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RESEARCH ARTICLE

Unveiling phytostimulation abilities and antifungal metabolites of *Trichoderma* spp. from avocado rhizosphere soil

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Abstract

Trichoderma spp. is a beneficial fungus with agricultural significance, known for its role in plant health enhancement and disease control. This study explores the characterization and phytostimulation abilities of *Trichoderma* species isolated from avocado plant rhizosphere soil. Ten *Trichoderma* isolates were obtained through isolation and purification. Their cultural characteristics, such as rapid colony growth and green conidial zones, were assessed, confirming their identity as *Trichoderma* spp. Biochemical tests revealed their abilities in indole acetic acid (IAA) production (10.2-27.4 µg/mL), phosphate solubilization (17.3-35.8 µg/mL), siderophore production and ammonia production Genomic deoxyribonucleic acid (DNA) extraction and polymerase chain reaction (PCR) amplification using universal primers (ITS1 and ITS4) confirmed their molecular identity, with amplicon sizes ranging from 550 bp to 650 bp. Metabolite analysis of TI-3 via GC-MS uncovered bioactive compounds, including Palmitic acid, 6-pentyl-2H-pyran-2-one, quinoline, phenol, 2-(6-hydrazino-3-pyridazinyl) and heptadecane. Thin Layer Chromatography (TLC) identified distinct antifungal compounds, with an Rf value of 0.84 for chitinase analysis. These findings highlight the multifaceted potential of *Trichoderma* spp. in promoting plant health and management of diseases, offering valuable insights for sustainable agricultural practices in avocado cultivation.

Keywords

antifungal compounds; biochemical characterization; GC-MS analysis; rhizosphere isolation; *Trichoderma* sp.; TLC

Introduction

Trichoderma spp. is widely recognized as a non-pathogenic and beneficial partner for plants, capable of colonizing roots and enhancing plant health. It is a versatile fungal genus with agricultural relevance as a biological agent against plant pathogens. *Trichoderma* employs direct antagonism and competition, primarily in the rhizosphere, influencing microbial interactions (1). Key *Trichoderma* species, including *T. atroviride*, *T. harzianum*, *T. asperellum*, *T. virens*, *T. longibrachiatum* and *T. viride*, are known for their biocontrol capabilities, often used in eco-friendly formulations to combat plant diseases, reducing the reliance on chemical pesticides and fertilizers (2, 3). *Trichoderma* stands out in biological control due to its diverse traits, including parasitism, antibiosis, secondary metabolite production and plant defense system activation. While some *Trichoderma* species are mycoparasites, others

effectively target nematodes and plant pests, making them versatile biological control agents (4). The rhizosphere soil of avocado plants is a dynamic environment rich in microbial diversity, playing a critical role in plant growth and health. It harbors beneficial microorganisms, such as *Trichoderma* spp., which enhance nutrient availability, promote phytostimulation and provide biocontrol against soil-borne pathogens. *Trichoderma* finds applications in agriculture, either alone or in combination with plant growth-promoting bacteria (PGPB), communicating with plant roots through compounds and regulating stress hormones. These beneficial *Trichoderma* species also produce phytohormones, ammonia, solubilize phosphate and generate siderophores, promoting plant growth. They employ various mechanisms to protect plants, including parasitism, antibiotic production, secondary metabolites and induced systemic resistance (ISR). *Trichoderma* stimulates both plant growth and defense responses by eliciting pathways dependent on salicylic acid (SA), ethylene (ET) and jasmonic acid (JA) against potential plant pathogens like nematodes and fungi (5). Among the volatile organic compounds (VOCs) emitted by *Trichoderma*, 6 -pentyl-2H-pyran-2-one (6-pentyl-α-pyrone, 6-PP) from T. atroviride is notable for enhancing plant growth and regulating sugar transport in Arabidopsis roots, along with other VOCs (6). This study explores the morphological and molecular characterization of Trichoderma species isolated from the rhizosphere soil of avocado plants. It further evaluates their plant growth-promoting abilities through biochemical tests and identifies key bioactive metabolites using Thin Layer Chromatography (TLC) and Gas Chromatography-Mass Spectrometry (GC-MS) analysis.

Materials and Methods

Isolation of Trichoderma sp.

