Journal of Phytopathology

WILEY

Journal of Phytopathology

ORIGINAL ARTICLE OPEN ACCESS

In Vitro Antagonistic Activity of *Trichoderma gamsii* Isolated From Hazelnut Twigs Against *Colletotrichum* spp.

Federico Brugneti[1] | Antonella Cardacino[1] | Giuseppina Fanelli[2] | Sara Rinalducci[2] | Angelo Mazzaglia[1] | Silvia Turco[1]

[1]Dipartimento di Scienze Agrarie e Forestali, Università Degli Studi Della Tuscia, Viterbo, Italy | [2]Dipartimento di Scienze Ecologiche e Biologiche, Università Degli Studi Della Tuscia, Viterbo, Italy

Correspondence: Antonella Cardacino (antonella.cardacino@unitus.it) | Angelo Mazzaglia (angmazza@unitus.it)

Received: 7 October 2025 | **Revised:** 7 November 2025 | **Accepted:** 2 December 2025

Keywords: antagonistic activity | anthracnose management | biocontrol | sustainable plant protection | *Trichoderma gamsii*

ABSTRACT

Trichoderma species are widely recognised for their antagonistic activity against phytopathogenic fungi and for their ability to promote plant growth, hence representing promising candidates for sustainable crop protection. In this study, a *Trichoderma gamsii* isolate was recovered from the phyllosphere of hazelnut (*Corylus avellana*) and characterised through morphological, molecular and phylogenetic approaches. Its antagonistic potential was evaluated in vitro against six *Colletotrichum* species using dual culture assays, as well as through exposure to volatile and non-volatile metabolites. To elucidate the chemical basis of its antagonism, volatile organic compounds (VOCs) and non-volatile metabolites (non-VOCs) produced in pure culture were profiled by gas chromatography–mass spectrometry (GC–MS) and liquid chromatography–mass spectrometry (LC–MS/MS), respectively. The isolate exhibited strong inhibitory effects, reducing pathogen growth by up to 79%, depending on the *Colletotrichum* species. These results expand current knowledge of the metabolic repertoire of *T. gamsii* and support its potential as an effective and eco-friendly biocontrol agent for the sustainable management of anthracnose diseases.

1 | Introduction

The increasing incidence of crop pests and diseases, driven by global trade and climate change, has resulted in substantial economic losses, forcing farmers to intensify control measures. However, strict international and national regulations on pesticide use underscore the urgent need for alternative and sustainable management strategies (Olowe et al. 2022; Guzmán-Guzmán et al. 2023). Fungal pathogens, in particular, impose significant global economic burdens, destroying an estimated 20%–40% of annual crop yields, corresponding to losses of $100–200 billion worldwide (Nizamani et al. 2024). These pathogens, therefore, pose serious threats to food security and economic stability.

Among fungal diseases, anthracnose, caused by species of the genus *Colletotrichum*, is one of the most destructive globally, ranking among the top ten fungal genera in economic and scientific importance. With over 340 recognised species infecting more than 3400 host plants (Talhinhas and Baroncelli 2023; Brugneti, Rossini, et al. 2024; Brugneti, Turco, et al. 2024), the genus *Colletotrichum* represents a major challenge for disease management. Its remarkable adaptability, including the ability to infect multiple hosts or harbour multiple species on a single host, further complicates control strategies (Talhinhas and Baroncelli 2023). The economic impact of anthracnose is particularly severe in perennial and fruit crops, as this disease affects bananas, blueberries, citrus, cacao, coffee, walnuts and strawberries, resulting in substantial yield and quality losses (Brugneti, Rossini, et al. 2024; Brugneti, Turco, et al. 2024). Of remarkable importance is the olive anthracnose, which in some producing countries causes annual losses exceeding $100 million due to premature fruit drop and

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reduced oil quality. Caused mainly by *C. gloeosporioides* and *C. acutatum*, the disease induces fruit rot and mummification while degrading oil quality by producing turbid, highly acidic and reddish oils that are unsuitable for commercial standards (Moral et al. 2021). Beyond olives, anthracnose is a major constraint in tropical and subtropical regions, and its relevance is increasing in Europe, affecting not only olives but also hazelnuts, citrus, UNESCO-protected forests and ornamental plants (Giubilei et al. 2023; Brugneti, Rossini, et al. 2024; Brugneti, Turco, et al. 2024). The disease thrives under warm, humid conditions with frequent rainfall and heavy dews, with severity influenced by cultivar susceptibility, pathogen virulence and environmental factors.

Current management strategies rely on cultural practices (e.g., early harvesting, irrigation control) and copper-based fungicides; however, these approaches are often insufficient and raise concerns about sustainability. Consequently, research is increasingly focused on alternative strategies, such as natural extracts and biological control agents, which offer the potential for more effective and environmentally friendly solutions. In Europe, although detailed quantitative data on losses are scarce, high sales of fungicides and bactericides across countries such as France, Spain, Italy and Germany reflect the magnitude of the problem. Among the most promising approaches are natural antagonists, which provide low-impact, eco-friendly tools for plant protection.

Within this context, the fungal genus *Trichoderma* has emerged as one of the most important groups of biological control agents (BCAs), widely applied to suppress plant pathogens, enhance crop productivity and reduce reliance on chemical pesticides and fertilisers (Guzmán-Guzmán et al. 2023). Several species, including *T. harzianum, T. virens, T. atroviride, T. asperellum, T. hamatum, T. gamsii* and *T. viride*, are commonly used in agriculture (Olowe et al. 2022). These fungi exhibit broad-spectrum antagonistic activity against soilborne and foliar pathogens, including *Fusarium* spp., *Rhizoctonia solani*, *Botrytis cinerea* and *Colletotrichum* spp., which affect crops such as cereals, vegetables, fruit trees and vines (Martinez et al. 2023).

The beneficial effects of *Trichoderma* on plants are mediated through multiple mechanisms: direct antagonism via mycoparasitism, competition for nutrients and space, production of antimicrobial secondary metabolites, and induction of systemic resistance in the host (Olowe et al. 2022; Guzmán-Guzmán et al. 2023). In addition to disease suppression, *Trichoderma* spp. stimulate plant growth, improve nutrient uptake and enhance overall crop resilience and productivity (Shetty et al. 2025).

Several strains have been successfully applied under European agricultural conditions. *T. atroviride* SC1 provides long-lasting control of grapevine trunk diseases, reducing pathogen incidence from nursery propagation to mature vines (Berbegal et al. 2020). In solanaceous crops such as tomato, strains of *T. longibrachiatum, T. harzianum* and *T. asperellum* suppress leaf spot diseases while promoting plant growth and yield (Hajji-Hedfi et al. 2025). *T. gamsii*, a species closely related to *T. viride* and *T. atroviride*, has recently gained attention for its biocontrol potential (Zhang et al. 2015). It has been included in commercial formulations and has been successfully tested against pathogens such as *Gaeumannomyces graminis* in cereals and *Rhizoctonia* and *Pythium* species associated with grapevine trunk diseases (Aloi et al. 2017; Bigot et al. 2020; Di Marco et al. 2022). In Italy and other European countries, *Trichoderma* formulations are also employed in olive and grapevine cultivation to reduce fungal infections and improve fruit quality, demonstrating their practical relevance in sustainable agriculture (Guzmán-Guzmán et al. 2023). Recent advances in formulation technologies, as well as the optimization of carriers and drying processes, have enhanced the viability, shelf life and field efficacy of *Trichoderma*-based products (Martinez et al. 2023). Nevertheless, field performance can be influenced by environmental conditions and soil microbial interactions, posing challenges for consistent application. Despite these limitations, the multifaceted benefits of *Trichoderma* support its integration into integrated pest and disease management strategies, aligning with efforts to reduce chemical inputs and promote sustainable crop production across European agricultural systems (Guzmán-Guzmán et al. 2023; Pozo et al. 2024).

In this study, we investigated the antagonistic activity of a *T. gamsii* TGH_24 strain isolated from hazelnut twigs collected in Viterbo province (Italy) against six *Colletotrichum* species. Specifically, we assessed its inhibitory effects through volatile organic compounds (VOCs), non-volatile metabolites (N-VOCs), and dual culture assays. Our findings showed a strong in vitro antagonistic potential of this *T. gamsii* TGH_24 isolate, providing detailed insights into its metabolic repertoire. This study represents a preliminary step in the metabolic characterisation of the strain. Future work will assess its phytocompatibility and further elucidate the molecular mechanisms underlying its biocontrol activity to support the development of safe and effective plant protection strategies.

