Evaluation of Indigenous *Trichoderma asperellum* Isolates for Suppression of Soil-Borne Plant Pathogens

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Received: 15 May 2025
Revised: 30 May 2025
Accepted: 20 Jun 2025
Published: 10 Jul 2025

E-ISSN: 2989-5340
DOI: https://doi.org/10.54660/jafi.2025.5.2.27-31

ABSTRACT

Soil-borne plant pathogens cause substantial agricultural yield losses globally, traditionally managed through the heavy application of synthetic fungicides. Due to the ecological degradation, human health risks, and development of pathogen resistance associated with chemical control, biological alternatives are urgently required. This study aimed to isolate, characterize, and evaluate indigenous isolates of *Trichoderma asperellum* for their antagonistic potential against three major soil-borne phytopathogens: *Fusarium oxysporum* f. sp. *lycopersici* (Fol), *Rhizoctonia solani* (Rs), and *Sclerotium rolfsii* (Sr). Five indigenous isolates (designated TA1 through TA5) were recovered from the rhizosphere of healthy tomato plants and identified using morphological traits and internal transcribed spacer (ITS) sequencing. *in vitro* dual culture assays demonstrated that isolate TA3 exhibited the highest mycelial growth inhibition across all tested pathogens, achieving 78.4% inhibition against Rs. Assays for volatile and non-volatile metabolites further confirmed the superior antibiosis capacity of TA3. Subsequent *in vivo* greenhouse experiments on tomato plants revealed that soil application of TA3 significantly reduced disease incidence by up to 65.2% compared to untreated controls, while concurrently promoting plant growth metrics such as height and biomass. These findings suggest that the indigenous *T. asperellum* isolate TA3 is a highly effective, ecologically adapted biocontrol agent suitable for integrated disease management programs in local agro-ecosystems.

Keywords: *Trichoderma asperellum*, Indigenous isolates, biological control, Soil-borne pathogens, *Fusarium oxysporum* f. sp. *lycopersici*, *Rhizoctonia solani*, *Sclerotium rolfsii*, Antagonistic activity, Integrated disease management, Tomato, Rhizosphere, Sustainable agriculture

1. Introduction

1.1. The Threat of Soil-Borne Pathogens

Soil-borne plant pathogens represent a formidable challenge to global food security (Agrios, 2005) [1]. Fungal pathogens such as *Fusarium oxysporum*, *Rhizoctonia solani*, and *Sclerotium rolfsii* are ubiquitous in agricultural soils and possess a broad host range, causing destructive diseases like vascular wilts, root rots, and damping-off. These pathogens produce resilient resting structures, such as chlamydospores and sclerotia, allowing them to survive in the soil for decades even in the absence of a suitable host plant (Agrios, 2005) [1]. The conventional paradigm for managing these pathogens has relied heavily on soil fumigation and the prophylactic application of synthetic fungicides. However, the continuous and indiscriminate use of these chemicals has led to severe environmental repercussions, including the disruption of non-target beneficial microbiota, contamination of groundwater aquifers, and the emergence of fungicide-resistant pathogen biotypes (Gisi and Sierotzki, 2008) [3].

1.2. Biological Control and *Trichoderma* Species

In response to the limitations and hazards of chemical control, the utilization of microbial antagonists for biological control has gained immense traction. Among these, fungi belonging to the genus *Trichoderma* are recognized as some of the most versatile and effective biocontrol agents (BCAs) (Harman *et al*., 2004) [4]. *Trichoderma* species are ubiquitous, free-living soil fungi that colonize plant roots and establish symbiotic relationships. They suppress pathogens through a multitude of mechanisms, primarily mycoparasitism, spatial and nutrient competition, and antibiosis via the secretion of secondary metabolites and cell-wall-degrading enzymes (CWDEs) such as chitinases (Benítez *et al*., 2004) [5] [14].