Soil samples were collected from the healthy avocado rhizosphere at ten distinct locations, ensuring geographic diversity, with three replicates collected from each site to enhance sampling reliability. Ten grams of rhizosphere soil from each sample were mixed with 100 milliliters of sterile distilled water in a conical flask. Trichoderma isolation involved serial dilution, starting with 1-milliliter portions of soil diluted to 10^3 and 10^4 levels.These dilutions were then transferred to Petri plates containing *Trichoderma* selective medium (TSM), following the protocol of Elad and Chet, (7). The plates were gently rotated for even distribution and incubated at 28 ± 2°C for seven days. After incubation, isolated Trichoderma colonies were purified using the single hyphal tip method and subcultured on Potato Dextrose Agar (PDA) medium. Cultures were transferred to PDA slants and stored at 4°C . In total, 15 *Trichoderma* sp. isolates were obtained through this process.

Cultural characteristics of Trichoderma spp. isolates

The ten *Trichoderma* isolates were assessed for cultural characteristics on the 7th day post-inoculation on the Potato Dextrose Agar (PDA) medium. Colony appearances observed notable diversity, including various colours ranging from pale green to dark green, yellowish hues and subdued white shades. Additionally, some colonies exhibited ring-like growth patterns, while others displayed elevated mycelial growth. Confirmation of these isolates as *Trichoderma* spp. was based on examining their conidia and phialidic characteristics (8).

Biochemical characterization of Trichoderma spp.

In the present study, all Ten *Trichoderma* spp. *viz.*, TI-1, TI-2, TI-3, TI-4, TI-5, TI-6, TI-7, TI-8, TI-9 and TI-10 were assessed for the biochemical characteristics such as siderophore production, IAA production, phosphate solubilization and ammonia production.

a. Siderophore production in *Trichoderma* sp. was detected using Chrom Azurol S (CAS) blue agar medium. An 8 mm plug of *Trichoderma* sp. was placed on a Petri dish containing CAS medium and incubated at 28°C for five days. A color change from yellow to an orange zone surrounding the colony indicated siderophore production (9, 10).

b. For the detection and quantification of Indole-3-acetic acid (IAA), Salkowski reagent was utilized, as per the method described by Hartmann *et al*. (11). Each of the six isolates of *Trichoderma* spp. was individually inoculated into a 20 mL PDA broth-containing conical flask. Additionally, 0.1 % tryptophan, a precursor for IAA, was added to each flask as a treatment, while a control was maintained without the addition of tryptophan, following the protocol outlined by Gordon and Paleg (12). The broths were then incubated at 28±2°C for five days. After incubation, the broths were centrifuged at 10000 rpm for 10 min and 1 mL of supernatant was mixed with 2 mL of Salkowski's reagent (comprising 1 mL of 0.5 M FeC_{l2} in 50 mL of 35 % perchloric acid) in the dark for 30 min. The development of a pink color indicated the production of IAA.

c. Phosphate solubilization was assessed using Pikovskayc's broth supplemented with 5 g/L of tricalcium phosphate, following a method similar to that described by King (13). The broth was inoculated with a 7-day-old culture of *Trichoderma* sp. and incubated at 28±2°C for four days. Trichoderma filtrate (50 µL) was mixed with 5 µL of ammonium molybdate and shaken after the addition of 13 µL of chlorostannous acid and 2.5 mL of distilled water. Different concentrations of K_2HPO_4 (0.2 ppm, 0.4 ppm, 0.6 ppm, 0.8 ppm and 1 ppm) were added to create standard solutions. The development of a blue color indicated phosphate solubilization and the intensity of the blue color was measured at 600 nm using a spectrophotometer.

d. Ammonia production was assessed by inoculating *Trichoderma* spp. culture into peptone water-amended boiling tubes. After 72 hours of incubation, the addition of Nessler's reagent resulted in a color change from yellow to brown, indicating a positive result for ammonia production (14).

Molecular confirmation of Trichoderma sp.

Genomic DNA was extracted from all the ten *Trichoderma* isolates used in phytostimulation studies. First, these isolates were inoculated into PDA broth and incubated for seven days. The mycelial mat was harvested and dried on sterilized blotter

paper. DNA extraction followed the CTAB method (15). Specifically, 100 mg of Trichoderma mycelia was ground with liquid nitrogen to obtain DNA powder. This powder added 5 mm of extraction buffer containing 700 mM NaCl, 50 mM Tris-HCl (pH 8.0), 10 mM EDTA, 2 % CTAB and 1 % mercapto-ethanol. After one hour of incubation at 60°C, the sample was extracted twice with chloroform-isoamyl alcohol (24:1). The resulting emulsions were centrifuged to separate the aqueous phase and DNA was precipitated by adding ice-cold isopropanol. The DNA pellet was obtained by centrifugation, air -dried and then dissolved in TE buffer, followed by treatment with RNAase A and storage at -20°C for further analysis.

