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Evaluation of Native Tanzanian *Trichoderma* Isolates for Biocontrol of *Fusarium oxysporum* f. sp. *lycopersici* In Vitro

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Abstract

Trichoderma species are commonly used as biological control agents against soil-borne pathogens, such as *Fusarium oxysporum* f. sp. *lycopersici* (FoL), which causes tomato wilt. However, their effectiveness varies based on isolate traits and bioassay conditions. This research tested 46 native Tanzanian *Trichoderma* isolates, along with one commercial strain, against FoL strain PD- 87/91 using various in vitro bioassays. Dual culture tests were performed with both simultaneous and staggered inoculations, and pathogen growth inhibition (PGI) was analyzed via two-way ANOVA in R software. Significant impacts of the isolate, inoculation method, and their interaction on PGI were found ($p < 0.001$). Under simultaneous inoculation, PGI ranged from 62. 1% (*Trichoderma erinaceum* MG 3- 1) to 79. 9% (*Trichoderma harzianum* MG 7- 2), while under staggered inoculation, PGI varied from 47. 6% (*Trichoderma* virens NH 7- 3) to 58. 6% (*Trichoderma harzianum* SH 10- 2). Several native isolates performed better than the commercial control in both methods. Bell' s scale assessment showed mostly class 1 interactions during simultaneous inoculation, indicating complete pathogen suppression by *Trichoderma*, whereas class 2 interactions dominated during staggered inoculation. No class 4 or 5 interactions were detected. During interactions, yellow coloration was highly common (>80%) under both methods, though Pearson' s Chi-square test found no significant link between color and inoculation method ($X2 = 0.018. 018$, $p = 0.892. 892$). VOC assays revealed significant differences among isolates ($p < 0. 001$), with SH 11- 2 (66. 22%) and KT 2- 2 (65. 92%) showing the highest VOC- mediated inhibition, outperforming the commercial strain (52. 23%). The findings indicate that *Trichoderma*' s antagonistic activity depends on both the specific isolate and the method, involving direct competition and VOC- based antibiosis. Combining PGI data, Bell' s scale, colour interaction, and VOC tests offers a comprehensive approach to selecting effective isolates for further research on biological control of tomato wilt in planta.

Keywords: *Trichoderma*; *Fusarium oxysporum*; biological control; antagonistic activity; volatile organic compounds

1. Introduction

Soil-borne fungal pathogens remain a significant constraint to global agricultural productivity, especially in tropical regions where high temperature and humidity accelerate disease progression. Among these, *Fusarium oxysporum* f. sp. *lycopersici* (FoL), is exceptionally devastating to tomato cultivation, causing substantial yield losses that threaten smallholder farmers[1,2]. While chemical fungicides have traditionally been used, their efficacy is increasingly compromised by rising pathogen resistance and environmental and food safety concerns. Consequently, there is an urgent need to identify native, ecologically adapted biocontrol agents, such as *Trichoderma* species, which offer a sustainable and locally viable alternative for disease suppression [3].

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Trichoderma species are premier biocontrol agents characterised by rapid growth, robust rhizosphere colonisation, and multifaceted antagonism against a broad spectrum of plant pathogens. Their suppressive power is mediated through direct mechanisms, including mycoparasitism, antibiosis, competition for nutrients, as well as induction of systemic resistance in host plants [4–6] . Beyond these direct interactions, *Trichoderma spp*. synthesises a variety of volatile organic compounds (VOCs) that play a crucial role in suppressing pathogens via non-contact mechanisms. These metabolites inhibit pathogen growth through non-contact mechanisms, meaning they can reduce pathogen development even without physical contact [7–9]. Several VOCs, such as 6-pentyl-2H-pyran-2-one, 2-pentylfuran, and 1-octen-3-ol, have been shown to significantly inhibit key plant pathogens, including *Fusarium oxysporum* and *Botrytis cinerea*. Besides their inhibitory effects, *Trichoderma* VOCs also act as signalling molecules, affecting plant physiological responses [10–13]. They can promote plant growth, activate defence-related genes, and influence the composition and behaviour of soil microbial communities. Overall, these volatile compounds underscore the complex role of *Trichoderma* in biological control and its broader ecological impacts.

While research has traditionally prioritised established commercial *Trichoderma* isolates, these non-native strains often underperform in diverse ecological settings [14]; consequently, there is a critical need to isolate and utilise indigenous species, as their superior local adaptation and ecological fitness provide enhanced competitive advantages within their native environments [15–17]. Although Sokoine University of Agriculture maintains a diverse collection of native *Trichoderma* isolates, their antagonistic potential against soil-borne pathogens remains largely uncharacterized. Consequently, there is an urgent need to evaluate these specific accessions to identify superior biocontrol agents tailored for suppressing *Fusarium* wilt in Tanzanian tomato cultivation.

This study evaluates the antagonistic potential of native *Trichoderma* isolates from the Sokoine University of Agriculture collection against FoL using dual-culture assays with both simultaneous and staggered inoculation methods. By assessing both direct competitive interactions and the inhibitory effects of volatile organic compounds (VOCs), this research provides the first comprehensive biocontrol profiling of Tanzanian *Trichoderma* strains. Ultimately, these findings establish a vital baseline for developing locally adapted, sustainable biological control products tailored to the Tanzanian horticultural sector.

2. Materials and Methods

2.1. Sampling and Pathogen Isolation Procedures

Tomato plants exhibiting typical symptoms of *Fusarium* wilt, such as yellowing, leaf drooping, and vascular browning (Figure 1), were collected from tomato fields at Mlali village (6°56′37″ S, 37°33′03″ E), Morogoro Region, Tanzania, on 26 August 2025. Samples were placed in sterile polyethylene bags, labelled, and transported to the Plant Pathology Laboratory, Department of Crop Science and Horticulture, Sokoine University of Agriculture, for analysis.

Figure 1. Symptoms of tomato wilt disease and root damage observed under field conditions.(A) Tomato plants showing typical wilt symptoms in the field. (B) Infected tomato plant exhibiting stem collapse and reduced vigour after uprooting. (C) Severely damaged root system with extensive root rot. (D) Cross-section of a tomato

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stem showing internal vascular discolouration. (E) Longitudinal section of a tomato stem showing brown vascular tissues associated with wilt infection.

Infected tomato stems were surface sterilized using 1% sodium hypochlorite for 2 minutes, rinsed three times with sterile distilled water, and dried on sterile paper towels. Small sections (3 mm) from the margins of diseased tissue were plated on Potato Dextrose Agar (PDA) amended with chloramphenicol (50 mg/L) to inhibit bacterial growth. Plates were incubated at room temperature for 7 days (Figure 2 A&B).

Figure 2. Isolation of Fusarium oxysporum associated with tomato wilt. (A) Surface-sterilised tomato stem fragments used for fungal isolation. (B) Initial fungal growth emerging from infected stem tissues on culture medium.

2.2. Morphological Identification

Emerging fungal colonies with morphological characteristics of *Fusarium spp*. were sub-cultured on fresh PDA to obtain pure cultures through hyphal-tip transfer. Identification of *F. oxysporum* was based on colony morphology, pigmentation, and microscopic characteristics such as the shape of macroconidia and microconidia, following Tilahun et al., 2024.

2.3. Molecular Identification of Pathogen

i. DNA extraction and PCR amplification

Genomic DNA was extracted from pure fungal cultures using CTAB-based method. Briefly, a CTAB/NaCl solution was added to the fungal biomass, followed by centrifugation, and the resulting pellet was washed twice with NE buffer. The washed pellet was then resuspended in 2× CTAB buffer supplemented with β-mercaptoethanol and polyvinylpyrrolidone (PVP), mechanically ground, vortexed, and incubated at 65 °C to facilitate cell lysis.

