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Biocontrol Potential of Native *Trichoderma* Species against *Fusarium verticillioides* and Fumonisin Contamination of Maize

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ABSTRACT

Fusarium verticillioides is the most prevalent fungal pathogen of maize, causing stalk and ear rot diseases and producing fumonisins, toxic secondary metabolites harmful to humans and animals. This study evaluated the biological control potential of *Trichoderma* species against this pathogen under *both in vitro* and field conditions. A total of 18 *Trichoderma* isolates were tested *in vitro* for their antagonistic potential against the pathogen using the dual culture interaction. Based on percent radial growth inhibition, eleven *Trichoderma* isolates were selected for further evaluation under field conditions using seed biopriming for controlling *F. verticillioides* and fumonisin contamination of maize grains. The field experiment was arranged in a randomized complete block design with three replications. Dual culture assays showed that all 18 *Trichoderma* isolates significantly ($p < 0.05$) inhibited the mycelial growth of *F. verticillioides*, with inhibition ranging from 34.45% to 77.78%. *T. harzianum* isolates (Thr1 and Thr2) and *T. hamatum* isolates (Thm5 and Thm6) showed the highest antagonistic activity against the pathogen *in vitro*. Field experiments conducted over two cropping seasons demonstrated that *Trichoderma* treatments significantly ($p < 0.05$) reduced kernel fungi colonization by 38.3–86.4% and fumonisin contamination by 44–89.8% compared with the untreated control. *Trichoderma harzianum* strains (Thr2, Thr5 and Thr6) and *T. hamatum* strains (Thm3 and Thm6) were the most effective compared to others. In addition, Trichoderma-treated plots exhibited improved germination, seedling vigor, seed weight, and grain yield. These results demonstrate the strong potential of native *Trichoderma* isolates as biological control agents for managing *Fusarium* infection and fumonisin contamination in maize.

Keywords: Maize, *Fusarium verticillioides*, Fumonisin, *Trichoderma*, Biological control.

1. INTRODUCTION

Fusarium verticillioides (Nectriaceae, Ascomycota) is among the most prevalent fungal pathogen affecting maize (*Zea mays* L.) worldwide, including Ethiopia (Tsehaye et al., 2017). The pathogen causes seedling decay, stalk rot, ear rot and kernel rots and can lead to significant losses in maize yield and grain quality (Munkvold et al., 1997; Presello et al., 2008). In addition to causing plant disease, the fungus produces several mycotoxins, including fumonisins, moniliformes, fusaric acid, and fusarins (Bacon et al., 2008; Darnetty

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and Salleh, 2013). Fumonisins are the most common toxins found associated with maize kernels and almost all *F. verticillioides* strains have the ability to produce fumonisins, but at different quantities (Bacon et al., 2008). Fumonisin contamination of grains is a major concern for the safety of food and feed since these toxins have a variety of negative impacts on human and domestic animal health. Animal illnesses such as pulmonary oedema in pigs and leukoencephalomalacia in horses have been linked to fumonisin (Kellerman et al., 1990; Harrison et al., 1990). In humans, ingestion of fumonisin-contaminated maize-based food has been epidemiologically associated to neural tube abnormalities (Missmer et al., 2006) and oesophageal cancer (Rheeder et al., 1992).

Fumonisin contamination in maize in Ethiopia is highly prevalent, often exceeding international safety limits. Previous studies indicated that up to 70% of samples are positive for the toxin. *Fusarium verticillioides* is the primary fungi responsible, with contamination levels ranging from low to as high as 4500 to 6770 μg km^{-1} (Atnafu et al., 2024; Tsehaye et al., 2017).

Management of *F. verticillioides* is very challenging because of its complex infection cycle. This pathogen can spread vertically from seed-to-plant or from soil to roots, causing systemic stalk infection, and subsequent plant-to-seed transmission (Bacon et al., 2008; Munkvold et al., 1997). Propagules of this fungus can also infest maize plants through insect vectors, rain splash and wind dispersal (Munkvold et al., 1997). Fungicide treatments are not effective enough to control the pathogen since it resides internally in the plant (Bacon et al., 2008). Due to the high cost of fungicides for small-scale farmers, growing concerns about their negative effects on beneficial species in the environment, and concerns about human safety, the use of fungicides is also discouraged. Management using host plant resistance is not yet feasible since there are no maize varieties with sufficient levels of resistance to control this pathogen. Therefore, in order to manage soil and seedborne pathogens like *F. verticillioides*, it is important to look for alternate and potentially sustainable strategies, such as the use of self-sustaining biological control agents.

Among the biocontrol agents getting great attention due to their multiple actions and promise to suppress fungal pathogens in various crops are the filamentous fungi in the genus *Trichoderma* (Yao et al., 2023). *Trichoderma* species have been widely used as effective biocontrol agents over other bacterial agents such as *Bacillus* or *Pseudomonas* species because of their superior ability to act as mycoparasites (directly parasitize and break down hyphae of fungal pathogens), their fast growth rate and aggressive rhizosphere colonization

(Harman et al., 2004; Ng et al., 2015). These groups of fungi also have the capacity to producer antifungal substances such as hydrolytic enzymes and antibiotics to suppress plant pathogenic fungi (Harman et al., 2004; Yao et al., 2023). Besides, certain strains can induce systemic host defense response to several plant pathogens (Harman et al., 2004), and they may promote plant growth and development, often resulting in higher yield increases (Entesari et al., 2013; Moya et al., 2020). Previous studies have shown success in the control of seed and soil borne pathogens of cereal crops using *Trichoderma* spp. (Chandra Nayaka et al., 2010; Ferrigoet al., 2014; Moya et al., 2020). Despite the widespread occurrence of *Fusarium verticillioides* in Ethiopian maize production systems, limited research has investigated about the potential of indigenous *Trichoderma* isolates for the biological control of this pathogen. The identification and selection of highly effective, locally adapted *Trichoderma* strains is desirable for the successful management of a particular plant pathogen. Thus, the first objective of the present study was to assess the antagonistic potential of native *Trichoderma* species in suppressing the growth of *F. verticillioides* pathogen under in vitro assays, and to select the most efficient strains for field-testing. The second objective of this study was to evaluate the effectiveness of selected *Trichoderma* isolates as biocontrol agents to reduce *F. verticillioides* colonization and fumonisin contamination of maize grains following seed biopriming under field conditions.

