



Trichoderma asperelloides TSUxPT3.5 Exhibits Antifungal Activity Against Plant Pathogenic Fungi and Enhances the Growth of Mini Cos Lettuce (*Lactuca sativa* L.)

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Abstract: *Trichoderma* species are widely recognized as effective biological control agents due to their multifaceted antagonistic mechanisms and plant growth-promoting activities. In this study, an indigenous fungal strain, TSUxPT3.5, was isolated from organic agricultural soil in southern Thailand. Molecular identification based on multi-locus sequence analysis of the ITS, *tef1*, and *rpb2* regions confirmed the strain as *Trichoderma asperelloides*. This fungus was further characterized for its plant growth-promoting traits through both direct and indirect mechanisms. *Trichoderma asperelloides* TSUxPT3.5 produced indole compounds and exhibited antagonistic activity against economically important plant pathogenic fungi, including *Curvularia aeria* and *Corynespora cassiicola*, causal agents of leaf spot disease in lettuce. Its biocontrol mechanisms involved competition for nutrients and space, production of cell wall-degrading enzymes (chitinase and cellulase), and emission of volatile organic compounds (VOCs). Among the detected VOCs, 2-ethylhexan-1-ol was the predominant compound. Additionally, 6-pentyl-2H-pyran-2-one, 2,4-Di-tert-butylphenol, and 2-methyl-1-propanol, a well-known antifungal metabolite, were also identified. Application of *T. asperelloides* TSUxPT3.5 significantly enhanced the growth of mini cos lettuce (*Lactuca sativa* L.), increasing fresh weight and improving plant vigor compared with the untreated control. In addition, disease severity was markedly reduced in treated plants. These findings demonstrate that the indigenous *T. asperelloides* TSUxPT3.5 possesses multifunctional antagonistic and plant growth-promoting traits, highlighting its potential as an eco-friendly biological control agent for sustainable lettuce production.

Keywords: Biological control; indole compounds; plant growth promotion; *Trichoderma*; volatile organic compounds

1. Introduction

Plant diseases caused by fungal pathogens represent a major constraint on global agricultural productivity, resulting in substantial yield losses and posing serious threats to food security worldwide [1]. In recent years, the

incidence and severity of fungal plant diseases have increased markedly, largely due to climate change-related factors such as rising temperatures, altered precipitation, increased humidity, and more frequent extreme climate events [2, 3]. These changing environmental conditions can induce pathogen germination, survival, reproduction, and spread, while simultaneously weakening host plant resistance [4-6]. For instance, crops grown under protected cultivation systems, including leafy vegetables such as lettuce (*Lactuca sativa*), have become increasingly vulnerable to fungal diseases [7-9]. Addressing fungal diseases in the context of climate change has therefore become a critical challenge for sustainable agricultural production.

Effective plant disease management relies on accurate disease diagnosis and the application of appropriate control strategies, including biological, chemical, and mechanical approaches. Chemical control provides rapid, broad-spectrum efficacy in suppressing fungal pathogens and inducing plant defense responses [10]. However, the extensive use of synthetic fungicides has led to several negative consequences, including environmental contamination, adverse effects on non-target microorganisms, risks to human health, and the rapid emergence of fungicide-resistant pathogens [11-13]. Given these limitations, there is increasing interest in alternative, environmentally friendly, and sustainable disease management strategies. Among them, biological control using beneficial microorganisms has emerged as an effective and sustainable approach for suppressing plant pathogens while reducing reliance on chemical inputs [14].

Among the diverse group of antagonistic biological control agents (BCAs), species of the genus *Trichoderma* are among the most extensively studied and commercially exploited fungi for plant disease control. *Trichoderma* spp. suppress plant pathogenic fungi through multiple mechanisms, including competition for nutrients and space, mycoparasitism, production of antimicrobial secondary metabolites, emission of volatile organic compounds (VOCs), and secretion of hydrolysis enzymes [15-18]. Furthermore, *Trichoderma* spp. are well recognized for their ability to promote plant growth and development by producing phytohormones, including indole compounds (e.g., indole-3-acetic acid), and to enhance systemic resistance against several pathogens [19-21]. Interestingly, accumulating evidence revealed that these biocontrol and plant growth-promoting traits are highly strain-dependent, even within the same *Trichoderma* species, highlighting the importance of identifying and characterizing effective indigenous strains adapted to local environmental conditions [22]. Although several strains of *T. asperelloides* have previously been reported for their biocontrol activity and volatile-mediated antifungal effects [16-18], information regarding the biological characteristics and plant growth-promoting potential of *T. asperelloides* strain TSUXPT3.5 remains limited. In particular, its antifungal activity, extracellular enzyme production, volatile organic compound emission, indole compound production, and plant growth-promoting potential in lettuce systems have not been comprehensively characterized.

Lettuce (*Lactuca sativa* L.) is one of the most widely cultivated and consumed leafy vegetables worldwide and represents an economically important crop in the fresh vegetable market. It includes a wide range of horticultural types, such as mini cos (baby romaine), crisphead (iceberg), butterhead, loose-leaf, and stem lettuce, which vary in morphology, texture, and consumer preference. Global production of lettuce and chicory exceeds 27 million tonnes annually [23], reflecting its high market demand and economic importance. Among lettuce types, mini cos lettuce has gained increasing popularity due to its compact size, crisp texture, and suitability for fresh consumption. Mini cos lettuce is increasingly cultivated as a high-value leafy vegetable under protected and semi-protected cultivation systems to meet the growing demand for fresh produce. However, fungal diseases such as leaf spot and leaf blight remain major constraints on lettuce production, causing both quality and yield losses under warm, humid conditions [7, 24]. *Trichoderma* species are widely recognized as effective BCAs and plant growth promoters, owing to their multiple mechanisms of antagonism against plant pathogens. Therefore, the objective of this study was to identify and characterize an indigenous *Trichoderma* isolate from agricultural soil and evaluate its antagonistic activity against economically important plant pathogenic fungi associated with lettuce diseases, as well as its plant growth-promoting potential.