Following lysis, chloroform–isoamyl alcohol (24:1, v/v) was added, mixed thoroughly, and centrifuged to allow phase separation. The aqueous phase was transferred to a new tube and treated with RNase, followed by incubation at 37 °C for 1 h. Genomic DNA was precipitated using cold isopropanol in the presence of sodium and incubated either at −20 °C. The DNA pellet was recovered by centrifugation, washed with chilled 70% ethanol, air-dried, and resuspended in distilled water. Extracted DNA was stored at −20 °C until further use.

The internal transcribed spacer (ITS) region was amplified using the universal primer pairs ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) [19–21]. PCR reactions were carried out in a total volume of 25 µl, containing 11.5 µl nuclease-free water, 0.5 µl of each primer (10 µM), and the appropriate PCR master mix. Amplification was performed in a thermal cycler with an initial denaturation at 94 °C for 30 s, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 54.2 °C for 45 s, and extension at 68 °C for 59 s, with a final extension at 68 °C for 5 min and a hold at 4 °C.

PCR products (5 µl) were separated on a 1% (w/v) agarose gel prepared in Tris–acetate–EDTA (TAE) buffer, alongside a 100 bp Plus DNA ladder (Bioneer). Purified PCR products were submitted

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for sequencing to Macrogen (Netherlands) using the same primers as those employed for amplification.

ii. Sequence analysis and phylogenetic reconstruction

Sequence chromatograms were examined, trimmed, and cleaned using Sequence Scanner Software version 2 (Applied Biosystems, Thermo Fisher Scientific) to remove low-quality bases and ambiguous reads. High-quality consensus sequences were then subjected to similarity searches against the NCBI GenBank database using the BLASTn algorithm [22,23]. To further validate species identification and assess evolutionary relationships, a phylogenetic analysis was conducted using the Maximum Likelihood (ML) method implemented in MEGA version 12. The analysis employed the Jukes–Cantor nucleotide substitution model and was based on six nucleotide sequences comprising 264 aligned positions [24]. The robustness of the phylogenetic tree was evaluated using an adaptive bootstrap analysis with 25 replicates, and bootstrap support values were displayed at the branch nodes. The initial tree topology was created by comparing Neighbour-Joining and Maximum Parsimony methods. The tree with the highest log-likelihood (−1,032.41) was chosen. The maximum likelihood phylogenetic tree was constructed using the Jukes–Cantor nucleotide substitution model in MEGA 12. Bootstrap support values from 25 replicates are displayed at the branch nodes. The isolates from this study group were compared with reference *Fusarium oxysporum* sequences, confirming their species-level identification.

2.4. Pathogenicity Tests

The pathogenicity of a selected *Fusarium oxysporum* f. sp. *lycopersici* isolate was tested on tomato seedlings grown in a greenhouse. Tomato seeds of the Money Maker variety at the four-true-leaf stage were carefully uprooted and gently rinsed with tap water to remove soil residues. The root system was trimmed about 7 mm below the taproot tip to promote infection. A microconidial suspension was prepared from actively growing fungal cultures and diluted with sterile distilled water to a concentration of 10^6 conidia per ml. The roots of the tomato seedlings were immersed in this suspension for 15 minutes, while control plants were dipped in sterile water only. After inoculation, the seedlings were transplanted into sterilised soil in plastic pots and kept under greenhouse conditions [25,26]. The experiment used one pathogenic *Fusarium oxysporum* isolate (strain PD-87/91) with four replicates, along with non-inoculated control plants. Plants were watered regularly and observed for symptom development. Disease was assessed three weeks after inoculation, focusing on both external and internal symptoms. Inoculated plants showed typical *Fusarium* wilt signs, such as leaf yellowing, wilting, curling, reduced vigour, and progressive chlorosis. Control plants remained healthy without visible symptoms.The pathogenicity test confirmed the isolate's virulence by inducing characteristic *Fusarium* wilt symptoms in tomato plants under greenhouse conditions (Figure 3).

Figure 3. Pathogenicity test of Fusarium oxysporum f. sp. lycopersici on tomato plants under greenhouse conditions. (A) Healthy non-inoculated control plant; (B) inoculated tomato plants exhibiting Fusarium wilt symptoms including chlorosis, wilting, and reduced vigor.

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2.5. Native Trichoderma Isolates

Forty-six *Trichoderma* isolates (Table 1), previously identified by Nyakeko et al. (in press) as *Trichoderma harzianum*, *T. viride*, *T. longibrachiatum*, *T. erinaceum*, *T. koningiopsis*, *T. atroviride*, *T. paraviridescens*, *T. pleuroti*, *T. virens* and *T. asperellum*, were isolated from lowland rice agroecosystems across six agroecological zones of Tanzania and used in the present study. The isolates were preserved on PDA slants at 4 °C under refrigeration before the experiment. Before use, each isolate was aseptically subcultured onto fresh potato dextrose agar (PDA) plates and incubated under laboratory conditions to obtain actively growing cultures for subsequent antagonistic assays.

Table 1. Isolates used in this experiment.

Isolate ID	Isolate identity	Nativity
MG7-2	*T. harzianum*	Semi-arid zone
SH11-2	*T. harzianum*	Southern Highland zone
KT7-1	*T. paraviridescens*	Coastal zone
BH10-2	*T. harzianum*	Central zone
KT2-2	*T. harzianum*	Coastal zone
SH11-4	*T. harzianum*	Southern Highland zone
MS3-3	*T. asperellum*	Alluvial zone
MG1-2	*T. harzianum*	Semi-arid zone
BH8-2b	*T. harzianum*	Central zone
SH10-2	*T. harzianum*	Southern Highland zone
THC	*Commercial*	Agro Shop
KT8-1	*T. pleuroti*	Coastal zone
MS5-3A	*T. harzianum*	Alluvial zone
MG2-3	*T. harzianum*	Semi-arid zone
NH9-2	*T. virens*	Northern Highland zone
KH6-1	*T. koningiopsis*	Semi-arid zone
NH5-2	*T. harzianum*	Northern Highland zone
SH5-2	*T. harzianum*	Southern Highland zone
KH3-2	*T. harzianum*	Semi-arid zone
BH3-1	*T. longibrachiatum*	Central zone
NH7-3	*T. virens*	Northern Highland zone
BH7-2	*T. harzianum*	Central zone
KT4-3	*T. virens*	Coastal zone
KT10-2	*T. harzianum*	Coastal zone
SH4-4	*T. harzianum*	Southern Highland zone
KT5-2	*T. harzianum*	Coastal zone
MS4-3	*T. harzianum*	Alluvial zone
BH2-3	*T. harzianum*	Central zone
NH2-2	*T. harzianum*	Northern Highland zone
MS8-4	*T. harzianum*	Alluvial zone
MR5-4	*T. atroviride*	Semi-arid zone
SH10-3	*T. harzianum*	Southern Highland zone
MR2-4	*T. harzianum*	Semi-arid zone
KT6-4	*T. harzianum*	Coastal zone
KH5-1	*T. harzianum*	Semi-arid zone
MS2-3	*T. harzianum*	Alluvial zone
SH3-3	*T. harzianum*	Southern Highland zone
KT8-4	*T. virens*	Coastal zone
BH5-1	*T. longibrachiatum*	Central zone