PCR amplification

PCR amplification of *Trichoderma* sp. DNA was used in a 10 µL reaction mixture containing a master mix, forward and reverse primers (ITS 1 and 4), DNA and sterile distilled water. The PCR program consisted of initial denaturation, followed by 35 cycles of denaturation, annealing and extension, with a final extension step. Gel electrophoresis on a 1.2 % agarose gel was used to visualize and document the PCR results under UV light (16).

Phylogenetic analysis

The PCR results were sequenced at Eurofins Genomics India Pvt Ltd in Bangalore, India and underwent phylogenetic analysis. The sequences were assembled into contigs using DNA Baser V.4 software and aligned with ClustalW in Bio-Edit software. The resulting sequences were then submitted to NCBI GenBank. Homologous sequences were identified through BLAST analysis on the NCBI website. Subsequently, a phylogenetic tree was constructed using the neighbour-joining approach with 1000 replications for each bootstrap value, utilizing MEGA 11.0 software, incorporating both the research sequences and reference strains from GenBank (17). Evolutionary distances were computed in base substitutions per site using the Maximum Composite Likelihood technique. The investigation involved twelve nucleotide sequences, covering first, second, third and noncoding codon positions. Ambiguous positions were removed for each sequence pair using pairwise deletion. The final dataset comprised a total of 1693 positions. Evolutionary analyses were conducted using MEGA11.

Extraction of metabolites from Trichoderma sp.

Metabolites were extracted from promising *Trichoderma* sp. (TI-3) by cultivating them in 100 mL of PDA broth for 15 days at 28 ± 2°C. After incubation, the supernatant was collected by centrifugation at 8000 rpm for 30 min. Ethyl acetate was added to the supernatant and the mixture was shaken for 2 hours at 200 rpm. This extraction process was repeated twice using ethyl acetate. The resulting concentrated crude metabolites, containing extracellular antifungal compounds, were obtained using a rotary flask evaporator at 80 rpm and 60°C. These concentrated metabolites were then dissolved in 1 mL of ethyl acetate for further GC-MS analysis.

Determining antifungal compounds by TLC

Thin Layer Chromatography (TLC) was employed to identify antifungal compounds in the *Trichoderma* sp. (TI-3) extract. The procedure involved setting up a TLC tank filled with a mixture of acetone and chloroform in a 3:1 ratio and sealed. A TLC plate (60 F254, Merck, India) was taken and a reference point was marked 0.5 cm above the plate lower corner. Carefully labeled samples were applied with a 1 cm separation. The TLC plate was then placed into the tank for chromatography, removed and exposed to UV fluorescence at 254 nm in a laminar environment. Regions emitting a distinct dark purple fluorescence were marked using a pencil. Rf values, a key parameter for compound identification, were calculated involving the quotient of substance and solvent travel distances (18). This technique facilitated the detection of antifungal compounds.

GC-MS analysis

The Shimadzu GC-MS QP-2020 gas chromatograph system was used to analyze crude ethyl acetate extracts from *Trichoderma* sp. (TI-3) samples. This system featured an auto-sampler gas chromatograph combined with a mass spectrophotometer instrument and utilized a silica capillary column. The GC-MS detection employed an electron ionization system with an energy level of 70 electron volts (eV). The temperature program began at 80 °C for 2 min, then gradually increased at 9 °C per min until reaching 200 °C, where it was maintained for 4 min. Subsequently, the temperature was raised at 10 °C per min until it reached 300 °C, holding this temperature for 5 min. Helium gas was the constant carrier at a flow rate of 1.5 ml/min, with an injection volume of 1 µL. The injector temperature remained at 250 °C and the ion source temperature was at 230 °C. Mass spectra were recorded at an energy of 70 eV, with a scan interval of 0.2 sec, covering the 50-550 atomic mass units (AMU) range. The entire GC process typically took approximately 35 min. The relative percentage of each component was determined by comparing the average peak area for each element to the total areas. This analysis allowed for identifying specific compounds by matching their characteristics with those found in the NIST libraries (19).

Statistical analysis

Statistical analysis was performed to evaluate the differences in means among the various treatments. This analysis was conducted using Analysis of Variance (ANOVA) and further assessed using Duncans' Multiple Range Test at a significance level of 5 % (20).

