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RESEARCH ARTICLE

# Biocontrol Potential of Native Trichoderma Isolates Against *Phytophthora infestans* in Tomato (*Solanum lycopersicum*): Isolation, Characterization, and Antagonistic Efficacy

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**ABSTRACT:** Tomato (*Solanum lycopersicum*) is a globally significant crop, yet its production is severely hampered by soil-borne diseases, particularly late blight caused by *Phytophthora infestans*. This pathogen leads to substantial yield and quality losses, necessitating sustainable alternatives to chemical fungicides. The rhizosphere microbiome, especially *Trichoderma* species, plays a vital role in plant health by suppressing pathogens through antibiosis, competition, and mycoparasitism. This study aimed to isolate and characterize native *Trichoderma* strains from tomato rhizospheres in Dedo and Goma districts, Ethiopia, and evaluate their antagonistic potential against *P. infestans*. Forty samples were collected, yielding 36 *P. infestans* isolates from infected tomato tissues and 10 *Trichoderma* isolates from rhizospheric soil. Dual-culture assays revealed that *Trichoderma* strains Ju-TGDa-2, Ju-TGDb-3, Ju-TDWc-2, and Ju-TGDb-2 exhibited significant inhibitory effects, suppressing pathogen growth by 10.2–15% after three days, 38–43.4% after six days, and 58–61.4% after nine days. Morphological characterization confirmed their rapid growth and competitive dominance over *P. infestans*. Statistical analysis (ANOVA, DMRT) validated the efficacy of these strains, with Ju-TGDb-2 showing the highest inhibition (61.4%). The findings demonstrate that native *Trichoderma* isolates possess strong biocontrol potential against *P. infestans*, offering an eco-friendly alternative for managing late blight in tomato cultivation. Further field trials and molecular characterization are recommended to optimize their application in integrated disease management strategies.

**Keywords:** *Solanum lycopersicum, Trichoderma* spp., *Phytophthora infestans,* Biocontrol agents, Rhizosphere microbiome, Antagonistic activity.

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## 1. INTRODUCTION

The tomato (*Solanum lycopersicum*) is one of the most widely cultivated vegetable crops globally, valued for its nutritional benefits and economic significance in both small-scale and commercial farming systems [1]. Despite its adaptability to diverse climates, tomato production faces persistent challenges from soil-borne diseases that severely reduce yield and fruit quality [2-4]. Among these diseases, late blight caused by *Phytophthora infestans* is particularly devastating, leading to significant pre- and post-harvest losses. Conventional disease management relies heavily on synthetic fungicides, which pose risks to human health, beneficial soil microbiota, and the environment. Consequently, there is an urgent need for sustainable alternatives, such as biological control agents (BCAs), to mitigate pathogen impact while maintaining ecological balance.

The rhizosphere, the soil region directly influenced by

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plant roots, harbors a dynamic microbial community that plays a pivotal role in plant health and disease suppression [5-7]. Within this microbiome, *Trichoderma* species have emerged as key beneficial fungi due to their multifaceted mechanisms of action against phytopathogens [8-10]. These fungi exhibit antagonistic properties through antibiosis, secreting secondary metabolites like peptaibols and polyketides that inhibit pathogen growth [11-15]. Additionally, *Trichoderma* engages in direct mycoparasitism by coiling around pathogenic hyphae, producing cell wall-degrading enzymes such as chitinases and glucanases, and ultimately lysing the target fungi [15, 16]. Competition for nutrients and ecological niches further enhances their biocontrol efficacy, as *Trichoderma* rapidly colonizes root surfaces, depriving pathogens of essential resources [17, 18]. Beyond pathogen suppression, *Trichoderma* species promote plant growth by enhancing nutrient uptake, stimulating root development, and inducing systemic resistance, making them invaluable for sustainable agriculture.

Isolating and characterizing *Trichoderma* strains from native agroecosystems is critical for developing region-specific biocontrol strategies [19-21]. Morphological identification, including colony characteristics, conidiation patterns, and microscopic features, remains a fundamental approach for preliminary classification [22, 23]. However, molecular techniques such as ITS sequencing are increasingly employed for precise species delineation [24, 25]. Selecting strains with high antagonistic potential involves in vitro screening against target pathogens, particularly *P. infestans*, which is notorious for its rapid spread and adaptability [26-29]. Previous studies have demonstrated the efficacy of *Trichoderma harzianum*, *T. viride*, and *T. asperellum* in suppressing *Phytophthora* spp., but the performance of native isolates from understudied regions like Ethiopia remains underexplored.