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KH7-1	*T. harzianum*	Semi-arid zone
BH6-2	*T. harzianum*	Central zone
NH1-2	*T. harzianum*	Northern Highland zone
NH4-4	*T. harzianum*	Northern Highland zone
NH3-3	*T. harzianum*	Northern Highland zone
KL7-4	*T. harzianum*	Alluvial zone
NH6-3	*T. harzianum*	Northern Highland zone
MG3-1	*T. erinaceum*	Semi-arid zone

2.6. Dual Culture Assay

The antagonistic potential of 47 *Trichoderma* isolates (both native and commercial) against *Fusarium oxysporum* f. sp. *lycopersici* (FoL) was tested using a dual culture assay on Potato Dextrose Agar (PDA), following a Completely Randomized Design (CRD) with four replications per treatment [27,28]. The experiment was carried out on 84 mm diameter Petri dishes. Two approaches were used: simultaneous inoculation, where 5 mm mycelial discs of *Trichoderma* and FoL were placed on opposite sides of the plate at an equal distance from the center, about 74 mm apart edge-to-edge, the same day; and staggered inoculation, where FoL was first inoculated and allowed to grow for 48 hours before adding *Trichoderma* on the opposite side. The plates were incubated at 25±2°C for 10 days, after which the radial growth of FoL was measured along two perpendicular axes, and its growth inhibition was calculated relative to controls. The Percentage Growth Inhibition (PGI) was calculated using the formula:

$$\text{PGI} = \frac{(\text{R1} - \text{R2})}{\text{R1}} x\ 100$$

where R1 represents the radial growth of the pathogen in the control and R2 represents the growth of the same in the dual culture.

Additionally, the degree of antagonism was ranked using Bell's Scale [29]: *1 = Trichoderma completely overgrew the pathogen (100% overgrowth); 2 = Trichoderma overgrew at least two-thirds of the pathogen (75% overgrowth); 3 = Trichoderma colonized half of the pathogen (50% overgrowth); 4 = Trichoderma and the pathogen met at the contact point after inoculation; 5 = the pathogen overgrew the Trichoderma isolate.*

The occurrence of yellow coloration during the interaction between *Trichoderma* isolates and *Fusarium oxysporum* was visually assessed throughout the dual culture assay. Following incubation, each Petri dish was examined for the presence or absence of yellow pigmentation at the interaction zone between the antagonist and the pathogen. Colour development was recorded qualitatively as either "Yes" (presence of yellow coloration) or "No" (absence of yellow coloration). The observations were documented for both simultaneous and staggered inoculation methods and subsequently analyzed to determine the frequency of colour occurrence under each inoculation condition.

2.7. Volatile Organic Compounds (Vocs)

From the dual culture assay, the eight best-performing isolates and THC (commercial *Trichoderma harzianum*) were used to test their non-contact antagonism against FoL. This aimed to evaluate the biocontrol efficacy of *Trichoderma* by testing if its gaseous metabolites can inhibit *Fusarium* growth. In this experiment, selected *Trichoderma* isolates were tested using a double-plate (sealed-base) method. Each *Trichoderma* isolate was cultured on Potato Dextrose Agar (PDA) and incubated at room temperature for five days. A 5-mm mycelial disc from the edge of the colony was placed at the centre of a new PDA plate. *Fusarium oxysporum* was grown on PDA for seven days, and a similar 5-mm mycelial plug was transferred to the centre of a fresh PDA plate as the target. To assess the effects of VOCS, a plate with *Trichoderma* was paired face-to-face with a *Fusarium* plate, and both were tightly sealed with a sealing tape to prevent gas exchange from outside and to avoid physical contact [30,31].

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A control was prepared by placing an empty PDA plate alongside a *Fusarium* plate under identical conditions. All plates were incubated in the dark at room temperature. At the 7th day, the radial growth of *Fusarium* was measured along two perpendicular axes, and the percentage growth inhibition (PGI) was calculated using the formula above.

2.8. Data Analysis

All experimental data were analyzed statistically with R software (version 4.5.0). The Shapiro–Wilk test checked the normality of residuals. For the dual culture assay, the effects of *Trichoderma* isolate, inoculation method (simultaneous and staggered), and their interaction on pathogen growth inhibition (PGI) were examined using two-way ANOVA under a Completely Randomized Design (CRD). When significant differences appeared, treatment means were compared using Tukey's Honest Significant Difference (HSD) test at ($p < 0.05$). In the volatile organic compounds (VOC) assay, differences in PGI among isolates were analyzed with one-way ANOVA followed by Tukey's HSD test at ($p < 0.05$). Bell's scale data were descriptively summarized with means and standard deviations for each isolate and inoculation method. Graphs illustrated variations in antagonistic performance among isolates under different conditions. Color differences between inoculation methods were analyzed using Pearson's Chi-square test. Frequencies and percentages were displayed with pie charts.

3. Results

3.1. Identification of Fusarium oxysporum

i. Morphological identification

The three fungal isolates exhibited morphological characteristics consistent with *Fusarium oxysporum* when cultured on Potato Dextrose Agar (PDA). All isolates produced abundant white aerial mycelia with varying degrees of pink pigmentation at the colony center. Isolate A (*Fusarium oxysporum* strain NIDHL-MOHA) showed dense cottony white mycelia with slight pinkish-purple coloration, isolate B (*Fusarium oxysporum* isolate T23) exhibited pale pink pigmentation with relatively lighter aerial growth, while isolate C (*Fusarium oxysporum* strain PD-87/91) developed profuse white mycelia with pronounced pink pigmentation and abundant sporulation. Microscopic examination revealed hyaline and septate hyphae together with characteristic curved fusiform conidia typical of *Fusarium oxysporum*.(Figure 4 A-D)

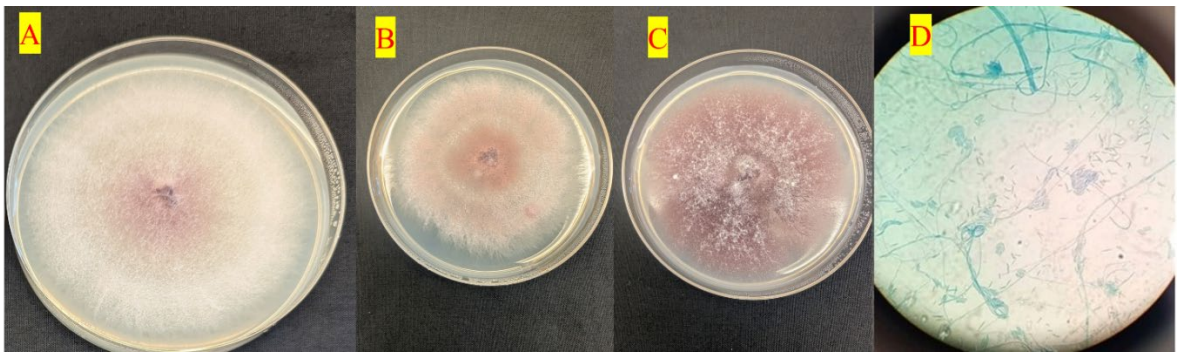

Figure 4. Morphological characteristics of Fusarium oxysporum isolates cultured on Potato Dextrose Agar (PDA). (A) Fusarium oxysporum strain NIDHL-MOHA, (B) Fusarium oxysporum isolate T23, (C) Fusarium oxysporum strain PD-87/91 (D) septate hyphae and characteristic conidia structures of Fusarium oxysporum.

ii. Molecular identification

BLAST analysis showed complete query coverage and very high sequence similarity (99.63–100%) to verified *Fusarium oxysporum* reference sequences in GenBank. The top matches included F. *oxysporum* strain NIDHL-MOHA (OK037502.1), strain PD-87/91 (MZ416903.1), and isolate T23

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(MZ868618.1), confirming the pathogen as *Fusarium oxysporum*. The ML phylogenetic tree demonstrated that the isolates from this study clustered tightly with reference F. *oxysporum* sequences from GenBank, with strong bootstrap support, confirming that the pathogen used in the antagonistic assays was *Fusarium oxysporum* (Figure 5).

