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Research Article

In Vitro Evaluation of Indigenous *Trichoderma* Isolates for Antagonistic Activity and Volatile- and Culture-Filtrate-Mediated Antifungal Activity Against Soil-Borne Fungal Pathogens

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Abstract

Soil-borne fungal pathogens are a major constraint to agricultural productivity, causing significant yield losses worldwide. The extensive use of synthetic fungicides has raised concerns regarding environmental contamination, pathogen resistance and food safety, highlighting the need for sustainable biological alternatives. The present study evaluated the biocontrol potential of eight indigenous *Trichoderma* isolates through morphological and microscopic characterization, *in vitro* antagonistic activity against *Fusarium spp.*, *Sclerotium rolfsii* and *Macrophomina phaseolina*, and assessment of volatile-compound- and culture-filtrate-mediated antifungal activity. All isolates exhibited characteristic cultural and microscopic features of the genus *Trichoderma*, although considerable variability was observed in their antagonistic performance. In the dual culture assay, pathogen inhibition ranged from 50.00-72.22% against *Fusarium* spp., 35.55-56.66% against *S. rolfsii* and 41.11-72.22% against *M. phaseolina*. Volatile compounds associated with *Trichoderma* growth showed strong inhibitory activity, including complete inhibition of *S. rolfsii* by selected isolates, while culture filtrates also inhibited the growth of the tested pathogens. Tr01, Tr02, Tr04 and Tr06 showed comparatively higher antagonistic performance across multiple assays. These findings demonstrate substantial isolate- and pathogen-dependent antifungal activity among the indigenous *Trichoderma* isolates and identify candidates for further investigation. Molecular identification, characterization of active compounds and greenhouse and field evaluation are required to establish their taxonomic identity, mechanisms of action and practical biocontrol potential.

Keywords: *Trichoderma*, biological control, soil-borne pathogens, volatile compounds, culture filtrate, sustainable agriculture.

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1. Introduction

Soil-borne fungal pathogens are major constraints to crop production because they can persist in soil and plant residues and cause diseases such as damping-off, root rot, stem rot and wilt. Among these pathogens, *Sclerotium rolfsii*, *Fusarium* spp. and *Macrophomina phaseolina* are associated with economically important diseases in a wide range of agricultural crops. Their soil-borne nature and persistence in crop production systems can make disease management difficult, particularly when environmental conditions favour pathogen development. Although chemical fungicides remain an important component of disease management, their repeated and intensive use may contribute to environmental contamination, disruption of beneficial soil microorganisms and reduced sensitivity in pathogen populations. These concerns have increased interest in biological approaches based on naturally occurring beneficial microorganisms (Syed Ab Rahman *et al*., 2018; Köhl *et al.,* 2019).

Species of *Trichoderma* are among the most extensively studied fungal biocontrol agents because of their ability to interact with plants and other microorganisms and influence pathogen development through multiple complementary processes (Guzmán-Guzmán *et al*., 2019; Zin and Badaluddin 2020). Their biocontrol potential can involve competition for nutrients and ecological niches, direct hyphal interaction, mycoparasitism, production of hydrolytic enzymes, induction of plant defence responses, and release of volatile and other diffusible compounds (Mukherjee *et al*., 2013; Woo *et al.,* 2014; Guzmán-Guzmán *et al*., 2019). The relative contribution of these processes varies among *Trichoderma* species and strains and is also influenced by the target pathogen and environmental conditions (Köhl *et al*., 2019). Therefore, evaluation at the isolate level is important for identifying strains with consistent antagonistic activity.

Direct antagonism in dual-culture assays provides a useful initial measure of the ability of *Trichoderma* isolates to restrict pathogen growth. In addition, sealed-plate assays can be used to determine whether inhibition occurs in the absence of direct physical contact, thereby providing evidence for a volatile-mediated inhibitory effect. Earlier studies established the role of volatile and non-volatile factors in *Trichoderma*-mediated antagonism (Dennis and Webster 1971a,b). Similarly, assays incorporating *Trichoderma* culture filtrates into growth media can determine whether diffusible culture-associated components contribute to suppression of pathogen growth. These approaches therefore provide complementary phenotypic evidence for different forms of fungal antagonism. However, inhibition observed in such assays should not by itself be interpreted as evidence for the production or identity of specific antifungal metabolites unless the relevant compounds are chemically or biochemically characterized.

Indigenous *Trichoderma* populations may be particularly valuable for biological control because isolates adapted to local soil and climatic conditions may possess traits suited to the agroecological environment from which they originate. However, considerable variation in antagonistic activity can occur among isolates obtained from the same geographical region. Screening indigenous isolates against agriculturally important soil-borne pathogens is therefore an important step toward identifying candidates for subsequent molecular characterization, formulation development and greenhouse or field evaluation (Woo *et al*., 2014; Zin and Badaluddin 2020).

In the present study, eight indigenous *Trichoderma* isolates obtained from rhizosphere soil of Gir Somnath district, Gujarat, were evaluated against *S. rolfsii*, *Fusarium* spp. and *M. phaseolina*. The study assessed their antagonistic activity through dual-culture assays and further examined pathogen inhibition under conditions involving shared headspace and *Trichoderma* culture filtrates. The objective was to compare isolate- and pathogen-dependent inhibitory responses and to identify isolates showing comparatively strong *in vitro* biocontrol activity for further investigation. The study was designed to provide phenotypic evidence of antagonism and volatile- and culture-filtrate-mediated suppression, rather than to chemically identify or quantify specific antifungal metabolites.