1.3. Focus on *Trichoderma asperellum*

Within the genus, *Trichoderma asperellum* has emerged as a particularly potent species. Previously grouped within the *Trichoderma viride* cryptic complex, *T. asperellum* was differentiated based on molecular phylogenetics (Samuels *et al*., 1999) [6]. It is highly aggressive against a wide array of phytopathogens and is also known to induce systemic resistance (ISR) in host plants, priming their defense transcriptomes against subsequent pathogen attacks (Shoresh *et al*., 2010) [7].

1.4. The Importance of Indigenous Isolates

While commercial formulations of *Trichoderma* are available, their field efficacy is frequently inconsistent. This variability is often attributed to the ecological mismatch between the introduced, non-native strains and the specific edaphic and climatic conditions of the target environment (Howell, 2003) [8]. Exogenous strains frequently fail to establish a stable population or compete with the indigenous microflora. Therefore, bioprospecting for native, locally adapted *Trichoderma* isolates is critical. Indigenous isolates are evolutionarily tailored to survive local abiotic stresses (such as pH fluctuations, extreme temperatures, and varying soil moisture) and are more likely to establish persistent, beneficial populations in the rhizosphere (Mukherjee *et al*., 2013) [9].

This research was undertaken with the explicit objective of isolating indigenous strains of *T. asperellum* from local agricultural soils, rigorously evaluating their antagonistic mechanisms *in vitro*, and validating their disease-suppressive and plant-growth-promoting efficacy *in vivo*.

2. Materials and Methods

2.1. Soil Sampling and Isolation of Trichoderma

Soil samples were collected from the rhizosphere of healthy, vigorously growing tomato (*Solanum lycopersicum*) plants in fields with a history of intensive cultivation but no visible disease symptoms. Samples were taken from a depth of 10 to 15 cm, placed in sterile polyethylene bags, transported to the laboratory in coolers at 4°C, and processed within 24 hours.

Isolation was performed using the serial dilution technique on *Trichoderma* Specific Medium (TSM). Ten grams of soil were suspended in 90 mL of sterile distilled water and agitated on a rotary shaker at 150 rpm for 30 minutes. Serial dilutions up to were prepared. A 0.1 mL aliquot of the dilutions was spread onto TSM plates. The plates were incubated in the dark at 28°C for 5 to 7 days. Putative *Trichoderma* colonies, identified by their characteristic rapid growth and concentric rings of green conidiation, were purified by transferring single hyphal tips to fresh Potato Dextrose Agar (PDA) plates (Samuels *et al*., 1999) [6].

2.2. Morphological and Molecular Identification

Initial identification was based on macroscopic and microscopic morphological characteristics (Samuels *et al*., 1999) [6]. Colony morphology, growth rate, and pigmentation were recorded on PDA. Microscopic features, including the branching pattern of conidiophores, the shape of phialides, and conidial morphology, were observed under a compound microscope at 400x magnification using lactophenol cotton blue staining.

For precise species-level identification, molecular characterization targeting the internal transcribed spacer (ITS) region of the ribosomal DNA was conducted (Samuels *et al*., 1999) [6]. Genomic DNA was extracted from 4-day-old mycelial mats using a standard CTAB (Cetyltrimethylammonium bromide) extraction protocol. The ITS region was amplified using the universal primer pair ITS1 and ITS4. The PCR cycling conditions consisted of an initial denaturation at 94°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute, and extension at 72°C for 1 minute, with a final extension at 72°C for 10 minutes. The amplified products were purified and sequenced. The resulting sequences were aligned and compared with existing sequences in the NCBI GenBank database using the BLAST algorithm to confirm the identity as *T. asperellum* (Samuels *et al*., 1999) [6].

2.3. Procurement and Maintenance of Pathogens

Virulent strains of *Fusarium oxysporum* f. sp. *lycopersici* (Fol), *Rhizoctonia solani* (Rs), and *Sclerotium rolfsii* (Sr) were obtained from the National Fungal Culture Collection. The pathogens were cultured on PDA and maintained at 4°C for short-term storage. Pathogenicity was confirmed by performing Koch's postulates on susceptible tomato seedlings prior to their use in the antagonism assays (Agrios, 2005) [1].