2. Materials and Methods

2.1 Source of *Trichoderma* and fungal pathogens

Trichoderma sp. strain TSUxPT3.5 were isolated from organic agricultural soil collected from Tha Chamuang subdistrict, Rattaphum district, Songkhla province, Thailand, by the serial dilution spread plate method. It has been preserved at the Culture Collection of the Department of Biological Science, Faculty of Science and Digital Innovation, Thaksin University, Phatthalung, Thailand. *Curvularia aeria* and *Corynespora cassiicola*, causal agents of leaf spot disease in lettuce [7, 25], were obtained from the Culture Collection of the Department of Plant Pathology, Faculty of Natural Resources, Prince of Songkla University, Thailand. Both *Trichoderma* and fungal pathogens were cultured on potato dextrose agar (PDA) and incubated at $30 \pm 2^\circ\text{C}$ for 5 days before use in the bioassay.

2.2 Identification of the *Trichoderma* sp. strain TSUxPT3.5

The *Trichoderma* sp. strain TSUxPT3.5 was identified using morphology and molecular taxonomic methods. For morphological identification, the fungus was cultured on PDA to assess growth and macroscopic features. Microscopic features of hyphae, conidiophores, phialides, and conidia were observed under a compound microscope. For molecular identification, genomic DNA was extracted from fungal mycelia using the FavoPrep® DNA Extraction Mini Kit (Favorgen, Taiwan) according to the manufacturer's instructions. Genus-level identification was performed using the internal transcribed spacer (ITS) region, which was PCR-amplified from fungal genomic DNA with the primers ITS4 and ITS5 [26]. Species-level identification was conducted using partial sequences of the translation elongation factor 1-alpha (*tef1*) and the second largest subunit of RNA polymerase II (*rpb2*). The *tef1* gene was amplified using the primer pairs EF1/EF2 [27] and EF1-728F/*tef1*-LLErevR [28, 29], while the *rpb2* gene was amplified using the primer pair RPB2-5F/RPB2-7cR [30]. The obtained ITS, *tef1*, and *rpb2* sequences were compared with reference sequences of type strains available in the GenBank database (<https://www.ncbi.nlm.nih.gov>). Phylogenetic trees based on *tef1* and *rpb2* sequences were constructed from evolutionary distance data using the general time-reversible (GTR) model and maximum likelihood methods [31] with the MEGA12 (molecular evolutionary genetics analysis) software [32]. Bootstrap confidence values were obtained using 1000 resamplings [33]. The sequence was aligned with the closest strains of the species using multiple sequence comparison by log-expectation (MUSCLE) [34]. *Nectria aurantiaca* was used as an outgroup.

2.3 Evaluation of antagonistic activity of *Trichoderma* sp. strain TSUxPT3.5 against plant pathogenic fungi

In vitro antagonistic activity was evaluated using the dual culture method [35]. A mycelial plug of *Trichoderma* sp. strain TSUxPT3.5, and a plug of the tested phytopathogen were placed 65 mm apart on 90 mm Petri dishes containing PDA and incubated at $30 \pm 2^\circ\text{C}$. The phytopathogenic fungi used in this study are *C. aeria* and *Co. cassiicola*. Control plates containing only the phytopathogen were included for comparison. Radial mycelial growth was measured after 3 and 5 days of incubation, and the percentage inhibition of mycelial growth was calculated using Equation (1):

$$\text{Percentage inhibition} = [(R_1 - R_2)/R_1] \times 100 \quad (1)$$

where R_1 represents the radial growth of the phytopathogen in the control plates, and R_2 represents the radial growth in the presence of *Trichoderma* sp. strain TSUxPT3.5. All assays were performed in triplicate.

2.4 Qualitative chitinase and cellulase activity of *Trichoderma* sp. strain TSUxPT3.5

The inoculum of the *Trichoderma* sp. strain TSUxPT3.5 was prepared on PDA and incubated at $30 \pm 2^\circ\text{C}$ for 5 days. Mycelial plugs (5 mm in diameter) obtained from the actively growing margins of the colonies were transferred onto carboxymethyl cellulose (CMC) agar and chitin agar, as described by Kamala and Indira [36], for the primary detection of cellulase and chitinase activities, respectively. For cellulase activity analysis, the agar plates were flooded with 0.1% (w/v) Congo red solution for 10 min, then destained with 1 N NaCl. The formation of clear hydrolysis zones around the fungal colonies indicated enzyme activity. Chitinase activity was detected by the formation of purple-colored zones on chitin agar, indicating chitin degradation and dye-substrate complex formation. All experiments were conducted in triplicate.

2.5 *In vitro* antifungal activity of VOCs produced by *Trichoderma* sp. strain TSUxPT3.5

The antifungal activity of volatile organic compounds (VOCs) produced by *Trichoderma* sp. strain TSUxPT3.5 was examined using a sealed plate assay. A mycelial plug of *Trichoderma* sp. strain TSUxPT3.5, and a plug of the fungal pathogen were placed at the center of two separate PDA plates of the same size. The plates were paired face-to-face, sealed with parafilm, and incubated at 30 ± 2 °C for 3 and 5 days. Control plates were prepared in the same manner without inoculation of *Trichoderma*. The percentage inhibition of mycelial growth was calculated using Equation (2):

$$\text{Percentage inhibition (\%)} = (C - T) / C \times 100 \quad (2)$$

where C is the colony diameter of the fungal pathogen in the control, and T is the colony diameter in the treatment.