Results and Discussion

Isolation and phenotypic characterization of Trichoderma sp.

Trichoderma exhibited a distinctive green-colored ring-like zone and a subtle light greenish ring-like growth pattern in the culture on Petri plates (Fig 1, 2a). The distinct cultural traits among different *Trichoderma* species vary. The mycelial pattern of the isolates ranged in color from dull green to dark green (TI-1). Additionally, some isolates exhibited a mycelial pattern of light green (TI-3) to yellowish green (TI-5). Isolates produced cultures ranging from light dark green (TI-7) to dull green (TI-8) characterized by abundantly developing mycelia. Likewise, various Trichoderma species were obtained from the rhizosphere region using the soil dilution plate technique (21). Our findings similar with a previous study which reported an in

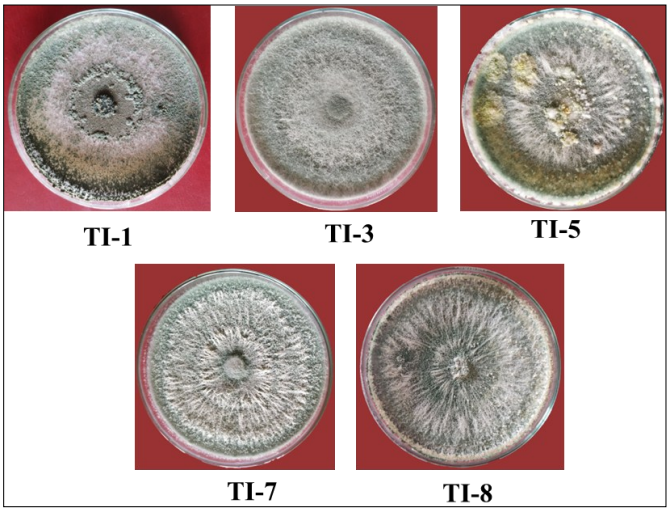

Fig. 1. *Trichoderma* sp. isolated from the Rhizospheric region of avocado.

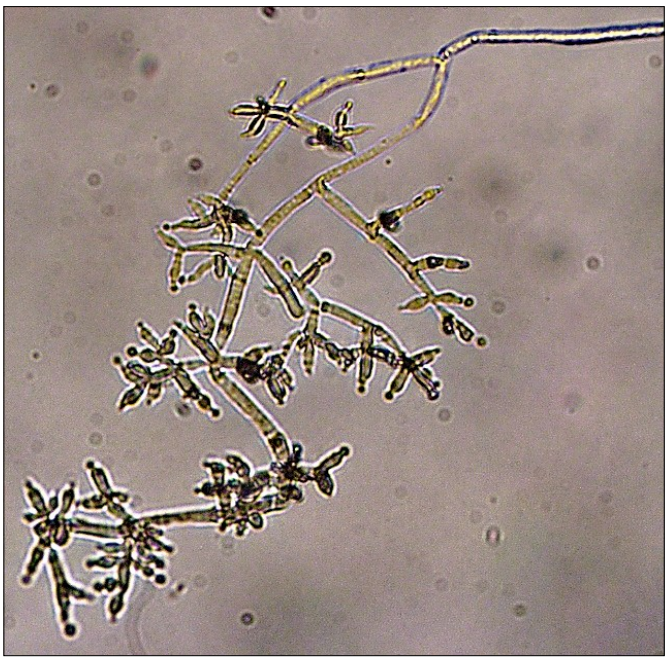

Fig. 2a. Morphological confirmation of *Trichoderma* sp. (TI-3).

-depth description of the cultural traits of *Trichoderma* spp. (22). Isolates, highlighting their color variation spanning from pale green to dark green and the colony growth pattern characterized by concentric rings. Another study observed dark green colonies with dense mycelium in the center and sparse mycelium at the edges (23).

Molecular confirmation of Trichoderma sp.

All ten *Trichoderma* spp. underwent molecular characterization via PCR, utilizing the universal primers ITS1 and ITS4, resulting in amplicon sizes ranging from 550 bp to 650 bp and then promising *Trichoderma* sp. TI-3 sent for sequencing, which sequence showed 100 % similarity with the reference sequences of *T. asperellum* isolate PHR3 from NCBI database (Fig. 2b). Similarly, in a study, PCR amplification of *T. koningii*, *T. longibrachiatum*, *T. citrinoviride*, *T. koningii* and *T. atroviride* isolates using the same universal primers (ITS1 and ITS4) consistently produced a uniform amplified band size of 700 bp (24). Additionally, universal primers ITS-1 and ITS-4 were used to amplify the 28S rRNA gene fragment of *T. longibrachiatum* (25). The outcome revealed a distinct band on the gel, sharp and measuring approximately 654-700 bp.