2.4. Dual Culture Antagonism Assay

The antagonistic potential of the isolated *T. asperellum* strains was evaluated using the dual culture technique (Benítez *et al*., 2004) [5]. Mycelial plugs (5 mm in diameter) were excised from the actively growing margins of 5-day-old cultures of both the pathogen and the antagonist. These plugs were placed on opposite sides of a 90 mm Petri dish containing PDA, approximately 1 cm from the edge. Plates inoculated solely with the pathogen served as the control.

The plates were incubated at 28°C for 7 days. The radial growth of the pathogen was measured continuously until the control plates were fully covered. The percentage of mycelial growth inhibition was calculated.

2.5. Evaluation of Volatile Organic Compounds (VOCs)

The effect of volatile metabolites produced by the isolates on pathogen growth was assessed using inverted plate methodology (Minerdi *et al*., 2009) [15]. A 5 mm plug of the *T. asperellum* isolate was placed centrally on a PDA plate and incubated for 48 hours at 28°C. Following this, the lid of the Petri dish was replaced with the bottom half of another Petri dish containing a freshly inoculated 5 mm plug of the target pathogen. The two plate bases were sealed together tightly with Parafilm to prevent the escape of volatile compounds. Control assemblies consisted of a pathogen plate inverted over an uninoculated PDA plate. Radial growth was measured after 5 days, and inhibition was calculated.

2.6. Evaluation of Non-Volatile Metabolites (Culture Filtrate)

To evaluate the efficacy of non-volatile metabolites, *T. asperellum* isolates were grown in Potato Dextrose Broth (PDB). Flasks containing 100 mL of PDB were inoculated with five mycelial plugs of the antagonist and incubated on a rotary shaker at 150 rpm at 28°C for 14 days. The culture was then filtered through Whatman No. 1 filter paper and subsequently sterilized using a 0.22 µm Millipore syringe filter. The sterile culture filtrate was added to molten PDA at a concentration of 10% (v/v) just before pouring into Petri dishes. Pathogen plugs were inoculated centrally, and growth was compared against control plates containing PDA amended with 10% sterile PDB (Benítez *et al*., 2004) [5] [13].

2.7. Greenhouse Assay

The most potent isolate, designated TA3, was selected for *in vivo* evaluation in a greenhouse setting. The experiment was conducted using a randomized block design with five replications per treatment.

Preparation of Inoculum: Pathogen inoculum was multiplied on a sterilized sand-cornmeal medium (9:1 w/w) for 15 days. *T. asperellum* TA3 was multiplied on sterilized sorghum grains for 10 days.

Potting Media and Sowing: Plastic pots (15 cm diameter) were filled with 1 kg of a sterilized soil mixture (soil:sand:compost in a 2:1:1 ratio). The soil was artificially infested with the pathogen inoculum at a rate of 20 g/kg of soil. After 48 hours, the *Trichoderma* formulation was incorporated into the soil at 10 g/kg. Surface-sterilized tomato seeds (susceptible variety) were sown in the pots (5 seeds/pot).

Treatments:

- T1: Uninoculated control (Healthy)
- T2: Pathogen only (Infected control)
- T3: Pathogen + *T. asperellum* TA3
- T4: *T. asperellum* TA3 only

Plants were maintained in a greenhouse at 25°C to 30°C with a 14-hour photoperiod. Disease incidence (%) was recorded after 30 days. Plant growth parameters, including shoot height, root length, and total fresh biomass, were also quantified at the end of the experiment.

2.8. Statistical Analysis

All *in vitro* experiments were conducted in triplicate. The data obtained were subjected to one-way Analysis of Variance (ANOVA) using statistical software. Treatment means were separated and compared using Tukey's Honestly Significant Difference (HSD) test at a significance level.

3. Results

3.1. Isolation and Identification of *T. asperellum*

A total of twelve putative *Trichoderma* isolates were initially recovered from the rhizosphere soil samples. Following rigorous morphological screening, five isolates, designated as TA1, TA2, TA3, TA4, and TA5, were selected for further study based on their rapid mycelial growth and profuse sporulation.

