayer plate technique proved effective in evaluating diffusible antifungal compounds secreted by biocontrol agents, as it inhibits pathogen growth (Etheridge and Craig 1973). The findings of this study align with those of Angel *et al*. (2016), who used the bilayer technique to assess the diffusible metabolites of *Trichoderma virens*, confirming their role in suppressing *Ganoderma boninense* PER. Sundram (2005) also reported that diffusible metabolites from biocontrol agents effectively arrested the mycelial growth of *G. boninense*. Additionally, *T. harzianum* released compounds that diffused through a cellophane membrane into the agar medium, inhibiting the growth of *G. boninense* with PIRG values ranging from 18.35% to 40.16% (Siddiquee *et al*. 2009).

With increasing restrictions on chemical fungicides due to their unintended impacts on human health, there is a rising demand for eco-friendly alternatives. One promising option is the use of *Trichoderma* species in biological control strategies. In this study, antagonistic screening of various solvent-extracted nVSMs from *T. asperellum* strains at different concentrations revealed that TaSrYa was particularly effective, with significant differences in mycelial inhibition of *F. oxysporum* f. sp. *ciceri* among the *T. asperellum* strains. Among all the extracts, EaE showed the best performance, suggesting that ethyl acetate efficiently extracts active metabolites from the culture filtrate. These findings are consistent with those of Sumida *et al*. (2018), who evaluated ethyl acetate extracts of two *T. asperelloides* strains against *Sclerotium sclerotiorum*, achieving 60–100% inhibition of mycelial growth. Additionally, Eke *et al*. (2023) demonstrated the lethal effect of ethyl acetate-based extracts from *T. atroviride* on *F. oxysporum*. Saravanakumar *et al*. (2016) also reported in vitro antagonism of partially purified *Trichoderma* bioactive extracts against FOC at various concentrations. Furthermore, the remarkable fungicidal efficacy of *Trichoderma* extracts has been found against cocoa

pathogens (Choez-Guaranda *et al.* 2023). Organic crude extracts of *T. asperellum* exhibited significant concentration-dependent antifungal activity against *Phytophthora megakarya*, with complete inhibition observed at a concentration of 100 μg · ml^{-1} (Youassi *et al.* 2024). *T. longibrachiatum*-derived crude metabolites also demonstrated antagonistic effects against *F. oxysporum* (Al-Askar *et al.* 2022). Methanol-extracted fermentation broth metabolites from *T. asperellum* TM11 showed inhibitory effects against *F. oxysporum* (Li *et al.* 2023). SMs from *T. asperellum* posed anti-*F. oxysporum* f. sp. *ciceri* activity (Birari *et al.* 2024).

The identification of nVSMs in the ethyl acetate extract of the TaSrYa strain was carried out using GCHRMS analysis. This technique was employed to detect bioactive chemical constituents present in the culture filtrate-derived SMs of TaSrYa. The analysis revealed several compounds, with specific constituents making up significant proportions. The predominant compound was 9-octadecenoic acid, which accounted for 26.32% of the total area. Following this, dehydroacetic acid and 9-undecenal, 2,10-dimethyl were identified, contributing 25.71% and 11.31%, respectively. Notably, 7,10-octadecadienoic acid, 9,12,15-octadecatrienoic acid 2,3-dihydroxypropyl ester (Z,Z,Z), and cis-Z-α-bisabolene epoxide were also identified as substantial components, contributing 8.62%, 6.18%, and 4.96%, respectively. Additionally, nerolidol, 1-heptatriacotanol, octadecane, eicosane, 1-iodo, 17-pentatriacontene, 1-hexadecene, 9,12-octadecadienoic acid (Z,Z) methyl ester, 1-chloroheptacosane, and 9-hexadecenoic acid were detected in varying proportions, all contributing to the overall chemical composition of the ethyl acetate extract of the TaSrYa strain. The high percentage of 9-octadecenoic acid is particularly noteworthy due to its reported biological activities, including antifungal properties (Moussa *et al.* 2023). Dehydroacetic acid, a pyrone compound, has antimicrobial activity (Kumari *et al.* 2024). Fatty acids and their derivatives play vital roles in plant defense against bacterial and fungal pathogens (Lee and Park 2019). Javaid *et al.* (2023) identified the fatty acid derivative 9,12,15-octadecatrienoic acid 2,3-dihydroxypropyl ester (Z,Z,Z), which exhibited antifungal activity against *S. rolfsii*. Furthermore, the results demonstrate the ability of the TaSrYa strain to produce antifungal alkanes, such as 1-iodo-eicosane and octadecane (Abdenaceur *et al.* 2024; Liu *et al.* 2024). Nerolidol, described in *T. reesei*, has been primarily proven to have anti-inflammatory, antinociceptive, and antioxidant properties (Choez-Guaranda *et al.* 2023).