2 | Materials and Methods

2.1 | *Trichoderma* spp. Strains Isolation

In June 2023, during a monitoring survey carried out in a hazelnut (*Corylus avellana* L.) orchard located in Bassano in Teverina (Viterbo Province, Central Italy; 42°45′54.4″ N, 12°18′34.4″ E), ten symptomatic twig samples were collected for pathogen isolation and identification. Tissue fragments from affected twigs were first surface sterilised in a 2% sodium hypochlorite (NaOCl) solution for 2 min, rinsed three times with sterile distilled water (SDW), and transferred onto fresh Potato Dextrose Agar (PDA; Oxoid, UK) plates. The plates were incubated at 25°C in the dark and monitored daily for fungal growth. After 5 days, one Petri dish was colonised by a rapidly growing green mycelium. A single pure culture was obtained by subculturing small agar plugs from the actively growing margin onto new PDA plates. After 10 days of incubation at 25°C, the colony morphology, particularly the growth rate, texture and pigmentation, was consistent with features typical of the *Trichoderma* genus. Microscopic observations were performed using a light microscope (Leitz, Wetzlar, Germany) at 40× magnification. Phialides and conidia were

examined and photographed, and the length and width of 30 randomly selected conidia were measured. The morphology of the conidiogenous cells was also recorded to support taxonomic identification.

2.2 | Molecular Identification of *Trichoderma* Strains

For molecular characterisation, mycelium from a 5-day-old *Trichoderma* culture grown on Potato Dextrose Agar (PDA) was scraped from the agar surface. Genomic DNA was extracted from 100 mg of fresh mycelium using the NucleoSpin Plant II Midi DNA Extraction Kit (Macherey-Nagel, Germany), following the manufacturer's protocol. The internal transcribed spacer (*ITS*) region of the ribosomal DNA was amplified using primers ITS1 and ITS4 (White et al. 1990). The PCR reactions were performed under the following conditions: initial denaturation at 95°C for 2 min; 35 cycles of denaturation at 95°C for 30 s, annealing at 57°C for 30 s and extension at 72°C for 30 s; followed by a final extension step at 72°C for 5 min. Amplification of the translation elongation factor 1-alpha (*EF-1α*) gene was carried out using primers EF728F and EF1LLErev (Jaklitsch et al. 2005). The cycling programme consisted of an initial denaturation at 95°C for 5 min; 35 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 50 s, and extension at 72°C for 3 min; followed by a final extension at 72°C for 10 min. PCR products were separated on a 1.2% agarose gel and visualised under UV light. Amplicons of the expected size were purified and sent to Eurofins Genomics (Konstanz, Germany) for Sanger sequencing.

2.3 | Phylogenetic Analysis

For phylogenetic analysis, the ITS and EF-1α sequences obtained from the *Trichoderma* isolate were analysed together with 45 reference sequences retrieved from GenBank (Table S1). *Hypocrea rodmanii* (C.P.K. 2852) was included as the outgroup. Sequences were quality-checked and trimmed using UGENE v48.1, aligned with MUSCLE v3.8.31 (Edgar 2004), and concatenated with SeqKit v0.16 (Shen et al. 2016). The concatenated alignment included *Trichoderma* isolates representing diverse species. A maximum likelihood (ML) phylogenetic tree was inferred using RAxML-HPC v8.2.12, applying the GTRCATI substitution model and 1000 bootstrap replicates to assess branch support. The resulting tree was visualised with FigTree v1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/, accessed on 24 April 2025) and graphically refined using Inkscape v0.92 (https://inkscape.org, accessed on 24 April 2025).

2.4 | In Vitro Antagonism Assay Against *Colletotrichum* spp.

Six *Colletotrichum* isolates, *C. acutatum, C. fioriniae, C. gloeosporioides, C. nymphaeae, C. karstii* and *C. kahawae* subsp. *cigarro*, respectively, were obtained from the fungal collection of the Department of Agriculture and Forest Sciences (DAFNE), University of Tuscia (Viterbo, Italy). Each isolate was cultured on Potato Dextrose Agar (PDA) and incubated at 25 C for 10 days to promote active growth. To evaluate the competitive ability of antagonistic fungi toward phytopathogens, the antagonistic activity of *T. gamsii* against the *Colletotrichum* isolates was assessed using the dual culture method, following the procedure described by da Costa et al. (2021). Ten-millimetre diameter mycelial plugs were cut from the margins of actively growing *Colletotrichum* colonies and placed on fresh PDA plates, 4 cm from the centre. After 48 h, a plug of *T. gamsii* was inoculated on the opposite side of each plate. All plates were incubated at 25 C in the dark, and five replicates were used for each pathogen-treatment combination. The degree of fungal inhibition was evaluated once the pathogen had fully colonised the control plate (without *Trichoderma*). Inhibition (%) was calculated according to Gao et al. (2017), using the following formula:

$$\%I = \left[\mathrm{Cd} - \mathrm{Td}\,/\,\mathrm{Cd} - 10\,\mathrm{mm}\right] \times 100$$

where Cd is the radial growth of the pathogen in the control plate, and Td is its growth in dual culture.

Additionally, the interaction between *T. gamsii* and each *Colletotrichum* species was assessed using the 1–5 rating scale proposed by Bell et al. (1982), where 1 = *T. gamsii* completely overgrowing the pathogen, and 5 = the pathogen completely overgrowing *T. gamsii*.

2.5 | In Vitro Assessment of VOC and Non-VOC Compounds Produced by *Trichoderma Gamsii*

The antagonistic activity of volatile organic compounds (VOCs) produced by *T. gamsii* TGH_24 against *Colletotrichum acutatum, C. fioriniae, C. gloeosporioides, C. nymphaeae, C. karstii* and *C. kahawae* subsp. *cigarro* was evaluated using the method described by Qualhato et al. (2013), with minor modifications. Briefly, 90 mm Petri dishes containing PDA medium were inoculated at the centre with a 10 mm mycelial plug of either *T. gamsii* or the target pathogen. The lids were removed, and one plate was inverted over the other to allow gaseous exchange between the two fungal cultures without physical contact. The paired plates were sealed with a double layer of Parafilm and incubated at 25°C in the dark. Five replicates were prepared for each treatment, and the experiment was repeated twice. Colony diameters of *Colletotrichum* isolates were measured once the control plates (without *T. gamsii*) were fully colonised. The percentage of growth inhibition was calculated according to the formula proposed by Gao et al. (2017). The effect of non-volatile compounds (N-VOCs) was assessed following the method of Dennis and Webster (1971). The *T. gamsii* isolate was grown for 48 h on a sterile cellophane membrane placed on the surface of PDA in 90 mm Petri dishes. In control plates, a sterile agar disc replaced the antagonist. After incubation, the cellophane membrane bearing *T. gamsii* mycelium was carefully removed, and a 10 mm plug of each *Colletotrichum* isolate was transferred to the treated medium. Plates were incubated at 25°C in the dark until the control colonies reached the edge of the dish. Colony diameters were recorded, and the percentage inhibition of mycelial growth was calculated relative to the control.

2.6 | VOCs Extraction and GC–MS Analysis

The volatile organic compounds (VOCs) produced by *Trichoderma gamsii* TGH_24 were characterised following the method of Stoppacher et al. (2010), with minor modifications. Briefly, a mycelial plug measuring 10 mm in diameter was obtained from the actively growing margin of a PDA culture and subsequently inoculated into 25 mL of sterile Potato Dextrose Broth (PDB). The tubes were sealed with Parafilm and incubated at 25°C for 10 days, with the tubes being shaken at 150 rpm. After incubation, the headspace of each culture was analysed for volatile metabolites. An 80 μm DVB/CWR/PDMS bipolar fibre, suitable for the extraction of aromatic compounds, was used for solid-phase microextraction (SPME). Each sample vial was equilibrated at 80°C for 5 min before the fibre was exposed to the headspace for 10 min using a PAL RSI 120 autosampler. The fibre was then desorbed in the injector for 10 min. Chromatographic separation and detection were performed on an Agilent 8890 GC system coupled with a 7010B triple quadrupole mass spectrometer (Agilent Technologies, Santa Clara, CA, USA). The GC oven temperature programme was as follows: initial temperature of 50°C (held for 5 min), increased at 5°C min^{-1} to 150°C, and then at 10°C min^{-1} to 250°C. The injector and ion source temperatures were maintained at 250°C and 230°C, respectively. Mass spectra were recorded in a scanning range of 20–400 m/z, and compound identification was achieved by comparison of chromatographic peaks with the NIST spectral library (https://chemdata.nist.gov/).