Macroscopically, all five isolates exhibited rapid growth, completely colonizing a 90 mm Petri dish within 72 to 96 hours. The colonies initially appeared white and floccose, gradually turning dark green as conidiation initiated, often forming distinct concentric rings. Microscopically, highly branched conidiophores with paired phialides and globose to subglobose, finely roughened conidia were observed, characteristic of the *T. asperellum* clade. Molecular identification via ITS sequencing confirmed the morphological identification; the sequences exhibited 99% to 100% homology with *T. asperellum* reference strains in the GenBank database.

Table 1: Morphological characteristics and growth rate of indigenous *Trichoderma asperellum* isolates

Isolate Code	Colony Color (Mature)	Growth Rate (mm/day)	Conidial Shape	Sporulation Density
TA1	Light Green	22.4±1.2	Globose	Moderate
TA2	Dark Yellow-Green	24.1±0.8	Subglobose	High
TA3	Dark Green	28.5±0.5	Globose	Very High
TA4	Green	21.0±1.4	Globose	Moderate
TA5	Pale Green	23.6±1.1	Subglobose	High

3.2. Antagonism in Dual Culture

The antagonistic potential of the five isolates varied significantly against the tested pathogens. In all dual culture plates, the *T. asperellum* isolates grew rapidly, restricting the growth of the pathogens, and in most cases, overgrowing the pathogen colonies (mycoparasitism).

Isolate TA3 demonstrated the highest antagonistic activity across the board. It completely halted the growth of *Rhizoctonia solani*, achieving a maximum inhibition of 78.4%. Against *Fusarium oxysporum* and *Sclerotium rolfsii*, TA3 achieved 72.1% and 69.5% inhibition, respectively. Isolates TA2 and TA5 also showed strong antagonistic capabilities, whereas TA1 and TA4 were comparatively less effective.

Table 2: Percentage of radial growth inhibition of soil-borne pathogens by *T. asperellum* isolates in dual culture assay

Antagonist Isolate	Inhibition of F. oxysporum (%)	Inhibition of R. solani (%)	Inhibition of S. rolfsii (%)
TA1	54.2±2.1	61.5±1.8	48.3±2.4
TA2	68.5±1.5	70.2±2.0	62.1±1.7
TA3	72.1±1.2	78.4±1.1	69.5±1.5
TA4	51.4±2.5	58.9±2.2	45.6±2.8
TA5	65.3±1.8	72.0±1.6	60.8±1.9

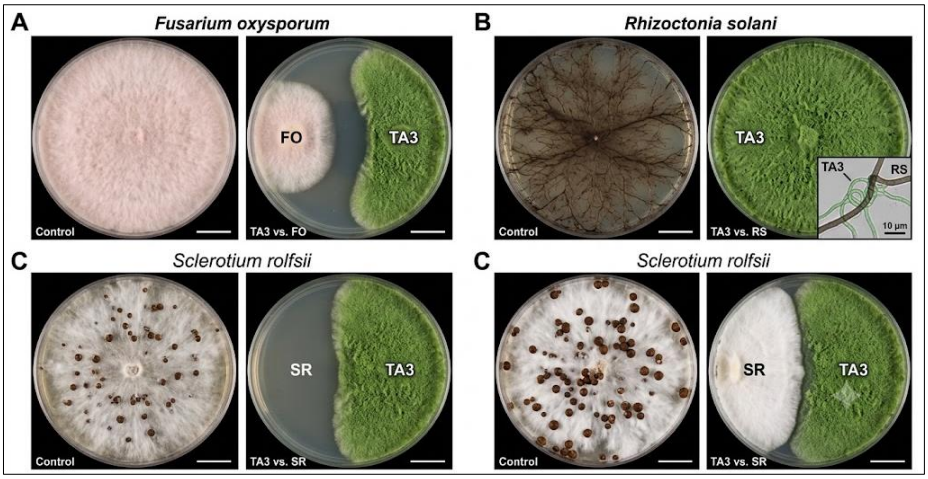

Fig 1: Dual culture assay showcasing the antagonistic activity of *T. asperellum* (TA3) against soil-borne pathogens.