This study contributes to the existing knowledge by isolating and characterizing indigenous *Trichoderma* strains from tomato rhizospheres in Ethiopia's Dedo and Goma districts, regions with significant tomato production but limited research on biocontrol agents. Unlike previous studies that often focus on well-known *Trichoderma* species, this work evaluates lesser-studied native isolates, providing insights into their unique antagonistic mechanisms against *P. infestans*. The rigorous in vitro screening, including time-dependent inhibition assays, offers a detailed understanding of the dynamics between *Trichoderma* and *Phytophthora*, highlighting strains with rapid mycelial growth and sustained suppression. Furthermore, the integration of morphological and statistical analyses ensures robust selection of candidate BCAs for future field applications. By emphasizing locally adapted strains, this research supports the development of tailored, eco-friendly solutions for smallholder farmers, reducing reliance on chemical inputs while enhancing crop resilience.

The persistent threat of *P. infestans* to global tomato production necessitates innovative and sustainable management approaches. While chemical control remains prevalent, its drawbacks—including pathogen resistance, environmental contamination, and non-target toxicity—underscore the importance of biological alternatives. *Trichoderma*-based biocontrol presents a promising solution, yet its success depends on identifying highly antagonistic strains suited to specific agroecological conditions. This study addresses this gap by systematically evaluating native *Trichoderma* isolates, combining traditional and empirical methods to validate their efficacy. The findings not only expand the repertoire of potential BCAs but also reinforce the importance of conserving and utilizing indigenous microbial diversity for sustainable agriculture. Future research should explore field trials, molecular mechanisms of antagonism, and formulation technologies to optimize the practical deployment of these biocontrol agents.

## 2. EXPERIMENTAL DETAILS

### 2.1. Study Area and Period

The study was conducted at Jimma University, located in Jimma town, Oromiya Regional State, Ethiopia (GPS coordinates: 7°40′N 36°50′E). This region is situated 352 km southwest of Addis Ababa and is characterized by a humid subtropical climate, with an average annual rainfall of 1,500 mm and temperatures ranging between 15°C and 30°C, providing favorable conditions for fungal growth and tomato cultivation. The research was carried out from March to October 2019 in the Research and Postgraduate Laboratory, Department of Biology, ensuring controlled environmental conditions for microbiological experiments.

### 2.2. Study Design

A cross-sectional and laboratory-based experimental design was employed to assess the antagonistic potential of *Trichoderma* isolates against *Phytophthora infestans*. Sampling followed a purposive non-probability technique, targeting tomato plantations in Dedo and Goma districts, which are major tomato-producing areas in southwestern Ethiopia. These regions were selected due to their high incidence of late blight disease, providing an optimal source for pathogen and biocontrol agent isolation.

### 2.3. Sample Collection and Transportation

A total of 40 samples were collected, comprising 30 rhizospheric soil samples and 10 symptomatic plant tissues (leaves, stems, and fruits) displaying characteristic late blight lesions. Rhizospheric soil was collected at a depth of 10 cm using a sterilized spatula, ensuring minimal disturbance to root-associated microbial communities. Diseased plant tissues were excised from the margins of active lesions to maximize pathogen recovery. All samples were placed in sterile polyethylene bags, labeled, and transported under cool

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conditions (4°C) to the laboratory to prevent microbial contamination and degradation.

### 2.4. Sample Preparation and Incubation

#### *2.4.1. Isolation of* Phytophthora infestans *from Plant Tissues*

Plant tissues were surface-sterilized by rinsing in 70% ethanol for 5 seconds, followed by three washes with sterile distilled water to remove residual disinfectant [30]. After air-drying under a laminar flow hood, small sections (5 mm²) from lesion margins were aseptically transferred onto carrot agar (CA) plates amended with chloramphenicol (250 mg/L) to suppress bacterial growth. The CA medium consisted of 200 g blended carrots, 20 g agar, and 1 L distilled water, autoclaved at 121°C for 15 min. Twenty plates, each inoculated with 4–5 tissue fragments, were sealed with parafilm and incubated at 18–20°C for 3–7 days. Emerging hyphae were subcultured onto potato dextrose agar (PDA) supplemented with chloramphenicol (250 mg/L) and purified through successive streaking.