2.6 Evaluation of volatile organic compounds produced by *Trichoderma* sp. strain TSUxPT3.5

Volatile organic compounds (VOCs) produced by *Trichoderma* sp. strain TSUxPT3.5 were analyzed using solid-phase microextraction (SPME) coupled with gas chromatography–mass spectrometry (GC–MS), following the method described by Suwannarach et al. [37]. A 50/30 µm divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) StableFlex fiber (Supelco, USA) was exposed to the headspace above each fungal culture for 45 min. The adsorbed fiber was then inserted into the splitless injection port of a gas chromatograph (GC-2010, Shimadzu, Japan) equipped with a DB-Wax capillary column (0.25 mm × 30 m, 0.25 µm film thickness, Supelco, USA). The column temperature was programmed to 40°C for 2 min and then increased to 200 °C at 5°C min⁻¹. Helium (ultra-high purity) was used as the carrier gas at an initial column head pressure of 60 kPa. The fiber was desorbed at 250°C for 57 min under a helium flow, then the volatiles were trapped. A 30 s injection time was used to introduce the adsorbed volatiles into the gas chromatograph (GC) interfaced with a mass spectrometer (MS-QP2010 Plus, Shimadzu, Japan). The mass spectrometer was operated at unit mass resolution. Software systems performed data acquisition and data processing. The VOCs produced by *Trichoderma* sp. strain TSUxPT3.5 were tentatively identified through a library comparison with the NIST database.

2.7 Detection of indole compounds produced by *Trichoderma* sp. strain TSUxPT3.5

Trichoderma sp. strain TSUxPT3.5 was cultured on PDA and incubated at 30 ± 2 °C for 5 days. After incubation, five mycelial plugs (5 mm in diameter) obtained from the actively growing margins of the colonies were transferred into 50 mL of potato dextrose broth (PDB) supplemented with 0.1% (w/v) L-tryptophan. The cultures were incubated on an orbital shaker at 170 rpm and 30 °C for 7 days. The culture supernatants were collected by centrifugation and used to determine indole compounds [38]. Briefly, 1 mL of culture supernatant was mixed with 2 mL of Salkowski's reagent (containing 0.5 M FeCl₃, 2 mL, and 35% HClO₄, 98 mL and incubated in the dark for 30 min. The development of a pink-to-red color indicated the presence of a positive indole compound. All experiments were conducted in triplicate.

2.8 Effects of *Trichoderma* sp. strain TSUxPT3.5 on plant growth parameters and reduction of leaf disease caused by natural infection

Mini cos lettuce seedlings aged 18 days after sowing were transplanted into experimental plots at a spacing of 20 × 20 cm, with one plant per planting hole. Plants were irrigated using a drip irrigation system, and additional overhead misting was applied three times daily to reduce temperature and increase relative humidity. The experiment was conducted in a protected greenhouse under conditions of 28–34 °C and approximately 70–85% relative humidity. The experiment consisted of three treatments: (i) *Trichoderma* sp. strain TSUxPT3.5, (ii) *Trichoderma asperellum* (commercial strain used to serve as a positive control), and (iii) untreated control. *Trichoderma* suspensions, adjusted to 1×10^6 spores mL⁻¹ using a hemocytometer according to standard fungal inoculum preparation procedures [15], were applied as foliar sprays every three days in the evening, starting 14 days after transplanting. Plant growth parameters, including plant height, number of leaves per plant, and fresh weight, were recorded during the experiment. Each treatment consisted of ten replicates, and the experiment was conducted twice. Natural infection and leaf disease were observed in protected cultivation. Disease severity was assessed using a rating scale of 0–5, where 0 = no spots, 1 = spot

area of 0–20%, 2 = 20–40%, 3 = 40–60%, 4 = 60–80% with approximately 50% spot coalescence, and 5 = 80–100% with more than 75% spot coalescence. Disease severity scores were converted to a disease severity index (DSI) according to Equation (3):

$$\text{DSI (\%)} = \frac{\sum (\text{scale} \times \text{amount of plants})}{\text{maximum level} \times \text{total number of plant}} \times 100 \quad (3)$$

2.9 Statistical Analysis

The effects of *Trichoderma* treatment on plant growth were analyzed using one-way analysis of variance (ANOVA). When significant differences were detected, treatment means were separated using the least significant difference (LSD) test at $P < 0.05$.

3. Results

3.1 Identification of *Trichoderma* sp. strain TSUxPT3.5

Morphological observations of *Trichoderma* sp. strain TSUxPT3.5 were conducted on PDA at $28 \pm 2^\circ\text{C}$ after 1–4 days of incubation. Conidia germinated on PDA within 24 h, and colonies reached a diameter of 85 mm after four days of incubation. Colonies initially produced white, downy mycelia that gradually turned pale green and, with age, dark green. Conidiophores were branched and produced in pustules with the formation of secondary branches. These secondary branches were typically paired and asymmetric, terminating in either a single phialide or a whorl of two to three divergent phialides. Phialides were ampulliform. Conidia were dark green and subglobose (Figure 1).

