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In vitro activities assessment of *Trichoderma asperellum* against soilborne pathogenic fungi

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ABSTRACT

Increasing awareness of the environmental and health risks associated with chemical pesticides has intensified the demand for sustainable alternatives to manage harmful plant pathogens. Biological control using *Trichoderma* species has emerged as a promising approach, but detailed evaluations of their antagonistic mechanisms remain essential for developing effective biocontrol products. The in vitro antagonistic activity of a Moroccan strain of *Trichoderma asperellum* (KU987252) is investigated in this study, isolated from compost, against four major soilborne pathogens affecting several vegetable crops: *Rhizoctonia solani*, *Fusarium oxysporum*, *Phytophthora* sp., and *Verticillium dahliae*. This work represents the first evaluation of a *T. asperellum* strain simultaneously against these four key pathogens, providing a comprehensive understanding of its antagonistic spectrum. The objective was to determine the efficiency and mechanisms of antagonism through direct confrontation, volatile-mediated, and diffusible metabolite assays, as a crucial step toward the development of a biological product based on this strain. We hypothesized that the strain would express multiple antagonistic strategies, both in direct contact and at a distance, resulting in strong suppression of pathogen development. The results confirmed that *T. asperellum* significantly inhibited pathogen growth, with inhibition ranging from 55.3% against *V. dahliae* to 86.7% against *F. oxysporum* in direct assays. Spatial colonization reached 85% against *F. oxysporum* and 82% against *Phytophthora* sp., highlighting its strong competitive ability. In indirect assays, *Phytophthora* sp. showed the highest inhibition of sporulation (96.0%), while *V. dahliae* exhibited the greatest inhibition of germination (84.9%). These pathogen-specific responses demonstrate the versatility of *T. asperellum* through both contact and metabolite-mediated interactions. These findings emphasize both the originality and applied potential of this Moroccan strain. By demonstrating its broad-spectrum antagonism, this study lays the foundation for developing compost-derived *T. asperellum* as a biocontrol product for sustainable tomato disease management.

Keywords: *Trichoderma asperellum*, antagonism, inhibition, soilborne pathogens, sporulation, germination, mycelial growth.

INTRODUCTION

Growing dependence on chemical pesticides in agriculture has become a critical issue, as their persistence in ecosystems and risks to human health continue to raise concern [Baweja et al., 2020; FAO, 2020]. Pesticides have been reported to cause soil pollution, biodiversity loss, and direct toxicity to non-target organisms. Additionally, they contribute to persistent organic pollutants in agricultural soils, impacting soil fertility and long-term crop productivity [Jat et al., 2022]. Among the soilborne fungal pathogens threatening crops, *Rhizoctonia solani* [El Haddadi et al., 2020], *Fusarium oxysporum* [El Haddadi et al., 2019], *Verticillium dahliae*, [Boukharta et al., 2012; Mouria et al., 2014] and *Phytophthora* spp. have proven particularly

destructive [Albatnan et al., 2018, 2020; El Haddadi et al., 2021]. These pathogens persist in the soil through resistant structures such as sclerotia, chlamydospores, and microsclerotia [Hoitink et al., 1991; McKeen and Wensley, 1961; Wilhelm, 1955; Douira et al., 1989], enabling them to survive for years without host plants [Chliyeh et al., 2017; Abdellaoui et al., 2017, 2020; Qostal et al., 2025]. Their aggressiveness and broad host range make them especially problematic in horticultural systems like tomato cultivation. In response, research has increasingly focused on using biological control agents (BCAs) as environmentally friendly alternatives [Elbouazaoui et al., 2022a, 2022b; Benjelloun et al., 2021; Errifi et al., 2024a, 20234b; Sellal et al., 2024; El Rhoch et al., 2025]. Among these, fungi from the genus Trichoderma have shown strong antagonistic activity against plant pathogens [Kribel et al., 2025]. Trichoderma spp. act through multiple mechanisms, such as competition for space, producing hydrolytic enzymes, synthesis of antimicrobial compounds, and boosting plants' natural defenses [Fravel, 2005; Zheng et al., 2021]. Moreover, *Trichoderma asperellum* has been recognized for its ability to inhibit pathogenic growth and sporulation *in vitro*, demonstrating promise for early-stage disease control [Hmouni et al., 2006; Mouria et al., 2007; Qostal et al., 2021; Mouden et al., 2023; El Kaissoum et al., 2022, 2024]. Despite numerous studies highlighting the biocontrol and growth-promoting potential of the *Trichoderma asperellum* strain KU987252 (Moroccan isolate), most investigations have remained limited to specific pathogens or single plant systems. For instance, the strain was evaluated for its antagonistic potential toward *Colletotrichum gloeosporioides*. [El Kaissoumi et al., 2022; Kaissoumi et al., 2024], against *Rhizoctonia solani* [Azeddine et al., 2024], or assessed for its potential to enhance plant growth [Najoua et al., 2022; Chahdi et al., 2025]. Its potential for phosphate solubilization has also been documented [Kribel et al., 2019]. However, no study to date has simultaneously examined its antagonistic activity against the four most destructive soilborne fungi, namely *Verticillium dahliae, Rhizoctonia solani, Phytophthora* sp. and *Fusarium oxysporum.*