2. MATERIAL AND METHODS

2.1. Isolation and Identification of *Trichoderma* species

Trichoderma species were isolated from post-harvest maize grains and soil taken from the rhizosphere of growing maize plants. Twenty-one composite soil samples were collected from Ethiopia's main maize-growing areas, and the *Trichoderma* strains were isolated using the soil dilution plate method on Trichoderma selective medium (TSM) according to Eladet al. (1981). Additional isolates of *Trichoderma* were recovered from maize kernels during the isolation of *Fusarium* species on CZPD agar, which is a modified version of Czapek-Dox Iprodione Dichloran Agar (Halstensen et al., 2006). The emergent colonies, which were suspected as *Trichoderma*, were pure-cultured onto PDA using the single-spore isolation method (Leslie and Summerell, 2006). Isolated strains were preserved in a culture collection on -80 °C at Norwegian Institute of Bio-economy Research (NIBIO), Norway and on – 4 °C on PDA cultures in the plant pathology laboratory, Mekelle University, Ethiopia for future studies.

Trichoderma species were identified based on morphological features and culture characteristics, using the methods and interactive identification keys (http://nt.ars-grin.gov/taxadescriptions/keys/TrichodermaIndex.cfm; Gams and Bissett 1998; Samuels et al., 2006). For this, single spore isolates were cultured for 7-10 days at 25 °C on PDA and SNA (Nirenberg, 1976). Each isolate's culture features, including growth rate, mycelial dispersion pattern, color, and medium changes throughout time, were investigated. The shape and size of conidia, conidiophores, phialides and chlamydospores, and the presence or absence of sterile hairs, are among the microscopic morphological features that were noticed.

2.2. Isolation and Identification of *Fusarium verticillioides*

Fusarium verticillioides, the pathogen, was isolated from infected maize kernels that were collected in Ethiopia. The identification was initially achieved using morphological and cultural characters as outlined by Leslie and Summerell (2006). Sequencing of the translation elongation factor 1-alpha (*EF-1α*) gene region was performed according to O'Donnell et al., (1998) to support the morphological identification. The CLC Main Workbench software 6.9 (Aarhus, Denmark) was used to assemble and evaluate the sequence data. The consensus sequences were then checked for similarity with entries from the *Fusarium*-ID database (http://isolate.fusariumdb.org/index.php) and the GenBank (NCBI-National Centre for Biotechnology Information) (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The GenBank accession number and sequence with best much for the tested *F. verticillioides* isolate was FD_01387_EF-1a.

2.3. *In-vitro* Screening of Antagonistic *Trichoderma* isolates using Dual Culture Interaction

The *Trichoderma* isolates were evaluated against *F. verticillioides* isolate following the dual culture method as described by Hajieghrari et al. (2008) to select the most efficient antagonists. A 6 mm diameter mycelial disc from a 7-day-old culture of *F. verticillioides* and each of the 18 *Trichoderma* isolates were added to 90 mm plates containing PDA (Table 1). The isolates were placed equally apart (1 cm) from the periphery along the same diameter but on opposite sides of the plates. PDA plates inoculated with the *F. verticillioides* isolate without the antagonists served as controls. Inoculated petri dish plats were incubated for 15 days at 25 °C with alternating 12- hours of photoperiod and dark condition. The assays were replicated three times. The inhibitory effect of *Trichoderma* isolates against *F. verticillioides* was evaluated as the percentage of mycelial growth inhibition, calculated by the difference

between the growth of the pathogen confronted with the antagonistic strain and the growth of pathogen without antagonist (control).

Table 1. District of origin and source material of Trichoderma isolates evaluated against Fusarium verticillioides in this study.

Code of Trichoderma isolates	***District of origin***	***Source material***	***Identity and names used in field trial***
Tr. G-5	Gibe	Soil	*T. hamatum*-1 (Thm1)
Tr. G-6	Gibe	Soil	*T. hamatum*-2 (Thm2)
Tr. Hal-3	Halaba	Soil	*T. hamatum*-3 (Thm3)
Tr. KB-1	Kobo	Soil	*T. hamatum*-4 (Thm4)
Tr. J-21A	Jimma	Seed	*T. hamatum*-5 (Thm5)
Tr. Km-1	Kemissie	Soil	*T. hamatum*-6 (Thm6)
Tr. Km-2	Kemissie	Soil	*T. hamatum* -7 (Thm7)
Tr. Mk-1	Mekelle	Soil	*T. hamatum*-8 (Thm8)
Tr. Ko-3	Korem	Soil	*T. hamatum*-9 (Thm9)
Tr. B-1	Bako	Seed	*T. harzianum*-1 (Thr1)
Tr. BD-1	Bedele	Seed	*T. harzianum*-2 (Thr2)
Tr. BD-2	Bedele	Seed	*T. harzianum*-3 (Thr3)
Tr. J-1	Jimma	Soil	*T. harzianum*-4 (Thr4)
Tr. NK-1	Nekemte	Seed	*T. harzianum*-5 (Thr5)
Tr. S-2A	Sire	Seed	*T. harzianum*-6 (Thr6)
Tr. Z-1B	Ziway	Soil	*T. harzianum*-7 (Thr7)
Tr. Ax-1	Axum	Soil	*T. spirale*-1 (Tsp1)
Tr. Ml-68E	Melkassa	Seed	*T. spirale*-2 (Tsp2)

Four and seven days after incubation, the *F. verticillioides* isolate's radial growth was measured toward the opposing colony in multi-directions. Ten days after incubation, plate area occupied by mycelia growth of the *F. verticillioides* isolate in dual culture were measured to compare to the plate area covered on control plates. The percentage inhibition of radial growth (PIRG) of the *F. verticillioides* pathogen was calculated using the following equation

$$PIRG = \left(\frac{R1-R2}{R1}\right) x100$$

Where, R_1 represents the radial growth the pathogen in control plates, and
R_2 represents its growth in the presence of antagonist (Rahman et al., 2009).