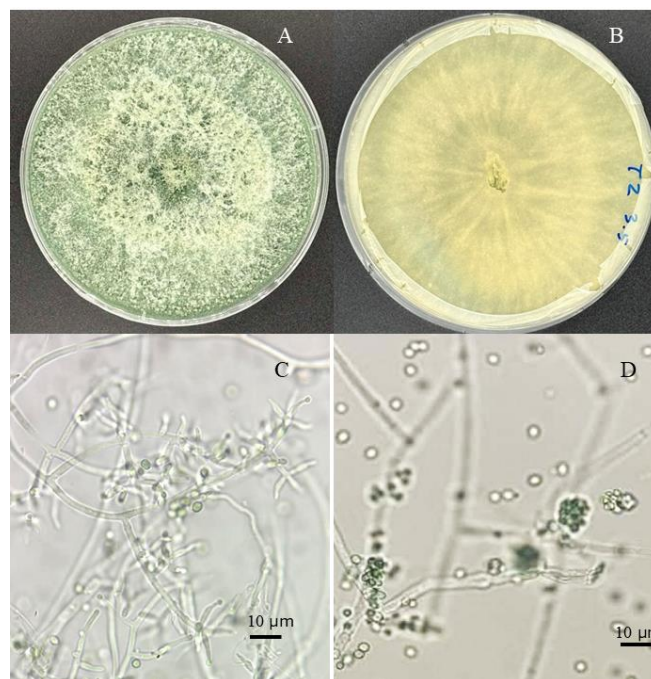


Figure 1. Morphological characteristics of the fungal strain TSUxPT3.5. Colony morphology on potato dextrose agar (PDA) showing the top view (A) and bottom view (B); microscopic structures including conidiophores and phialides (C); and conidia (D). Scale bars = 10 μm .

Based on ITS region sequence analysis, the fungal strain TSUxPT3.5 was identified as a member of the genus *Trichoderma*. Species-level identification was subsequently performed using multi-locus phylogenetic analysis of the *tef1* and *rpb2* gene regions. Strain TSUxPT3.5 exhibited high sequence similarity to *Trichoderma asperelloides*, showing 99.81% and 100% identity for the *tef1* and *rpb2* genes, respectively. According to the molecular identification guidelines proposed by Cai and Druzhinina [39], a query strain can be reliably assigned to an existing *Trichoderma* species when sequence similarity is $\geq 97\%$ for *tef1* and $\geq 99\%$ for *rpb2*, provided that both loci indicate the same species. Phylogenetic analysis further revealed that strain TSUxPT3.5

clustered within a strongly supported monophyletic clade of *Trichoderma asperelloides* with 100% bootstrap support (Figure 2). In the present study, multi-locus phylogenetic analysis consistently assigned strain TSUxPT3.5 to *T. asperelloides*, thereby confirming its taxonomic placement in accordance with established molecular identification criteria.