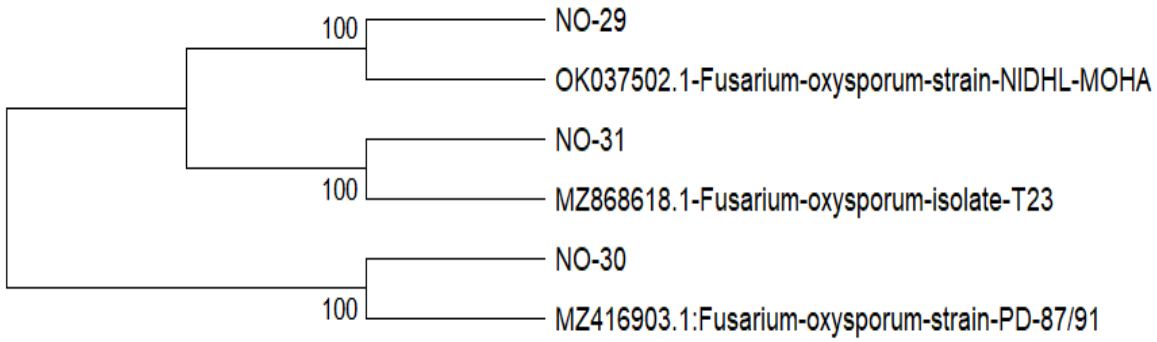

Figure 5. Maximum Likelihood phylogenetic tree of Fusarium oxysporum.

3.2. Effect of Trichoderma Isolates on Pathogen Growth Inhibition (Pgi %)

The effect of *Trichoderma* isolates on pathogen growth inhibition (PGI) was highly significant (p=2e−16, <0.001). As shown in Figure 6, isolates exhibited varying levels of antagonistic activity against the pathogen, with PGI ranging from 56.8% to 66.1%. MG7-2 had the highest average PGI (66.1%), closely followed by SH10-2 (65.6%), MG1-2 (65.1%), SH11-4 (64.5%), and SH11-2 (63.5%). Other isolates, such as KT7-1, SH4-4, and NH5-2, also showed strong inhibition, with PGI above 63%. The lowest inhibition was observed in BH6-2 at 56.8%, followed by KL7-4 and NH4-4, both at 57.6%. Despite these differences, most isolates showed moderate to high antagonistic activity, with PGI mostly between 58% and 63%.

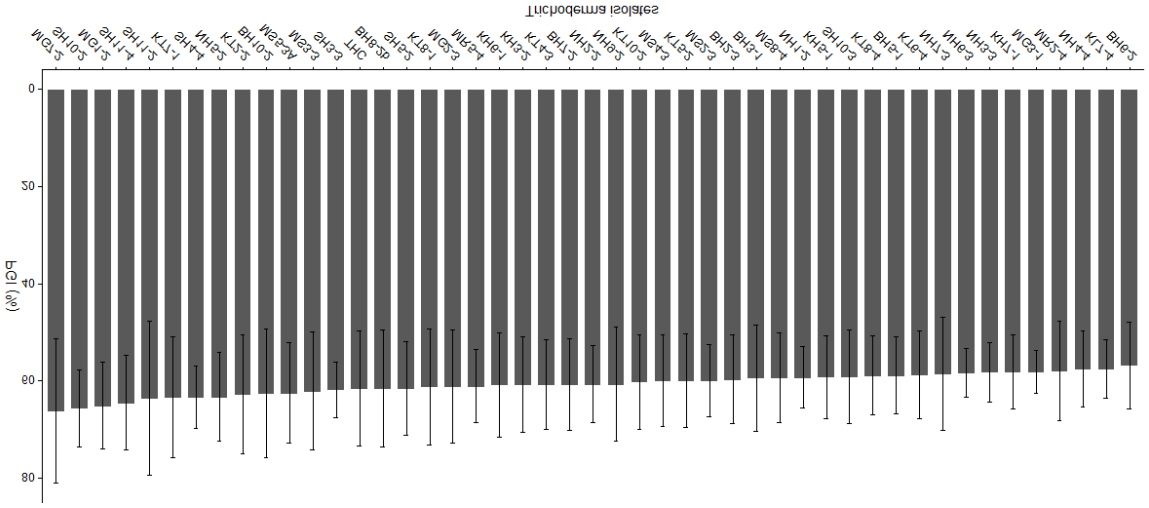

Figure 6. Effect of Trichoderma isolates on pathogen growth inhibition (PGI %). Bars represent mean PGI values, while error bars indicate standard deviation (SD).

3.3. Effect of Inoculation Method on Pathogen Growth Inhibition (Pgi %)

The inoculation method had a significant impact on pathogen growth inhibition (p=2e−16). Simultaneous inoculation led to notably greater inhibition than staggered inoculation. The mean PGI for simultaneous inoculation was 69.41%, while staggered inoculation showed a reduced average inhibition of 51.94% (Figure 7).

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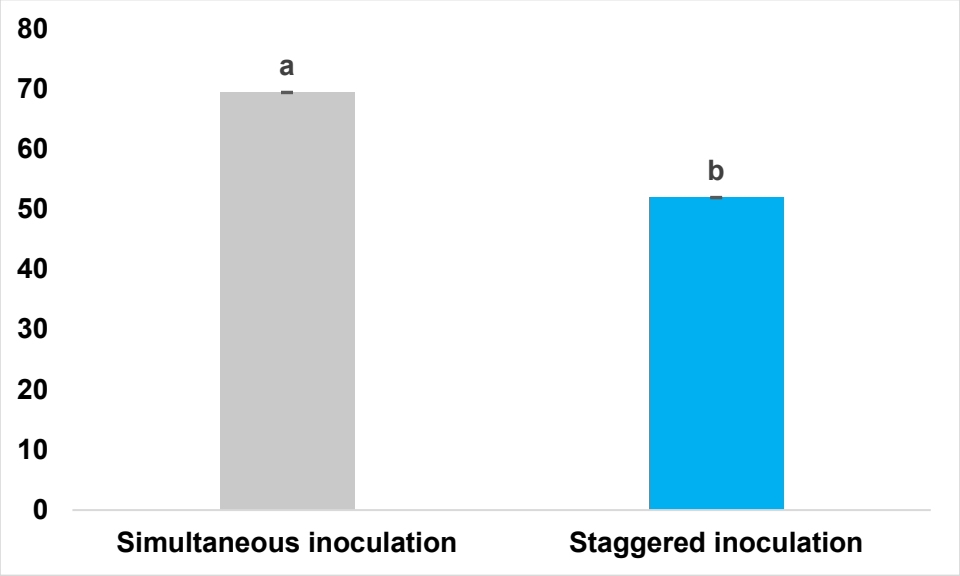

Figure 7. Effect of inoculation method on pathogen growth inhibition (PGI %) by Trichoderma isolates Values represent mean PGI percentages obtained under simultaneous and staggered inoculation methods.