The pathogen-*Trichoderma* isolate interaction was directly observed in the dual culture plates for signs of hyphal barrier formation (thickened mycelial growth between cultures after contact), abnormal exudate production in comparison to axenic culture, and whether or not the *Trichoderma* isolate overgrew the entire *F. verticillioides* colony. The inhibition zone, pathogen's extent of growth retardation in dual culture, was estimated in terms of presence (+) and absence (-) after four days of pairing using the following ratings:

+++ = ≥11 mm, ++ = 6–10 mm, and + = 1–5 mm. Preparations were made from the antagonist fungi contact region and observed for hyphal interaction (presence of mycelial coiling and coiling frequency, penetration and destruction of hyphae) using an optical microscope equipped with a digital camera. The coiling frequency was determined by counting loops in 10 squares with three replications (Almeida et al., 2007) and categorized as: low/less (1-2 coiling/square), medium (3-5 coiling/square), or high frequent (≥ 6 coiling/square).

2.4. Field Experiment

The field study was conducted on the experimental field site of Mekelle University, northern Ethiopia. The location of the site is 13º 29' N latitudes and 39º 30' E longitudes, with an altitude of 2130 meters above sea level. According to Araya et al. (2011), the experimental field's soil type was Cambisols with a silt-clay textural class, and the region's climate is classified as limited moisture semi-arid.

2.4.1. Fungal Inoculum Preparation and Field Experiments

The top-performing *Trichoderma* isolates were chosen based on their *in vitro* antagonistic behavior towards the *F. verticillioides* isolate in the dual culture assay and used in the field experiment. A *F. verticillioides* strain that had been previously isolated from infected maize kernels collected in Ethiopia was used for the field experiment. This strain was chosen for its capacity to produce fumonisin, because it was one of the strains with the highest fumonisin production among the field-collected isolates tested on autoclaved maize cultures (Tsehaye et al., 2023). The pathogen was arterially inoculated using colonized maize seeds. For the purpose of producing spores, the *F. verticillioides* strain was cultivated on Mung Bean Agar (MBA) as suggested by Dill-Macky et al. (2003). The strain was grown at 23±2 for 10 days, with 12 hours of near ultraviolet/white light and 12 hours of darkness alternating (Leslie and Summerell, 2006). The *Trichoderma* species isolates was grown on PDA under the same circumstances for the inoculum production.

Spore suspension was prepared by removing conidia from the agar medium's surface using sterile distilled water, which was then filtered through two layers of cheesecloth. The spore concentration was adjusted to 10^7 spores /ml after counting the concentrations using the Kova glasstic slides. Tween 20 (polyoxyethylenesorbitan monolaurate; Sigma-Aldaich, St. Louis, Missouri), a surfactant, was added to the conidial suspension at a rate of 0.2 ml/liter to improve its dispersion and adhesion to the seed surface. Maize seeds were bioprimed using spore suspension from the various *Trichoderma* isolates in Erlenmeyer flasks. Following an

overnight soaking period at room temperature, the seeds were taken out of the spore suspension and immediately planted without being allowed to air-dry. Conidial suspension of the *F. verticillioide*s pathogen was applied onto autoclaved maize seeds in separate flasks. In every instance, two viable maize seeds treated with the *Trichoderma* isolates and three autoclaved seeds colonized with the *F. verticillioides* strain were placed closely together per planting hill in the field.

Two years of field experiments were carried out in 2013 and 2014 during the rainy season. The experimental design was randomized complete block design with thirteen treatments and three replications for each treatment. Eleven *Trichoderma* isolates, five from *T. harzianum*, five from *T. hamatum*, and one from *T. spirale*, were examined using the seed biopriming under field condition. Seeds treated with a fungicide recommended for protection of seedborne and soilborne pathogens in Ethiopia, Apron Star 45 WS at the rate of 2.5 g/kg, were also included in the treatments. Other seeds soaked in Erlenmeyer flasks containing sterile distilled water without any biological control agent but planted together with *F. verticillioides*-colonized autoclaved seeds were used as untreated control. Maize variety Melkassa-4 which is one of the recommended cultivars for the study area was used. Each plot was 13.5 m^2, with 3 m length and 4.5 m width. Spacing was 0.75 m between rows and 0.25 m between plants. Spacing between blocks and between plots were 1.5 m and 1.0 m, respectively. All crop management techniques were according to recommended practices for maize production in the region. Thus, Di-ammonium phosphate (DAP) and urea fertilizer were applied at a rate of 100 kg per ha each i.e, 64 kg N and 46 kg P per hectare. Nitrogen (N) was applied three times: one third at sowing and the remaining, two times, 4 and 6 weeks after crop emergence.

2.4.2. Assessment of Kernel Fungal Contamination and Plant-Growth Parameters

Fungal contamination and plant-growth parameters (germination percentage, shoot height, seedling vigor index, seed weight and grain yield) were recorded. The total number of emerging plants that broke the soil surface in each plot 10 days after sowing was counted in order to determine the percentage of germination as follows.

$$Germination\ percentage = \frac{number\ of\ seedlings\ emerged * 100}{Total\ number\ of\ seeds\ planted}$$

At 45 days after emergence, shoot height was measured on 10 randomly chosen plants per plot by measuring the distance from soil to the base of final leaf with visible collar. Then seedling vigorindex (VI) was calculated as:

$$VI = average\ shoot\ length\ (cm) * percentage\ of\ seed\ germination$$

When plants reached physiological maturity, all cobs were harvested per individual plot. After threshing and drying to a moisture level of about 15%, kernels per plot were weighed separately to determine grain yield. Thousand seed weight was also determined for each treatment-replica. Grain samples (200 seeds per plot) were randomly selected, and fungal infection level in the maize kernels was determined using the deep-freeze blotter method (ISTA, 2003).