3.3. Effect of Volatile and Non-Volatile Metabolites

The production of secondary metabolites plays a crucial role in antibiosis (Sivasithamparam and Ghisalberti, 1998) [13] [5]. In the inverted plate assay for volatile organic compounds (VOCs), isolate TA3 again outperformed the others, causing a 55.3% reduction in the growth of *R. solani* without physical contact. The volatile metabolites were slightly less effective against *S. rolfsii* (42.1% inhibition) (Minerdi *et al*., 2009) [15]. The culture filtrate assay, which tests for thermostable and thermolabile non-volatile compounds (including secreted enzymes and antibiotics), revealed profound pathogen suppression. The 10% culture filtrate of TA3 restricted the mycelial growth of *F. oxysporum* by 68.2%, indicating the presence of highly potent antifungal exudates in the liquid medium (Benítez *et al*., 2004) [5] [13].

3.4. Greenhouse Efficacy

Based on its superior *in vitro* performance, isolate TA3 was evaluated in the greenhouse to determine its capacity to suppress disease and promote growth in tomato plants. The application of TA3 significantly mitigated the adverse effects of the pathogens (Vinale *et al*., 2008) [10].

In soils infested with *R. solani* (which typically causes severe damping-off), the disease incidence in the untreated control was 85.4%. However, treatment with TA3 reduced this incidence drastically to 29.7%, representing a disease suppression rate of over 65%. Similar significant reductions were observed for *Fusarium* wilt and *Sclerotium* rot (Harman *et al*., 2004) [4] [10].

Furthermore, TA3 demonstrated a clear plant growth-promoting (PGP) effect. Plants treated exclusively with TA3 (T4) exhibited the highest growth metrics across all treatments, significantly surpassing the uninoculated control (T1), indicating that the fungus provides benefits beyond mere disease suppression (Contreras-Cornejo *et al*., 2009) [17] [18].

Table 3: Effect of *T. asperellum* TA3 application on disease incidence and plant growth parameters of tomato under greenhouse conditions

Treatment	Pathogen	Disease Incidence (%)	Plant Height (cm)	Fresh Biomass (g)
T1 (Healthy Control)	None	0.0	45.2±2.4	38.5±1.8
T2 (Pathogen Only)	*F. oxysporum*	78.5±3.2	22.1±1.5	15.2±1.2
T3 (TA3 + Pathogen)	*F. oxysporum*	32.4±2.1	41.5±2.0	34.1±1.5
T2 (Pathogen Only)	*R. solani*	85.4±2.5	18.4±1.1	12.8±0.9
T3 (TA3 + Pathogen)	*R. solani*	29.7±1.8	43.0±2.2	35.6±1.7
T4 (TA3 Only)	None	0.0	52.6±2.5	46.3±2.1

Note: Data represent the mean of five replicates±standard deviation. Means within columns differ significantly at $p \le 0.05$ according to Tukey's HSD.

4. Discussion

The transition from chemical dependency to sustainable agricultural practices requires the discovery and integration of highly efficient biological control agents. The present study meticulously evaluated indigenous isolates of *Trichoderma asperellum* for their ability to combat three devastating soil-borne pathogens. The utilization of indigenous strains is a strategic approach, as these microorganisms are already acclimated to the local pedoclimatic conditions, thereby ensuring superior ecological fitness, colonization efficiency, and long-term persistence in the soil microbiome compared to exotic strains (Vinale *et al*., 2008) [10].