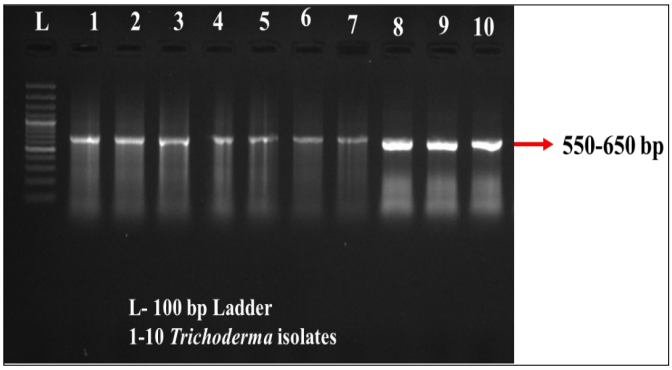

Fig. 2b. PCR amplification of *Trichoderma* spp.

Phylogenetic analysis

In the ITS 1and 4 phylogenetic analysis, the promising isolate Trichoderma asperellum TI-3 [PP469744] from our study showed 100 % sequence similarity with *Trichoderma asperellum* isolate PHR3 [OP831322.1] from Karnataka, India. Trichoderma asperellum formed a distinct cluster separate from other genera, sharing a 54 % relationship with them. *Coniothyrium minitans* strain CBS 861.71 AY642526.1 was used as an outgroup (Fig. 3). Similarly, universal primers (Internal Transcribed Spacer, ITS) were used to amplify the 18S rRNA gene in identified strains *T. koningii* Tk-5201/CSAU and *T. virens* Tvi-4177/CSAU, with sequence deposits in GenBank under accession numbers KC800923 and KC800924, respectively (26). This integrated approach, which combines morphological and molecular markers, demonstrates a viable method for commercialising superior strains of *Trichoderma*. Similar results have shown that *Trichoderma* isolates were categorized based on morphological traits and PCA (Principal Component Analysis) revealed genetic variations of 31.53 %, 61.95 %, 62.22 % and 60.25 %. Additionally, distinctions between isolates were determined through element analysis, RAPD, REP, ERIC and BOX methods (27). Particularly, ERG-1 gene based phylogenetic analysis linked with *Hypocrea lixii*, *T. arundinaceum* and *T. reesei* having biocontrol mechanism of squalene epoxidase-driven triterpene production.

Plant growth promoting activities of Trichoderma sp.

Certain *Trichoderma* isolates, specifically TI-3 and TI-1, displayed robust phosphate solubilization indices ranging from 8.72 to 1.24 (Fig. 4). This aligns with findings of enhanced

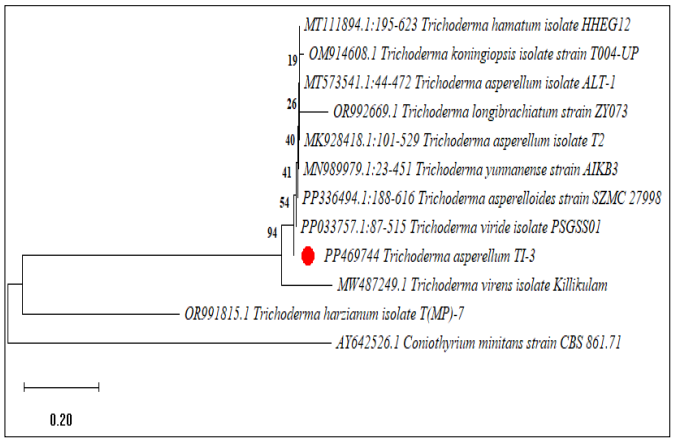

Fig. 3. Phylogenetic tree generated based on sequences of ITS using the neighbour-joining method in Mega 11 software.

The isolates of this study were indicated with a dot mark. Bootstrap values obtained from 1000 replicates are indicated at the nodes. *Coniothyrium minitans* is used as outgroup.