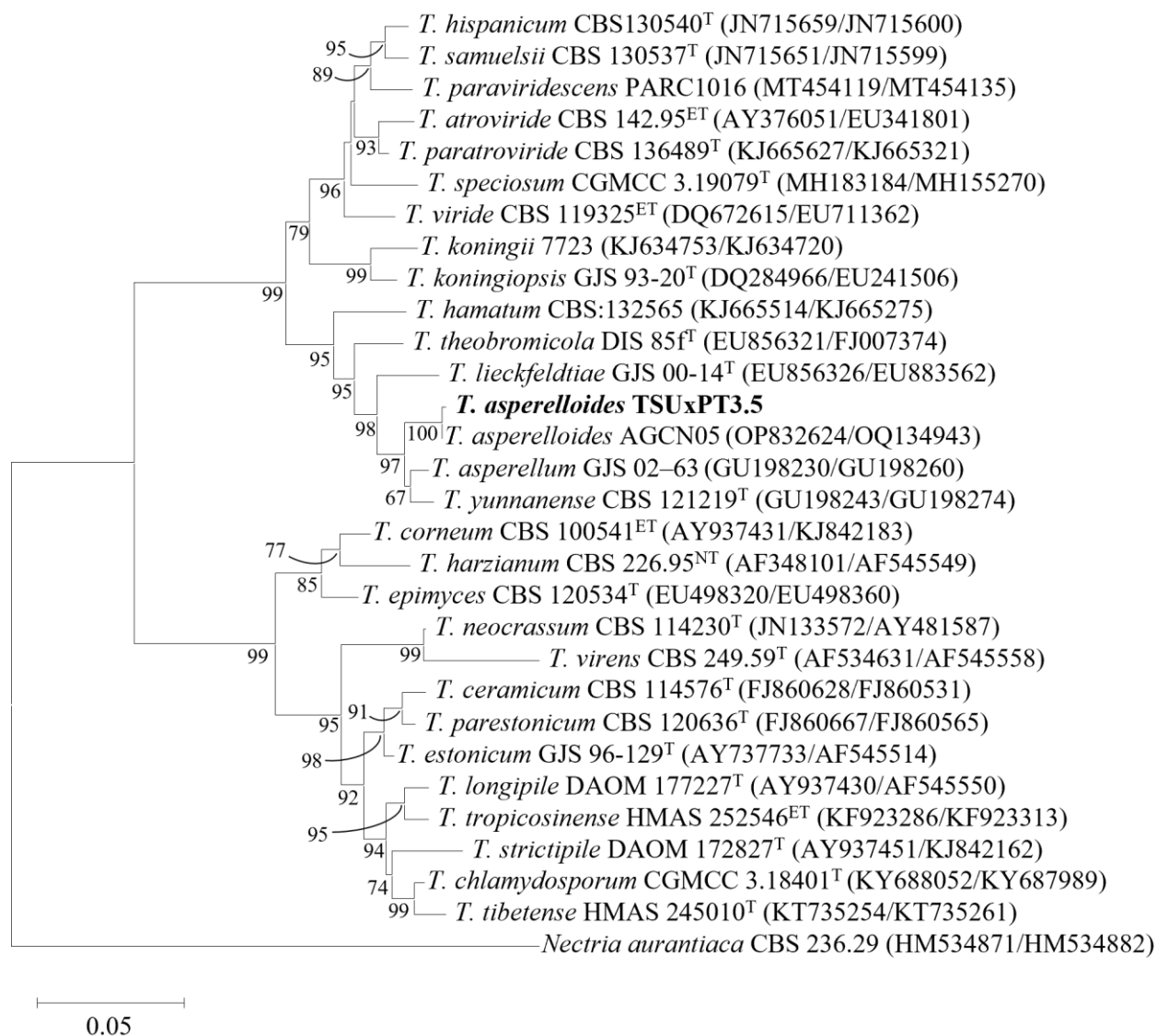


Figure 2. Phylogenetic tree based on combined *tef1* and *rpb2* gene sequences, constructed using the maximum likelihood method with the General Time Reversible (GTR) model, showing the phylogenetic relationships of *Trichoderma* sp. strain TSUxPT3.5 with representative species of the genus *Trichoderma* and related taxa. The numbers in parentheses are GenBank accession numbers of the *tef1/rpb2* sequences. *Nectria aurantiaca* was used as an outgroup in these analyses. Sequences of fungal species obtained in this study are bold. Superscript T, ET, and NT represent type, epitype, and neotype culture, respectively.

3.2 Competitive ability of *Trichoderma asperelloides* strain TSUxPT3.5 in dual culture assay

The results showed that strain TSUxPT3.5 inhibited the mycelial growth of the tested pathogens, *C. aerea* and *Co. cassiicola*. After 3 days of incubation, *T. asperelloides* TSUxPT3.5 began to grow toward the pathogen colonies, and an interaction zone was observed between the mycelia of the antagonist and the pathogens (Figure 3). The growth of both pathogens was noticeably restricted compared with the control

plates. After 5 days of incubation, *T. asperelloides* TSUxPT3.5 rapidly colonized the culture medium and overgrew the pathogen colonies. The mycelia of *Trichoderma* intertwined with those of the pathogens, and browning was observed at the interaction zone. The inhibition rates of *T. asperelloides* TSUxPT3.5 against *C. aeria* and *Co. cassiicola* were 49.78% and 59.81%, respectively, indicating stronger antagonistic activity against *Co. cassiicola*.

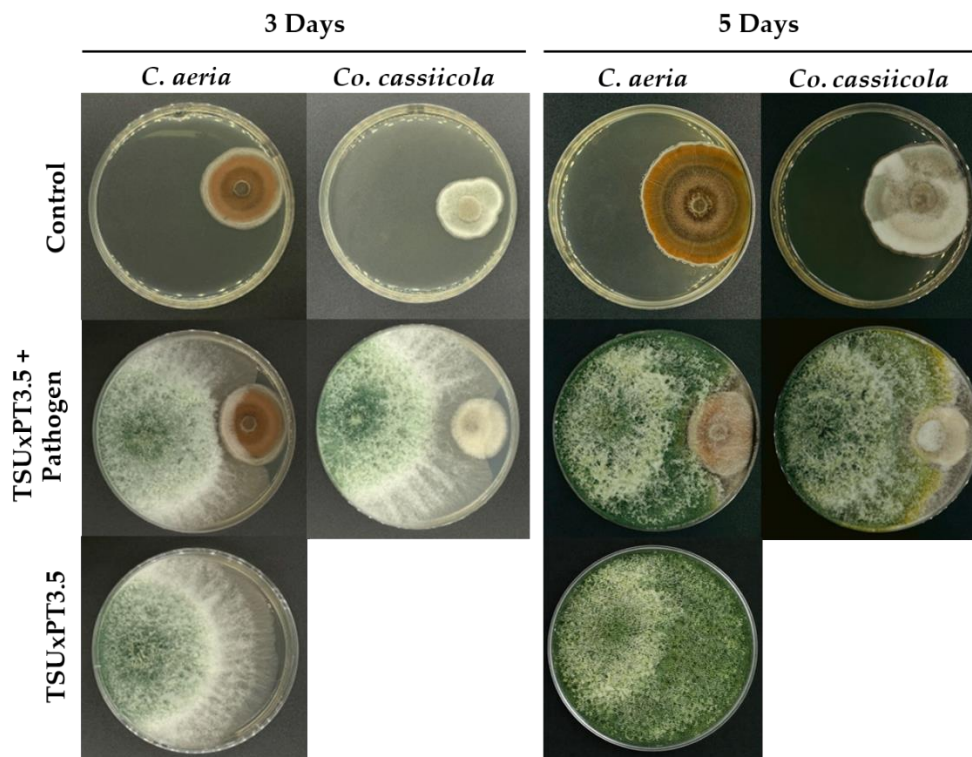


Figure 3. Dual culture assay illustrating the antagonistic interaction between *T. asperelloides* strain TSUxPT3.5 and the plant pathogenic fungi *C. aeria* and *Co. cassiicola* on potato dextrose agar after 3 and 5 days of incubation. Control plates containing only the pathogens and plates containing *T. asperelloides* TSUxPT3.5 alone are included for comparison.

3.3 Qualitative chitinase and cellulase activity of *Trichoderma asperelloides* strain TSUxPT3.5

In the present study, *T. asperelloides* TSUxPT3.5 exhibited positive activity for both cellulase and chitinase production. However, cellulolytic activity was relatively weak, as indicated by the formation of a narrow substrate degradation zone beneath the fungal colony. In contrast, chitinase activity was more pronounced, as evidenced by a broader clear zone on chitin agar (Figure 4).

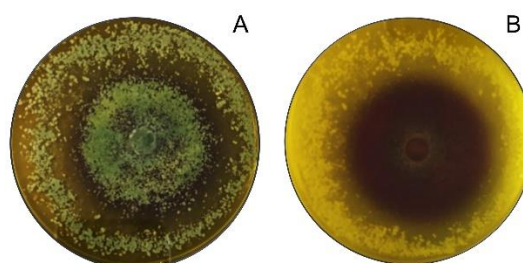


Figure 4. Chitinase activity of *T. asperelloides* TSUxPT3.5 on chitin agar: (A) top view and (B) bottom view.