#### *2.4.2. Isolation of* Trichoderma *spp. from Rhizospheric Soil*

Soil samples were processed using a serial dilution technique [30]. Briefly, 10 g of air-dried soil was suspended in 90 mL of sterile distilled water and agitated for 10 min on a rotary shaker (150 rpm). After sedimentation for 20 min, serial dilutions ($10^{-1}$ to $10^{-6}$) were prepared, and 0.1 mL aliquots from the $10^{-4}$, $10^{-5}$, and $10^{-6}$ dilutions were spread onto Czapek-Dox agar plates (composition: sucrose 30 g, $NaNO_3$ 2 g, $K_2HPO_4$ 1 g, $MgSO_4$ 0.5 g, KCl 0.5 g, $FeSO_4$ 0.01 g, agar 15 g, pH 7.3 ± 0.2). Plates were incubated at 25°C for 7 days, and fungal colonies exhibiting *Trichoderma*-like morphology (rapid growth, green conidiation) were subcultured onto PDA for purification.

### 2.5. Pathogen Identification and Pathogenicity Testing

#### *2.5.1. Morphological Characterization of* Phytophthora infestans

Presumptive *P. infestans* isolates were identified based on colony morphology and microscopic features [27, 31]. Mycelia from 21-day-old CA cultures were mounted in lactophenol cotton blue and examined under a light microscope (400× magnification). Key diagnostic traits included semi-papillate, lemon-shaped sporangia (caducous with short pedicels), amphigynous antheridia, and thick-walled oogonia. Sporangial dimensions were quantified using Piximetre 5.9 software, with confirmatory measurements of 30 spores per isolate.

#### *2.5.2. Pathogenicity Assay*

Virulence of *P. infestans* isolates was confirmed using detached leaflet assays on the susceptible tomato cultivar 'Moneymaker' [30, 31]. Sporangial suspensions ($1 \times 10^5$ sporangia/mL) were prepared by flooding 7-day-old CA cultures with sterile water and scraping with a glass slide. Droplets (10 µL) were inoculated onto the abaxial surface of healthy leaflets, which were then placed on moist filter paper in Petri dishes and incubated at 15–18°C in darkness. Disease progression (lesion expansion, sporulation) was monitored for 6 days, and Koch's postulates were fulfilled by reisolating the pathogen from infected tissues.

### 2.6. Antagonistic Activity Screening

#### *2.6.1. Dual-Culture Assay*

The biocontrol potential of *Trichoderma* isolates was evaluated using a dual-culture technique [32]. Mycelial plugs (6 mm diameter) from the leading edge of 5-day-old *Trichoderma* and *P. infestans* cultures were placed 4 cm apart on PDA plates amended with chloramphenicol (250 mg/L). Controls consisted of *P. infestans* alone. Plates were incubated at 24 ± 2°C, and radial growth inhibition was calculated after 3, 6, and 9 days using the formula [33]:

$$I = \frac{(C - T)}{C} \times 100$$

where $I$ = inhibition percentage, $C$ = radial growth of *P. infestans* in control, and $T$ = radial growth in co-culture. Three replicates per isolate were maintained.

#### *2.6.2. Morphological Characterization of Trichoderma Isolates*

Antagonistic *Trichoderma* strains were further characterized by culturing on PDA at 24 ± 2°C for 7 days. Colony morphology (texture, pigmentation, conidial color) and microscopic features (hyphal septation, phialide arrangement, conidial shape) were documented. Conidial dimensions were measured from lactophenol cotton blue-stained slides using Piximetre 5.9.

### 2.7. Statistical Analysis

Data were analyzed using SAS 9.1 (SAS Institute, USA). One-way ANOVA followed by Duncan's Multiple Range Test (DMRT) at $*p* \leq 0.05$ was applied to compare inhibition percentages among *Trichoderma* isolates. Assumptions of normality (Shapiro-Wilk test) and homogeneity of variance (Levene's test) were verified prior to analysis. This rigorous methodological framework ensured reliable isolation, characterization, and evaluation of indigenous *Trichoderma* strains as biocontrol agents against *P. infestans*, contributing to sustainable tomato

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disease management strategies.