2.4.3. Fumonisin Analysis

For fumonisin analysis, 100 g maize grains from each plot were finely ground using a laboratory mill (IKA®-Werke, GmbH & Co. KG., Germany) and passed through a 1 mm mesh. Enzyme Linked Immunosorbent Assay (ELISA) kits (RIDASCREEN®Fumonisin, R-Biopharm AG, Darmstadt, Germany) were used, in accordance with the manufacturer's instructions, to evaluate the level of fumonisin contamination in the samples. The minimum detection limit of the kit was 0.025 ppm and all samples were analyzed in duplicate. Maize samples spiked with known fumonisin content that obtained from Bari, Italy were used as a standard to validate the precision of the ELISA kits.

2.5. Statistical Analysis

All data were subjected to analysis of variance (ANOVA) using Statistical Analysis System (SAS) software (SAS institute, Cary, NC). Data on percentage of maize kernels colonized by fungi were log-transformed before statistical analysis. A combined analysis was conducted for the agronomic data (germination, seedling vigor index, plant height, seed weight, and grain yield) that were recorded during the two years. Fisher's protected least significance (LSD) test was used to separate differences between treatments at $p < 0.05$ significance level.

3. RESULTS

3.1. Isolation and Identification of *Trichoderma* species

Trichoderma species were isolated in 9 out of the 21 soil samples taken from the rhizosphere and root zone of growing maize plants. Among the 200 maize kernel samples assessed,

Trichoderma species were recovered from 7 samples. A total of 18 *Trichoderma* isolates were isolated, 11 from soil samples and 7 from maize seeds. The *Trichoderma* isolates were found to belong to three distinct species: *T. hamatum* (9 isolates or 50% of the total isolates), *T. harzianum* (7 isolates or 38.9%), and *T. spirale* (2 isolates or 11.1%).

3.2. *In vitro* Antagonism Assays

Dual culture interaction results showed that all *Trichoderma* isolates tested inhibited mycelial growth of the *F. verticillioides* isolate. Significant differences ($p < 0.001$) in the degree of inhibition of mycelial growth of the pathogen were observed among the *Trichoderma* species isolates examined. The Trichoderma species isolates were growing faster compared to the pathogen occupied larger areas of the plats, and leaving very limited space for growth of the pathogen. Four days after pairing, aclear zone of inhibition before hyphal contact was observed when the different *Trichoderma* isolates were grown in dual cultures with the *F. verticillioides* isolate (Fig 1).

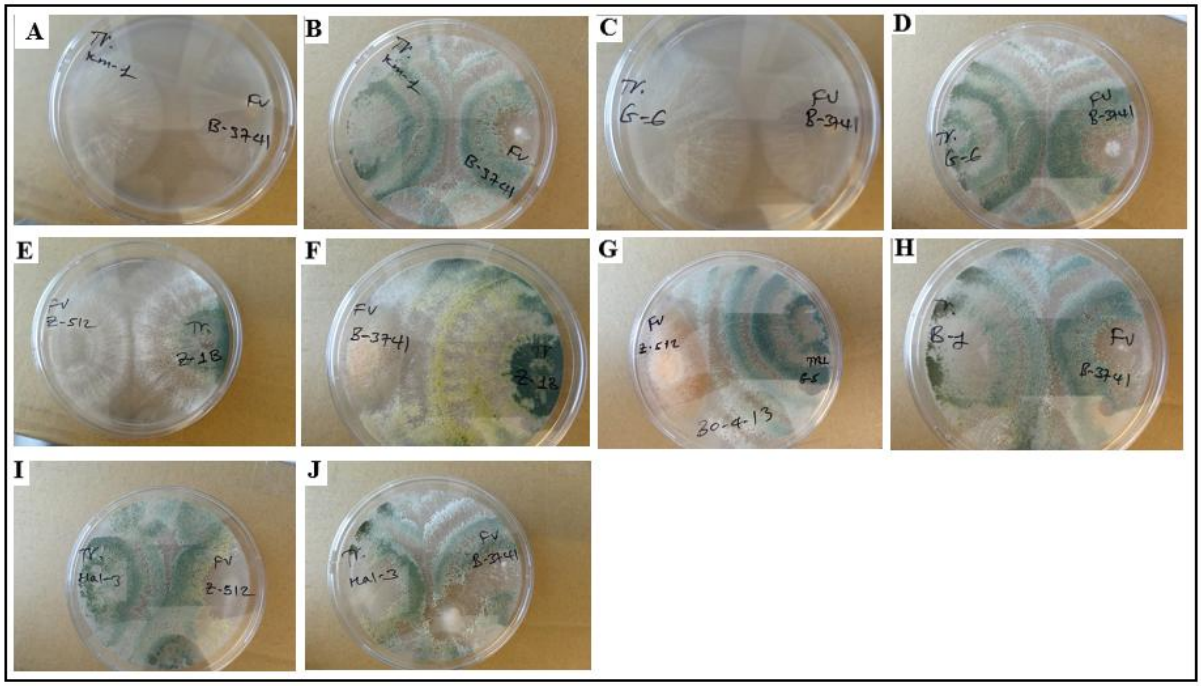


Figure 1. Dual culture of Trichoderma species isolates and F. verticillioides. Rapid growth of Trichoderma isolates and retarding the growth of the pathogen 4 days after pairing (A, C & E), and Trichoderma isolates growing over the pathogen 10 days after incubation at 25 °C (B, D, H, I & J), but not crossing the inhibition zone or little hyphal interaction (F, G). Fv: denotes for F. verticillioides and Tr: Trichoderma isolates.