The purpose of this research was to investigate the antagonistic effectiveness of *Trichoderma asperellum* KU987252 under in vitro conditions against four phytopathogenic fungi through direct and indirect confrontation assays. We hypothesize that this strain will deploy multiple antagonistic strategies and exhibit pathogen-specific inhibition patterns. By addressing this gap, the study provides the first integrated assessment of a Moroccan *T. asperellum* strain against several major soilborne pathogens, thereby offering novel insights and a foundation for the development of compost-based biocontrol products.

MATERIALS AND METHODS

Fungal isolates and culture conditions

Pathogens

Four soilborne fungal pathogens were used in this study: *Rhizoctonia solani* (RsPOB1), *Fusarium oxysporum* (FoBST3), *Verticillium dahliae* (OMV5), and *Phytophthora* sp. (PMT9). These isolates were collected between 2023–2024 from symptomatic plants in different agricultural zones of Morocco: olive petioles (Boufekrane), saffron bulbs (Taliouine), olive tree roots (Meknes), and melon roots (Tifelt), respectively [Albatnan et al., 2025]. At each location, five symptomatic plants were sampled randomly, and tissues showing characteristic disease symptoms (root necrosis, vascular discoloration, rot) were disinfected by immersion in 1% sodium hypochlorite for 2 min and subsequently rinsed three times with sterile distilled water. Mycelial fragments (5 × 5 mm) were transferred aseptically onto PSA medium (composed of 200 g potato, 20 g sucrose, and 15 g agar per liter of distilled water) and maintained at 28 °C in the dark for seven days. Fungal colonies that appeared were re-inoculated onto fresh PSA medium until purification was complete. Pathogens were identified morphologically using a light microscope (Olympus BX43) by observing colony characteristics, spore morphology, and reproductive structures, and confirmed by comparison with standard descriptions (Watanabe, 2002). For subsequent experiments, each pathogen was maintained on PSA slants at 4 °C and sub-cultured weekly. For confrontation assays. Mycelial discs (5 mm in diameter) were collected from the margin of 7-day-old cultures incubated at 28 °C in darkness. For each pathogen, assays were carried out using three distinct biological replicates, and every replicate was repeated twice to verify reproducibility (Figure 1) (Table 1).