2.7 | LC–MS/MS Analysis of Non-Volatile Metabolites

N-VOCs metabolites were extracted following a protocol proposed by Rouini et al. (2006). Briefly, two plugs of *T. gamsii* TGH_24, obtained from actively growing margins of PDA cultures, were used to inoculate 750 mL flasks containing 500 mL of sterile PDB. After 10 days at 25°C and 150 rpm, 250 mL of ethyl acetate (EtOAc) were added to the fungal liquid culture. The mixture was then mixed by shaking at 150 rpm for 10 min, after which it was incubated for 1 h without shaking. The top phase was subsequently transferred into a round flask and concentrated using a rotary vacuum evaporator at 36°C. Concentrated metabolites were transferred into 100 mL vials, completely dried in a fume hood and stored at −20°C. Three replicates were made for each sample. Concentrated metabolites were resuspended in water with 5% formic acid, and 20 μL of each sample was analysed in triplicate using LC–MS/MS using a Dionex UltiMate 3000 RSLCnano system (Thermo Fisher Scientific, Waltham, MA, USA) coupled to an Orbitrap Q Exactive mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). Metabolite separation was performed on a Reprosil C18 column (2.0 mm × 150 mm, 2.5 μm; Dr. Maisch, Ammerbuch, Germany) maintained at 30°C with a flow rate of 0.2 mL/min. Chromatographic separation was achieved with a gradient of solvent A (ddH2O with 0.1% formic acid) and solvent B (acetonitrile with 0.1% formic acid), starting at 0% B, increasing gradually to 60% over 15 min, ramping to 90% B in 18 min and returning to 0% B by 19 min with a total runtime of 23 min including re-equilibration. Mass spectrometric analysis was performed in positive ionisation mode using a Full MS/dd-MS^2 (Top 10) method with a total runtime of 23 min. Full MS scans were acquired at a resolution of 70,000 (at m/z 200) across a scan range of 60–900 m/z, with an AGC target of 3e6 and a maximum injection time of 100 ms. Data-dependent MS^2 scans of the top seven precursors were acquired at a resolution of 17,500 with an AGC target of 1e5, a maximum injection time of 50 ms and an isolation window of 4.0 m/z, applying stepped normalised collision energies of 15, 30 and 45. Ion source conditions included a spray voltage of 3.8 kV, sheath gas of 40 and auxiliary gas of 25. Raw data were acquired with Thermo Xcalibur software (Thermo Fisher Scientific, Waltham, MA, USA) and processed for metabolite identification and quantification. Metabolite profiling was carried out using Compound Discoverer 3.3 (Thermo Fisher Scientific, Waltham, MA, USA), which enabled peak detection, retention time alignment, background subtraction and compound grouping across all samples. Annotation of metabolites was performed by matching accurate mass (within 3 ppm), isotopic pattern and MS/MS spectra against built-in databases, including ChemSpider, KEGG, AraCyc and BioCyc. Mass lists of 'Extractables and Leachables HRAM Compound Database' were used as well, mostly to recognise contaminants.

2.8 | Statistical Analysis and Data Visualisation

All statistical analyses were performed in R (version 4.2.3). Differences in mycelial growth inhibition, as well as the effects of volatile (VOCs) and non-volatile (N-VOCs) compounds produced by *Trichoderma gamsii*, were evaluated using a Generalised Linear Model (GLM) to account for experimental replication. Pairwise comparisons among means were conducted using Tukey's Honest Significant Difference (HSD) test at a significance level of $\alpha = 0.05$, implemented with the *emmeans()* function from the *emmeans* package. Data visualisation was carried out using the *ggplot2* package, and results were presented as bar plots showing mean values ± standard deviation with distinct letters indicating statistically significant differences.

2.9 | N-VOCs Annotation and Functional Classification

All analyses were performed within the R environment, including packages from the *tidyverse* ecosystem (Wickham et al. 2019) as well as *webchem* (Szöcs et al. 2020) and *KEGGREST* (Tenenbaum et al. 2025). A total of 472 fungal non-VOC metabolites were included at the beginning of the analysis. Compound names were first standardised by removing special characters and redundant spaces to ensure consistency and facilitate reliable database matching. Each metabolite was then queried against the PubChem database (https://pubchem.ncbi.nlm.nih.gov/, accessed on 24 August 2025) to retrieve the corresponding Compound ID (CID), using tools provided by the *webchem* package. To ensure robustness and reduce redundancy, duplicated names and entries without matching CIDs were excluded from subsequent steps. Metabolites with valid PubChem CIDs were further matched to KEGG

compound identifiers using the *KEGGREST* package. KEGG annotations were used to extract information on associated biochemical pathways and chemical classes. Pathway data were expanded to enable compound-to-pathway associations and facilitate downstream interpretation. Only metabolites with valid annotations in both PubChem and KEGG databases were retained in the final dataset, which served as the basis for functional characterisation and classification of fungal metabolic profiles. Pathway-level representation was achieved by calculating the number and relative percentage of metabolites assigned to each KEGG pathway and associated functional category. Pathways contributing less than 2% of the total were aggregated into an 'Other' group to improve readability. A circular stacked bar plot was generated to visualise the distribution of pathways and their respective categories. To explore relationships among metabolites based on shared pathway annotation, a co-occurrence network was constructed. An incidence matrix was computed to represent the presence of metabolites across pathways, which was then transformed into a weighted adjacency matrix. A network graph was generated using a force-directed layout, where metabolites were represented as nodes and their shared pathways were represented as edges. Node attributes such as category and degree centrality were included to highlight connectivity patterns within and across functional groups. Visual elements such as edge width and node colour were used to encode edge weight and category, respectively.

2.10 | Volatile Organic Compound (VOC) Analysis

A total of 64 volatile organic compounds (VOCs) were identified from GC–MS chromatograms and included in the initial dataset. Relative abundances were calculated as the percentage of the total chromatographic area and expressed as the logarithm (base 10) of these values. Compound names were submitted to the MetaboAnalyst platform (https://www.metaboanalyst.ca, accessed on 27 August 2025) for automated compound name recognition and conversion to KEGG compound identifiers. The resulting KEGG IDs were used to classify the metabolites and to identify and exclude potential contaminants. Only compounds recognised as fungal-derived VOCs, based on information retrieved from scientific literature, were retained for data visualisation.

3 | Results

3.1 | Morphological and Molecular Identification

During a survey conducted to assess the health status of a hazelnut orchard, twig samples were collected for pathogen isolation. The isolation procedure yielded a colony showing typical *Trichoderma*-like morphological features. After purification, a mycelial plug was transferred onto a fresh PDA plate and incubated for 10 days at 25°C. The colony developed a dense, dirty-white mycelium at the centre with a pale yellowish tinge and a light green coloration at the margin. Microscopic examination revealed lageniform phialides measuring 7–18 × 2.5–3.5 μm at the widest point. Conidia were ellipsoidal to subglobose, 3.5–4.5 × 2.0–3.5 μm in size and produced singly at the tips of phialides. Abundant subglobose chlamydospores were observed at the ends of hyphae, measuring 4.4–12 × 6–12.5 μm (Figure 1).

Following genomic DNA extraction, the *ITS* and *EF-1α* regions were successfully amplified and sequenced, showing 100% sequence identity with *Trichoderma gamsii*. The obtained sequences were deposited in the NCBI GenBank database under accession numbers PP786234 (*ITS*) and PP793890 (*EF-1α*). Phylogenetic analysis based on the concatenated *ITS* and *EF-1α* dataset (Figure 2) confirmed the taxonomic placement of the isolate, which grouped within the *T. gamsii* clade together with five reference isolates from different hosts and geographic origins. The *T. gamsii* TGH_24 isolate showed the closest phylogenetic affinity to strain TU Graz 8TSM2, originally recovered from Tenerife (Canary Islands) (Zachow et al. 2009).

3.2 | Antagonist Activity of *Trichoderma Gamsii* Through the Double Culture Assay

The antagonist activity of the *T. gamsii* TGH_24 isolate was tested against six *Colletotrichum* isolates. The minimum inhibition observed was 66% against *Colletotrichum gloeosporioides*, while the maximum inhibition reached 81.5% against *Colletotrichum acutatum*. The inhibition percentages for the other four species, *C. kahawae* subsp. *cigarro*, *C. karstii*, *C. nymphaeae* and *C. fioriniae*, were 70.5%, 70.5%, 77.5% and 78.5%, respectively (Figures 3 and 4). These results demonstrate the effective antagonistic potential of the tested *T. gamsii* TGH_24 isolate against multiple *Colletotrichum* species. Specifically, the *T. gamsii* TGH_24 isolate tested exhibited strong antagonism toward all six *Colletotrichum* isolates, achieving the highest score according to the Bell classification scale (Bell et al. 1982) (grade 1 = *Trichoderma* completely overgrew the pathogen and completely covered the entire medium surface) (Figure 3).