## 3. RESULTS AND DISCUSSION

### 3.1. Isolation and Morphological Characterization of *Phytophthora infestans*

Among the 36 fungal isolates obtained from diseased tomato tissues (leaves, stems, and fruits), 29 exhibited morphological characteristics typical of Oomycetes. These isolates formed white, rapidly growing colonies with profusely branching aseptate mycelium on carrot agar medium. Microscopic examination revealed distinctive semi-papillate, caducous sporangia borne terminally on sympodial sporangiophores, along with amphigynous antheridia and thick-walled oogonia. The sporangial dimensions averaged 117.7 × 95.5 µm, while oogonia measured 84.4 × 77.3 µm. These morphological features are consistent with previous descriptions of *P. infestans* [34, 35], confirming the successful isolation of the late blight pathogen from infected tomato tissues in the study region.

The pathogenicity of these isolates was confirmed through detached leaf assays, where 18 out of 29 isolates demonstrated aggressive virulence on tomato cultivar 'Moneymaker'. This high percentage (62%) of virulent isolates suggests significant pathogenic diversity among *P. infestans* populations in southwestern Ethiopia, which may have implications for disease management strategies. The rapid lesion development (within 6 days post-inoculation) observed in the pathogenicity tests underscores the destructive potential of this pathogen in tomato production systems.

### 3.2. Isolation and Screening of *Trichoderma* Biocontrol Agents

From 102 fungal isolates obtained from tomato rhizosphere soils, 21 were preliminarily identified as Trichoderma spp. based on colony morphology and microscopic characteristics. These isolates exhibited the typical rapid growth and green conidiation associated with Trichoderma species. Subsequent dual-culture assays against *P. infestans* revealed significant variation in antagonistic potential among the isolates (Table 1). The inhibition percentages ranged from 0.28-15.86% after 3 days, increasing to 26.4-43.4% by day 6, and reaching 49.8-61.4% by day 9 of co-cultivation. This progressive inhibition pattern suggests that the antagonistic activity of Trichoderma involves both immediate and time-dependent mechanisms. Isolates Ju-TGDb-2, Ju-TGDb-3, and Ju-TDc-2 consistently showed the highest inhibition rates (>60% by day 9), surpassing the 40% threshold considered indicative of effective biocontrol agents [36].

Figure 1 illustrates the comparative antagonistic ability of selected Trichoderma isolates after 6 days of interaction with *P. infestans*. The differential inhibition patterns observed suggest varying modes of action among the isolates, with some showing clear zone formation (indicative of antibiosis) while others demonstrated overgrowth and mycoparasitism. These findings align with previous reports of Trichoderma's multifaceted antagonistic mechanisms against plant pathogens [37, 38].

### 3.3. Morphological Characterization of Effective Trichoderma Isolates

The five most effective Trichoderma isolates (Ju-TGDa-2, Ju-TGDb-1, Ju-TGDb-2, Ju-TGDb-3, and Ju-TDc-2) exhibited distinct morphological characteristics (Table 2). All isolates showed rapid colony expansion, covering 9 cm PDA plates within 4 days (mean ± SD of 9 ± 0.00 cm). Colony pigmentation varied from white (Ju-TGDa-2, Ju-TGDb-1) to deep green (Ju-TGDb-2, Ju-TGDb-3) and yellow with white margins (Ju-TDc-2), reflecting the morphological diversity within the genus.

Microscopic examination revealed septate, hyaline hyphae and abundant conidia production (Figure 2). Conidial morphology ranged from ovoid (22.6 × 20.4 µm to 36.7 × 31.0 µm) to circular (36.1 × 33.4 µm), with green pigmentation characteristic of Trichoderma species. These morphological features are consistent with descriptions of known biocontrol species such as *T. harzianum* and *T. viride* [39, 40], suggesting that the effective isolates may belong to these or related species.