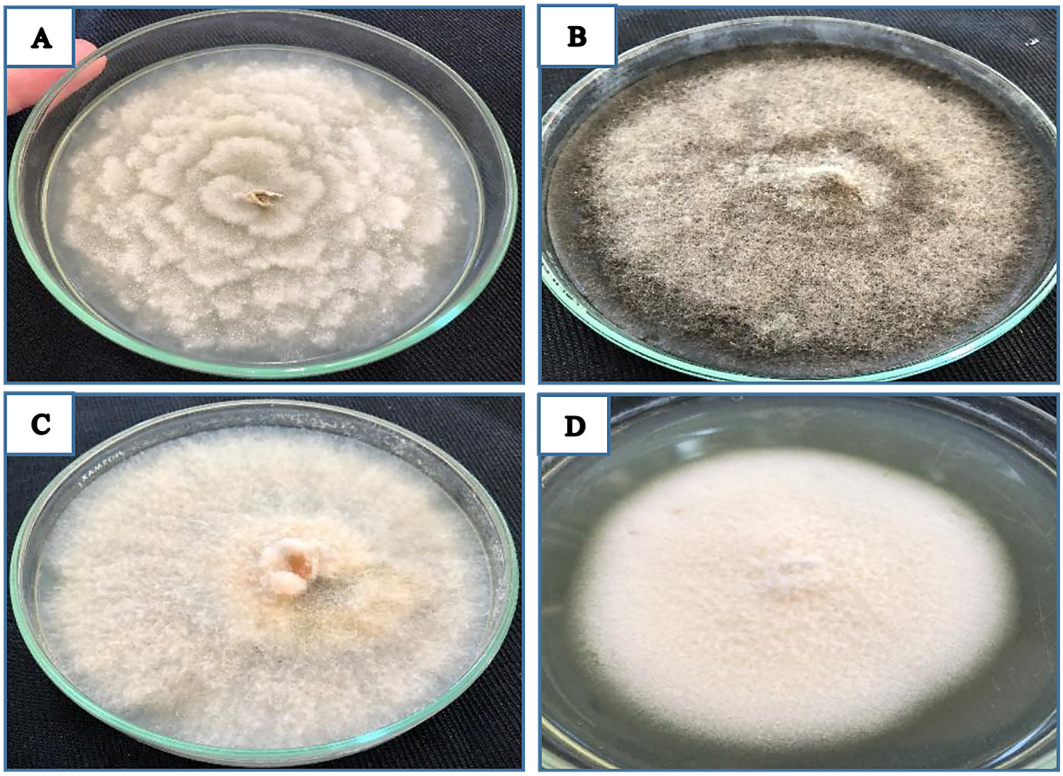

Figure 1. Soilborne fungal pathogens isolated in Morocco: (A) *Phytophthora* sp; (B) *Rhizoctonia solani*; (C) *Fusarium oxysporum*; (D)*Verticillium dahliae*

Table 1. Origin and characteristics of soilborne fungal pathogens used in this study

Pathogens	Code	Host plant (source tissue)	Sampling location	Symptoms observed	Reference
Rhizoctonia solani	RsPOB1	Olive (petioles)	Boufekrane	Necrotic lesions, leaf drop	Albatnan et al., 2025
Fusarium oxysporum	FoBST3	Saffron (bulbs)	Taliouine	Bulb rot, wilting	
Verticillium dahliae	OMV5	Olive tree (roots)	Meknes	Vascular discoloration, dieback	
Phytophthora sp.	PMT9	Melon (roots)	Tifelt	Root rot, damping-off	

Biocontrol agent

The *Trichoderma asperellum* strain KU987252, isolated from compost (Patent MA 41534) and preserved in the Mycotheque of Ibn Tofail University (Kenitra, Morocco), was used as the biocontrol agent.The strain was maintained on PSA medium (200 g potato, 20 g sucrose, 15 g agar, 1 L distilled water) at 25 °C in the dark for 7 days before use. [Khirallah et al., 2017]

For subsequent experiments, circular mycelial plugs (5 mm) were obtained from the growing border of colonies. The inoculum was standardized using three biological replicates per assay, with each replicate repeated twice to ensure reproducibility. Cultures were handled under sterile conditions in a laminar flow cabinet, and all manipulations were carried out using sterilized equipment (autoclaved Petri dishes, scalpels, and micropipettes).

Visual characterization of the strain was documented through colony morphology on PSA plates, and photographs were taken after 7 days of incubation (Figure 2).

Dual culture techniques

Pathogen – T. asperellum direct confrontation

Fungal interaction assay was performed *in vitro* to analyze the biocontrol ability of *Trichoderma asperellum* targeting several fungal species; *R. solani, F. oxysporum, Phytophthora* sp, and *V. dahliae.* This experiment was performed using PSA agar on Petri plates of 90 mm diameter. Two 5 mm diameter mycelium discs were

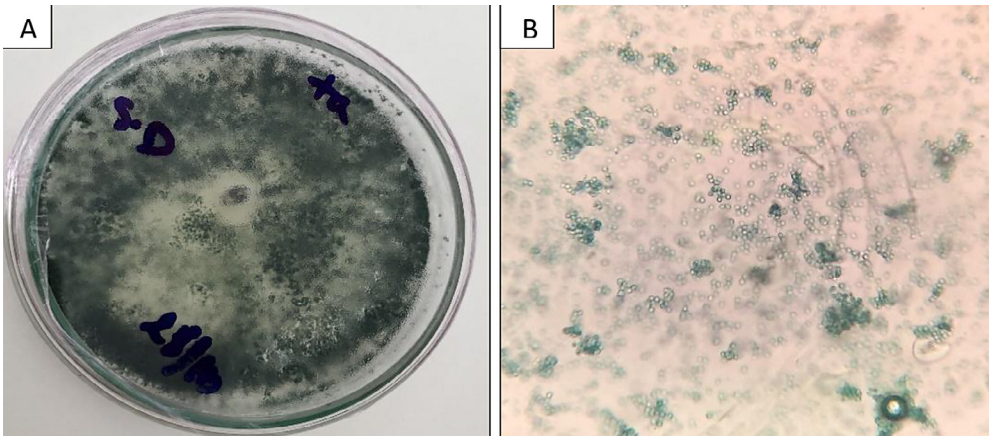

Figure 2. Morphology of *Trichoderma asperellum* KU987252: (A) colony on PSA, (B) microscopic view of conidia and mycelia

applied on each plate, one on the left side 40 mm in distance from the second disc and the second disc was positioned 25 mm from the edge of the Petri dish, as illustrated in Figure 3. This method was used for all the combinations (4): *R. s*+ *T. asperellum, F. o* + *T. asperellum, P.* sp + *T. asperellum,* and *V. d* + *T. asperellum.*

For the control treatment, a 5 mm diameter mycelial plug of each pathogen was inoculated at the center of a PSA plate. Incubation was carried out at 28 °C under dark conditions for 7 days. According to Comporta (1985), the percentage of space colonized by *T. asperellum* (%Cs) was determined as the ratio between the distance advanced by *T. asperellum* and the total distance (40 mm) separating it from the pathogen.

$$\%Cs = \left(\frac{\mathrm{DTa}}{D}\right) \times 100 \qquad (1)$$

where: D_{Ta} – distance covered by *T. asperellum* X, D – total distance (D: 40 mm).