3.3 | Effect of N-VOCs on *Colletotrichum* Isolates

The inhibitory activity of non-volatile compounds (N-VOCs) produced by *T. gamsii* was evaluated following the method by Dennis and Webster (1971). Radial growth of the *Colletotrichum* isolates was measured over 10 days, corresponding to the time required for the control colonies (without *Trichoderma*) to fully cover the Petri dish. Results showed that N-VOCs significantly suppressed the mycelial growth of all six *Colletotrichum* species tested (Figure 3 and Figure 5). The highest inhibition was observed against *C. nymphaeae* (71.2%), while the lowest was recorded for *C. kahawae* subsp. *cigarro* (42.5%). Intermediate levels of inhibition were observed for *C. fioriniae* (66.7%), *C. karstii* (65.7%), *C. acutatum* (64.2%) and *C. gloeosporioides* (58.2%).

3.4 | Effect of VOCs on *Colletotrichum* spp.

The inhibitory effects of volatile organic compounds (VOCs) produced by *Trichoderma gamsii* were assessed using the sandwiched Petri dish method described by Qualhato et al. (2013). Mycelial growth of each *Colletotrichum* isolate was recorded over a 10-day incubation period, during which the control

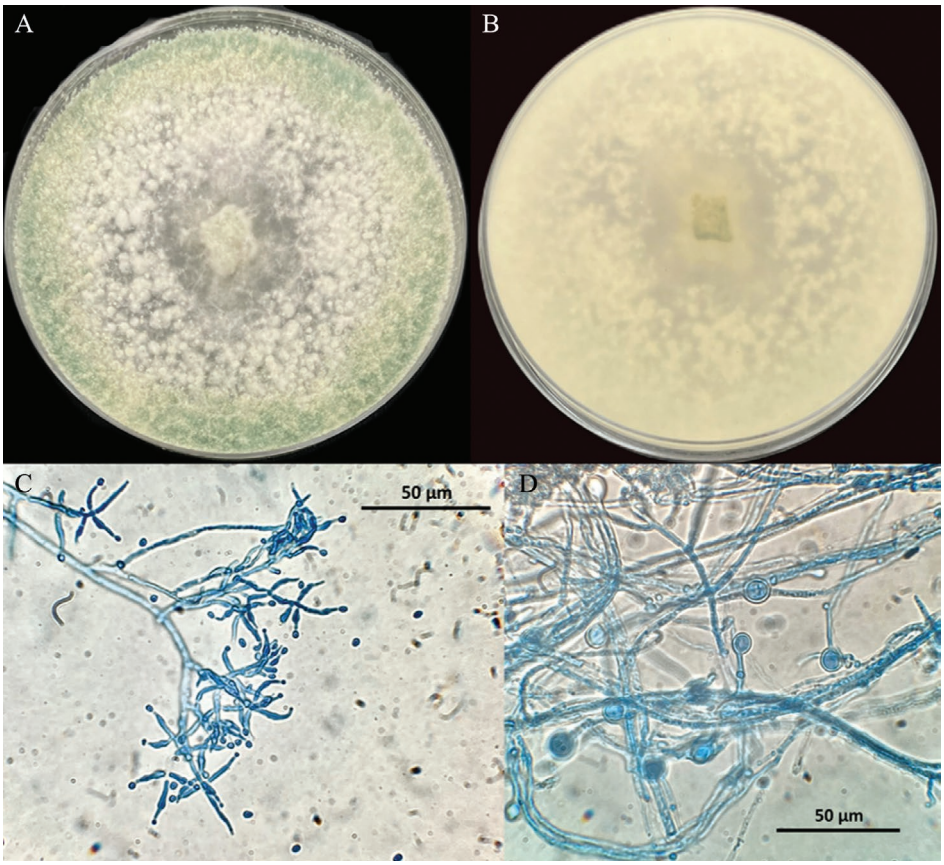

FIGURE 1 | Morphology and microscopy of *Trichoderma gamsii*; (A, B) colony morphology on PDA medium after incubation at 25°C for 10 days; (C) phialides and conidia; (D) chlamydospores.

colonies fully colonised the Petri dishes. The results revealed that VOCs from *T. gamsii* significantly inhibited the growth of all six *Colletotrichum* species tested (Figure 3 and Figure 5). The highest inhibition was observed for *C. nymphaeae* (73.1%), while *C. gloeosporioides* exhibited the lowest inhibition (51.6%). Intermediate inhibition percentages were recorded for *C. kahawae* subsp. *cigarro* (53.1%), *C. karstii* (68.6%), *C. acutatum* (69.6%) and *C. fioriniae* (70.6%). These findings are consistent with previous studies reporting strong antifungal activity of VOCs produced by different *Trichoderma* species against several phytopathogens (Bozdeveci and Karaoğlu 2023; da Costa et al. 2021; Poletto et al. 2024).

3.5 | N-VOCs Compound Profiling in *Trichoderma Gamsii* via LC–MS/MS

The annotated fungal metabolites were classified into diverse KEGG pathways, spanning multiple functional categories (Figure 6). The most represented categories included central metabolism, secondary metabolism, signal transduction, degradation, absorption, hormonal signalling, regulation and a substantial portion grouped as general/unclassified. Central metabolism pathways accounted for a large fraction of the dataset, encompassing amino acid biosynthesis and degradation, nucleotide metabolism, central carbon metabolism and cofactor biosynthesis. Secondary metabolism was also well represented, particularly through pathways involved in alkaloid biosynthesis, phenylpropanoid and plant hormone biosynthesis and various antibiotic production routes. Signal transduction pathways, including ABC transporters and MAPK signalling, highlighted the importance of molecular communication processes. Several degradation pathways indicated active metabolic recycling and detoxification mechanisms. The general/unclassified category contained broad metabolic pathways and microbial metabolism in diverse environments, reflecting compounds with widespread or less-specific functional roles.

To explore the potential functional relationships among the detected N-VOCs, we constructed a metabolite co-occurrence network based on the shared Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. This network consists of multiple interconnected nodes, each representing a metabolite, and edges indicating shared participation in metabolic or signalling pathways (Figure 7). A clear clustering of metabolites by functional category was observed, as reflected by node colours. Metabolites involved in central metabolism, such as amino acids (e.g., L-isoleucine and valine) and nucleotides (e.g., cytosine and guanine), formed a highly connected central core, suggesting their role as metabolic hubs. Conversely, secondary metabolites (e.g., tryptamine and beta-ethynylserine) and degradation-related compounds (e.g., benzaldehyde and 5-hydroxymethylfurfural) clustered more peripherally, suggesting specialised or context-dependent functions. Interestingly, metabolites involved in hormonal regulation (e.g., trans-zeatin) and signalling-related molecules (e.g., biotin and its derivatives) were integrated within the network, connecting central and peripheral pathways.