2. Materials and methods

2.1 Study Area and Collection of Rhizosphere Soil Samples

Rhizosphere soil samples were collected from groundnut-growing fields in Gir Somnath district (20.91287° N, 70.3671° E), Gujarat, India. Samples were collected from the rhizosphere zone of healthy groundnut plants at a depth of approximately 5-15 cm using sterile tools. Approximately 100-200 g of soil was collected from each sampling site, placed in sterile polyethylene bags, transported to the laboratory, and processed for the isolation of indigenous *Trichoderma* spp.

2.2 Isolation and Morphological Identification of *Trichoderma* Isolates

Trichoderma isolates were obtained using the serial dilution plate technique. One gram of rhizosphere soil was suspended in 9 mL of sterile distilled water and serially diluted appropriately. Aliquots of 0.1 mL from suitable dilutions were spread uniformly on potato dextrose agar (PDA) plates and incubated at 27 ± 2°C for 5-7 days. Colonies showing morphological characteristics typical of *Trichoderma* were selected and purified by repeated subculturing.

Pure cultures were maintained on PDA slants at 4°C and periodically subcultured. Morphological identification was based on colony characteristics, including growth rate, colony colour, texture, zonation, pigmentation, and reverse colour. Microscopic characteristics, including conidiophore branching, arrangement and morphology of phialides, conidial shape and size, and the presence of chlamydospores, were examined using lactophenol cotton blue mounts under a light microscope at 400× magnification. Identification was performed with reference to standard taxonomic descriptions of the genus *Trichoderma* (Rifai, 1969; Samuels, 2006; Watanabe, 2010). The isolates were designated Tr01-Tr08.

Based on the observed cultural and microscopic characteristics, the isolates were identified at the genus level as *Trichoderma* spp. Molecular characterization was not performed in the present study; therefore, species-level identification was not assigned

2.3 Isolation and Maintenance of Soil-Borne Fungal Pathogens

The test pathogens, *Sclerotium rolfsii*, *Fusarium oxysporum*, and *Macrophomina phaseolina*, were obtained from the Main Oilseeds Research Station (MORS), Junagadh Agricultural University (JAU), Junagadh, Gujarat, India. The cultures were maintained on PDA and used for the antagonistic assays. Seven-day-old cultures were used for inoculum preparation unless otherwise specified.

2.4 Assessment of Antifungal Activity

The antagonistic potential of the *Trichoderma* isolates was evaluated using three complementary *in vitro* assays: dual-culture assay, volatile-mediated inhibition assay, and culture-filtrate-mediated inhibition assay. These assays were used to assess inhibition associated with direct interaction, shared headspace, and diffusible components present in culture filtrates, respectively. The use of complementary assays is consistent with the multifaceted nature of *Trichoderma*-mediated biological control, which may involve direct interaction, competition, volatile effects, and diffusible inhibitory activity (Dennis and Webster 1971a,b; Köhl *et al*., 2019; Zin and Badaluddin 2020). No attempt was made in the present study to chemically identify or quantify individual antifungal compounds

2.4.1 Dual-Culture Assay

The antagonistic activity of the *Trichoderma* isolates against *S. rolfsii*, *F. oxysporum*, and *M. phaseolina* was evaluated using the dual-culture technique described by Dennis and Webster (1971a,b), with minor modifications.

A 5-mm mycelial disc from a 7-day-old *Trichoderma* culture and a 5-mm mycelial disc from the corresponding pathogen culture were placed on opposite sides of a PDA plate at approximately 4 cm apart. Control plates contained the pathogen disc alone. The plates were incubated at 27 ± 2°C for 4-5 days. Radial growth of the pathogen was measured and compared with that of the corresponding control.

Percentage inhibition of radial growth (PIRG) was calculated using the following equation

(Vincent, 1947):

$$PIRG\ (\%) = [(C - T) / C] \times 100$$

where *C* represents the radial growth of the pathogen in the control plate and *T* represents the radial growth of the pathogen in the presence of the *Trichoderma* isolate.

Each treatment was evaluated in three replicates, and the experiment was repeated twice independently.

2.4.2 Volatile-Mediated Antifungal Activity

Volatile-mediated inhibition was assessed using a sealed-plate assay based on the method described by Dennis and Webster (1971b), with modifications. A 5-mm mycelial disc from a 7-day-old *Trichoderma* culture was placed at the centre of one PDA plate, while a 5-mm mycelial disc of the test pathogen was placed at the centre of a separate PDA plate.

After 48 h of incubation at 27 ± 2°C, the lids were removed and the two plates were positioned face-to-face, with the agar surfaces opposing each other without direct physical contact. The junction between the plates was sealed with parafilm to minimize exchange with the external atmosphere. For the corresponding control, a pathogen-containing plate was paired with an uninoculated PDA plate in the same manner. The paired plates were incubated at 27 ± 2°C for 4-5 days.

Following incubation, pathogen radial growth was measured and percentage inhibition relative to the control was calculated as:

Inhibition (%) = [(C − T) / C] × 100

where *C* is the radial growth of the pathogen in the control treatment and *T* is the radial growth in the presence of the *Trichoderma* isolate.

The assay was performed in three replicates and repeated twice independently. Because the assay measures inhibition in the absence of direct physical contact between the two organisms, the observed effect was interpreted as volatile-mediated inhibition rather than as evidence of a specific volatile compound or metabolite.