Mycelial growth inhibition (%) in the dual culture assay was calculated according to the method of Sy [12]:

$$IC\ dc\ (\%) = (Dc1 - Dp1\ /Dc1) \qquad (2)$$

where: D_{c1} – diametric growth of control. (mm), D_{p1} – diametric growth of pathogen X in confrontation with *Ta*. (mm)

Pathogen – T. asperellum interaction through volatile compounds

This method involves placing a 4 mm diameter mycelial disc of each pathogen and the strain of Trichoderma at the center of each Petri dish containing PSA medium. The plate with *Trichoderma* is aseptically positioned below the plate with pathogens and both are sealed with Para film to prevent contamination and retain gases following the protocol of Camporota, 1985 and described by Adnani et al., (2024). The control was grown on potato saccharose agar (PSA) plates incubated at 28 °C and dark for 7 days.

The percentages of mycelial growth, germination and sporulation inhibition in the presence of the volatile substances (IC vs, IGvs % and (ISvs%) were calculated according to the formula previously mentioned in the direct confrontation section.

Pathogen – *T. asperellum* interaction through diffusible compounds

Diffusible compounds produced by the *Trichoderma asperellum* strain were examined using the cellophane technique as outlined by Adnani et al. (2024). PSA plates were overlaid with sterile cellophane sheets 24 h before inoculation. At the midpoint of the cellophane surface, a 4 mm disc removed from a 7-day-old *T. asperellum* colony was placed. Plates were incubated at 24 °C in darkness for 48 h. The cellophane sheet with the *Trichoderma* colony was carefully taken off, and a 7-day-old pathogen mycelial plug was set in the middle of the medium. A plug of pathogen mycelium was placed in the center of the control plates. Incubation was carried out at 28 °C under dark conditions for 7 days.

The percentage inhibition of pathogen growth by diffusible metabolites (PIDs%) was calculated relative to the control using the same formula as in the direct confrontation assay after three days

Evaluation of pathogen sporulation and germination in confrontation with *T. asperellum*

Sporulation

- Isolates were cultured on PSA plates at 25 °C for 7 days to induce sporulation, alone and in co-culture with *T. asperellum*.
- Mycelial plugs were transferred into a test tube with 1ml of sterile distilled water and vortexed.
- A 0.1 mL volume of the conidial suspension was deposited onto a Malassez counting chamber to quantify the spores, expressed as Nsp (number of conidia per mm²)

The inhibition percentage of pathogen sporulation when in co-culture with *T. asperellum* was calculated as follows:

$$Isp = \frac{X - PTa}{X} \times 100 \qquad (3)$$

where: X – spore number per mm² in untreated control, PTa – spore number per mm² of pathogen (X +*T.a*).

Germination

- The tested isolates were maintained on potato sucrose agar (PSA) plates and incubated at 25 °C for seven days.
- Mycelial plugs harvested from three-day-old cultures were transferred into sterile distilled water contained in test tubes.
- After vortexing suspensions were prepared as follows: conidial suspensions (Fo, Vd), a sporangial suspension (*P*), and a mycelial suspension (Rs).
- Two milliliters of each suspension were then spread onto PSA plates to obtain polyspore cultures. Incubation was carried out at 25 °C under dark conditions conditions for a period of four days, 0.2 mL of each pathogen suspension (10^3 conidia·mL^{-1} or equivalent) as well as the control suspension was inoculated onto PSA plates previously seeded with *T. asperellum* (10^3 conidia·mL^{-1}) (Figure 4).
- Spore germination was determined after 24 h of incubation at 28 °C under dark conditions, using the following equation.:

$$\%\, G = (NCG\ /NTC) \qquad (4)$$

where: N_{CG} – count of conidia germinated, N_{TC} – total conidia examinated (NTC = 200).

The Inhibition percentage of germination when in co-culture with *T. asperellum* was calculated as follows:

$$\%IG = (\%Gcc - \%Gcp/\%Gcc) \qquad (5)$$

where: G_{cc} – count of pathogen conidia germinated, G_{Cp} – conidia germination of pathogen (X +*T.a*).