Trichoderma spirale isolate Tsp2 and *T. hamatum* Thm4 displayed the lowest zone of inhibition (<6 mm) compared to the others (Table 2). After 7 days of incubation, the inhibitory activity of Trichoderma isolates against *F. verticillioides* ranged from 34.45% to

77.78%. The highest inhibitory effect on radial growth of the *F. verticillioides* pathogen was achieved by *T. harzianum* isolates Thr6 (77.78%), Thr2 (77.74%) and Thr5 (76.48%), while the lowest inhibitory effect on radial growth was observed using the *T. spirale* isolate Tsp1 (34.45%). Ten days after pairing, a clear zone with no/little physical contact between the fungal colonies were observed in *T. hamatum* isolate Thm1 and *T. harzianum* Thr7 (Fig 1 F and G).

All tested *Trichoderma* isolates, except Thm9 and Tsp1, formed a hyphal barrier when growth began to overlap with the pathogen. After contact, most antagonistic *Trichoderma* isolates were observed to grow-over on the pathogen and produce abundant spores in concentric rings. Abnormal color (orang-yellow) or exudate production was observed in *Trichoderma* isolates (Tsp2, Thm3 and Thm8) in the contact area with *F. verticillioides*. Ten days after incubation, mycelial growth of the *F. verticillioides* isolate was confined to a very small portion of the plate surface in the dual culture. At the last observation date, the largest area/space occupied by *Trichoderma* strains in terms of mycelial growth territory was observed in plates of *T. harzianum*Thr2, Thr5 and Thr6 (96% each) as well as *T. hamatum* Thm5 = 93.4% and Thm6 = 94.5% (Table 2).

3.3. Microscopic Observations

Microscopic observation showed that most of the *Trichoderma* isolates tested in this study exhibited coiling structures around the hyphae of the *F. verticillioides* isolate and created disintegrated and deformed hyphae, suggesting the use of hyperparasitisim to suppress growth of the pathogen. Variations in mycelial coiling frequency were also observed among the *Trichoderma* strains (Table 2). These variations were not only between species but also between isolates of the same species. Some *Trichoderma* isolates (*T. hamatum* isolate Thm1 and *T. harzianum* Thr7) showed bigger empty space with no physical contact between the fungal colonies and no hyphal interaction were observed in these isolates (Table 2).

3.4. Effect of Seed Treatment with *Trichoderma* species on Fungal and Fumonisin Contamination of Maize Kernels

The colony counts of *Fusarium* species on maize kernels did not significantly differ from year to year. Differences among treatments were significant for percentage of kernels contaminated with fungal agents assessed after crop harvest. When maize seeds were treated with the *Trichoderma* isolates, *F. verticillioides* colonization of kernels was reduced by 38.3% - 86.4% in 2013 and by 36.8% - 69.6% in 2014 compared to the control, i.e plots that received only *F. verticillioides* treatment (Fig 2).

Table 2. In vitro antagonistic activity of Trichoderma isolates against F. verticillioides pathogen.

***Trichoderma* isolates [a]**	**Growth inhibition (%)[b]**		**Plate area coverage (%), 10 days [c]**	**Zone of inhibition[d]**	**Overgrowth on Fv and sporulation [e]**	**Abnormal color [f]**	**Mycelial coiling frequency[g]**	**Formation of a hyphal barrier**
	4 Days	7 Days						
T. hamatum-1 (Thm1)	35.43de	55.56e	77.78f	++	No	none	-	Yes
T. hamatum-2 (Thm2)	49.47c	65.28c	92.22c	+++	Yes	none	High	Yes
T. hamatum-3 (Thm3)	50.67b	73.61b	92.23c	+++	Yes	Present	High	Yes
T. hamatum-4 (Thm4)	34.53f	51.37gh	73.34gh	+	Yes	none	Medium	Yes
T. hamatum-5 (Thm5)	36.87d	65.59c	93.34bc	++	Yes	none	High	Yes
T. hamatum-6 (Thm6)	50.10c	73.59b	94.45ab	+++	Yes	none	High	Yes
T. hamatum-7 (Thm7)	29.93f	52.77fg	74.44g	++	Yes	none	Medium	Yes
T. hamatum-8 (Thm8)	22.93g	58.31d	83.32d	++	Yes	Present	Medium	Yes
T. hamatum-9 (Thm9)	9.43h	35.67j	66.29j	-	Yes	none	Low/less	No
T. harzianum-1 (Thr1)	52.07b	76.11a	95.45a	++	Yes	Present	High	Yes
T. harzianum-2 (Thr2)	50.97bc	77.74a	96.12a	+++	Yes	none	High	Yes
T. harzianum-3 (Thr3)	31.03f	48.61i	71.12i	++	Yes	none	Medium	Yes
T. harzianum-4 (Thr4)	30.20g	49.98hi	72.22hi	++	Yes	none	Medium	Yes
T. harzianum-5 (Thr5)	53.30a	76.48a	96.11a	+++	Yes	none	High	Yes
T. harzianum-6 (Thr6)	53.93a	77.78a	96.11a	+++	Yes	none	High	Yes
T. harzianum-7 (Thr7)	36.17d	57.37d	80.00e	++	No	none	-	Yes
T. spirale-1 (Tsp1)	8.80h	34.45j	66.67j	-	Yes	none	Low/less	No
T. spirale-2 (Tsp2)	22.78g	54.16ef	77.76f	+	Yes	Present	Medium	Yes

***Note*:**[a]Thm: *T. hamatum*, Thr: *T. harzianum*, Tsp: *T. spirale*; [b] radial growth inhibition of the pathogen; [c]Reduction in mycelial growth territory (plate area) of the pathogen in dual culture compared to mycelial growth area on control; [d]Inhibition zone (mm) after 4 days of pairing: +++ represents = ≥11mm; ++ = 6–10 mm; + = 1–5 mm; – = no inhibition; [e] Fv: *F. verticillioides*; [f]none: no abnormal pigmentation or exudate production observed; [g]– no hyphal coiling observed; low/less =1-2 coiling square^{-1}; medium =3-5 coiling square^{-1}; high frequent =≥ 6 coiling square^{-1}. All values are averages of three determinations; within columns, means followed by the same letter are not statistically different according to LSD (0.05).