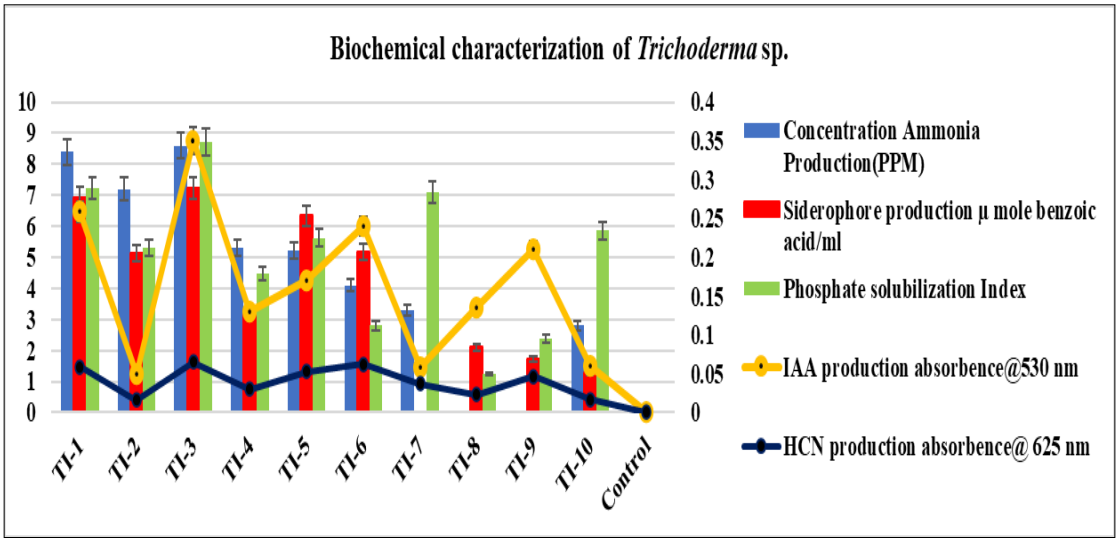

Fig. 4. Biochemical characterization of *Trichoderma* sp.

phosphorus and nutrient content in tomato seedlings with *T. harzianum* (28). In ammonia production tests, all *Trichoderma* spp. isolates exhibited a yellowish to brown color, indicating ammonia production ranging from 8.6 to 0. TI -3 and TI-1 showed particularly dark brown coloration, consistent with positive results reported in ten *Trichoderma* spp., excluding *T. harzianum* (29). All ten isolates demonstrated positive indole acetic acid (IAA) and HCN production, noticeable as a reddish-pink color, with TI-3 (0.354), TI-1 (0.259) and TI-9 (0.21) exhibiting the highest IAA production. Likewise, HCN production was highest in TI-3 (0.065), TI-1 (0.059) and TI-9 (0.047). This study aligns with observations and findings on increased biomass production in *Arabidopsis* seedlings treated with certain *Trichoderma* species (30). Siderophore production was evident in all isolates, marked by a prominent yellow color zone. TI-3 (7.23) and TI-1 (6.94) exhibited notably high siderophore production, consistent with findings on a siderophore-producing *T. asperellum* strain (31, 32). These results support positive outcomes for ammonia, IAA and siderophore production, along with effective inhibition (95.1 %) of *S. rolfsii* mycelial growth (29). High siderophore production by *Trichoderma* spp. plays a crucial role in enhancing plant growth by improving iron availability in the rhizosphere, particularly in iron-limited soils. Siderophores chelate iron, making it more accessible to plants and reducing competition from pathogenic microbes. This activity not only promotes plant health but also strengthens *Trichoderma*'s role as an effective biocontrol agent by limiting pathogen proliferation through nutrient competition.

GC-MS analysis of effective Trichoderma sp.

The GC-MS analysis of *Trichoderma* sp. (TI-3) identified several bioactive compounds, including 6-pentyl-2H-pyran-2-one and 2 -(6-hydrazino-3-pyridazinyl), the latter being potentially novel for *Trichoderma* isolates. 6-pentyl-2H-pyran-2-one, a well-documented antifungal metabolite, disrupts fungal cell walls and shows significant promise for development as a biopesticide. The identification of 2-(6-hydrazino-3-pyridazinyl), which has been rarely reported in *Trichoderma*, opens up new avenues for research into its antimicrobial or antifungal properties. Additionally, other metabolites such as palmitic acid and quinoline further enhance the antifungal profile of *Trichoderma* sp. (TI-3), supporting its potential applications in sustainable agriculture and pathogen management. These findings highlight the diverse metabolic capabilities of Trichoderma and its potential for producing novel bioactive compounds.