3.4. Interaction Effect of Trichoderma Isolates and Inoculation Methods on Pathogen Growth Inhibition (Pgi %)

The interaction between *Trichoderma* isolate and inoculation method significantly influenced pathogen growth inhibition (PGI) (p=2 x 10−16). Simultaneous inoculation resulted in higher inhibition levels than staggered inoculation for most isolates. Among all treatment combinations, isolate MG7-2 achieved the highest PGI at 79.89%, followed by SH11-2 at 78.24%, KT7-1 at 74.98%, and BH10-2 at 74.93% (Table 2). Conversely, lower inhibition values were generally observed with staggered inoculation, with NH7-3 registering the lowest PGI at 47.6%. The results also indicated that some isolates showed a notable decline in antagonistic activity when inoculated in a staggered manner, MG7-2's PGI dropped from 79.9% with simultaneous inoculation to 52.4% with staggered inoculation, while SH11-2's PGI decreased from 78.24% to 48.81% (Figure 8, 9A-D).

Table 2. Interaction Effect of Trichoderma Isolates and Inoculation Methods on Pathogen Growth Inhibition.

Isolate x Inoculation method	PGI (%) Mean ± SD	Group
MG7-2 x Simultaneous	79.9 ± 0.57	a
SH11-2 x Simultaneous	78.2 ± 0.80	ab
KT7-1 x Simultaneous	75.0 ± 1.09	abc
BH10-2 x Simultaneous	74.9 ± 0.44	abc
KT2-2 x Simultaneous	74.1 ± 0.46	bcd
SH11-4 x Simultaneous	73.5 ± 0.48	bcde
MS3-3 x Simultaneous	73.3 ± 0.39	bcde
MG1-2 x Simultaneous	73.3 ± 0.68	bcde
BH8-2b x Simultaneous	72.7 ± 0.79	cdef
SH10-2 x Simultaneous	72.6 ± 0.46	cdefg
THC x Simultaneous	72.6 ± 0.44	cdefg
KT8-1 x Simultaneous	72.4 ± 0.45	cdefg
MS5-3A x Simultaneous	71.9 ± 0.48	cdefgh
MG2-3 x Simultaneous	71.9 ± 2.13	cdefghi
NH9-2 x Simultaneous	71.6 ± 0.67	cdefghij
KH6-1 x Simultaneous	70.8 ± 0.55	cdefghijk
NH5-2 x Simultaneous	70.4 ± 0.54	cdefghijk

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SH5-2 x Simultaneous	70.4 ± 0.35	cdefghijk
KH3-2 x Simultaneous	69.8 ± 0.21	defghijkl
BH3-1 x Simultaneous	69.5 ± 0.53	defghijkl
NH7-3 x Simultaneous	69.4 ± 0.91	defghijkl
BH7-2 x Simultaneous	69.3 ± 0.40	defghijkl
KT4-3 x Simultaneous	69.3 ± 0.54	defghijkl
KT10-2 x Simultaneous	69.2 ± 0.67	defghijkl
SH4-4 x Simultaneous	69.2 ± 0.69	defghijkl
KT5-2 x Simultaneous	69.0 ± 0.28	efghijkl
MS4-3 x Simultaneous	68.7 ± 0.65	efghijklm
BH2-3 x Simultaneous	68.1 ± 0.65	fghijklmn
NH2-2 x Simultaneous	68.0 ± 0.20	fghijklmn
MS8-4 x Simultaneous	67.9 ± 0.56	fghijklmn
MR5-4 x Simultaneous	67.9 ± 0.77	fghijklmn
SH10-3 x Simultaneous	67.8 ± 0.77	fghijklmn
MR2-4 x Simultaneous	67.5 ± 0.80	ghijklmn
KT6-4 x Simultaneous	67.1 ± 0.23	hijklmno
KH5-1 x Simultaneous	66.9 ± 1.73	hijklmno
MS2-3 x Simultaneous	66.8 ± 1.72	ijklmno
SH3-3 x Simultaneous	66.6 ± 0.47	jklmno
KT8-4 x Simultaneous	66.4 ± 0.64	klmno
BH5-1 x Simultaneous	66.2 ± 0.46	Klmno
KH7-1 x Simultaneous	65.2 ± 0.54	lmno
BH6-2 x Simultaneous	65.1 ± 0.83	lmno
NH1-2 x Simultaneous	65.1 ± 0.47	lmno
NH4-4x Simultaneous	64.9 ± 0.72	lmno
NH3-3 x Simultaneous	63.7 ± 0.23	mnop
KL7-4 x Simultaneous	63.2 ± 0.60	nop
NH6-3 x Simultaneous	63.0 ± 0.18	nop
MG3-1 x Simultaneous	62.1 ± 0.83[s]	opq
SH10-2 x Staggered	58.6 ± 4.16	pqr
SH4-4 x Staggered	57.4 ± 2.20	qrs
SH3-3 x Staggered	57.0 ± 3.93	qrst
MG1-2 x Staggered	56.8 ± 1.59	rstu
NH5-2 x Staggered	56.1 ± 7.55	rstuv
SH11-4 x Staggered	55.5 ± 2.50	rstuvw
MR5-4 x Staggered	54.2 ± 1.94	rstuvwx
MG3-1 x Staggered	54.0 ± 1.32	rstuvwx
NH6-3 x Staggered	53.7 ± 1.32	rstuvwxy
NH1-2 x Staggered	53.4 ± 0.75	stuvwxyza
NH2-2 x Staggered	53.3 ± 1.85	stuvwxyza
MS2-3 x Staggered	53.1 ± 1.02	stuvwxyza
MS5-3A x Staggered	53.0 ± 2.75	stuvwxyza
NH3-3 x Staggered	52.7 ± 2.68	stuvwxyzazb
SH5-2 x Staggered	52.5 ± 2.24	stuvwxyzazb
MG7-2 x Staggered	52.4 ± 2.53	stuvwxyzazb
KT4-3 x Staggered	52.1 ± 1.24	tuvwxyzazb
BH7-2 x Staggered	51.9 ± 2.14	tuvwxyzazb
KL7-4 x Staggered	51.9 ± 0.57	tuvwxyzazb
KT7-1 x Staggered	51.8 ± 1.98	uvwxyzazb
KH3-2 x Staggered	51.6 ± 2.09	vwxyzazb

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KH5-1 x Staggered	51.5 ± 3.24	vwxyzazb
BH5-1 x Staggered	51.5 ± 2.41	vwxyzazb
KT2-2 x Staggered	51.3 ± 0.57	vwxyzazb
KT8-4 x Staggered	51.3 ± 1.02	vwxyzazb
BH2-3 x Staggered	51.2 ± 2.06	vwxyzazb
KT10-2 x Staggered	51.2 ± 1.61	vwxyzazb
MS4-3 x Staggered	51.2 ± 2.01	vwxyzazb
KH7-1 x Staggered	51.0 ± 2.25	vwxyzazb
KT5-2 x Staggered	50.9 ± 0.77	wxyzazb
MS3-3 x Staggered	50.8 ± 0.92	wxyzazb
MS8-4 x Staggered	50.7 ± 1.85	wxyzazb
KH6-1 x Staggered	50.7 ± 1.49	wxyzazb
THC x Staggered	50.4 ± 0.75	wxyzazb
SH10-3 x Staggered	50.4 ± 3.42	wxyzazb
KT6-4 x Staggered	50.3 ± 1.76	xyzazb
BH8-2b x Staggered	50.3 ± 1.85	xyzazb
MG2-3 x Staggered	50.3 ± 0.35	xyzazb
NH4-4 x Staggered	50.3 ± 0.60	xyzazb
BH10-2 x Staggered	50.3 ± 2.12	xyzazb
KT8-1 x Staggered	50.0 ± 1.09	Xyzazb
NH9-2 x Staggered	49.7 ± 1.55	Xyzazb
BH3-1 x Staggered	49.3 ± 2.43	Xyzazb
SH11-2 x Staggered	48.8 ± 1.75	Yzazb
BH6-2 x Staggered	48.5 ± 1.42	Zazb
MR2-4 x Staggered	48.4 ± 1.02	Zazb
NH7-3 x Staggered	47.6 ± 0.97	Zb
Pvalue	2e-16 ***	

Means with different letters are significantly different as per Tukey's HSD test at .test at $p < 0.05$.Significance code: '***' 0.001.