The fungicide (Apron Star 45 WS) used in this study also reduced *F. verticillioides* colonization of maize kernels by 64.2% in 2013 and by 52.6% in 2014 compared to the control (Fig 2). Total fumonisin content was significantly higher in maize grains grown from seeds treated with the *F. verticillioides* alone (*Trichoderma* untreated control) compared with *Trichoderma*-treated and Apron Star-fungicide-treated plots (Fig 3). Seeds treated with *Trichoderma* species reduced fumonisin contamination by 44% - 89.8% in 2013 and 48.6% - 88.8% in 2014. The highest reductions in fumonisin content in maize kernels were achieved by seed treatment with *T. harzianum* isolate Thr2 (89%), followed by isolate Thr5 (87.4%). The fungicide, Apron Star, also reduced fumonisin contamination by 38.5% in 2013 and by 57.3% in 2014 compared to plots received with *F. verticillioides*-treatment alone (Fig 3).

Trichoderma strains tested in this study differed in their overall performance with respect to all the parameters measured under field conditions. The efficacy of *T. hamatum* isolates (Thm3, Thm6) and *T. harzianum* (Thr2, Thr5, Thr6) were better than others considered in this study. Moreover, a slight difference was observed in efficacy performance ranking of *Trichoderma* isolates under *in vitro* and field conditions (Table 2, Fig 2 and Fig 3).

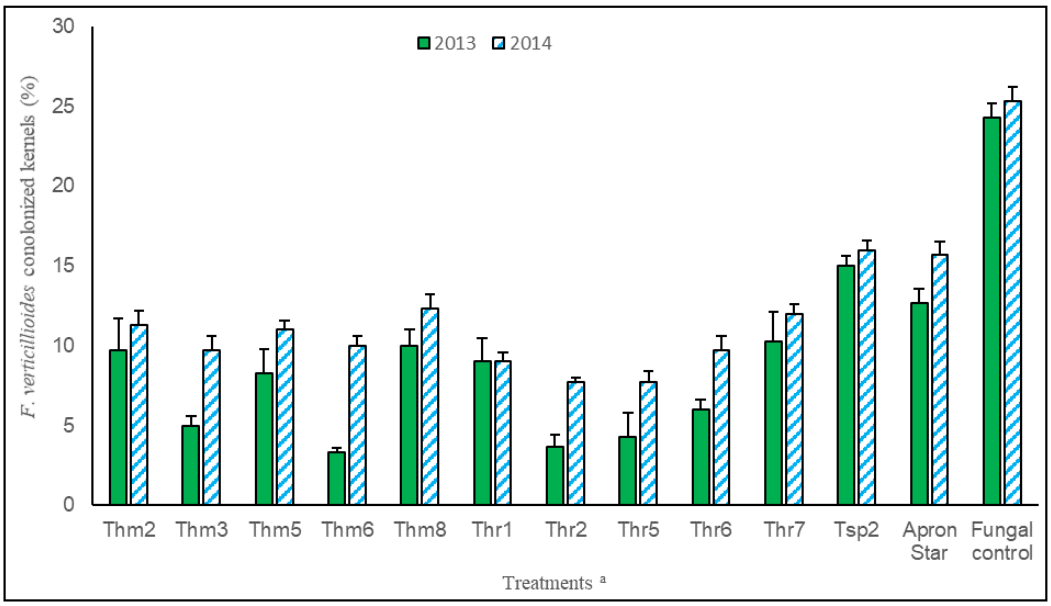


Figure 2. Effect of seed-treatment with different Trichoderma species on F. verticillioides colonization of maize kernels, during the 2013 and 2014 cropping season. Note: [a]=Thm refers to T. hamatum, Thr: T. harzianum, Tsp: T. spirale, Fungal control (untreated control): seeds only received F. verticillioides treatment but with no biocontrol agent, Apron Star: fungicide used for seed treatment (positive control).

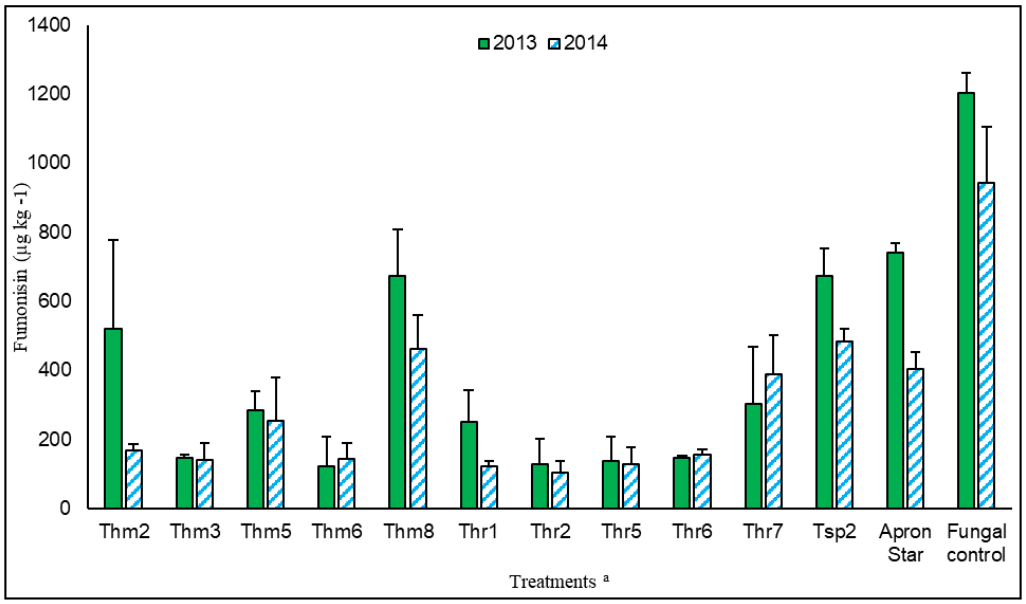


Figure 3. Effect of seed-treatment with Trichoderma species isolates on fumonisin contamination of maize grains, during 2013 and 2014 cropping season. Note: [b]= Thm refers to T. hamatum, Thr: T. harzianum, Tsp: T. spirale, Fungal control (untreated control), Apron Star: fungicide used for seed treatment (positive control).