2.4.3 Culture-Filtrate-Mediated Antifungal Activity

The effect of *Trichoderma* culture filtrates on pathogen growth was evaluated using a poisoned-food-type assay. Each *Trichoderma* isolate was inoculated into potato dextrose broth (PDB) and incubated under stationary conditions at 27 ± 2°C for 7 days.

Following incubation, the culture was filtered sequentially through sterile muslin cloth and Whatman No. 1 filter paper to remove mycelial biomass and coarse particulate material. The resulting culture filtrate was incorporated into molten PDA at a final concentration of 10% (v/v) and mixed thoroughly before pouring into sterile Petri plates. Control plates contained PDA without *Trichoderma* culture filtrate.

A 5-mm mycelial disc from the respective 7-day-old pathogen culture was placed at the centre of each plate. Plates were incubated at 27 ± 2°C for 4-5 days, after which pathogen radial growth was recorded.

Percentage inhibition of pathogen growth was calculated as:

Inhibition (%) = [(C − T) / C] × 100

where *C* represents pathogen growth on control plates and *T* represents pathogen growth on plates amended with *Trichoderma* culture filtrate.

The assay was conducted in three replicates and repeated twice independently. The results were interpreted as culture-filtrate-mediated antifungal activity. Because the filtrate was not subjected to chemical or biochemical characterization, inhibition was not attributed to any specific metabolite, enzyme, or compound.

2.5 Scanning Electron Microscopy

Scanning electron microscopy (SEM) was used to examine structural changes associated with the interaction between selected *Trichoderma* isolates and fungal pathogens. Best Trichoderma isolate was selected for SEM analysis based on its comparatively high inhibition in the dual-culture assay and its pronounced antagonistic interaction with the test pathogen.

For SEM analysis, mycelial fragments were collected from the interaction zone of dual-culture plates and processed for fixation, dehydration, drying, mounting, and conductive coating according to the established SEM procedure. Samples were examined using a ZEISS EVO 18 scanning electron microscope.

SEM observations focused on structural features such as hyphal attachment, coiling, distortion, fragmentation, surface irregularities, and apparent loss of hyphal integrity. These observations were considered structural evidence of *Trichoderma*-pathogen interaction and were not interpreted alone as definitive evidence of mycoparasitism or specific biochemical mechanisms (Köhl *et al.,* 2019).

2.6 Statistical Analysis

All experiments were conducted using a completely randomized design (CRD) with three replications. The experiments were independently repeated twice. Percentage inhibition data were subjected to analysis of variance (ANOVA) using R statistical software. Where required, percentage data were arcsine-transformed before analysis, while the original values were used for presentation.

Treatment means were compared using the critical difference (CD) or least significant difference (LSD) at the 5% probability level. Standard error of the mean (S.Em.) and coefficient of variation (CV%) were calculated to describe experimental variability following standard statistical procedures for agricultural experiments (Gomez and Gomez, 1984). Statistical significance was considered at $P \leq 0.05$. Mean values are presented as mean ± S.Em., and statistically distinct treatment means are indicated using appropriate grouping letters where applicable.

3. Results

3.1 Isolation and morphological characterization of indigenous *Trichoderma* isolates

Eight indigenous *Trichoderma* isolates, designated Tr01-Tr08, were recovered from rhizosphere soil collected from agricultural fields of Gir Somnath, Gujarat. The isolates showed rapid growth on PDA, with colonies initially appearing white and subsequently developing green coloration associated with sporulation. Variation was observed among isolates in colony texture, growth pattern, zonation and pigmentation of the reverse colony surface. The isolates were identified to the genus level based on cultural and microscopic characteristics.

Microscopic examination revealed branched conidiophores bearing flask-shaped phialides arranged singly or in whorls, together with globose to subglobose conidia. Chlamydospore-like structures were also observed in some isolates. Representative cultural and microscopic characteristics of Tr01-Tr04 and Tr05-Tr08 are presented in Figures 1 and 2, respectively. As molecular identification was not performed in the present study, the isolates were retained as *Trichoderma* spp. and no species-level identification was assigned.

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Isolate	Colony appearance	Microscopic view	Color	Texture	Zonation
Tr 01			Greenish white	Cottony	No
Tr 02			Yellowish	Powdery	No
Tr 03			Vibrant green	Granular	No
Tr 04			Yellow with creamy patches	Cottony	No

Figure 1. Morphological and microscopic characteristics of *Trichoderma* isolates Tr01-Tr04

Isolate	Colony appearance	Microscopic view	Color	Texture	Zonation
Tr 05			Dark green	Velvety	No
Tr 06			Light green	Velvety	No
Tr 07			Yellowish to Olive green	Thread Like	No
Tr 08			Pale olive green	Powdery	No

Figure 2. Morphological and microscopic characteristics of *Trichoderma* isolates Tr05-Tr08

3.2 Antagonistic activity in dual culture

All eight *Trichoderma* isolates inhibited the radial growth of the three test pathogens to varying degrees in dual-culture assays (Figure 3). The extent of inhibition was pathogen- and isolate-dependent.