### 3.4. Mechanisms of Antagonism and Biocontrol Potential

The dual-culture assays revealed three distinct interaction patterns between Trichoderma isolates and *P. infestans*: (1) formation of inhibition zones with concave margins toward the pathogen (e.g., Ju-TGDb-1, Ju-TGDb-2), suggesting antibiosis through metabolite production; (2) complete overgrowth of the pathogen colony (e.g., Ju-TGDa-2, Ju-TDc-2), indicating competitive exclusion and mycoparasitism; and (3) intermediate interactions with partial overgrowth and inhibition zones.

The superior performance of isolates Ju-TGDb-2 and Ju-TDc-2 (61.4% and 60.9% inhibition, respectively) may be attributed to their combination of rapid growth (competitive advantage) and metabolite production. These results corroborate findings by ref. [41], which reported varying antagonistic capabilities among different Trichoderma species, with *T. viride* showing particularly strong inhibition against soilborne pathogens.

The progressive increase in inhibition percentages over time (Table 1) suggests that multiple mechanisms contribute to pathogen suppression. Early inhibition (days 1-3) likely results from antibiosis through diffusible metabolites, while later stages (days 6-9) involve mycoparasitism and nutrient competition. This temporal pattern of antagonism is particularly relevant for field applications, as it suggests that Trichoderma applications would need to precede pathogen establishment for optimal control.

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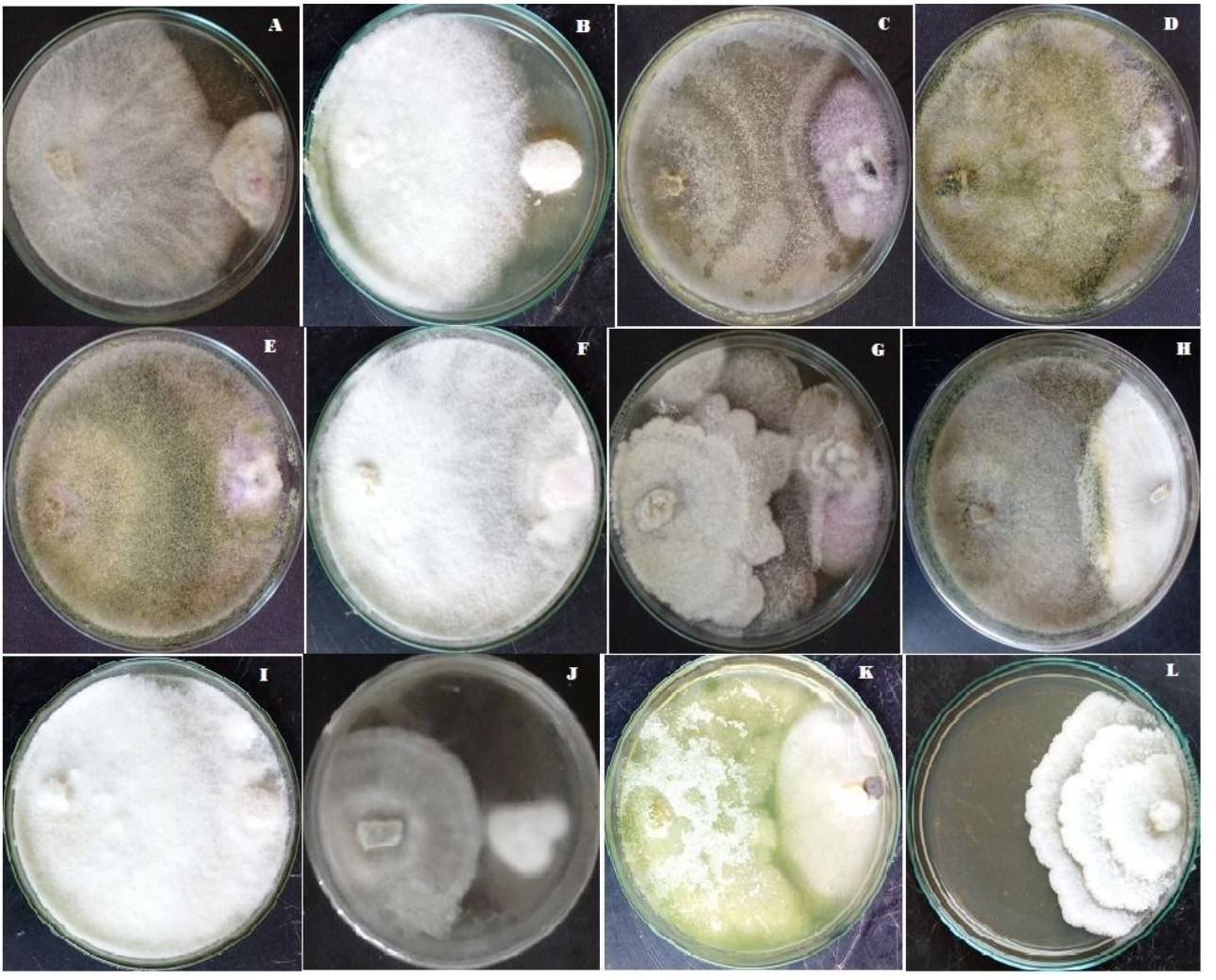