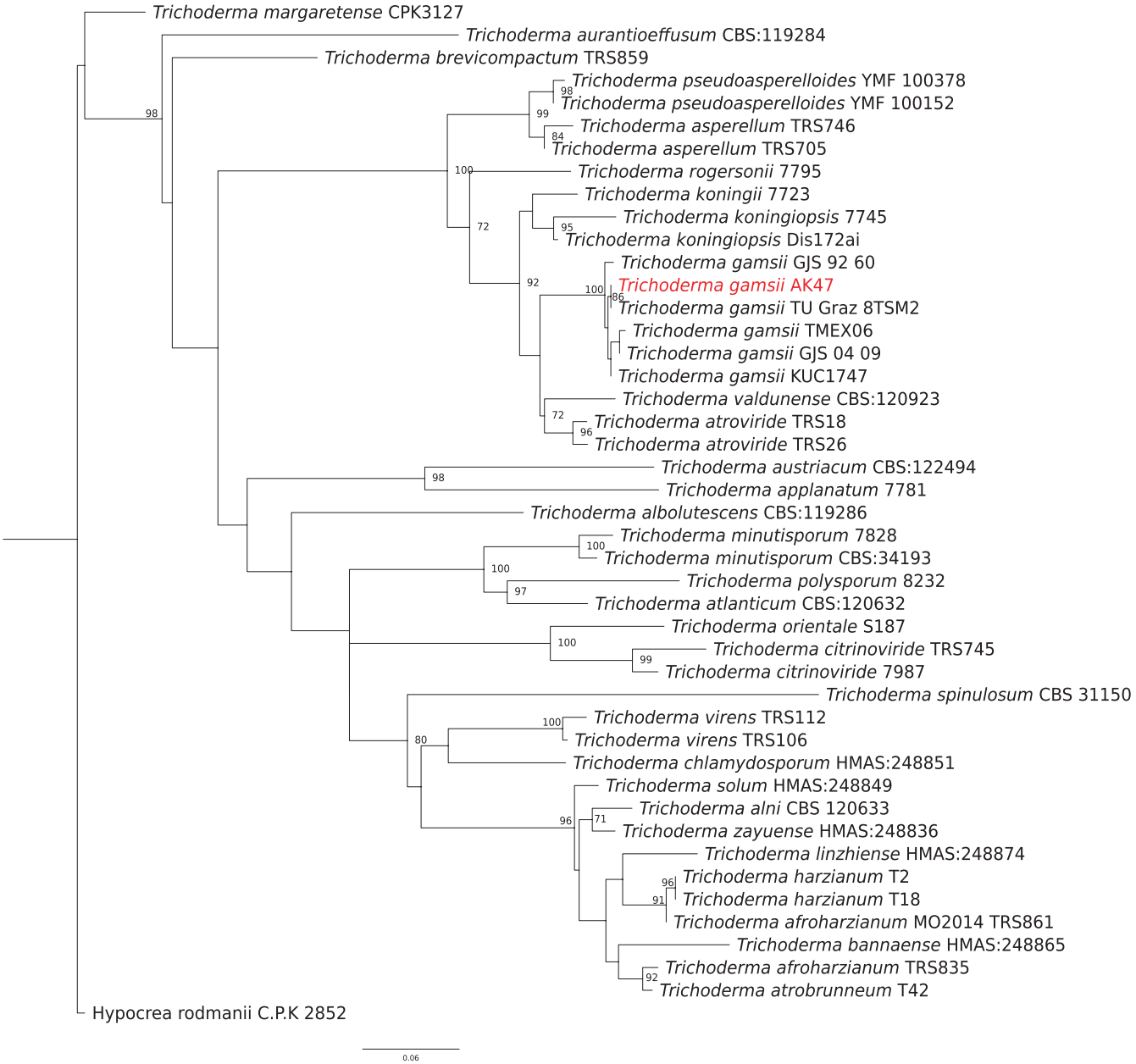

FIGURE 2 | Maximum Likelihood (ML) phylogenetic tree on the concatenated sequences of *ITS* and *EF-1α* genes among different species of *Trichoderma*. Bootstrap values that indicate clustering robustness are reported as node values. The *Trichoderma gamsii* TGH_24 isolate (in red) is perfectly clustered within the *Trichoderma gamsii* clade.

This integration implies that *T. gamsii* may modulate core and specialised functions through its metabolic outputs, which have implications for its antagonistic and symbiotic behaviour. Overall, the network topology reveals a core set of metabolites that co-occur across major metabolic categories. Peripheral nodes indicate potential niche- or strain-specific metabolites. Several metabolites detected in *T. gamsii*, including benzaldehyde, camptothecin and nicotinamide, are associated with antifungal or antagonistic functions according to previous studies (Kim et al. 2011; Chen et al. 2016; Khan et al. 2020). These compounds, along with aromatic aldehydes and alkaloid precursors, suggest that the metabolite network may contribute to inhibition mechanisms relevant to biocontrol. A summary of metabolites with their putative antifungal roles, evidence level and mechanisms is provided in Table S2.

3.6 | GC–MS-Based Characterisation of VOCs Produced by *Trichoderma Gamsii*

The gas chromatography–mass spectrometry (GC–MS) analysis revealed a total of 64 volatile organic compounds (VOCs), of which 15 were confirmed as fungal VOCs based on KEGG classification and literature curation. These were selected for downstream analysis. Among the identified compounds, 1-butanol, 3-methyl- (iso-butanol) was the most abundant (27.2%), representing a prominent component of the volatilome. This branched-chain alcohol is commonly produced by *Trichoderma* species and is associated with microbial interactions (Stracquadanio et al. 2020). Phenylethyl alcohol (2.87%), an aromatic alcohol with a floral odour, and β-phellandrene (1.72%), a monoterpene involved in plant–fungus interactions, were also abundant. Other

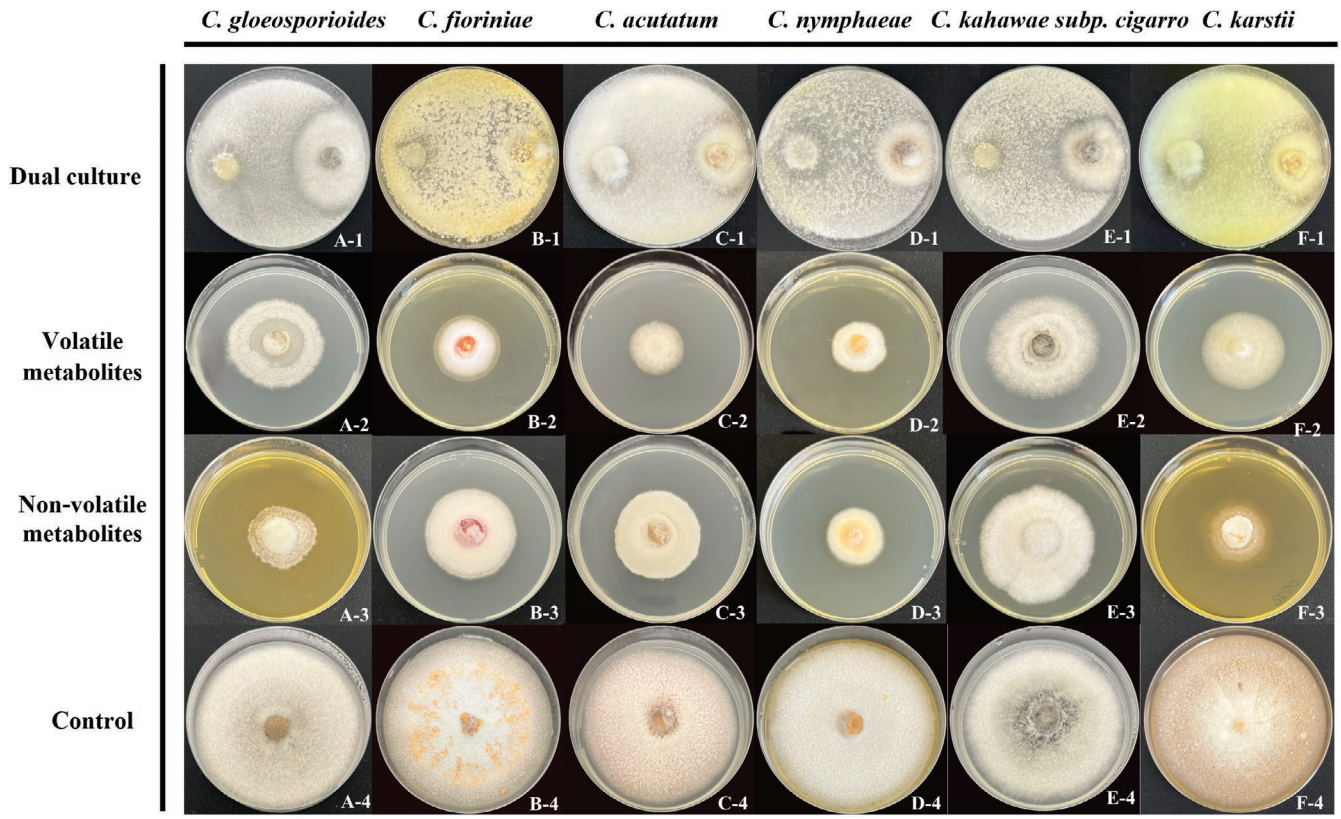

FIGURE 3 | Effect of *T. gamsii* against *C. gloeosporioides* (A-1–A-3), *C. fioriniae* (B-1–B-3), *C. acutatum* (C-1–C-3), *C. nymphaeae* (D-1–D-3), *C. kahawae* subsp. *cigarro* (E-1–E-3) and *C. karstii* (F-1–F-3) with respect to the controls (A-4, B-4, C-4, D-4, E-4 and F-4) with different in vitro tests.