Each treatment in vitro was repeated three times.

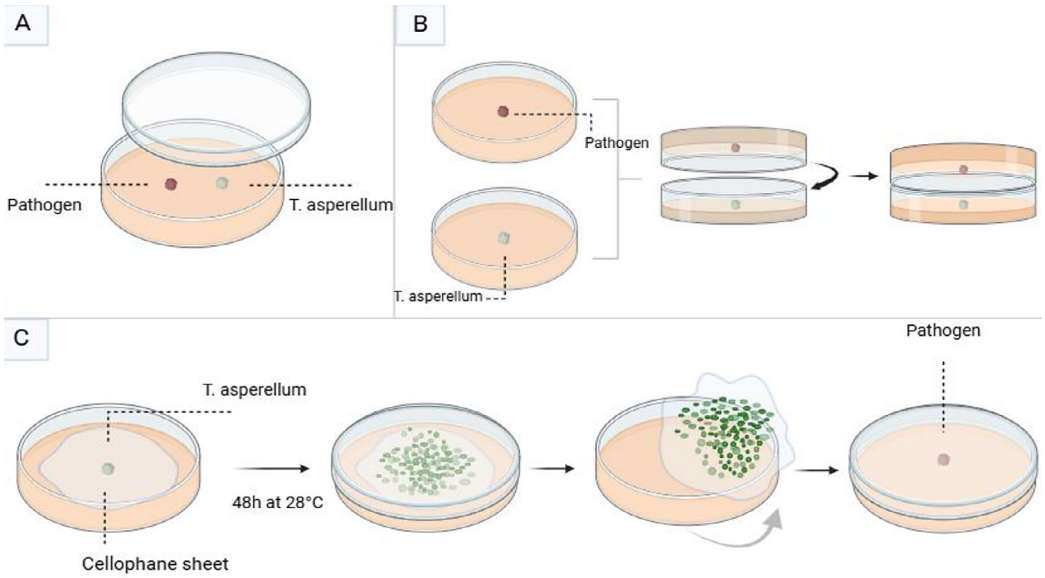

Figure 3. Confrontation techniques employed: direct dual culture (a), volatile-mediated interaction (b), and non-volatile metabolite assay (c), created with BioRender.com

RESULTS

Direct confrontation and spatial colonization

The dual culture assay revealed strong antagonistic activity of *Trichoderma asperellum* against *Rhizoctonia solani, Fusarium oxysporum, Phytophthora* sp., *and Verticillium dahliae.* In all cases, *T. asperellum* rapidly overgrew the available surface, limiting pathogen spread (Figure 5). Colonization reached 85% against *F. oxysporum*, 82% against *Phytophthora* sp., and about 68% against both *R. solani* and *V. dahliae* (Table 2). These results highlight the strong competitive ability of *T. asperellum* to occupy space and suppress pathogen development.

Microscopic observations (Figure 6) further confirm the antagonistic effect of *Trichoderma asperellum.* In the case of *Rhizoctonia solani*, the pathogen was completely overgrown and its hyphae showed clear structural damage, demonstrating direct mycoparasitism. For *Fusarium oxysporum*, the number of conidia was visibly reduced under the effect of diffusible metabolites, highlighting its sensitivity to biochemical inhibition.

Several types of interactions were observed, all leading to a decrease in pathogen growth, as shown in Figure 1. In direct confrontation, *T. asperellum* strongly inhibited the mycelial growth of *F. oxysporum* and *Phytophthora* sp. In the case of *R. solani, T. asperellum* exhibited a different interaction by completely overgrowing its mycelium. Conversely, *V. dahliae* showed only slight inhibition, with *T. asperellum* circumventing its mycelial growth rather than directly inhibiting it. (Figure 5)

Mycelial growth inhibition

The inhibition of pathogen growth varied according to the confrontation method (Table 2). In direct confrontation, the highest inhibition was observed in *F. oxysporum* (86.7%), while the lowest was recorded for *V. dahliae* (55.3%). Under volatile-mediated inhibition, *Phytophthora* sp. showed the strongest effect (79.7%), whereas *R. solani* displayed the lowest (55.8%). For diffusible substances, inhibition ranged from 23% (*F. oxysporum*) to 48% (*R. solani*).

Sporulation inhibition

Sporulation was significantly affected by *T. asperellum* (Table 2). In direct confrontation, the highest inhibition was observed for *V. dahliae* (68.8%), while the lowest occurred in *F. oxysporum* (41.2%). With diffusible metabolites, inhibition peaked for *Phytophthora* sp. (96.1%) but remained low for *F. oxysporum* (41.3%). In the case of volatile-mediated confrontation, inhibition values were moderate and relatively uniform across the four pathogens.