**Fig. 1.** Comparative antagonistic activity of Trichoderma isolates against *P. infestans* after 6 days of dual culture. Inhibition percentages represent mean values of three replicates. Different letters indicate significant differences according to Duncan's Multiple Range Test ($p < 0.05$).

**Table 1.** Inhibition of *P. infestans* growth by Trichoderma isolates in dual-culture assays over time. Values represent mean radial growth (cm) ± standard deviation and corresponding percentage inhibition from three replicate experiments.

| Treatments | 3 days | | 6 days | | 9 days | |
|---|---|---|---|---|---|---|
| | Mean ± SD | % | Mean ± SD | % | Mean ± SD | % |
| **Ju-TGDa-2** | $3.17 \pm .058^{c}$ | 10.2 | $3.37 \pm .058^{b}$ | 38.52 | $3.40 \pm .00^{bc}$ | 58 |
| **Ju-TGDa-14** | $3.31 \pm .138^{c}$ | 6.23 | $3.64 \pm .112^{bc}$ | 34.17 | $3.64 \pm .112^{bc}$ | 55 |
| **Ju-TGDb-1** | $2.97 \pm .058^{a}$ | 15.86 | $3.23 \pm .058^{b}$ | 41.59 | $3.23 \pm .058^{b}$ | 60.1 |
| **Ju-TGDb-2** | $3.00 \pm .00^{bc}$ | 15.01 | $3.13 \pm .058^{a}$ | 43.4 | $3.13 \pm .058^{a}$ | 61.4 |
| **Ju-TGDb-3** | $3.07 \pm .115^{bc}$ | 13.03 | $3.20 \pm .100^{b}$ | 42 | $3.20 \pm .100^{c}$ | 60.5 |
| **Ju-TDa-9** | $3.06 \pm .211^{bc}$ | 13.3 | $3.44 \pm .09^{b}$ | 37.8 | $3.44 \pm .09^{bc}$ | 57.5 |
| **Ju-TDa-11** | $3.22 \pm .54^{c}$ | 8.78 | $4.07 \pm .058^{bc}$ | 26.4 | $4.07 \pm .058^{bac}$ | 49.8 |
| **Ju-TGDc-3** | $3.52 \pm .058^{c}$ | 0.28 | $3.97 \pm .05^{bc}$ | 28.21 | $3.97 \pm .05^{bac}$ | 51 |
| **Ju-TDc-2** | $3.10 \pm .10^{bc}$ | 12.18 | $3.17 \pm .06^{b}$ | 42.7 | $3.17 \pm .06^{c}$ | 60.9 |
| **Ju-TDd-4** | $3.09 \pm .112^{bc}$ | 12.2 | $3.8 \pm .058^{bc}$ | 31.3 | $3.8 \pm .058^{bac}$ | 53 |
| **JUT-9** | $3.18 \pm .00^{c}$ | 10 | $3.73 \pm .058^{bc}$ | 32.6 | $3.73 \pm .058^{bac}$ | 54 |
| **P** | $3.53 \pm .115^{d}$ | | $5.53 \pm .451^{c}$ | | $8.10 \pm .20^{e}$ | |

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**Table 2.** Morphological characteristics of selected Trichoderma biocontrol agents and *P. infestans*. Measurements represent mean values from 30 observations per isolate.