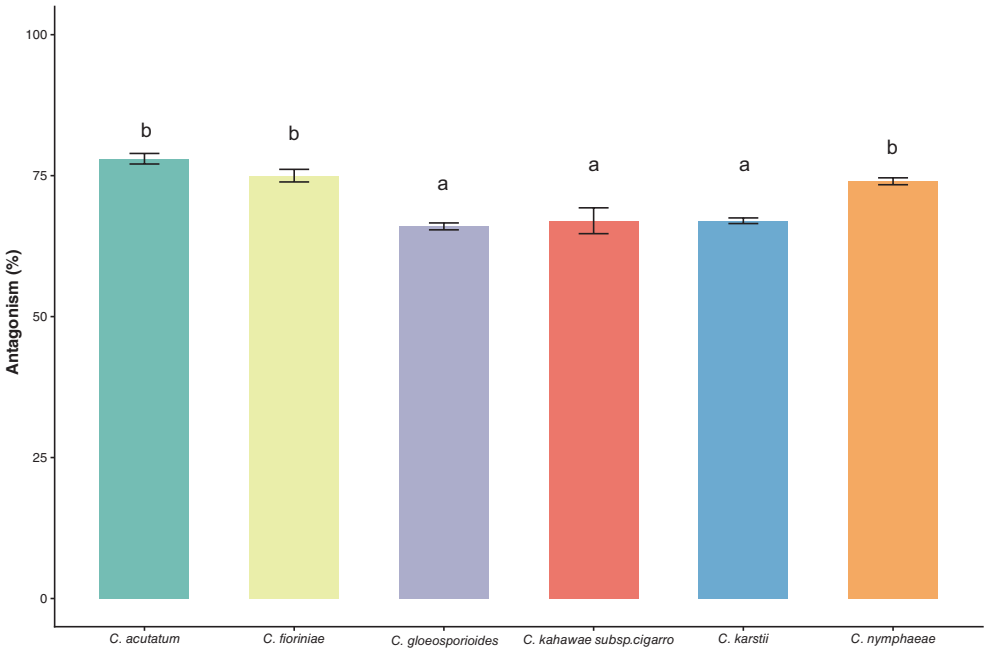

FIGURE 4 | Antagonistic activity of *Trichoderma gamsii* TGH_24 strain against six *Colletotrichum* isolates. Bars represent mean inhibition percentages ± standard error ($n = 5$). Different letters above the bars indicate statistically significant differences among isolates according to Tukey's HSD test ($p < 0.05$).

detected VOCs included acetoin (0.43%), (−)-myrtenol (0.37%), 2-heptanone (0.24%), benzaldehyde (0.17%) and α-phellandrene (0.27%), all of which have been reported in fungal VOC profiles, particularly those of *Trichoderma* spp. Their chemical nature ranged from alcohols and ketones to esters, aldehydes and terpenes, indicating a chemically diverse volatilome (Figure 8).

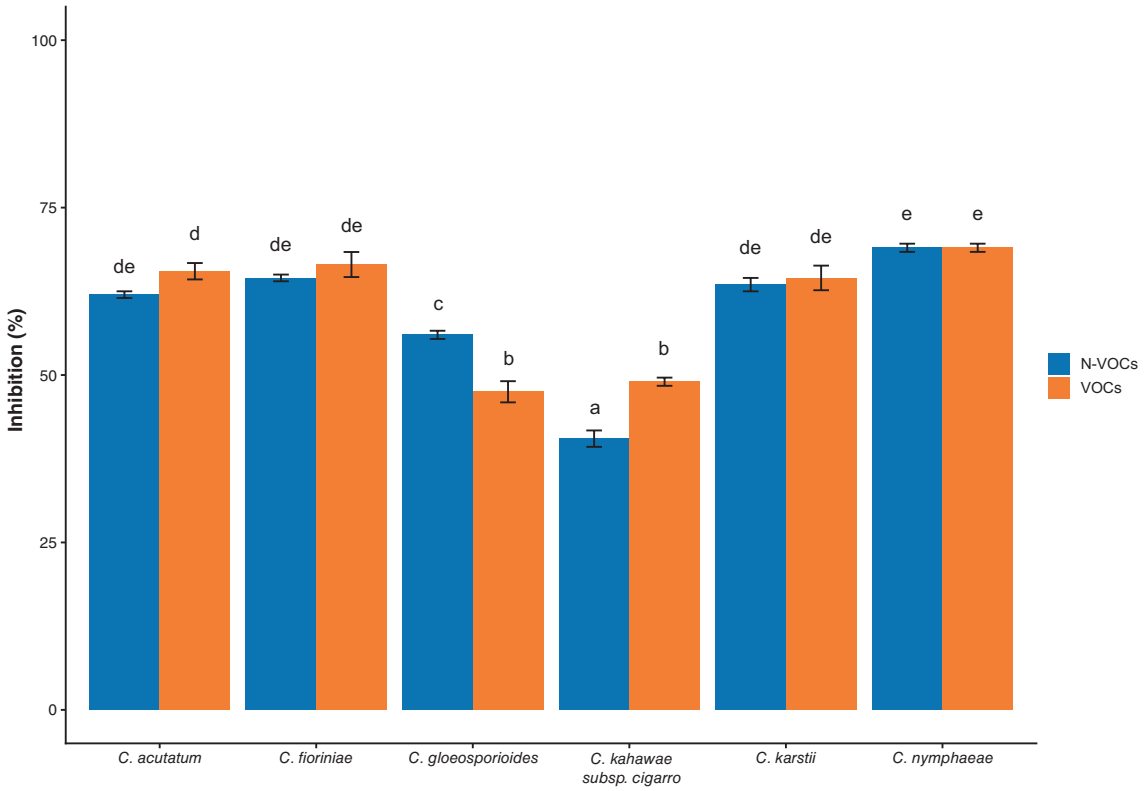

FIGURE 5 | Inhibition of six *Colletotrichum* isolates by volatile (VOCs) and non-volatile (no-VOCs) metabolites of *Trichoderma gamsii* TGH_24 strain. Bars represent mean inhibition percentages ± standard error ($n = 5$). Blue bars correspond to no-VOCs, and orange bars correspond to VOCs. Different lowercase letters above the bars indicate statistically significant differences among treatments according to Tukey's HSD test ($p < 0.05$).

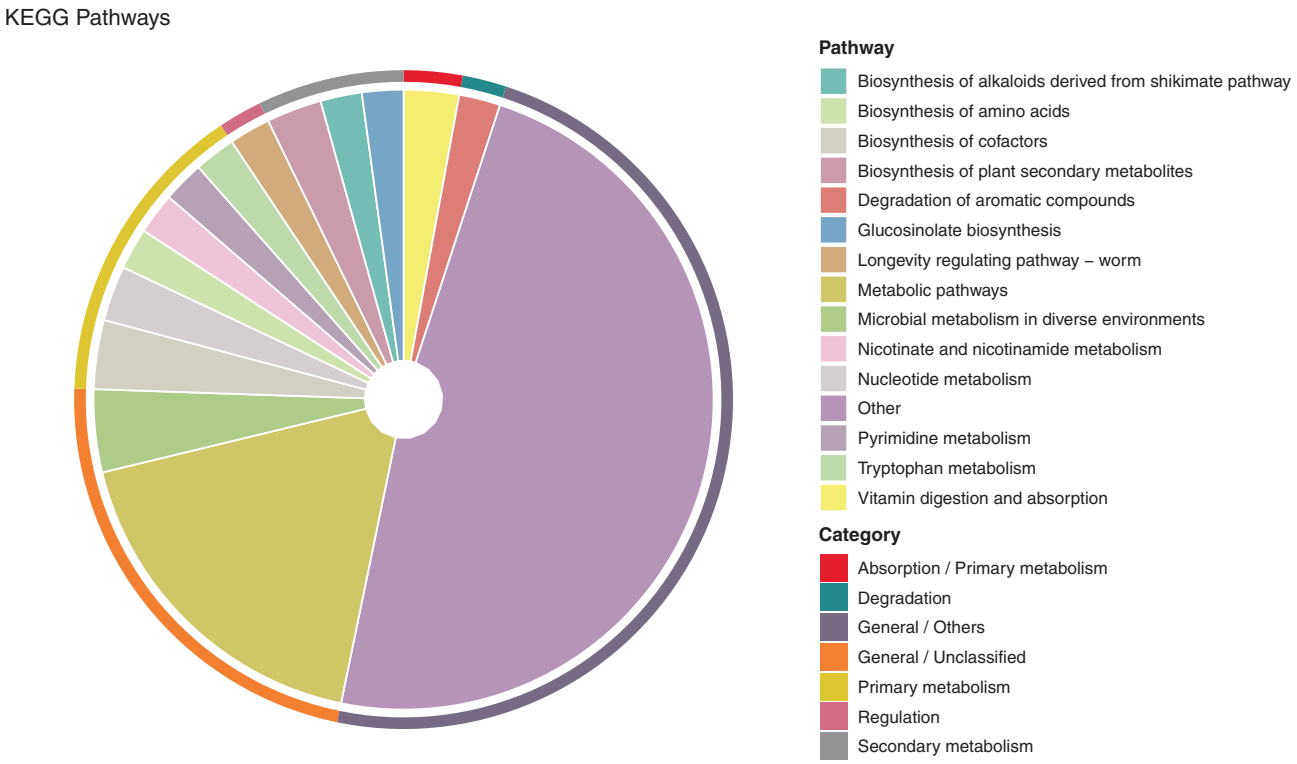

FIGURE 6 | Functional classification of non-volatile metabolites detected in *Trichoderma gamsii* TGH_24. A circular stacked bar plot (pie chart with concentric rings) illustrates the functional classification of approximately 150 curated fungal non-volatile metabolites based on KEGG pathway annotation. The inner ring represents broad metabolic pathways, while the outer ring shows the corresponding functional categories.