3.5. Effect of Seed Treatment with *Trichoderma* species Isolates on Plant-Growth Parameters

A significant ($p < 0.05$) difference was observed among the treatments in terms of the percentage of seeds emerged 10 days after sowing, shoot height and seedling vigor index 45 days after emergence. In comparison to seeds treated with the fungicide and untreated control, seeds treated with *Trichoderma* isolates showed increased rates of germination seedling growth (shoot height), and seedling vigor index (Table 3). On average an increase in speed of seed germination by 4.1% - 14.7% was observed when seeds were treated with various isolates of *Trichoderma* species compared to the untreated control. Seedlings produced from *Trichoderma*-treated seeds exhibited also higher levels of shoot growth (6.4% - 22.3%) and seedling vigor index (10.2% – 34.0%) as compared to seedlings from untreated control (Table 3). Likewise, a significant increase was observed for plant height, 1000 seed weight and grain yield in *Trichoderma*-treated seeds compared to control. In comparison to the untreated control, increments of plant height (4.3% - 6.7%), seed weight (3.0% – 10.6%) and grain yield (9.5% - 19.2%) were recorded in maize plants grown from *Trichoderma*-treated seeds.

Table 3. Effect of Trichoderma species on agronomic and yield performance of field grown maize, in northern Ethiopia.

Treatments [a]	***Germination***		***Shoot height***		***seedling vigour***		***Plant height***		***1000 seed weight***		***Grain yield***	
	(%)	***DCC (%)*** [b]	***(cm)***	***DCC (%)*** [b]	***index***	***DCC (%)*** [b]	***(cm)***	***DCC (%)*** [b]	***(g)***	***DCC (%)*** [b]	***(tons ha^{-1})***	***DCC (%)*** [2]
Thm2	91.0e	8.8	25.0cde	12.0	2304ef	20.9	164abc	5.9	359.5e	6.5	4.4bc	13.6
Thm3	95.8abc	13.4	26.3abc	16.4	2526bcd	27.8	163abcd	5.3	364.8cde	7.9	4.6a	17.4
Thm5	93.3cde	11.1	26.0bcd	15.4	2424cde	24.8	162abcd	4.8	365.0cd	7.6	4.3c	11.6
Thm6	97.2a	14.6	28.0ab	21.4	2729.5a	33.2	164.5abc	6.2	368.8bc	8.9	4.7a	19.2
Thm8	91.8de	9.6	23.8def	7.6	2183fg	16.5	160.8cd	4.1	359.2e	6.5	4.4bc	13.6
Thr1	94.5abcd	12.2	27.8ab	20.9	2621abc	30.5	161.2bcd	4.3	375.8a	10.6	4.6ab	17.4
Thr2	97.3a	14.7	28.3a	22.3	2762a	34.0	165.3a	6.7	370.7abc	9.4	4.6ab	17.4
Thr5	96.5ab	14.0	28.5a	21.4	2749a	33.7	164.8ab	6.4	372.5ab	9.8	4.6a	17.4
Thr6	94.0bcde	11.7	28.3a	22.3	2660ab	31.5	162.7abcd	5.2	369.8abc	9.2	4.7a	19.2
Thr7	92.3de	10.1	25.8bcd	14.3	2386def	23.6	161.3bcd	4.4	359.7de	6.6	4.3c	11.6
Tsp2	86.5f	4.1	23.5ef	6.4	2031g	10.2	161.2bcd	4.3	346.2c	3.0	4.2c	9.5
Apron Star	87.5f	5.1	23.8def	7.6	2210fg	17.5	160d	3.6	342.3f	1.8	4.2c	9.5
Control	83.0g	-	22.0f	-	1823h	-	154.3e	-	336.0g	-	3.8d	-
Mean	92.4	-	25.9	-	2414	-	161.9	-	360.08	-	4.4	-
LSD	3.08	-	2.17	-	202.4	-	4.4	-	6.2	-	2.1	-
CV (%)	2.6	-	7.3	-	7.3	-	2.1	-	1.5	-	4.2	-
P-value	0.0001	-	0.0001	-	0.0001	-	0.0001	-	0.0001	-	0.0001	-

***Note*:** [a]Thm: *T. hamatum*, Thr: *T. harzianum*, Tsp: *T. spirale*, [b] DCC: Differences of plant-growth parameters compared to the control. LSD: Least Significant Difference, CV: Coefficient of Variation. All data are pooled over two years; within columns, means followed by the same letter are not statistically different according to LSD (0.05).

4. DISCUSSION

Results of the present study showed that maize rhizosphere in the study areas supports a variety of potentially beneficial fungal species. The dominance of *Trichoderma hamatum* compared to the other species might be related to specific environmental conditions such as soil moisture and temperature conditions in the maize growing areas of Ethiopia. In this study, soil samples were taken in August when soil moisture was ample and this may contribute to the predominance of *T. hamatum.* In line with this, Kredics et al. (2014) was reported dominant occurrence of *T. hamatum* under excessive moisture conditions, while *Trichoderma viride* were found to be largely restricted to cool temperature areas and *T. harzianum* requires warm climate. The distribution of *Trichoderma* is influenced by multiple factors such as cropping systems, plant type and root exudates, water availability, low osmotic conditions, soil pH, temperature, and various biotic and abiotic stressors (Cripps-Guazzone et al., 2025; Okoth et al., 2009; Nandini et al., 2021).