| Isolate | Mycelia | | Spore | | |
|---|---|---|---|---|---|
| | **Color** | **Diameter** | **Types** | **Shape** | **Length:width (µm)** |
| **Ju-TGDa-2** | White | 9 ± .00 | *Conidia* | Ovoid | 22.6 × 20.4 |
| **Ju-TGDb-1** | White | 9 ± .00 | *Conidia* | Ovoid | 36.7 × 31.0 |
| **Ju-TGDb-2** | Deep green | 9 ± .00 | *Conidia* | Ovoid | 29.8 × 25.5 |
| **Ju-TGDb-3** | Green | 9 ± .00 | *Conidia* | Ovoid | 35.8 × 31.1 |
| **Ju-TDc-2** | Yellow to white | 9 ± .00 | *Conidia* | Circular | 36.1 × 33.4 |
| **Ju-PGD-5** | White | 8.87 ± .248 | *Sporangia* | Lemoniform | 117.7 × 95.5 |
| | | | *Oogonia* | Circular | 84.4 × 77.3 |

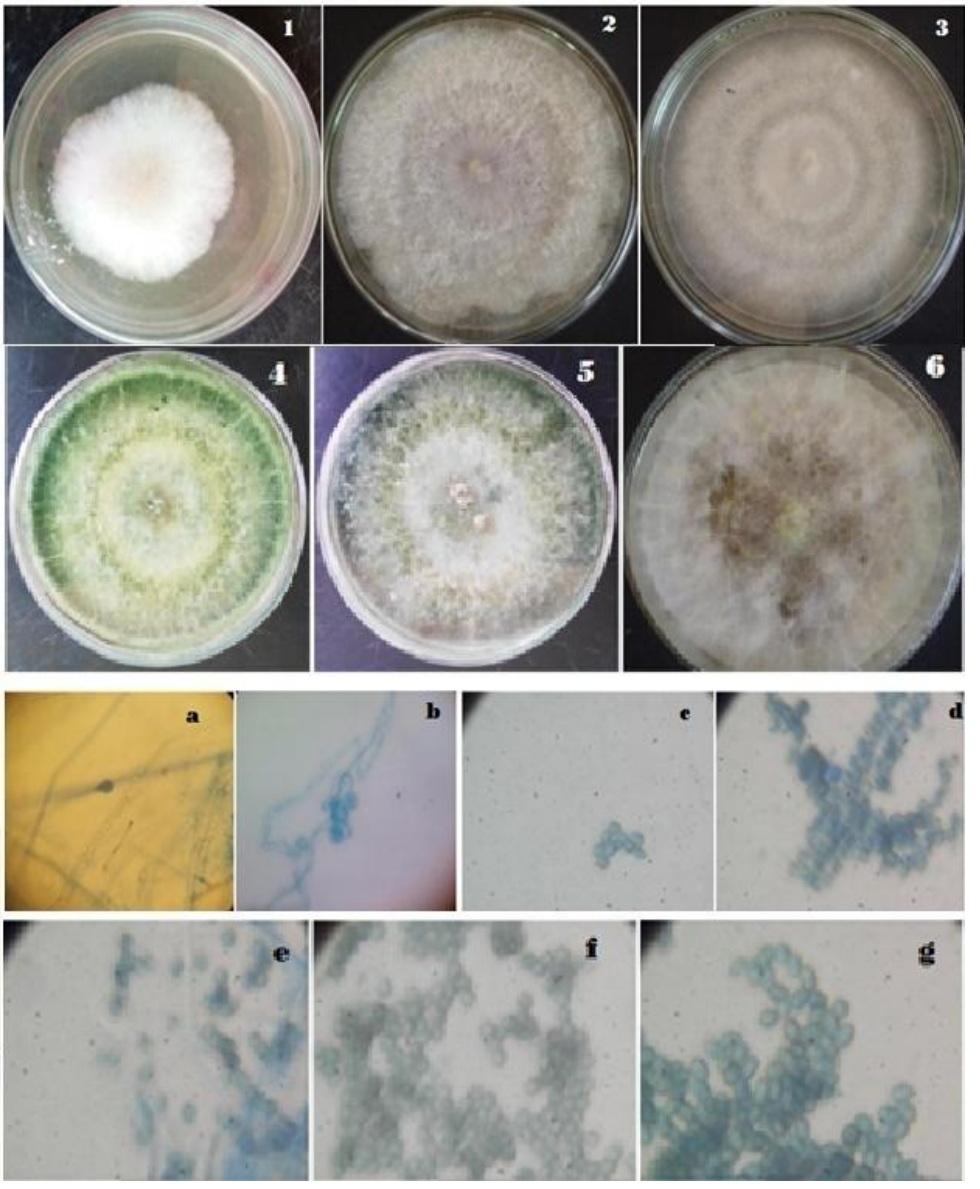


**Fig. 2.** Morphological characteristics of *P. infestans* and selected Trichoderma isolates. (A) *P. infestans* colony and reproductive structures; (B-F) Trichoderma colonies and conidia showing variation in pigmentation and morphology. Scale bars: 20 µm for microscopic images.

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### 3.5. Implications for Sustainable Tomato Production

The identification of native Trichoderma isolates with high antagonistic activity against *P. infestans* has significant implications for sustainable tomato production in Ethiopia. The use of locally adapted biocontrol agents offers several advantages over introduced strains, including better environmental adaptation and potentially longer persistence in the rhizosphere [19-21].

The morphological diversity observed among the effective Trichoderma isolates (Table 2, Figure 3) suggests that different strains may be suited for various agroecological conditions within the region. For instance, the white-pigmented Ju-TGDa-2 might be more tolerant to higher temperatures, while the deeply pigmented Ju-TGDb-2 could have enhanced UV resistance - properties that could be exploited for field applications.

These findings support the growing body of evidence demonstrating Trichoderma's potential as a biocontrol agent against oomycete pathogens [37-41]. However, the transition from in vitro efficacy to field performance requires additional research on formulation, application timing, and integration with other disease management practices. The next research steps should include molecular characterization of the most promising isolates, field efficacy trials, and investigations into their plant growth promotion capabilities.