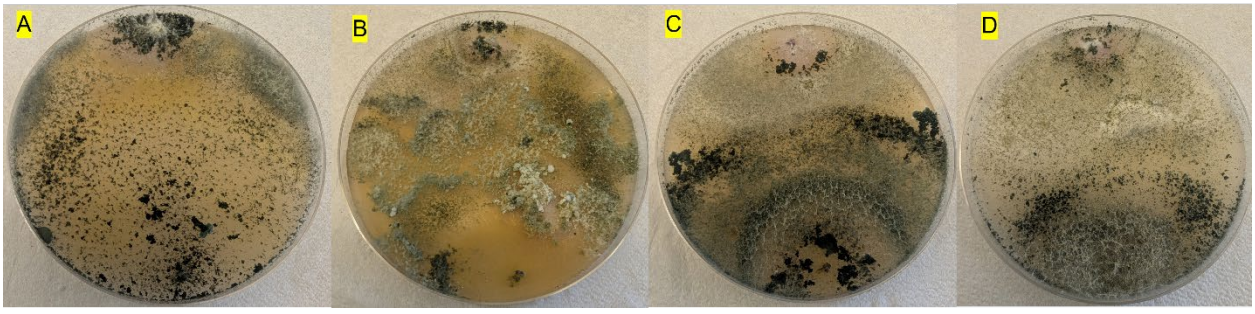

Figure 8. Antagonistic interaction between Trichoderma isolates and Fusarium oxysporum evaluated using a dual culture technique with simultaneous inoculation: (A) T. harzianum MG7-1 + F. oxysporum; (B) T. harzianum SH11-2 + F. oxysporum; (C) T. paraviridescens KT7-1 + F. oxysporum; (D) T. harzianum BH10-2 + F. oxysporum.

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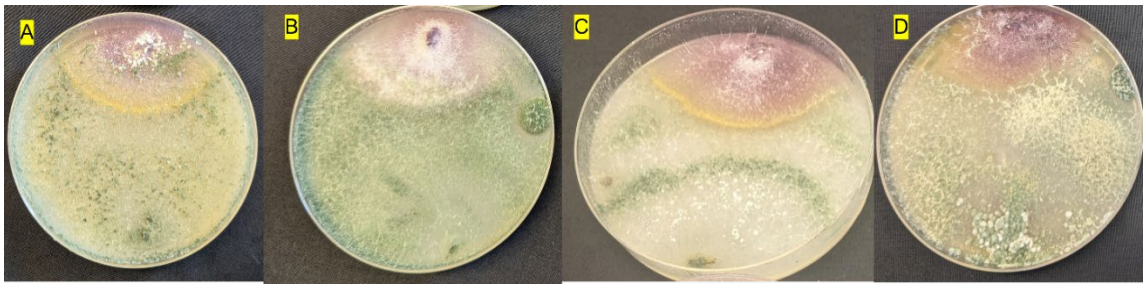

Figure 9. Antagonistic interaction between Trichoderma isolates and Fusarium oxysporum evaluated using a dual culture assay under staggered inoculation conditions. (A) T. harzianum MG7-1 + F. oxysporum; (B) T. harzianum SH11-2 + F. oxysporum; (C) T. paraviridescens KT7-1 + F. oxysporum; (D) T. harzianum BH10-2 + F. oxysporum.

3.5. Bell's Scale Performance and Occurrence of Yellow Colouration of Trichoderma Isolates Under Different Inoculation Methods

As shown in Table 3, most isolates treated with simultaneous inoculation exhibited Bell's scale values close to 1, indicating strong antagonistic activity and near-complete dominance over the pathogen. Isolates such as BH10-2, KT7-4, KT10-2, KT2-2, KT5-2, KT6-4, KT7-1, MG1-2, MG7-2, MR5-4, NH9-2, SH11-2, and THC consistently scored around 1 on Bell's scale under both inoculation methods, indicating highly effective antagonism. During staggered inoculation, some isolates showed slightly higher Bell's scale values between 1.5 and 2.0, pointing to a reduction but still significant pathogen overgrowth by *Trichoderma*. A few isolates, such as BH6-2 and MS3-3, had the highest Bell's scale values (3) in the staggered setup, suggesting weaker antagonistic effects and only partial pathogen colonization (Figure 10).

The presence of yellow colouration (Figure 11) was not significantly affected by the inoculation method ($p > 0.05$). Pearson's chi-squared test indicated no significant association between the inoculation method and colour development ($X2 = 0.018$, $df = 1$, $p = 0.892$). As shown in Table 3, yellow colouration was common under both methods. In the simultaneous inoculation group, 156 observations (83.0%) showed yellow colouration, while 32 (17.0%) did not. Similarly, in the staggered inoculation group, 154 observations (81.9%) displayed yellow colouration, with 34 (18.1%) lacking it. Isolates such as BH6-2, KT4-3, KT7-1, KT8-1, MR5-4, MS3-3, NH7-3, SH10-3, and SH3-3 did not produce yellow colouration under either method.

Table 3. Mean Bell's scale values and yellow pigmentation response of *Trichoderma* isolates under simultaneous and staggered inoculation methods.

Isolate	Bell's scale		Yellow colouration	
	Simultaneous	Staggered	Simultaneous	Staggered
BH10-2	1	1	+	+
BH2-3	1	2	+	+
BH3-1	2	2	+	+
BH5-1	2	2	+	+
BH6-2	1	3	-	-
BH7-2	1	2	+	+
BH8-2b	1	2	+	+
KH3-2	1	2	+	+
KH5-1	1	1.5	+	+
KH6-1	1	2	+	+
KH7-1	1	1.5	-	+
KL7-4	1	1	-	+

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KT10-2	1	1	+	+
KT2-2	1	1	+	+
KT4-3	1	1.75	-	-
KT5-2	1	1	+	+
KT6-4	1	1	+	+
KT7-1	1	1	-	-
KT8-1	1	1.25	-	-
KT8-4	1	2	+	+
MG1-2	1	1	+	+
MG2-3	1	1.5	+	+
MG3-1	1	2	-	+
MG7-2	1	1	+	+
MR2-4	1	2	+	+
MR5-4	1	1	-	-
MS2-3	1	2	+	+
MS3-3	1	3	-	-
MS4-3	1	2	+	+
MS5-3A	1	2	+	+
MS8-4	1	1.75	+	+
NH1-2	1	2	+	+
NH2-2	1	2	+	+
NH3-3	1	2	+	+
NH4-4	1	2	+	+
NH5-2	1	1.75	+	+
NH6-3	1	2	+	+
NH7-3	1	1.25	-	-
NH9-2	1	1	+	+
SH10-2	1	2	+	+
SH10-3	1	1.5	-	-
SH11-2	1	1	+	+
SH11-4	1	2	+	+
SH3-3	1	2	+	+
SH4-4	1	2	+	+
SH5-2	1	1.7	+	+
THC	1	1	+	+

Bell's scale interpretation is as follows: 1 = *Trichoderma* completely overgrew the pathogen (100% overgrowth); 2 = *Trichoderma* overgrew at least two-thirds of the pathogen (75% overgrowth); 3 = *Trichoderma* colonized half of the pathogen (50% overgrowth); 4 = *Trichoderma* and the pathogen met at the contact point after inoculation; 5 = the pathogen overgrew the *Trichoderma* isolate. Positive (+) and negative (−) signs indicate the presence or absence of the evaluated trait, respectively (n=4).