Germination inhibition

Conidial germination was strongly inhibited during direct confrontation (Table 2). *V. dahliae*

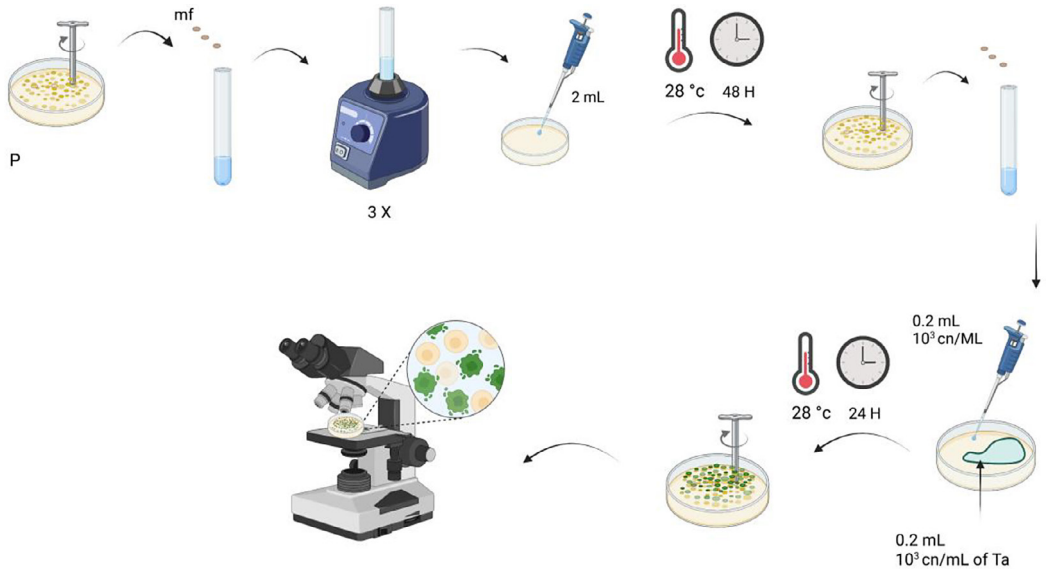

Figure 4. Conidial germination of soilborne pathogens under direct confrontation with *Trichoderma asperellum*. mf – mycelial fragments

Table 2. Antagonistic effect of *Trichoderma asperellum* on the four soil borne pathogenic fungi in direct and indirect confrontation and volatile molecule

Pathogenic Fungal species	Direct confrontation with *T. asperellum*				Indirect confrontation with *T. asperellum*					
					Diffusible substances			Volatile substances		
	Cs*T. Asperellum* (%)	ICdc (%)	IGdc (%)	ISdc (%)	ICds (%)	IGds (%)	%ISds (%)	ICvs (%)	IGvs (%)	ISvs (%)
RsPOB 1	68^{b}	71^{b}	*	*	48^{a}	*	*	55.89^{b}	*	*
FoBST 3	85^{a}	86.7^{a}	41.3^{c}	41.2^{c}	23^{c}	22.01^{ab}	41.34^{c}	83.36^{a}	92.02^{b}	29.7^{a}
PMT9	82^{a}	82^{a}	82.37^{b}	53.3^{b}	34^{b}	11.17^{c}	96.68^{a}	79.77^{a}	89.04^{c}	34.8^{a}
OMV5	68.45^{b}	55.3^{c}	84.88^{a}	68.83^{a}	23^{c}	55.41^{a}	86.05^{b}	63.41^{b}	95.37^{a}	18.4^{b}

Note: Two results followed by the same letter are not significantly different at the 5% level of significance. G – inhibition of germination; IS – inhibition of sporulation. * – *Mycelia sterilia* without conidia.

recorded the highest inhibition (84.9%), while *F. oxysporum* exhibited the lowest (41.3%). With diffusible substances, inhibition was again highest for *V. dahliae* (55.4%) and lowest for *Phytophthora* sp. (11.7%). Under volatile exposure, inhibition ranged from 18.4% (*V. dahliae*) to 34.8% (*Phytophthora* sp.).