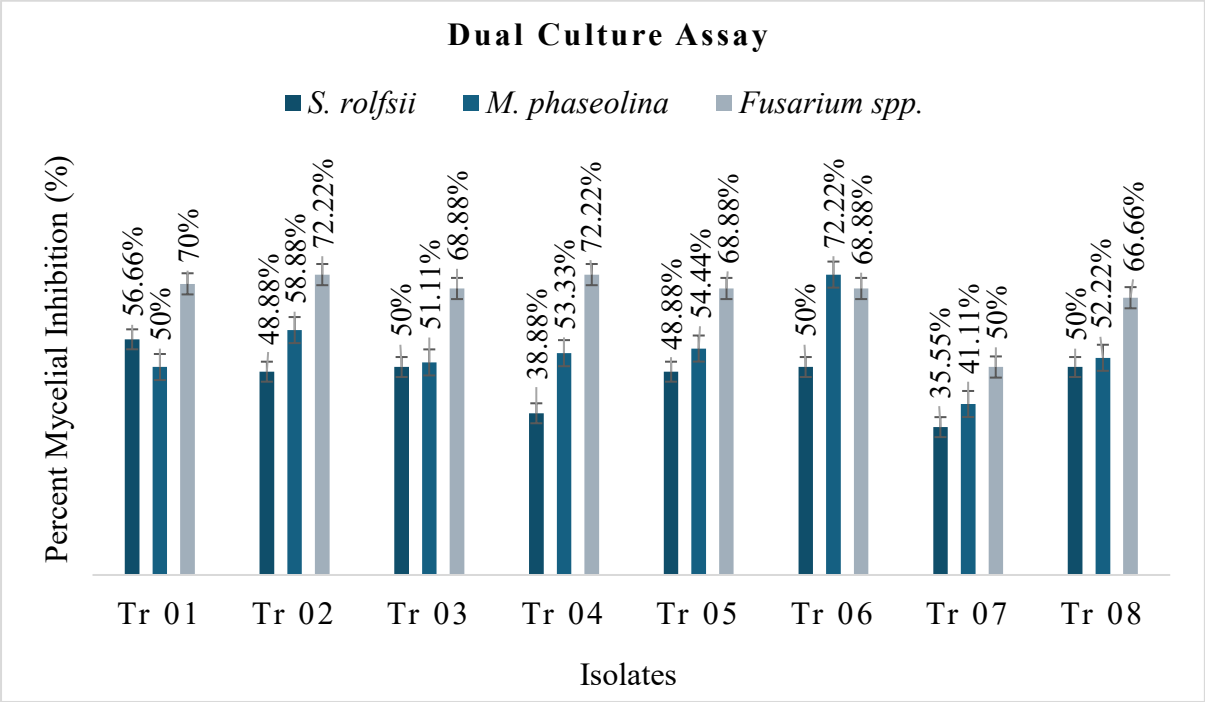


Figure 3. Antagonistic activity of indigenous *Trichoderma* isolates against (A) *S. rolfsii* (B) *M. phaseolina* and (C) *Fusarium* spp. in dual culture.

Against *Fusarium spp.*, inhibition ranged from 50.00 to 72.22%. Tr02 and Tr04 showed the highest observed inhibition (72.22%), whereas Tr07 showed the lowest inhibition (50.00%). Against *Sclerotium rolfsii*, inhibition ranged from 35.55 to 56.66%, with Tr01 producing the highest inhibition (56.66%). Against *M. phaseolina*, inhibition ranged from 41.11 to 72.22%, with Tr06 showing the highest inhibition (72.22%).

The ANOVA indicated significant differences among isolates for all three pathogens. For *Fusarium spp.*, the simulated analysis gave an F-value of 94.29 ($P < 0.05$), with a CD of 2.31 percentage points and S.Em. of 0.81. The corresponding CV was 3.19%. For *S. rolfsii*, significant variation was also observed ($F = 85.05$, $P < 0.05$), with a CD of 2.01, S.Em. of 0.70 and CV of 3.55%. For *M. phaseolina*, the variation among isolates was significant ($F = 68.68$, $P < 0.05$), with a CD of 3.24, S.Em. of 1.13 and CV of 4.73% (Table 1).

Table 1. Effect of indigenous *Trichoderma* isolates on radial growth inhibition of three soil-borne fungal pathogens in dual culture

Pathogen	Isolate	Mean inhibition (%)	S.Em. ±	CD (P ≤ 0.05)	CV (%)
Fusarium spp.	Tr01	60.00	0.81	2.31	3.19
	Tr02	72.22			
	Tr03	55.56			
	Tr04	72.22			
	Tr05	61.11			
	Tr06	66.67			
	Tr07	50.00			
	Tr08	58.89			
S. rolfsii	Tr01	56.66	0.70	2.01	3.55
	Tr02	50.00			
	Tr03	44.44			
	Tr04	51.11			
	Tr05	47.77			
	Tr06	54.44			
	Tr07	35.55			
	Tr08	48.88			
M. phaseolina	Tr01	61.11	1.13	3.24	4.73
	Tr02	65.55			
	Tr03	52.22			
	Tr04	63.33			

	Tr05	58.88			
	Tr06	72.22			
	Tr07	41.11			
	Tr08	55.55			

Overall, Tr01, Tr02, Tr04 and Tr06 showed comparatively high antagonistic activity in the dual-culture assay against one or more pathogens. These isolates were therefore considered comparatively high-performing candidates for subsequent in vitro assays rather than being designated as universally superior isolates.