3.4 *In vitro* antifungal activity of VOCs produced by *Trichoderma asperelloides* strain TSUxPT3.5

The inhibitory effect of VOCs produced by *T. asperelloides* TSUxPT3.5 against the tested pathogens differed between species. For *C. aeria*, the percentage inhibition was 7.15%. In contrast, *Co. cassiicola* showed a

higher level of inhibition, with a 27.24% inhibition. These results suggest that VOCs produced by *T. asperelloides* TSUxPT3.5 may contribute to inhibiting fungal pathogens (Figure 5).

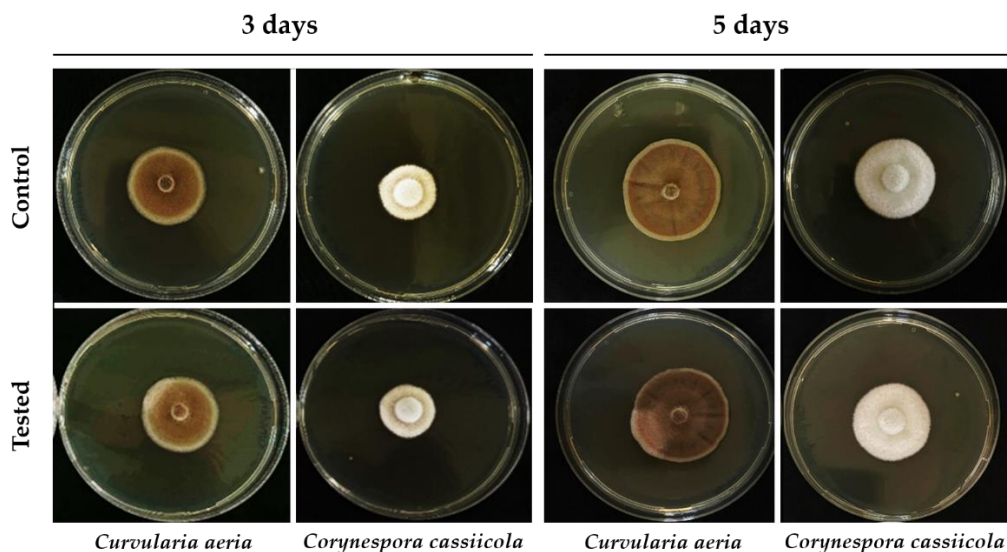


Figure 5. Antifungal activity of volatile organic compounds (VOCs) produced by *Trichoderma asperelloides* strain TSUxPT3.5 against *C. aeria* and *Co. cassiicola* using a sealed plate assay. Fungal growth was assessed after 3 and 5 days of incubation. Control plates represent pathogen growth without exposure to *Trichoderma*, while tested plates represent pathogen growth exposed to VOCs emitted by strain TSUxPT3.5.

3.5 Volatile organic compounds produced by *Trichoderma asperelloides* strain TSUxPT3.5

Volatile organic compounds (VOCs) produced by *Trichoderma* spp. have been widely reported to exhibit antifungal activity. In this study, the VOCs emitted by *T. asperelloides* TSUxPT3.5 were analyzed using solid-phase microextraction coupled with gas chromatography–mass spectrometry (SPME/GC–MS). A total of six volatile compounds with $\geq 90\%$ similarity were tentatively identified by comparison with the NIST mass spectral library (Table 1). Among the detected compounds, 2-ethylhexan-1-ol was the predominant, followed by 6-pentyl-2H-pyran-2-one, a well-known antifungal metabolite produced by *Trichoderma* species. These results indicate that strain TSUxPT3.5 can produce multiple VOCs, which may contribute to its antifungal activity.

Table 1. International Union of Pure and Applied Chemistry (IUPAC) names of volatile compounds produced by *T. asperelloides* TSUxPT3.5 identified by SPME/GC/MS analysis

Retention time (min)	Libraries identify with the IUPAC name	Formula	Similarity (%)	Area (%)
7.80	2-Methyl-1-propanol	C ₄ H ₁₀ O	90	1.34
13.52	2-Ethylhexan-1-ol	C ₈ H ₁₈ O	90	14.66
27.75	6-Pentyl-2H-pyran-2-one	C ₁₀ H ₁₄ O ₂	91	5.12
27.91	4-Isopropyl-1,6-dimethylnaphthalene	C ₁₅ H ₁₈	98	1.86
29.86	2,4-Di-tert-butylphenol	C ₁₄ H ₂₂ O	94	2.32
31.09	Diethyl benzene-1,2-dicarboxylate	C ₁₂ H ₁₄ O ₄	96	1.11

3.6 Indole compounds production by *Trichoderma asperelloides* strain TSUxPT3.5

Indole-producing fungi have been widely recognized for their potential to enhance plant growth and alleviate abiotic stress, thereby improving agricultural productivity. In this study, *T. asperelloides* TSUxPT3.5 was evaluated for its ability to produce the indole compounds. *Trichoderma asperelloides* TSUxPT3.5 exhibited positive indole compound production, as indicated by the development of pink coloration in the culture supernatant, whereas no visible color change was observed in the control (Figure 6).



Figure 6. Colorimetric assay of indole compound production of *T. asperelloides* TSUxPT3.5.