## 4. CONCLUSION

This study successfully isolated and characterized native *Trichoderma* strains from tomato rhizospheres, demonstrating their potential as biocontrol agents against *Phytophthora infestans*, the causative agent of late blight. The dual-culture assays revealed that *Trichoderma* isolates Ju-TGDa-2, Ju-TGDb-3, Ju-TDWc-2, and Ju-TGDb-2 exhibited significant antagonistic activity, with inhibition rates escalating over time, reaching up to 61.4% by the ninth day. These results align with previous studies highlighting *Trichoderma*'s role in suppressing soil-borne pathogens through competitive exclusion, mycoparasitism, and antibiosis. The morphological and growth dynamics of the selected *Trichoderma* strains further support their efficacy, as their rapid colonization of the growth medium restricted *P. infestans* expansion. Notably, Ju-TGDb-2 emerged as the most potent antagonist, suggesting its suitability for large-scale biocontrol applications. The study also confirmed the pathogenicity of *P. infestans* isolates from infected tomato tissues, reinforcing the need for sustainable disease management strategies in tomato farming. The findings underscore the importance of harnessing native microbial antagonists for plant disease control, reducing reliance on synthetic fungicides. However, while in vitro results are promising, field validation is essential to assess performance under varying environmental conditions. Future research should explore molecular mechanisms of antagonism, such as chitinase production and induced systemic resistance, to enhance biocontrol strategies. Additionally, formulating these *Trichoderma* strains into biofungicides could facilitate practical application in integrated pest management (IPM) systems. This study contributes to the growing body of evidence supporting *Trichoderma*-based biocontrol as a sustainable solution for late blight management in tomatoes. By leveraging indigenous microbial resources, farmers can mitigate crop losses while promoting environmentally friendly agricultural practices. Further investigations into strain optimization, delivery methods, and field efficacy will be critical for maximizing their practical implementation.

## DECLARATIONS

### Ethical Approval

We affirm that this manuscript is an original work, has not been previously published, and is not currently under consideration for publication in any other journal or conference proceedings. All authors have reviewed and approved the manuscript, and the order of authorship has been mutually agreed upon.

### Funding

Not applicable

### Availability of data and material

All of the data obtained or analyzed during this study is included in the report that was submitted.

### Conflicts of Interest

The authors declare that they have no financial or personal interests that could have influenced the research and findings presented in this paper. The authors alone are responsible for the content and writing of this article.

### Authors' contributions

All authors contributed equally to this work.

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