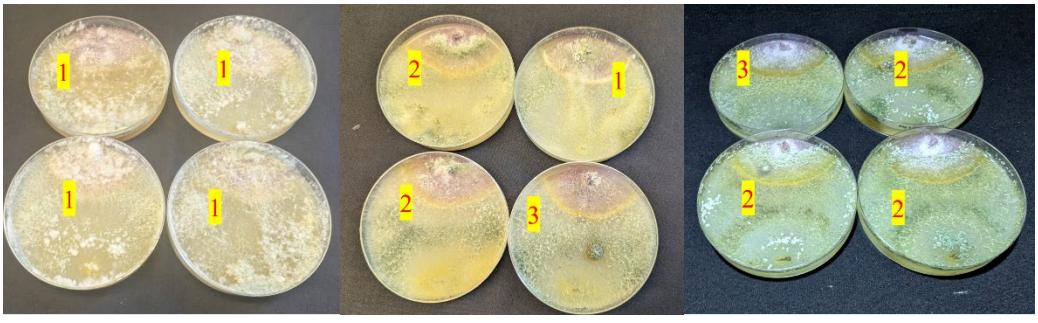

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Figure 10. Representative dual culture interactions showing different levels of pathogen overgrowth by Trichoderma isolates according to Bell's scale evaluation.

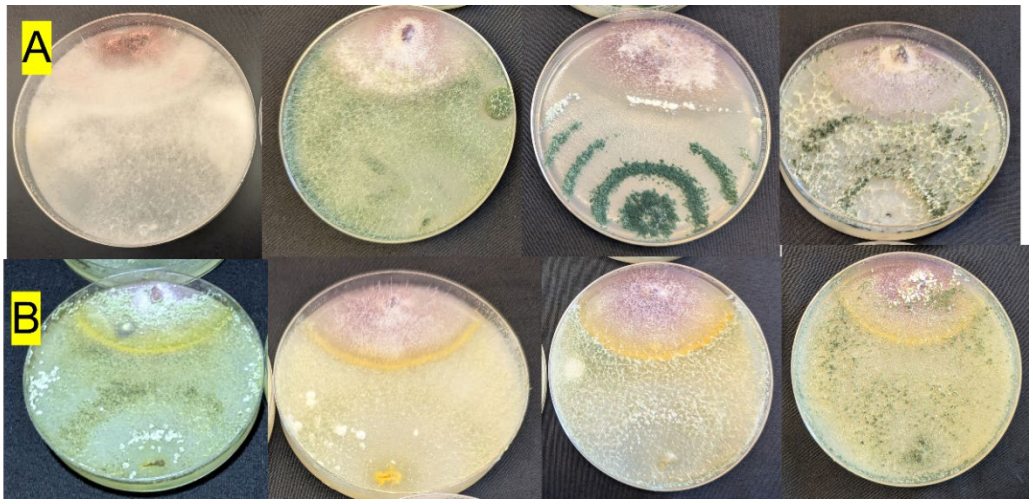

Figure 11. Representative observations of yellow coloration during dual culture assays. (A) Absence of yellow colouration. (B) presence of yellow colouration.

3.6. Effect of Trichoderma Isolates on Pathogen Growth Inhibition Under Voc Conditions

The analysis of variance showed a highly significant impact of *Trichoderma* isolates on pathogen growth inhibition (PGI) under VOC conditions ($F = 89.14,\ p < 0.001$). This suggests that different isolates vary notably in their ability to hinder pathogen growth through volatile organic compounds. As shown in Figure 15, SH11-2 and KT2-2 achieved the highest PGI values, around 66–67%, and were categorised in the statistical group "a," indicating strong antagonistic ability. BH10-2 and THC had moderate inhibition levels of about 52–54%, falling into group "b." Lower inhibition levels, between roughly 33% and 40%, were observed in KH7-1, MR5-4, SH10-2, MG7-2, and SH4-4. Of these, SH4-4 demonstrated the weakest inhibitory effect and was placed in group "d". and was classified in group "d" (Figure 12, 13).

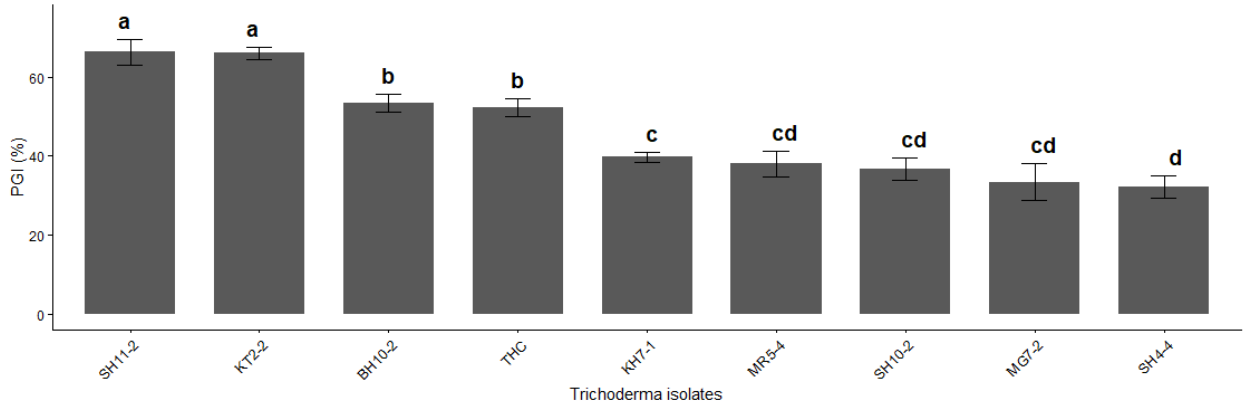

Figure 12. Impact of Trichoderma isolates on pathogen growth inhibition (PGI %) under VOC conditions. Bars show average PGI values, and error bars display standard deviation (SD). Values positioned above error bars denote SD values. Means with different letters are significantly different as per Tukey's HSD test at .test at $p < 0.05$.

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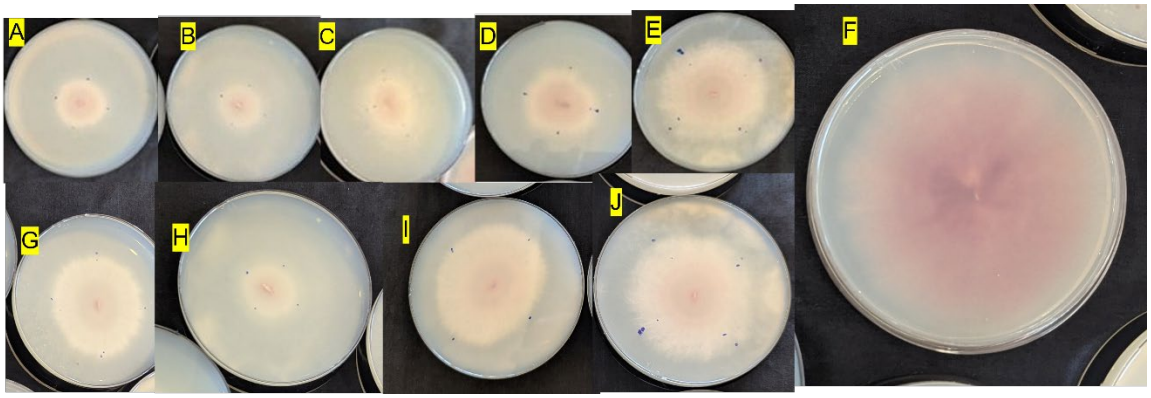

Figure 13. Inhibition of Fusarium oxysporum mycelial growth by volatile organic compounds (VOCs) emitted by Trichoderma isolates in a sealed plate assay compared with the untreated control (A) KT2-2 + F. oxysporum, (B) SH11-2 + F. oxysporum (C) BH10-2 + F. oxysporum, (D) KH7-1 + F. oxysporum, (E) SH10-2 + F. oxysporum, (F) Control (F. oxysporum), (G) MR5-4, (H) THC + F. oxysporum, (I) MG7-1 + F. oxysporum, (J) SH4-4 + F. oxysporum.