3.7 *Trichoderma asperelloides* strain TSUxPT3.5 enhances plant growth and reduces leaf disease severity

At 29 days after planting, plants treated with commercial *T. asperellum* (33.00 cm) and *T. asperelloides* TSUxPT3.5 (33.90 cm) showed no significant difference in plant height; however, both treatments produced significantly taller plants than the untreated control (27.53 cm). Similarly, the number of leaves per plant was higher in plants treated with *T. asperelloides* TSUxPT3.5 (28.40 leaves plant⁻¹) and *T. asperellum* (27.04 leaves plant⁻¹) than in the control (24.72 leaves plant⁻¹), with no significant difference between the two *Trichoderma* treatments (Table 2 and Figure 7). Fresh weight was significantly affected by the treatments, with *T. asperelloides* TSUxPT3.5 exhibiting the highest (81.00 g plant⁻¹), followed by *T. asperellum* (68.00 g plant⁻¹), while the untreated control showed the lowest (63.00 g plant⁻¹). For disease severity, no disease symptoms were observed in plants treated with *T. asperelloides* TSUxPT3.5 (0.00%), whereas low disease severity was observed in *T. asperellum*-treated plants (0.80%). The highest disease severity was found in the untreated control (2.20%). However, since the experiment was conducted under natural infection conditions with relatively low overall disease pressure, these results should be interpreted cautiously regarding the efficacy of disease suppression. The F-test indicated significant differences among treatments for all measured parameters at $P < 0.01$, indicating good treatment discrimination (Figure 7).

Table 2. Effects of *T. asperelloides* TSUxPT3.5 on plant growth parameters and disease severity of mini cos lettuce at 29 days after planting.

Treatment	Plant height (cm)	Leaves plant ⁻¹	Marketable fresh weight (g plant ⁻¹)	Disease severity (%)
<i>Trichoderma asperellum</i>	33.00 ± 0.83 ^a	27.04 ± 1.45 ^a	68.00 ± 0.44 ^b	0.80 ± 0.55 ^b
<i>Trichoderma asperelloides</i> TSUxPT3.5	33.90 ± 0.86 ^a	28.40 ± 1.53 ^a	81.00 ± 0.52 ^a	0.00 ± 0.00 ^b
Control	27.53 ± 0.70 ^b	24.72 ± 1.33 ^b	63.00 ± 0.40 ^c	2.20 ± 1.50 ^a
C.V. (%)	2.53	5.38	0.64	68.31
F-Test	**	**	**	**

**, significant at $P < 0.01$.

Means within a column followed by different letters are significantly different according to the least significant difference (LSD) test at $P < 0.05$.

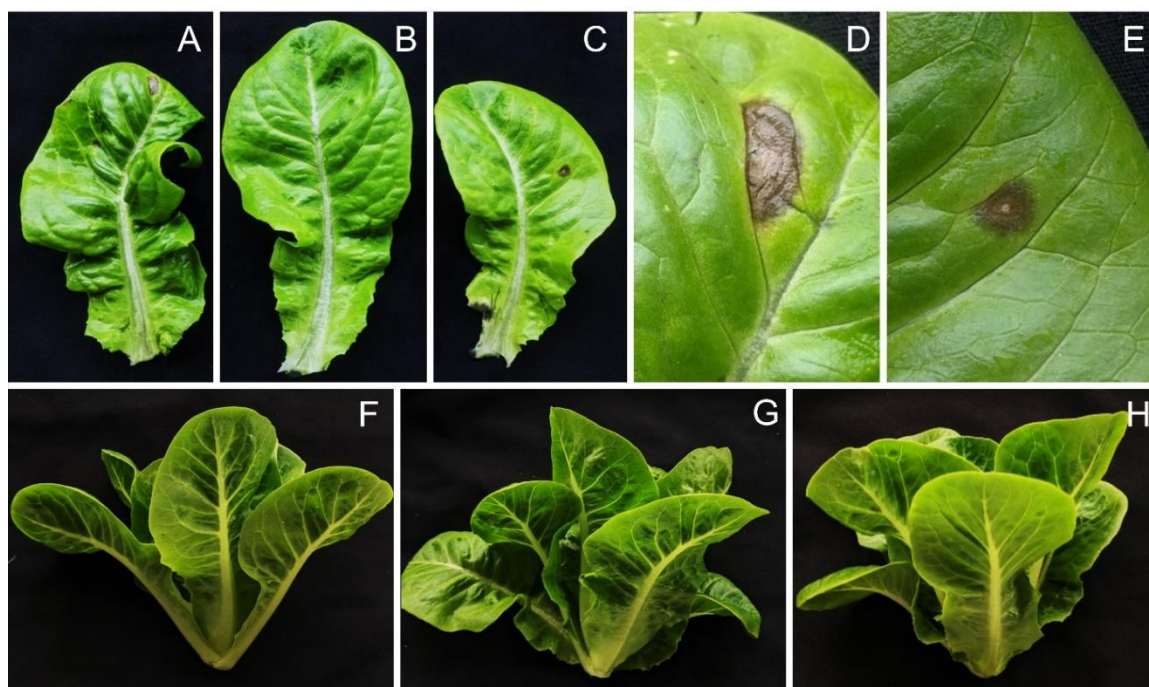


Figure 7. Disease symptoms and plant growth of mini cos lettuce (*Lactuca sativa* L.) under natural infection with different treatments. Control (A), *T. asperelloides* strain TSUxPT3.5 (B), and *Trichoderma asperellum* (C). Close-up of leaf spot symptoms in the control (D) and *T. asperellum*–treated plants (E) caused by *Corynespora cassiicola*. Growth of mini cos lettuce under different treatments: control (F), *T. asperelloides* TSUxPT3.5 (G), and *T. asperellum* (H).