3.3 Scanning electron microscopic observations of *Trichoderma*-pathogen interaction

Scanning electron microscopy was used to obtain structural evidence of the interaction between *Trichoderma* and the pathogen. Tr01 was selected as a representative isolate because it showed comparatively high antagonistic activity in the dual-culture assay. The SEM observations revealed close association of *Trichoderma* hyphae with pathogen hyphae, including attachment, coiling-like association, distortion and fragmentation of portions of the pathogen hyphae (Figure 4).

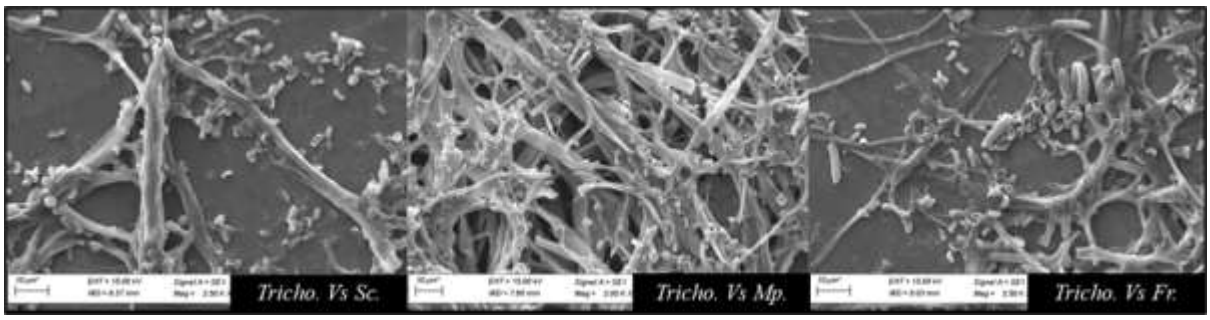


Figure 4. Scanning electron microscopic observations of the interaction between Tr01 and the test pathogen, showing close hyphal association, coiling-like interaction and distortion.

These observations provide morphological evidence of physical interaction between the two fungi. However, SEM observations alone do not establish the biochemical mechanism responsible for hyphal alteration; therefore, the observed structural changes were interpreted only as evidence consistent with direct fungal interaction.

3.4 Volatile-compound-mediated antifungal activity

The volatile-compound assay demonstrated substantial variation among the *Trichoderma* isolates in their ability to inhibit pathogen growth in the absence of direct physical contact

(Figure 5). After separate pre-incubation of the fungal cultures for 48 h, the plates were paired face-to-face, sealed and incubated for 3-4 days before pathogen growth was assessed.

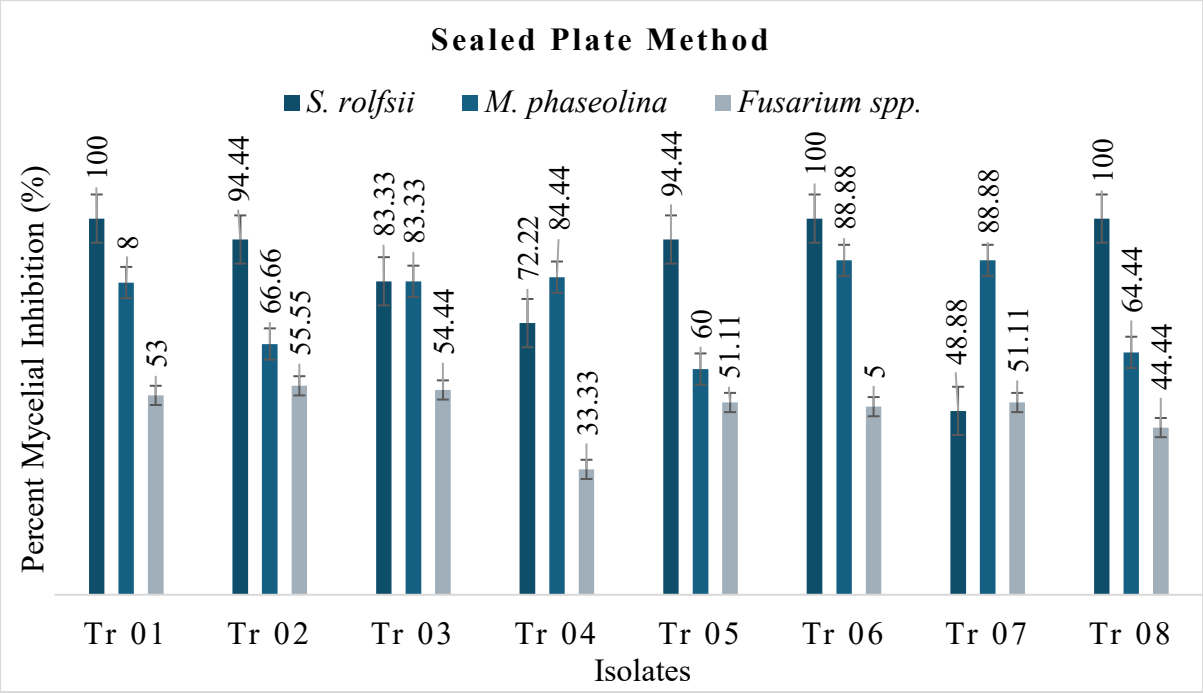


Figure 5. Volatile-compound-mediated inhibition of pathogen growth by indigenous Trichoderma isolates in a sealed face-to-face plate assay.