4.1. Antagonistic Mechanisms

The dual culture assays unequivocally demonstrated the robust antagonistic capabilities of the isolated strains, with TA3 exhibiting exceptional efficacy. The rapid growth rate of TA3 (28.5 mm/day) provides it with a distinct competitive advantage for space and nutrients in the rhizosphere, a fundamental mechanism of *Trichoderma*-mediated biocontrol (Elad, 2000) [11]. As observed in the interaction zones, TA3 did not merely halt pathogen growth but actively overgrew the pathogenic mycelia. This indicates aggressive mycoparasitism, a complex sequential process involving target recognition, physical coiling around the host hyphae, and the localized secretion of cell-wall-degrading enzymes (CWDEs) (Chet *et al*., 1997) [12]. The significant inhibition recorded in the culture filtrate assays further elucidates the biochemical arsenal of *T. asperellum*. The non-volatile secondary metabolites likely include a diverse array of peptaibols, gliotoxin, and viridin, which are known to disrupt the structural integrity of pathogenic fungal cell membranes (Sivasithamparam and Ghisalberti, 1998) [13]. Furthermore, the robust suppression observed is indicative of the prolific secretion of hydrolytic enzymes. *Trichoderma* species are renowned hyper-producers of chitinases, 1,3-glucanases, and proteases, which synergistically degrade the chitin and glucan polymers that constitute the structural backbone of fungal cell walls (Lorito *et al*., 1994) [14]. The volatile organic compounds (VOCs), primarily simple aromatic compounds and pyrones, also contributed to the fungistatic effect, providing a mechanism for long-distance spatial suppression in soil pores without requiring direct physical contact (Minerdi *et al*., 2009) [15].

4.2. Disease Suppression and Growth Promotion

The translation of *in vitro* antagonism to *in vivo* greenhouse efficacy is a critical step in validating a biocontrol agent. The application of TA3 significantly reduced the incidence of *Fusarium* wilt, *Rhizoctonia* root rot, and *Sclerotium* collar rot in tomato plants. The reduction in disease incidence is attributed to the combined effects of direct pathogen suppression in the bulk soil and the aggressive colonization of the tomato rhizosphere by TA3, which creates a protective microbiological shield around the roots (Yedidia *et al*., 1999) [16].

An equally important finding of this study is the significant enhancement of plant growth parameters in the presence of TA3. Plants treated solely with the antagonist (T4) exhibited superior height and fresh biomass compared to the uninoculated controls. This biostimulant effect is a hallmark of elite *Trichoderma* strains. The mechanisms underpinning this growth promotion include the solubilization of insoluble soil nutrients (such as phosphates and micronutrients), the production of phytohormones like indole-3-acetic acid (IAA) analogues, and the priming of the plant's innate immune system (Induced Systemic Resistance - ISR) (Contreras-Cornejo *et al*., 2009) [17] (Altomare *et al*., 1999) [18]. By upregulating defensive signaling pathways (salicylic acid and jasmonic acid/ethylene pathways), TA3 not only physically protects the plant but also biochemically fortifies it against subsequent pathogenic incursions (Shoresh *et al*., 2010) [7].

4.3. Practical Implications

The profound efficacy of isolate TA3 against multiple genetically divergent pathogens highlights its broad-spectrum biocontrol potential (Harman *et al*., 2004) [4] [10]. Given its indigenous origin, TA3 possesses a high probability of successful field application in regional agro-ecosystems. The development of stable, long shelf-life formulations (such as wettable powders or granular formulations based on chlamydospores) of TA3 could provide local farmers with a viable, environmentally benign alternative to synthetic soil fumigants and fungicides (Mukherjee *et al*., 2013) [9].

5. Conclusion

This comprehensive investigation confirms that the indigenous rhizosphere microflora harbors highly potent biological control agents. The isolated *Trichoderma asperellum* strain TA3 demonstrated exceptional multifaceted antagonistic activity against *Fusarium oxysporum*, *Rhizoctonia solani*, and *Sclerotium rolfsii* through rapid spatial competition, aggressive mycoparasitism, and the secretion of potent volatile and non-volatile inhibitory metabolites. Furthermore, its application in greenhouse environments resulted in significant disease suppression coupled with pronounced plant growth promotion. These findings firmly establish *T. asperellum* TA3 as an elite, broad-spectrum biocontrol candidate. Future research should focus on large-scale multi-location field trials, optimization of mass multiplication techniques, and transcriptomic studies to fully unravel the molecular dialogue during its tripartite interaction with the host plant and the pathogens.

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