4. Discussion

The antagonistic activity of *T. asperelloides* TSUxPT3.5 against the plant pathogenic fungi *C. aeria* and *Co. cassiicola* can be explained by several well-established biocontrol mechanisms. These include competition for nutrients and space, the production of extracellular cell wall-degrading enzymes such as chitinases and cellulases, and the emission of volatile organic compounds (VOCs) [15–18, 40]. Cell wall-degrading enzymes play a crucial role in weakening pathogen structures and facilitating mycoparasitism, whereas VOCs enable *Trichoderma* spp. to inhibit pathogen growth without direct physical contact [41]. Chitinase is a key cell wall-degrading enzyme that hydrolyzes chitin, a major structural component of fungal cell walls, including those of many phytopathogenic fungi. The production of chitinase is a well-established biocontrol mechanism employed by antagonistic fungi, which degrades the pathogen's cell wall by hydrolyzing β -1,4 linkages between N-acetylglucosamine residues, ultimately leading to cell lysis and death. In the present study, 2-ethylhexan-1-ol was the predominant VOC produced by strain TSUxPT3.5, which is consistent with previous reports identifying this compound among volatile metabolites emitted by *Trichoderma* species [42], followed by 6-pentyl-2H-pyran-2-one. Several of the detected VOCs, including 2-ethylhexan-1-ol [43–45], 6-pentyl-2H-pyran-2-one [46–49], 2,4-di-tert-butylphenol [50–52], and 2-methyl-1-propanol [53], have previously been reported to inhibit fungal growth. The combined presence of multiple VOCs may also result in synergistic antifungal effects, thereby enhancing the inhibitory activity against plant pathogenic fungi. Among the detected VOCs, 6-pentyl-2H-pyran-2-one, a well-known antifungal metabolite commonly produced by *Trichoderma*, and 2,4-di-tert-butylphenol, a phenolic compound previously reported to exhibit antifungal activity, may contribute to the inhibition of plant pathogenic fungi observed in this study. In addition, 2-ethylhexan-1-ol, the major VOC detected in this study, has been reported to exhibit antifungal activity against several plant pathogenic fungi. It should be noted that only VOCs with similarity scores $\geq 90\%$ were tentatively identified and included in this study. Therefore, additional unidentified volatile compounds may also contribute to the observed antifungal activity of *T. asperelloides* TSUxPT3.5. The inhibitory effects observed in the VOC assay were lower than those obtained in the dual culture assay, suggesting that volatile compounds

alone may contribute only partially to the antagonistic activity of *T. asperelloides* TSUxPT3.5. The stronger inhibition observed in dual culture assays may result from the combined effects of multiple biocontrol mechanisms, including nutrient and space competition, secretion of extracellular hydrolytic enzymes, and the production of both volatile and diffusible antifungal metabolites. The coexistence of multiple antagonistic mechanisms suggests that strain TSUxPT3.5 employs a multifaceted mode of action, which is advantageous for durable disease suppression and may reduce the risk of resistance development in target pathogens.

The present study demonstrated that *Trichoderma asperelloides* TSUxPT3.5 produced indole compounds, as detected by the Salkowski colorimetric assay. This assay is widely used as a rapid method for screening indole compound production by microorganisms [38, 54]. Although the reagent reacts with several indole compounds, including indole-3-acetic acid (IAA), indolepyruvic acid, and indoleacetamide [55], IAA has been reported as the predominant auxin produced by many plant-associated microorganisms [56]. Therefore, high-performance liquid chromatography (HPLC) or gas chromatography–mass spectrometry (GC–MS) may be used in future studies to precisely identify and quantify the indole compounds produced by strain TSUxPT3.5. Generally, *Trichoderma*-mediated plant growth promotion is associated with the production of fungal signaling metabolites that diffuse through the soil and exhibit hormone-like effects, including indole compounds [57]. Consistent with this observation, application of strain TSUxPT3.5 resulted in a statistically significant enhancement of mini cos lettuce growth compared with the untreated control. Similar observations have been reported in previous studies, where plant growth promotion by *Trichoderma* spp. was attributed to auxin-like compounds, modification of root system architecture, and improved nutrient acquisition, even in the absence of direct IAA quantification [58, 59]. Therefore, the observed growth-promoting effect supports the functional role of strain TSUxPT3.5 as a plant growth-promoting fungus under the experimental conditions.

Trichoderma asperelloides has been extensively reported as an effective biological control agent against a wide range of soilborne and foliar plant pathogens, as well as a promoter of plant growth in diverse cropping systems [16–18]. The findings of this study further support the concept that biocontrol efficacy is highly strain-dependent, underscoring the importance of selecting indigenous strains well adapted to local environmental conditions [60]. The multifunctional traits exhibited by strain TSUxPT3.5, including antagonistic activity, enzyme production, VOC emission, and plant growth promotion, underscore its strong potential for use in sustainable lettuce production. Future studies should focus on the precise identification and quantification of bioactive metabolites, validation under field conditions, and the development of suitable formulations to enhance shelf life and field efficacy. Such efforts will facilitate the integration of *T. asperelloides* TSUxPT3.5 into practical biological control programs and sustainable crop management strategies.

5. Conclusions

This study demonstrated that the indigenous fungus *Trichoderma asperelloides* strain TSUxPT3.5 possesses strong biocontrol potential against multiple economically important plant pathogenic fungi, as well as the ability to promote plant growth in mini cos lettuce (*Lactuca sativa*). Strain TSUxPT3.5 exhibited broad-spectrum antagonistic activity mediated by a combination of mechanisms, including competition for nutrients and space, production of fungal cell wall-degrading enzymes, and emission of antifungal volatile organic compounds. In addition to its antagonistic properties, *T. asperelloides* TSUxPT3.5 produced indole compounds and significantly enhanced lettuce growth, resulting in increased fresh weight and improved plant vigor compared with the untreated control. These findings highlight the potential of *T. asperelloides* TSUxPT3.5 as an eco-friendly biological control agent and plant growth promoter for sustainable lettuce production. Further studies focusing on metabolite characterization, formulation development, and field validation will be essential to support its practical application in integrated disease management programs and to maximize its contribution to sustainable agriculture.

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