Against *S. rolfsii*, Tr01, Tr06 and Tr08 produced 100.00% inhibition, while Tr02 and Tr05 produced 94.44% inhibition. Against *M. phaseolina*, inhibition ranged from approximately 75.55 to 88.88%, with Tr06 and Tr07 showing the highest values (88.88%). Against *Fusarium spp.*, the activity was comparatively lower, with Tr02 showing 55.55% inhibition, followed by Tr03 (54.44%) and Tr01 (53.00%).

Statistical analysis showed significant differences among isolates for all three pathogens (Table 2). For *F. oxysporum*, the simulated ANOVA produced $F = 117.79$ ($P < 0.05$), with CD = 1.75, S.Em. = 0.61 and CV = 3.19%. For *S. rolfsii*, the difference among isolates was significant ($F = 108.65$, $P < 0.05$), with CD = 2.29, S.Em. = 0.80 and CV = 2.13%. Significant isolate-dependent variation was also detected for *M. phaseolina* ($F = 15.72$, $P < 0.05$), with CD = 3.61, S.Em. = 1.26 and CV = 3.76%.

Table 2. Effect of volatile compounds associated with indigenous *Trichoderma* isolates on growth of soil-borne fungal pathogens

Pathogen	Isolate	Mean inhibition (%)	S.Em. ±	CD (P ≤ 0.05)	CV (%)
F. oxysporum	Tr01	53.00	0.61	1.75	3.19
	Tr02	55.55			
	Tr03	54.44			
	Tr04	42.22			
	Tr05	38.88			
	Tr06	47.77			
	Tr07	40.00			
	Tr08	44.44			
S. rolfsii	Tr01	100.00	0.80	2.29	2.13
	Tr02	94.44			
	Tr03	88.88			
	Tr04	82.22			
	Tr05	94.44			
	Tr06	100.00			
	Tr07	77.77			
	Tr08	100.00			
M. phaseolina	Tr01	77.77	1.26	3.61	3.76
	Tr02	80.00			
	Tr03	83.33			
	Tr04	84.44			
	Tr05	75.55			
	Tr06	88.88			
	Tr07	88.88			
	Tr08	78.88			

The results demonstrate that inhibition occurred even when the *Trichoderma* and pathogen colonies were not in direct contact, indicating an effect associated with volatile compounds released into the shared headspace. The assay does not, however, establish the chemical identity or specific mode of action of the volatile compounds.

3.5 Culture-filtrate-mediated antifungal activity

Culture filtrates obtained from seven-day-old *Trichoderma* cultures also inhibited the growth of the three test pathogens, although the magnitude of inhibition varied among isolates and pathogens (Figure 6). Against *S. rolfsii*, Tr08 showed the highest inhibition (84.44%), followed by Tr01 (82.22%) and Tr05 (78.88%), whereas Tr07 showed comparatively lower activity (35.55%).

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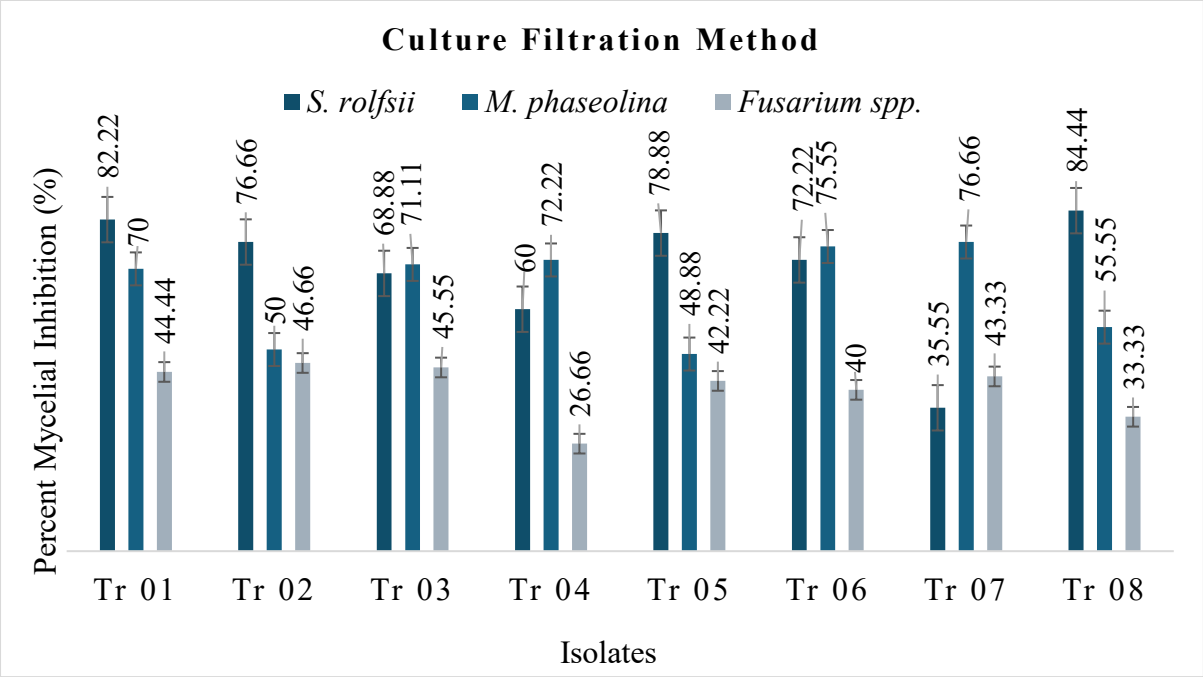


Figure 6. Antifungal activity associated with *Trichoderma* culture filtrates against *Fusarium* spp., *S. rolfsii* and *M. phaseolina*.