4. Discussion

The current study revealed significant variability in the antagonistic effects of 46 native *Trichoderma* isolates against *Fusarium oxysporum* f. sp. *lycopersici* . This varibility supports previous reports indicating that *Trichoderma*'s antagonistic capacity is highly strain-specific and affected by genetic, physiological, and ecological differences among isolates. [32–34].

The high-performing isolates in this study demonstrated strong antagonism consistent with strains previously reported in Africa and other regions. For example, in Ethiopia, strains used by [35] showed inhibition efficiency over 70% against *F. oxysporum*'s mycelial growth. Our top isolates MG7-2, SH11-2, and others, fall within the range reported by [36] who used endophytes like *Trichoderma asperellum* M2RT4, *Hypocrea lixii* F3ST1, *Trichoderma harzianum* KF2R41, and *Trichoderma atroviride* ICIPE 710, with PGI values between 68.84–99.61%.These results emphasise the value of exploring local biodiversity to find candidates adapted to regional soil and climate conditions. The strong inhibitory effects observed across many isolates are likely due to multiple antagonistic mechanisms, including rapid mycelial colonization, competition for space and nutrients, mycoparasitism, and the production of secondary metabolites such as enzymes and antibiotics [37,38].

Using the staggered inoculation method, a notable variation in PGI among *Trichoderma* isolates was observed. This variation was less pronounced than that observed under simultaneous inoculation. These findings indicate that although several isolates could still suppress *Fusarium oxysporum*, their effectiveness generally decreased once the pathogen had established itself. Our results suggest that only a few isolates have the competitive capacity, rapid colonisation ability, or strong antagonistic mechanisms needed to surpass *F. oxysporum* during early colonisation. Similar studies have shown that the biocontrol efficacy of *Trichoderma* largely depends on its ability to colonise roots and dominate the rhizosphere before pathogen establishes itself in the host tissues [39,40]. Additionally, they emphasise that the timing of *Trichoderma* application, such as seed treatments, is crucial to ensure it occupies the spermosphere or rhizosphere before soil-borne pathogen establishment.

The superior performance of native *Trichoderma* compared with the commercial strain demonstrates that native strains possess enhanced adaptive traits that enable them to function effectively suppress the pathogen growth after infecting the host. This highlights the potential value of screening indigenous isolates for biological control programs, as they may be better adapted to local pathogen populations and environmental conditions [41]. Conversely, the generally lower inhibition among most isolates reinforces the importance of early or preventive application strategies, since timely establishment of *Trichoderma* can provide a competitive advantage and more reliable disease suppression. The staggered inoculation results emphasize that application timing plays a

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critical role in determining the success of *Trichoderma*-based biocontrol, while also identifying promising isolates capable of maintaining antagonism even when introduced after pathogen establishment.

The significant interaction between isolate and culture method reveals that *Trichoderma* isolates' antagonistic effects vary with inoculation timing. Some isolates are more effective when competing directly with the pathogen through simultaneous inoculation, while others are less active if the pathogen has already established itself during staggered inoculation. This interaction aligns with previous research indicating that differences in growth, competitiveness, and metabolite production among *Trichoderma* isolates significantly impact their biocontrol effectiveness under different experimental conditions [42,43]

The Bell's scale evaluation further confirmed the antagonistic superiority of several isolates, particularly under simultaneous inoculation conditions, where most isolates achieved Bell's scale values close to 1. This indicates complete or near-complete overgrowth of the pathogen by *Trichoderma*. The generally lower Bell's scale scores observed under simultaneous inoculation suggest that direct competition established at the same time favored the antagonist and enhanced pathogen suppression. In contrast, some isolates exhibited reduced antagonistic performance under staggered inoculation, indicating that inoculation timing influenced the competitive balance between the antagonist and the pathogen. The absence of classes 4 and 5 in both methods indicates that none of the isolates were overtaken by the pathogen, underscoring the overall strong antagonistic potential of all isolates.

The occurrence of yellow coloration was highly prevalent under both inoculation methods, although no significant association was observed between inoculation method and colour development. Yellow pigmentation during isolate–pathogen interaction may be associated with the production of secondary metabolites or stress-related compounds induced during antagonistic confrontation [44]. However, since colour occurrence was similar between inoculation methods, the phenomenon may represent a common response during microbial interaction rather than a method-specific effect.

Under the VOC bioassay, the differences in *Fusarium oxysporum* f. sp. *lycopersici* inhibition can be linked to *Trichoderma* genetic differences suggesting variability not only in their metabolic profiles but also in the potency and composition of the VOCs they emit. Isolates SH11-2 and KT2-2, which exhibited the strongest VOC-mediated inhibition, likely produce compounds with strong antifungal activity, often acting as biofumigants that operate at a distance without direct physical contact. This observation aligns with recent research showing that isolates within the same *Trichoderma* species can vary widely in their antagonistic effectiveness [44,45]. For instance, in dual confrontation assays on PDA, different *T. asperellum* isolates exhibited highly variable VOC-mediated suppression of *Colletotrichum acutatum*, with the least effective strains inhibiting pathogen growth by only 12.82–25.46%, while the most effective isolates achieved 42.14–47.41% inhibition [46]. Remarkably, the top isolates in this study exceeded these values, demonstrating substantially higher inhibition. This suggests that our isolates possess superior antagonistic traits, possibly due to increased VOC production, enhanced colonisation ability, or stronger secondary metabolite expression, as reported for other highly suppressive *Trichoderma* strains [47,48].

The superior performance of SH11-2 and MG7-2 compared to the commercial strain highlights the importance of screening native isolates for VOC-producing strains with stronger antagonistic abilities. These findings suggest that VOC-mediated suppression is a promising biocontrol approach, but its success relies on the specific traits of each *Trichoderma* isolate rather than being a universal feature of the genus. Overall, the results emphasise that antagonistic effectiveness varies between strains, reinforcing the need for comprehensive screening to find the most effective *Trichoderma* candidates for biological control.

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5. Conclusion

The present study demonstrated that native *Trichoderma* isolates exhibit significant antagonistic activity against the pathogen in both dual-culture and VOC assays. Significant differences were observed among isolates in their ability to inhibit pathogen growth, indicating variability in antagonistic potential. Simultaneous inoculation generally resulted in greater inhibition of pathogen growth than staggered inoculation. Bell's scale evaluation further confirmed the strong overgrowth capacity of several isolates against the pathogen. Among the tested isolates, MG7-2, SH11-2, KT2-2, and SH10-2 consistently exhibited superior antagonistic performance and may represent promising candidates for biological control applications.

However, the most effective isolates identified in this study should be further evaluated under greenhouse and field conditions to confirm their biocontrol potential under natural environments. Further studies should investigate the production of antifungal metabolites and volatile organic compounds associated with antagonistic activity, and formulation and mass-production studies are recommended to facilitate the development of effective *Trichoderma*-based biocontrol products for sustainable disease management.

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