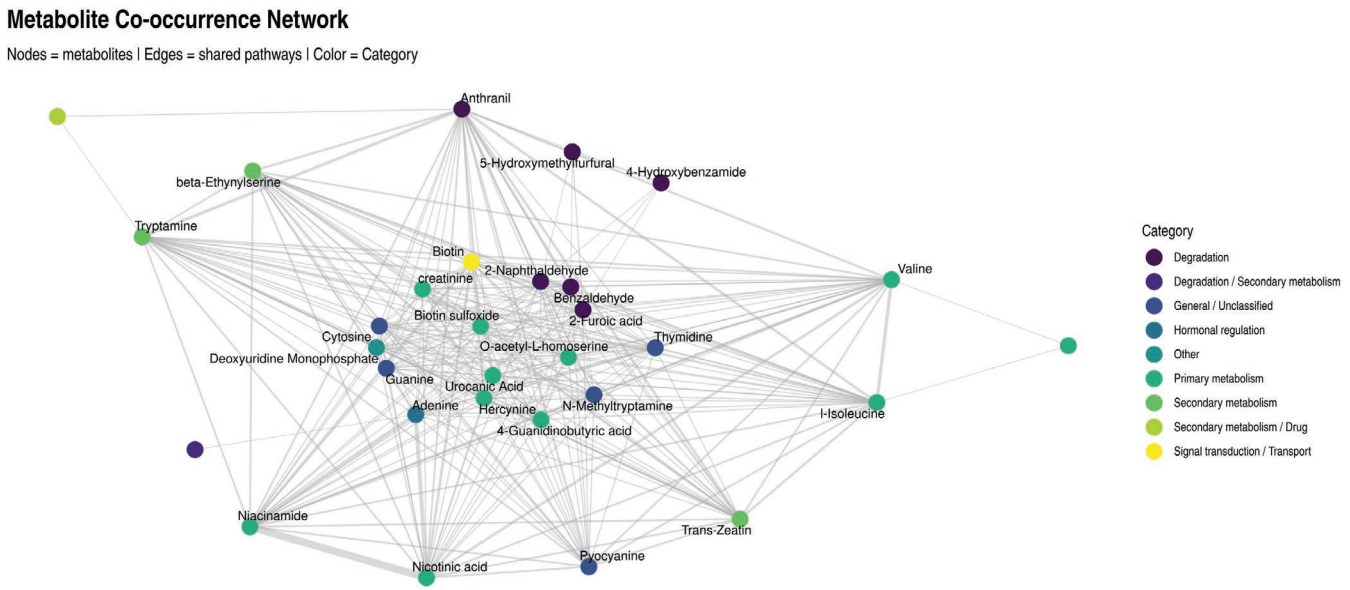

FIGURE 7 | Chemical similarity network of non-volatile fungal metabolites identified in *Trichoderma gamsii* TGH_24 strain. The network depicts chemical similarity relationships among curated fungal metabolites based on molecular structure and shared chemical features. Nodes represent individual metabolites and edges indicate structural similarity above a defined threshold. Clustered metabolites (indicated by proximity and edge connections) suggest shared biosynthetic origins or functional relatedness.

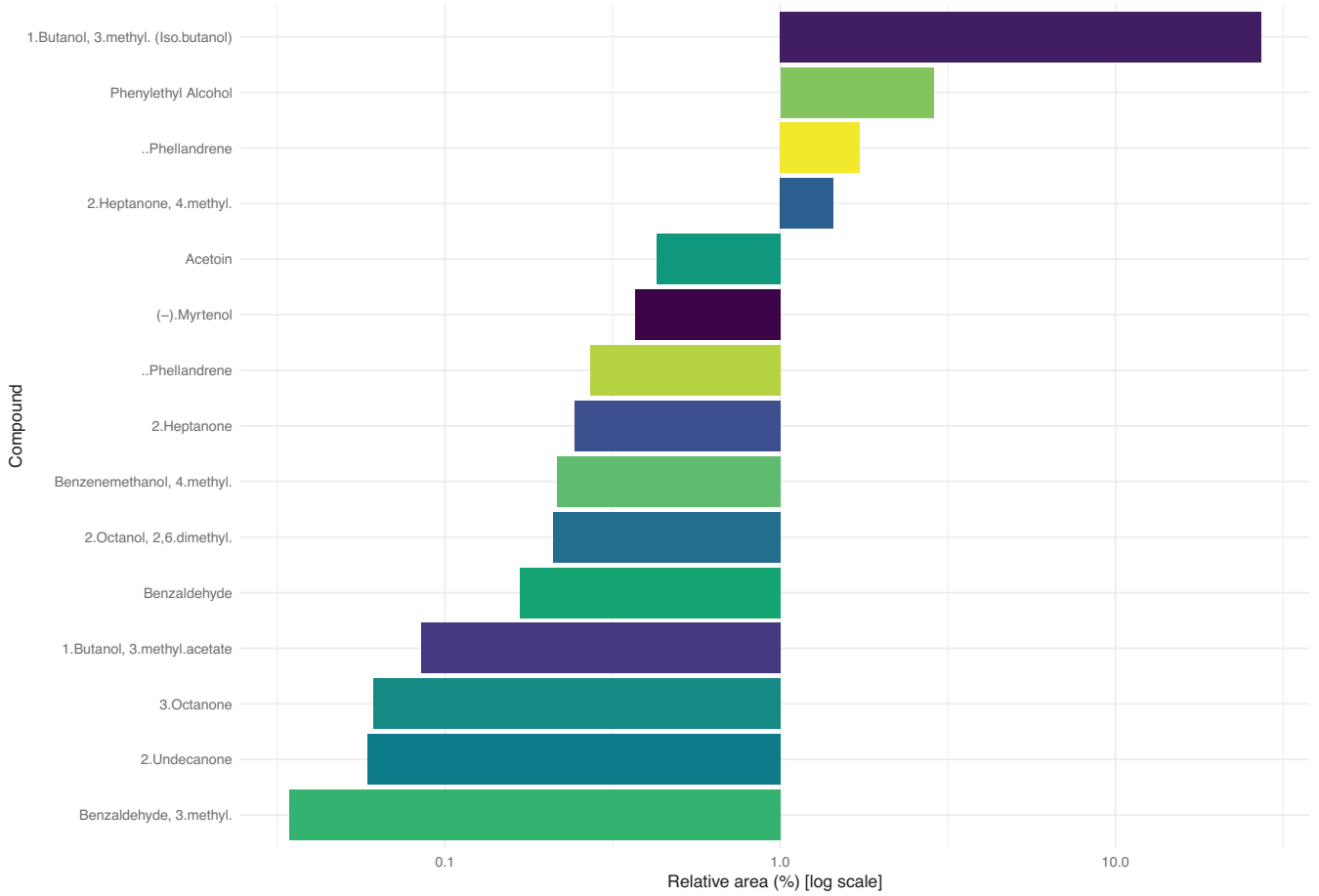

FIGURE 8 | Volatile organic compounds (VOCs) identified in *Trichoderma gamsii* TGH_24 isolate. Bar chart showing the $\log_{10}$-transformed percentage of the relative chromatographic area of 15 fungal-derived VOCs identified via GC–MS analysis.