DISCUSSION

The present study evaluated the antagonistic effects of *Trichoderma asperellum* KU987252, as well as the biocontrol and biostimulant efficacies of this strain's laboratory-made product against soil-borne plant pathogens *R. solani*, *F. oxysporum*, *Phytophthora* sp., and *V. dahliae*. The results of *in vitro* trials using various methods, such as direct and indirect confrontation, along with volatile molecules, confirmed the antagonistic effect of *T. asperellum* on different pathogens. Indeed, the percentage of mycelial growth inhibition varied depending on the method used and the specific pathogen. Several studies have shown that *T. asperellum* inhibits the mycelial growth of pathogens through various modes of action, including mycoparasitism, antifungal metabolite production [Chahdi et al., 2025; Elouark et al., 2025], and competition for resources, nutrition, and space [Adnani et al., 2024a, 2024b; Kribel et al., 2019] [Li et al., 2023; Chahdi et al., 2019; Ourras et al., 2025]. In this study, we observed that for *R. solani* and *V. dahliae*, the mechanism of mycelial growth inhibition employed by *T. asperellum* differed from that observed against *F. oxysporum* and *Phytophthora* sp. during direct confrontation. This variation may be related to their life cycles, morphology, infection strategies, and resistance to biocontrol agents. Due to their resistance and durability under stress, *R. solani* and *V. dahliae* exhibit different morphological characteristics, such as the production of sclerotia in *R. solani* and microsclerotia in *V. dahliae*. These protective barriers may render them less vulnerable to *T. asperellum*'s mycoparasitism. As confirmed by the study of Yao et al. (2023), *R. solani* and *V. dahliae* produce resilient structures that provide them with durability and resistance to biocontrol agents. In contrast, what might make *F. oxysporum* and *Phytophthora* spp. more vulnerable to the enzymatic activities of *T. asperellum* – such as chitinase and glucanase production – is the absence of such robust resting structures. This is supported by the results of Osorio-Hernández et al. (2016), which evaluated the *in vitro* activities of various *Trichoderma* species against *Phytophthora* and *F. oxysporum*. They found that *Trichoderma* exhibited significant chitinase and glucanase activities, leading to the inhibition of mycelial growth in these pathogens. Similarly, Li et al. (2023) demonstrated that hydrolytic enzymes produced by *T. asperellum* effectively broke down the cell walls of *F. oxysporum* during the biocontrol of blueberry root rot. The growth inhibition in indirect confrontation was markedly different, as *F. oxysporum* and Phytophthora sp. (34%) displayed the lowest inhibition rates, especially *F. oxysporum* (23%). The volatile organic compounds (VOCs) produced by *T. asperellum* are known to prevent the growth of various pathogens, even without direct contact. The effectiveness of the VOCs depends on the pathogens' sensitivity and the specific Trichoderma species. A study conducted by Li et al. (2023) found that VOCs emitted by *Trichoderma atroviride* effectively suppressed *F. oxysporum* in tomato seedlings, suggesting that the efficacy of VOCs can vary based on the *Trichoderma species* and the target pathogen. On the other hand,

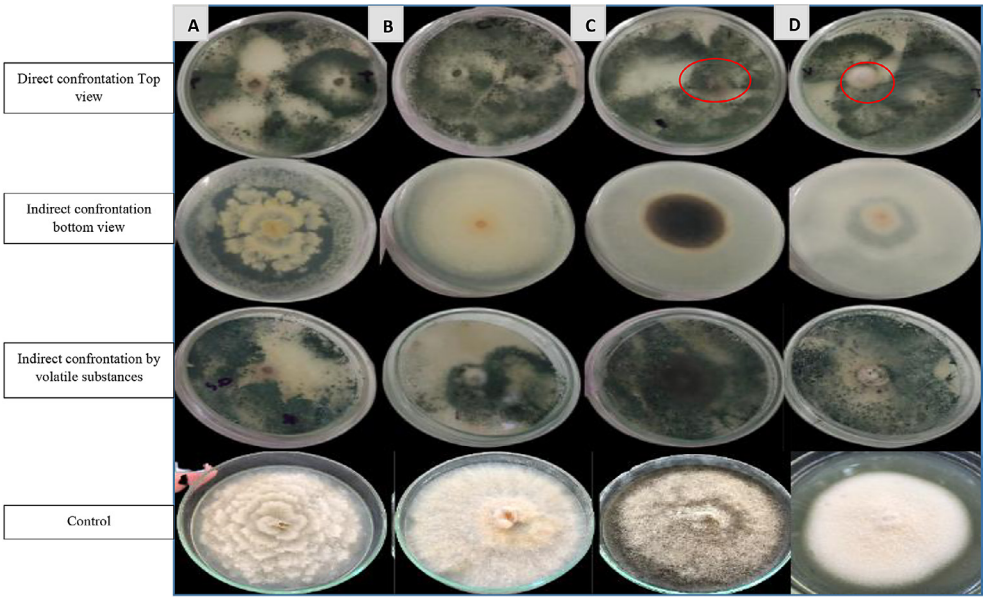

Figure 5. In vitro antagonistic activity of *T. asperellum* against four pathogens isolates in dual culture assay after 7 days of incubation at 28 °C. Top and bottom view of the Petri dish: (A) *Phytophthora* sp., (B) *F. oxysporum*, (C) *R. solani*, (D) *V. dahliae*