The presence of these diverse compounds underscores the potential of *Trichoderma* as a biocontrol agent, capable of suppressing a wide range of fungal pathogens. These bioactive compounds offer applications in sustainable agriculture, providing eco-friendly alternatives to chemical fungicides and supporting more sustainable farming practices (Fig. 5). One intriguing observation is the potential synergistic effect among the bioactive components in *Trichoderma* extracts. It was suggested that the antifungal efficacy of the extract might arise from the combined action of these compounds (33). Synergy among bioactive molecules has been documented in various contexts and in the case of *Trichoderma*, this synergy could contribute to enhanced antifungal activities, making them formidable biocontrol agents against plant pathogens.

Antifungal compounds confirmed through TLC plate

A study involving Thin Layer Chromatography (TLC) was performed on *Trichoderma* spp. (TI-3 & TI-8)The TLC assay identified antifungal compounds in *Trichoderma* sp. (TI-3) extract, with distinct dark purple fluorescence under UV light at 254 nm. Rf values calculated from the mobility of these compounds facilitated their characterization.The TLC results provide crucial insights into the antifungal activity observed in the study. The distinct spot with an Rf value of 0.84 likely corresponds to a bioactive compound, potentially chitinase or another antifungal metabolite, produced by *Trichoderma* spp. The clear separation without smudging indicates the purity and stability of the compound under analysis Chitinase is known to degrade fungal cell walls, which are primarily composed of chitin, thereby inhibiting fungal growth. The visibility of the spot under UV fluorescence suggests that the compound possesses structural characteristics that absorb UV light, further supporting its bioactive nature. These results align with the antifungal activity observed, where the compound might interfere with the structural integrity or enzymatic processes of pathogenic fungi, making *Trichoderma*

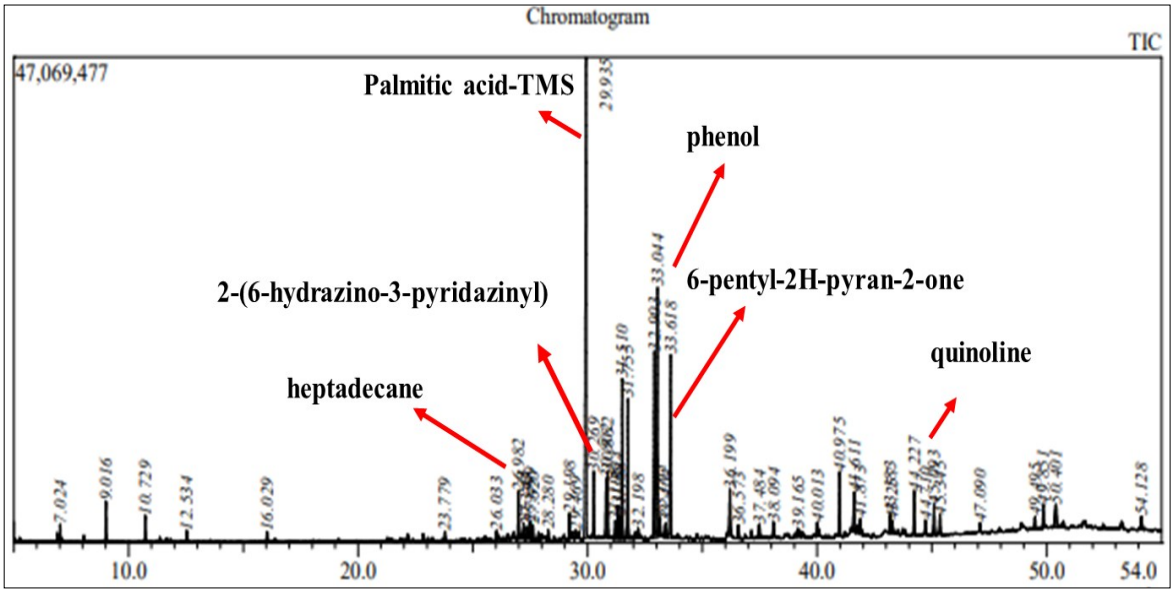

Fig. 5. GC-MS analysis of promising *Trichoderma* sp. (TI-3).

spp. a promising candidate for biocontrol formulations. For chitinase analysis, the use of an acetone and chloroform mixture in a 3:1 proportion resulted in clearly visible spots when exposed to UV light at 254 nm. These spots exhibited a calculated Rf value of 0.84. It is noteworthy that the substance's journey during the chromatographic process was measured at a distance of 5.1 cm (Fig. 6).