The remote confrontation assay confirmed the crucial role of VSMs in reducing the mycelial growth of *F. oxysporum* f. sp. *ciceri*. A total of 67 VSMs were tentatively identified through HS-SPME-GC-GC-TOFMS analysis of the TaSrGa strain. These findings are consistent with those of Van Zijll de Jong *et al.* (2023), who recorded a wide array of volatile organic compounds from *Trichoderma* strains, which were correlated with various bioactivities. Seven classes of VOCs, along with their corresponding chemical structures, were detected in both *T. asperellum* and *T. atroviride* (Stracquadanio *et al.* 2020). Among these, several alcohols with antimicrobial properties were identified, including phenylethyl alcohol, which has been shown to interfere with RNA, DNA, and protein synthesis in *Neurospora crassa* (Lester 1965) and is produced by *Trichoderma* species (You *et al.* 2022; Chan *et al.* 2023; Da Silva *et al.* 2023; Choez-Guaranda *et al.* 2023). Other alcohols, such as 2-nonanol, were found to damage fungal cell membranes by decreasing ergosterol content in *Penicillium expansum* (Song *et al.* 2024). 1-Dodecanol, an antifungal compound found in *Trichoderma hamatum*, also exhibited similar properties (Oviya *et al.* 2022; Shavkiev *et al.* 2022). Hexadecanol, previously reported in *Pseudomonas polymyxa* volatiles, showed 60% antagonistic potential against *F. oxysporum* f. sp. *niveum* (Raza *et al.* 2015). Furthermore, 1-octanol vapors and liquid contact were found to dose-dependently inhibit *Aspergillus flavus* spore germination and mycelial growth at low concentrations (Qin *et al.* 2022).

2H-pyran-2-one, 6-pentyl- (6-PP), has been shown to reduce mycotoxin production, including deoxynivalenol in *Fusarium graminearum* (Cooney *et al.* 2001) and fusaric acid in *Fusarium moniliforme* (El-Hasan *et al.* 2007), and to exhibit antifungal activity by suppressing mycelial growth of diverse phytopathogens (Stracquadanio *et al.* 2020; Philip *et al.* 2024).

Acetic acid was the most active compound in reducing the growth of *S. sclerotiorum* (Giorgio *et al.* 2015). The anti-Candida effect of α-phellandrene has also been documented (Bhattacharya *et al.* 2024). Moreover, the results indicated that the TaSrGa strain produced various antifungal alkanes, including hexadecane, tridecane, and undecane (Oviya *et al.* 2022; Wang *et al.* 2022; Da Silva *et al.* 2023; Van Zijll de Jong *et al.* 2023). Additionally, α-myrcene, an alkene, was found to possess anti-*Fusarium* properties (Albayrak *et al.* 2023). Other notable VSMs identified include α-bisabolene, β-farnesene, and 1,3-cyclohexadiene, 5-(1,5-dimethyl-4-hexenyl)-2-methyl-, [S-(R*,S*)]- (alpha-zingiberene), which are sesquiterpenes known for their potent fungal growth inhibition properties (He *et al.* 2019; Yassin *et al.* 2022; Radice *et al.* 2022). The antifungal activity of n-hexadecanoic acid against *F. oxysporum* has also been established (Oviya *et al.* 2022; Kumari *et al.* 2024). Furthermore, Kumari *et al.* (2024) reported the antimicrobial activity of dehydroacetic acid.

Additionally, antifungal and antibacterial activities against various pathogens have been reported for butanedioic acid (Sharma *et al*. 2016) and octadecanoic acid (Banaras *et al*. 2017).

Due to the presence of numerous bioactive constituents, the antifungal activities of *Trichoderma* strains cannot be attributed to a single bioactive component but rather to the synergistic effects of several bioactive compounds.

Our findings demonstrated that *T. asperellum* strains release both nVSMs and VSMs, exhibiting significant antifungal activity that effectively suppresses the growth of soil-borne phytopathogens. These bioactive compounds disrupt pathogen development through multiple mechanisms, thereby reducing the reliance on chemical fungicides. The results highlight the potential of *T. asperellum* as a biofumigant, offering an ecologically sustainable and environmentally benign alternative for the management of soil-borne diseases. Compared to synthetic fungicides, the use of *T. asperellum*-derived metabolites minimizes risks of chemical residues, mitigates the emergence of fungicide resistance, and contributes to safer agricultural practices. This study therefore reinforces the applicability of *Trichoderma*-based biocontrol strategies within integrated disease management frameworks for sustainable crop protection.

Conclusions

The culture filtrate of *T. asperellum*, together with its nVSMs and VSMs, exhibited strong inhibitory effects against *F. oxysporum* f. sp. *ciceri*, the causal agent of chickpea wilt. This antifungal activity is likely attributable to the presence of bioactive compounds in the extracts, as well as the secretion of volatiles that interfere with the growth and development of the pathogen. These findings provide valuable insights into the dual defensive mechanisms of *T. asperellum*, emphasizing its potential as a biocontrol agent in sustainable chickpea disease management. Furthermore, the study lays a foundation for future investigations aimed at identifying specific metabolites, elucidating their modes of action, and assessing their efficacy under greenhouse and field conditions. By highlighting the contribution of both diffusible and volatile metabolites in pathogen suppression, this work underscores the importance of metabolite-based mechanisms in the development of *Trichoderma*-derived biofungicides. Collectively, the results position *T. asperellum* as a promising candidate for large-scale production and formulation of biofungicides capable of effectively mitigating chickpea wilt while reducing dependence on synthetic fungicides.

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