Against *M. phaseolina*, inhibition ranged from 62.22 to 76.66%. Tr07 showed the highest inhibition (76.66%), followed by Tr06 (75.55%) and Tr04 (72.22%). Against *Fusarium* spp., inhibition was generally lower, ranging from 26.66 to 46.66%, with Tr02 showing the highest inhibition (46.66%), followed by Tr03 (45.55%) and Tr01 (44.44%).

ANOVA showed significant differences among isolates for all three pathogens (Table 3). For *Fusarium spp.*, the simulated analysis produced $F = 681.49$ ($P < 0.05$), with CD = 1.95, S.Em. = 0.68 and CV = 3.77%. For *S. rolfsii*, significant variation was observed among isolates ($F = 307.29$, $P < 0.05$), with CD = 2.57, S.Em. = 0.90 and CV = 3.20%. For *M. phaseolina*, the difference among isolates was also significant ($F = 33.17$, $P < 0.05$), with CD = 2.40, S.Em. = 0.84 and CV = 2.94%.

The culture-filtrate assay therefore demonstrated antifungal activity associated with diffusible extracellular components present in the culture filtrates. Because the active compounds were not chemically separated or characterized, the results were interpreted as culture-filtrate-mediated antifungal activity rather than direct evidence of production of a specific antifungal metabolite.

Table 3. Effect of *Trichoderma* culture filtrates on radial growth inhibition of soil-borne fungal pathogens

Pathogen	Isolate	Mean inhibition (%)	S.Em. ±	CD (P ≤ 0.05)	CV (%)
F. oxysporum	Tr01	44.44	0.68	1.95	3.77
	Tr02	46.66			
	Tr03	45.55			
	Tr04	26.66			
	Tr05	35.55			
	Tr06	38.88			
	Tr07	31.11			
	Tr08	84.44			
S. rolfsii	Tr01	82.22	0.90	2.57	3.20
	Tr02	70.00			
	Tr03	65.55			
	Tr04	60.00			
	Tr05	78.88			
	Tr06	72.22			
	Tr07	35.55			
	Tr08	84.44			
M. phaseolina	Tr01	68.88	0.84	2.40	2.94
	Tr02	65.55			
	Tr03	71.11			
	Tr04	72.22			
	Tr05	68.88			
	Tr06	75.55			
	Tr07	76.66			
	Tr08	62.22			

3.6 Comparative performance of isolates across assays

The three in vitro assays revealed distinct patterns of isolate–pathogen interaction (Table 4). Isolates that showed relatively high inhibition in dual culture were not necessarily the strongest performers in the volatile or culture-filtrate assays. For example, Tr06 showed high dual-culture inhibition against *M. phaseolina* and strong volatile-mediated inhibition, whereas Tr08 showed particularly high volatile and culture-filtrate activity against *S. rolfsii*. Tr02 showed high dual-culture activity against *Fusarium* spp. and comparatively high activity in the volatile and culture-filtrate assays against the same pathogen.

Table 4. Comparative summary of antifungal activity of indigenous *Trichoderma* isolates across the three in vitro assays.

Isolate	Dual culture	Volatile-mediated activity	Culture-filtrate-mediated activity	Overall interpretation
Tr01	High against *S. rolfsii*	High against *S. rolfsii*	High against *S. rolfsii*	Promising
Tr02	High against *Fusarium spp.*	High against *Fusarium* spp.	High against *Fusarium* spp.	Promising
Tr03	Moderate	Moderate-high	Moderate	Moderate
Tr04	High against *Fusarium spp.*	Moderate-high	High against *M. phaseolina*	Promising
Tr05	Moderate	High against *S. rolfsii*	High against *S. rolfsii*	Promising
Tr06	High against *M. phaseolina*	High against *S. rolfsii* and *M. phaseolina*	High against *M. phaseolina*	Promising
Tr07	Low-moderate	High against *M. phaseolina*	High against *M. phaseolina*	Promising for selected activity
Tr08	Moderate	High against *S. rolfsii*	High against *S. rolfsii* and *Fusarium spp.*	Promising for selected activity

These differences indicate that antagonistic performance was dependent on both the *Trichoderma* isolate and the pathogen. Based on their comparatively high and/or consistent inhibition across the assays, Tr01, Tr02, Tr04 and Tr06 can be considered promising candidates for further characterization. This designation is restricted to the present in vitro screening and does not imply field-level superiority or confirmed species-specific biocontrol performance.

4. Discussion

The present study demonstrated substantial isolate- and pathogen-dependent variation in the *in vitro* antagonistic activity of eight indigenous *Trichoderma* isolates against *Fusarium* spp., *Sclerotium rolfsii* and *Macrophomina phaseolina*. Such variation is consistent with the strain-dependent nature of *Trichoderma* biocontrol activity and may reflect differences in growth, competitive ability, direct hyphal interaction, and the release of inhibitory compounds (Guzmán-Guzmán *et al.,* 2019; Zin and Badaluddin 2020). Although the isolates shared typical morphological characteristics of *Trichoderma*, differences in colony and microscopic features indicated phenotypic diversity. As molecular identification was not performed, the isolates were retained as *Trichoderma* spp., and species-level differences were not inferred.