4 | Discussion

Trichoderma species are among the most extensively studied fungal biocontrol agents, owing to their ecological versatility and multiple mechanisms of action, which include mycoparasitism, nutrient competition, production of secondary metabolites and induction of plant defence responses (Guzmán-Guzmán et al. 2023). Beyond their role in the suppression of pathogens, several *Trichoderma* strains are also recognised as plant biostimulants, with the capacity to enhance nutrient uptake, root development and tolerance to abiotic stresses (Lugtenberg 2016). This dual functionality positions *Trichoderma* as a cornerstone in the transition toward sustainable agriculture. At the policy level, these characteristics align closely with the objectives of the European Green Deal and the Farm to Fork Strategy, which aim to reduce the overall use and risk of chemical pesticides by 50% by 2030 while promoting eco-friendly alternatives (European Commission, 2020). The recent EU Fertilising Products Regulation (EU) 2019/1009 has further consolidated the role of microbial-based products, officially recognising plant biostimulants as a distinct category and facilitating their placement on the European market (European Union, 2019; EBIC, 2022). Concurrently, the International Biocontrol Manufacturers Association (IBMA) has underscored the pressing imperative to expedite the implementation of biocontrol methodologies to align with sustainability objectives and bolster integrated pest management (IBMA, 2022). Our findings contribute to this broader framework by demonstrating the antagonistic potential of *T. gamsii* TGH_24 isolate against *Colletotrichum* spp., one of the most challenging fungal genera in agriculture due to its wide host range and adaptability.

The in vitro dual culture assays revealed high levels of inhibition, ranging from 66% against *C. gloeosporioides* to 81.5% against *C. acutatum*, with all six tested species showing substantial suppression. These findings confirm the broad-spectrum antifungal activity of *T. gamsii*, consistent with previous reports highlighting the efficacy of various *Trichoderma* species as biocontrol agents against fungal pathogens such as *Fusarium, Alternaria* and *Colletotrichum* (Kim et al. 2023; Peralta-Ruiz et al. 2023). The complete overgrowth observed in the Bell classification further underscores the aggressive colonisation and competitive advantage of this isolate, a trait crucial for successful biocontrol in natural or agricultural environments.

The evaluation of non-volatile compounds (N-VOCs) demonstrated a differential inhibitory effect, with maximal suppression against *C. nymphaeae* (71.2%) and minimal effect on *C. kahawae* subsp. *cigarro* (42.5%). This variability likely reflects differences in pathogen susceptibility and the spectrum of secondary metabolites produced. Non-volatile metabolites produced by *Trichoderma* are known to include hydrolytic enzymes, peptaibols, polyketides and other bioactive compounds that disrupt pathogen growth through cell wall degradation, membrane destabilisation and interference with metabolic processes (Chávez-Avilés et al. 2024; Cortez-Lázaro et al. 2025). Similarly, volatile organic compounds (VOCs) contributed significantly to pathogen suppression, with inhibition ranging from 51.6% to 73.1% across species. The comparable efficacy of VOCs and N-VOCs indicates that *T. gamsii* employs a multifaceted antagonistic strategy, combining both contact-dependent and volatile-mediated mechanisms, as previously documented for other *Trichoderma* strains (Lahlali et al. 2022; Peralta-Ruiz et al. 2023; Yao et al. 2023).

The metabolomic analyses provided further mechanistic insights. LC–MS/MS profiling revealed a diverse array of non-volatile metabolites spanning central metabolism, secondary metabolism, signal transduction, degradation pathways and hormonal signalling. Central metabolites, such as amino acids (L-isoleucine, valine) and nucleotides (cytosine, guanine), formed a highly interconnected network core, likely representing essential hubs for growth and metabolic integration. Peripheral metabolites, including tryptamine, beta-ethynylserine, benzaldehyde and 5-hydroxymethylfurfural, may contribute to specialised antagonistic or signalling functions. Notably, the presence of plant hormone-related compounds (e.g., trans-zeatin) and signalling molecules (e.g., biotin derivatives) suggests potential for modulation of host-pathogen interactions and systemic plant responses, a feature that aligns with emerging concepts of *Trichoderma*-mediated priming and growth promotion (Pozo et al. 2024; Yao et al. 2023; Shetty et al. 2025). The metabolite co-occurrence network underscores the coordinated integration of central and specialised pathways, highlighting the complexity of fungal secondary metabolism and its ecological relevance. Beyond pathway mapping, several nodes and edges in the co-occurrence network suggest potential inhibitory functions. For example, aromatic aldehydes such as benzaldehyde are well-documented antifungals that disrupt fungal redox homeostasis (Dhakshinamoorthy et al. 2020; Xing et al. 2023). Similarly, signatures of phenazine biosynthesis, including anthranilate and pyocyanine, are associated with antifungal activities against *Candida* and *Aspergillus* (Price-Whelan et al. 2006). Notably, the camptothecin node and its alkaloid context correspond with previous reports indicating that topoisomerase I inhibitors exhibit fungicidal properties and can control postharvest diseases (Banadka et al. 2024). Other studies have shown that nicotinamide exhibits potent anti-*Candida* effects, suppressing biofilm formation and remodelling the cell wall (Yan et al. 2022). Additionally, 5-hydroxymethylfurfural and related furanics are more accurately categorised as microbial growth inhibitors than targeted antifungals (Jilani and Olson 2023). Finally, regulatory connectors such as the plant hormone zeatin provide a plausible host-mediated component. *Trichoderma* can synthesise CKs and alter plant cytokinin dynamics, which are associated with enhanced resistance to pathogens (Sakakibara 2006). Together, these features explain why a dense central hub (amino acids/nucleotides) feeds peripheral inhibitory branches (aromatic aldehydes and indole/alkaloid routes), resulting in a topology where a few key nodes act as mediators of inhibition while core metabolism supplies precursors.

GC–MS analysis of VOCs further revealed a chemically diverse volatilome, dominated by iso-butanol (27.2%), phenylethyl alcohol (2.87%) and β-phellandrene (1.72%), alongside minor components such as acetoin, (−)-myrtenol, 2-heptanone, benzaldehyde and α-phellandrene. These compounds are consistent with known antifungal VOCs produced by *Trichoderma* species and are implicated in both direct pathogen inhibition and modulation of plant-microbe interactions (Chávez-Avilés et al. 2024). The prevalence of branched-chain alcohols and monoterpenes supports the notion that *T. gamsii* can exert inhibitory effects even at a distance from the pathogen, expanding its potential ecological impact.

Overall, these findings indicate that *T. gamsii* TGH_24 employs a multifactorial antagonistic strategy combining direct mycelial competition, secretion of inhibitory N-VOCs and emission of bioactive VOCs. The integration of metabolomic data with in vitro bioassays provides a mechanistic framework explaining its efficacy against diverse *Colletotrichum* species. While these results are promising, future studies are needed to assess phytocompatibility, *in planta* biocontrol efficacy and the molecular regulation of these metabolic pathways. Understanding these aspects will be crucial for the rational development of *T. gamsii* as a sustainable and effective biocontrol agent in European and Mediterranean agroecosystems.

Author Contributions

Federico Brugneti: conceptualization; data curation; formal analysis; investigation; methodology; validation; visualisation; writing – original draft; writing – review and editing. Antonella Cardacino: data curation; formal analysis; investigation; methodology; validation; visualisation; writing – original draft; writing – review and editing; Giuseppina Fanelli: formal analysis; investigation; methodology. Sara Rinalducci: formal analysis; investigation; methodology. Angelo Mazzaglia: conceptualization; funding acquisition; project administration; resources; supervision; writing – review and editing. Silvia Turco: conceptualization; data curation; formal analysis; project administration; visualisation; supervision; writing – review and editing.

Acknowledgements

All the bioinformatics calculations and analyses were carried out at the DAFNE HPC scientific computing centre of the Università degli Studi della Tuscia. The authors are grateful to Noemi Villanova for the technical support given during the preparation of the material for the HPLC analysis.

Funding

The research was carried out within the framework of the Ministry for University and Research (MUR) initiative 'Department of Excellence' (Law 232/2016) DAFNE Project 2023–27 'Digital, Intelligent, Green and Sustainable' (acronym: D.I.Ver.So).

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are openly available in NCBI GenBank at https://www.ncbi.nlm.nih.gov/genbank/, reference numbers P786234 and PP793890.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Table S1:** List of ITS and EF-1α sequences used for phylogenetic analysis. The sequences obtained from the *Trichoderma gamsii* isolate in this study were analysed together with 45 reference sequences retrieved from GenBank. Accession numbers and species names are reported as provided in the database. **Table S2:**. Annotated non-volatile metabolites detected in *Trichoderma gamsii* TGH_24 with documented or putative antifungal/antagonistic roles. The table summarises each metabolite, the level of evidence (high, medium, low, indirect) and the proposed mechanism of action.