Based on the Rf value of 0.84, the compound detected in the TLC assay is likely linked to 6-pentyl-2H-pyran-2-one (6-PAP), a well-known antifungal metabolite produced by *Trichoderma* spp. This metabolite is recognized for its ability to inhibit fungal growth by disrupting cell wall integrity and interfering with essential metabolic pathways. The UV fluorescence and distinct separation further support the identification of this bioactive compound, aligning with its reported characteristics in earlier studies.

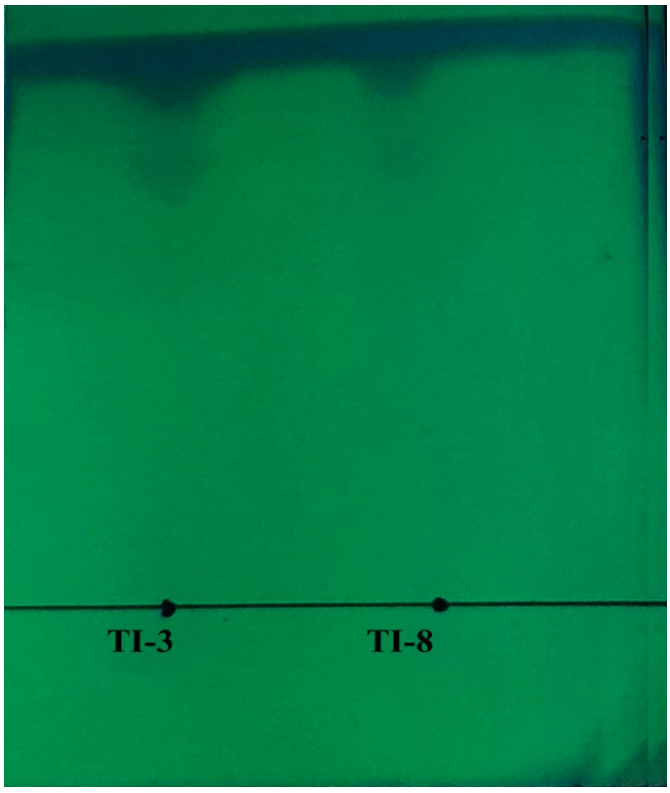

Fig. 6. The effective *Trichoderma* spp. antifungal compounds confirmed through TLC plate.

Thin Layer Chromatography (TLC) emerges as a valuable tool for investigating the antifungal compounds present in *Trichoderma* isolates. The use of TLC to separate and identify these compounds provides insights into their mobility and presence. Notably, the calculated Rf value, a measure of migration distance relative to the solvent front, offers valuable information about the mobility of specific compounds. Comparisons between Rf values obtained in different studies can provide a basis for identifying specific compounds and tracking their presence across different *Trichoderma* species. Interestingly, the Rf values observed in the TLC separations of antifungal compounds in *Trichoderma* isolates appear to be consistent with values reported in the separation of enzymes (34) and other metabolites (35). These findings suggest that similar compounds or metabolites may contribute to these processes across different studies and *Trichoderma* species. The commonality in Rf values underscores the relevance of TLC as a technique for identifying and characterizing metabolites in *Trichoderma* spp.

Conclusion

This study confirms the potential of *Trichoderma* species as effective plant growth promoters and biocontrol agents. Promising isolates, particularly *T. asperellum* TI-3, exhibited robust activities such as phosphate solubilization, ammonia, IAA, HCN and siderophore production. GC-MS analysis revealed bioactive antifungal compounds, while TLC validated their presence with consistent Rf values. These findings highlight *Trichoderma*'s role in enhancing plant health and managing diseases, offering a sustainable solution for improved crop productivity. The results provide a foundation for practical applications in real-world agricultural settings. For instance, the promising isolates can be developed into commercial biocontrol formulations to effectively manage crop diseases. Additionally, the use of these isolates in promoting sustainable farming practices, such as enhancing soil fertility and disease resistance, can significantly benefit crops like avocado. These approaches support eco-friendly agriculture by reducing reliance on

chemical pesticides and fertilizers, ultimately contributing to sustainable and resilient crop production systems.

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Authors' contributions

MA wrote the original draft and conceptualized the study. AS was involved in the revision of draft, inclusion of tables and figures and proof reading. All the authors read and approved the final version of the manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interests to declare.

Ethical issues: None

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