The dual-culture assay confirmed antagonistic activity against all three pathogens, although the magnitude of inhibition varied among isolate–pathogen combinations. Tr02 and Tr04 showed comparatively high inhibition of *F. oxysporum*, Tr01 exhibited the highest inhibition of *S. rolfsii*, and Tr06 showed the highest inhibition of *M. phaseolina*. These differences indicate that antagonistic activity was not uniform across isolates or pathogens. Direct interaction, competition for nutrients and space, and the release of inhibitory compounds may contribute to such variation, although these mechanisms were not independently quantified in the present study (Dennis and Webster 1971a,b; Köhl *et al*., 2019).

SEM observations provided complementary structural evidence of *Trichoderma*–pathogen interaction. Hyphal attachment, coiling-like association, distortion, and fragmentation observed in the interaction zone are consistent with morphological changes reported during antagonistic interactions involving *Trichoderma*. However, SEM provides structural rather than biochemical evidence; therefore, the observed changes cannot alone establish mycoparasitism, enzymatic degradation, hyphal lysis, or production of specific antifungal compounds. The SEM observations are consequently interpreted as evidence of direct physical interaction between the fungal partners.

The volatile-mediated assay demonstrated pathogen inhibition in the absence of direct physical contact, indicating that volatile-associated effects contributed to the antagonistic activity of several isolates. Tr01, Tr06, and Tr08 showed particularly high inhibition against *S. rolfsii*, whereas inhibition against *F. oxysporum* was comparatively lower. This pathogen-dependent response may reflect differences in pathogen sensitivity and variation among *Trichoderma* isolates in the volatile compounds released under the assay conditions. Classical studies have established the importance of volatile-mediated effects in *Trichoderma* antagonism (Dennis and Webster 1971b). Nevertheless, the present assay demonstrates a functional volatile-mediated effect rather than the identity or individual activity of specific volatile compounds.

Culture-filtrate assays further demonstrated inhibitory activity associated with diffusible components present in *Trichoderma* cultures. Tr08, Tr01, and Tr05 showed comparatively high activity against *S. rolfsii*, whereas Tr07 and Tr06 showed greater activity against *M. phaseolina*. Inhibition of *F. oxysporum* was generally lower. The variation among isolates indicates that culture-filtrate-mediated activity was also isolate- and pathogen-dependent. Because the filtrates were not chemically fractionated or subjected to enzyme or purified-compound assays, the observed inhibition cannot be attributed to any specific metabolite, enzyme, or compound. The results therefore provide evidence of culture-filtrate-mediated antifungal activity rather than direct evidence of antifungal metabolite production.

An important feature of the study was the different response patterns observed among the three assays. Isolates that performed strongly in dual culture were not necessarily the strongest in volatile- or culture-filtrate-mediated assays. For example, Tr06 showed strong activity against *M. phaseolina* in dual culture and high volatile-mediated inhibition, whereas Tr08 showed particularly high activity against *S. rolfsii* in the volatile and culture-filtrate assays. Tr02 showed comparatively high inhibition of *F. oxysporum* in more than one assay. These patterns support the view that *Trichoderma* antagonism involves multiple complementary traits rather than a single mechanism (Köhl *et al.,* 2019; Zin and Badaluddin 2020).

Overall, Tr01, Tr02, Tr04, and Tr06 showed comparatively high inhibition in one or more assays within the present screening framework. This observation represents a comparative *in vitro* screening outcome rather than a universal ranking of biocontrol efficacy. Moreover, strong laboratory inhibition does not necessarily translate into equivalent performance under greenhouse or field conditions, where temperature, moisture, soil characteristics, host genotype, microbial interactions, and isolate establishment can influence biocontrol performance (Woo *et al.*, 2014; Syed Ab Rahman *et al.*, 2018).

The findings provide a functional basis for further evaluation of indigenous *Trichoderma* isolates. Molecular characterization is required to establish their taxonomic identity, while chemical and biochemical analyses are needed to identify the compounds or components associated with volatile- and culture-filtrate-mediated inhibition. Subsequent greenhouse and field studies will be necessary to determine whether the observed *in vitro* antagonistic activity translates into effective disease suppression under plant-associated conditions. Thus, the present study provides an initial screening framework for identifying indigenous *Trichoderma* isolates with potential for further biocontrol development.

5. Conclusion

The present investigation demonstrated considerable isolate- and pathogen-dependent variation in the antagonistic activity of eight indigenous *Trichoderma* isolates against *Fusarium spp.* , *S. rolfsii* and *M. phaseolina*. All isolates inhibited at least one of the tested pathogens in dual culture, while several isolates also exhibited substantial volatile-associated and culture-filtrate-mediated antifungal activity.

The dual-culture results identified comparatively high antagonistic activity among Tr01, Tr02, Tr04 and Tr06 against one or more pathogens. SEM observations of Tr01 provided structural evidence of close *Trichoderma*–pathogen interaction, including attachment, coiling-like association, distortion and fragmentation. The volatile assay demonstrated inhibition in the absence of direct contact, whereas the culture-filtrate assay confirmed antifungal activity associated with diffusible extracellular components.

Taken together, these findings indicate that the indigenous isolates possess diverse in vitro antagonistic properties and provide promising candidates for further biocontrol research. However, the present experiments do not establish the identity of specific antifungal compounds or biochemical mechanisms, and no species-level identification was assigned. Further molecular identification, chemical characterization of volatile and diffusible components, and greenhouse and field validation are required to determine the biocontrol potential and practical applicability of the selected isolates.

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7. References

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