In this study *Trichoderma* species isolates, which obtained from the rehizoshpere of growing maize plants and postharvest maize kernels, had shown a great potential to control *F. verticillioides* under *in vitro* and field conditions. In the *in vitro* study, the *Trichoderma* isolates exhibited significant antagonistic activity against the mycelial growth of *F.verticillioides* and various antagonistic mechanisms were apparent for different *Trichoderma* isolates. Many of the *Trichoderma* isolates showed rapid growth while limiting the growth area of the pathogen to a very small portion, as observed by Santos et al. (2020) regarding the interaction between *Trichoderma* spp and *Fusarium verticilliodes*. The rapid growth of *Trichoderma* isolates may provide them an additional benefit to inhibit the pathogen's growth through competition for nutrients and space, even prior to the release of antimicrobial substances (Kumar et al., 2012). Almost all *Trichoderma* isolates tested in this study displayed formation of hyphal barrier between species as reported by Bunbury-Blanchette and Walker (2019) in the interaction zone between *Trichoderma* species and *Fusarium oxysporum* f. sp. cepae. In addition, Intense sporulation and some signs of hyphal coiling was observed in the interaction zone after growth overlaps in most of the *Trichoderma* isolates tested, which indicated that hyperparastisim played a very important role in the biocontrol mechanism to reduce the growth of the *F. verticillioides* pathogen, which is in line with the reports of Santos et al. (2017) and Moya et al. (2020). The

greatest inhibition zone detected in some isolates before hyphal contact clearly showed that diffusible chemical compounds with capability to reduce the growth of the pathogen were being produced by those *Trichoderma* isolates. Both volatile and non-volatile antibiotics might be effective against certain isolates, which are active prior to the hyphae (Hajieghrari et al., 2008; Kumar et al., 2012). Consistent with the results of this study, other antagonistic relationships have been documented in dual culture tests of fungal plant pathogens and *Trichoderma* species, such as abnormal pigment or exudate production, hyphal coiling and parasitism, vacuolization and mycelial breakdown, which have been interpreted as indicators of antagonism (Almeida et al., 2007; Bunbury-Blanchette and Walker, 2019).

During the field experiments, a substantial reduction of *F. verticillioides* colonization and fumonisin contamination of maize kernels was observed in plants treated with the different *Trichoderma* species isolates. These results demonstrate that the *Trichoderma* isolates tested in this study could provide control of *F. verticillioides,* which is the most common fungal pathogen of maize in Ethiopia. In field experiments conducted for three years, Ferrigo et al. (2014) has observed a considerable reduction of fungal infection and fumonisin contamination in maize kernels using *T. harzianum* seed treatment against *F. verticillioides*. Results of this study are similar to other previous studies elsewhere, where *Trichoderma* species was used successfully to control various diseases caused by *Fusarium* species (Bunbury-Blanchette and Walker, 2019; Chandra Nayaka et al., 2008; Santos et al., 2020).

Significant differences were noticed among the *Trichoderma* isolates in reducing *F. verticillioides* and fumonisin contamination in maize. *Trichoderma harzianum* strains Thr2, Thr5 and Thr6 as well as *T. hamatum* strains Thm3 and Thm6 were the most efficient compared to others trains. The superior performance of these *Trichoderma* isolates in controlling the pathogen compared to the others is probably driven by a combination of factors including specific genetic potential to produce antimicrobial metabolites, environmental adaptability, and the ability to rapidly colonize the rhizosphere. Some *Trichoderma* isolates possess enhanced mechanisms such as mycoparasitism, antibiosis and induction of plant defense that allow them to more aggressively suppress pathogens (Almeida et al., 2007; Yao et al., 2023). Synergism between different modes of action may occur in nature for the biocontrol of fungal pathogens, and efficient biological control agents should express more than one mode of action for suppressing

the pathogen. A good biocontrol agent should adapt and proliferate in the rhizosphere so that it can protect the seed as well as the seedling (Harman et al., 2004). In addition, combined application of *Trichoderma* isolates could produce synergetic effects. Chandra Nayaka et al. (2010) have also reported certain *T. harzianum* strains are more effective than others in reducing *F. verticillioides* infection and fumonisin contamination in maize, which is similar with results of this study.

Results of the present study show that seed treatments with *Trichoderma* spore suspension have various positive effects on the maize plant growth including increasing speed of seed germination, seedling vigor index, seed weight and grain yield. This is an additional advantage, compared to other pest management options such as pesticides that frequently cause phytotoxicity. In addition to their potential of biocontrol activities, *Trichoderma* species have been observed to promote plant growth and grain yield (Entesari et al., 2013; Moya et al., 2020). Several explanations have been suggested for the improved plant growth and yield including enhanced root growth and development, enhancing resistance to biotic stress, and improving nutrients uptake (Entesari et al., 2013; Mastouri et al., 2010).

5. CONCLUSION

The *in vitro* test revealed that *Trichoderma* species can suppress significantly growth of *F. verticillioides*. Besides, the *in vitro* dual culture studies contribute to the understanding about the biocontrol mechanisms involved in the *Trichoderma* strains and *F. verticillioides* interaction. Results of the field trial demonstrated that seed treatment with *Trichoderma* species isolates have the ability to reduce *F. verticillioides* and fumonisin contamination of maize kernels to a reasonable extent where the performance of *T. hamatum* isolates (Thm3, Thm6) and *T. harzianum* (Thr2, Thr5, Thr6) happened to be superior compared to the others. The results of the present study also indicate that *Trichoderma* species isolates enhances plant growth attributes and grain yield of maize. The *Trichoderma* strains with the best overall performance can be used as integral part of integrated management of *F. verticillioides.* Future research should evaluate the performance of these isolates across different agro-ecological regions and assess their potential for commercialization as biological control products.

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7. CONFLICT OF INTEREST

The authors declare that they have no conflict of interest regarding this manuscript.

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