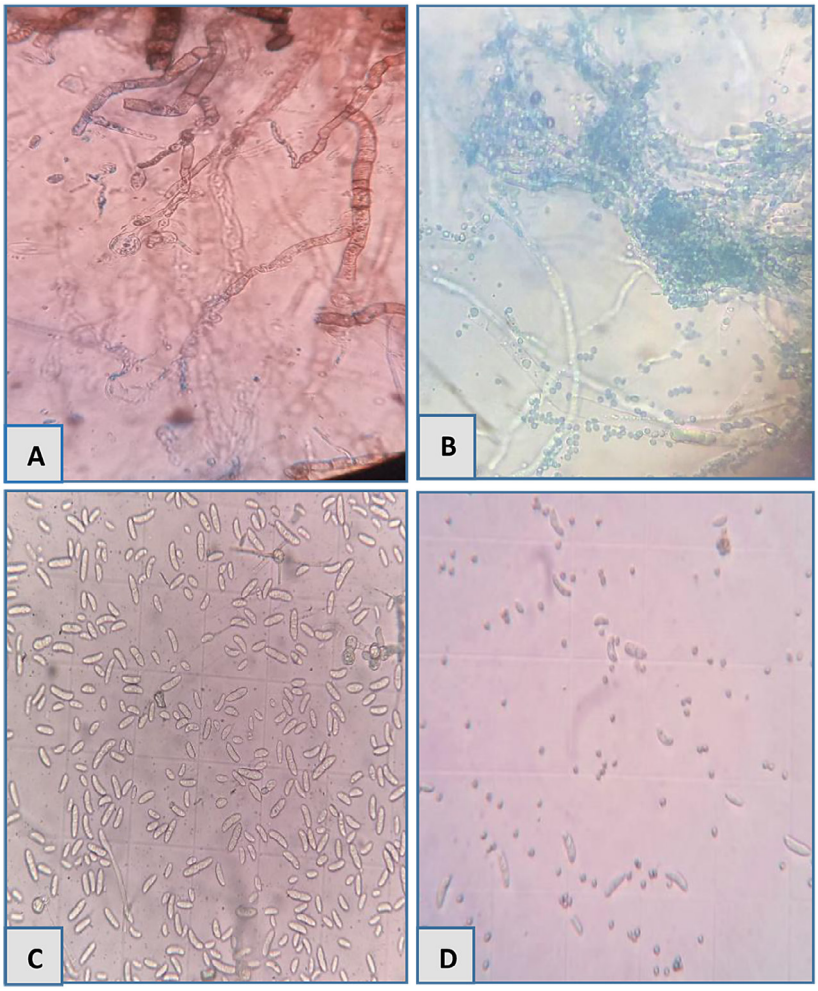

Figure 6. Microscopic views of *Rhizoctonia solani* (A – alone, B – with *Trichoderma asperellum*) direct confrontation and *Fusarium oxysporum* (C – alone, D – with *Trichoderma asperellum*) indirect confrontation by diffusible substances

another study by Zhang et al. (2014) demonstrated that *F. oxysporum* can induce the production of proteins and VOCs in *Trichoderma harzianum*, indicating a complex interaction that may influence the inhibitory efficacy. The culture media (PSA) might be another factor affecting the efficiency of VOCs against *F. oxysporum*. This was highlighted by the studies of Chávez-Avilés et al. (2024) and Rao et al. (2022), stating that *T. asperellum* strains produced different VOC profiles when cultured in various media, impacting their antifungal properties. However, in the same experiment, *V. dahliae* showed the highest inhibition rate, possibly due to its sensitivity to the VOCs produced by *Trichoderma* species. Some findings by Kong et al. (2022) suggest that VOCs emitted by *Trichoderma* species significantly inhibited the mycelial growth of *V. dahlia*e and reduced the severity of verticillium wilt in tobacco and cotton; one of the VOCs identified in the study, 3-octanone, was highly effective in suppressing *V. dahliae*. Similarly, another study by Morán-Diez et al. (2019) emphasized that VOCs produced by *Trichoderma* species played a crucial role in inhibiting *V. dahliae* growth, highlighting their potential as a biocontrol mechanism. Furthermore, the findings of

Carrero-Carrón et al. (2016) pointed out the sensitivity of *V. dahliae*, as they identified that *V. dahliae* is highly sensitive to small compounds produced by *T. asperellum*, and this strain has the strongest antagonistic potential against *V. dahliae*, at least *in vitro*.

CONCLUSION

The in vitro assays confirmed that *Trichoderma asperellum* effectively inhibited the growth, germination, and sporulation of key soilborne pathogens. These results highlight its strong potential as a biological control agent in controlled conditions. But would these antagonistic effects be maintained under greenhouse conditions, where plant-pathogen-microbe interactions become more complex? This question remains open for future investigation.

Acknowledgements

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