Original Research Article||

Isolation and Pathogenic diversity among Fusarium oxysporium f.sp. capsici isolates in southern Ethiopia and evaluation of Biocontrol agents against the Pathogen

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Abstract

Fusarium wilt is one of the major biotic factors affecting hot pepper production in Ethiopia. Therefore, this study was designed with the objectives of analyzing the pathogenic diversity of Fusarium oxysporium f.sp. capsici isolates and to evaluate the biocontrol potential of some antagonists for the management of the disease. For this purpose, twenty nine isolates of Fusarium oxysporium f.sp. capsici were identified from visibly diseased plant samples collected from the 80 surveyed fields based on macroscopic and microscopic characteristics. Pathogenicity test was done for the 29 Fusarium oxysporium f.sp. capsici isolates and based on diseases severity index value, isolates were classified as highly pathogenic, moderately pathogenic, and weak. Isolate LWS7 was found to be the most pathogenic isolate that induced disease and was used for the studies involving biocontrol agents. The in vitro and in vivo efficacy trial of six Trichoderma spp. and Bacillus subtilis was investigated under dual culture assays in the lab and in a greenhouse, respectively. The highest (81.8%) and lowest (48%) mycelial growth inhibition were estimated from Trichoderma harzianum and Trichoderma hamatum, respectively. Lower wilt incidence (31%) was recorded on pepper plants following seedling root dip inoculation by Trichoderma viride. In addition to their significant effect on mycelia growth in-vitro, the bioagents evaluated in the current experiment were also found to result in significant reduction of the disease and promotion of growth of hot pepper in soil application and seedling root dip tests. This finding indicated that bioagents are an important development direction for their role in the sustainable development of agriculture. The efficacy and economic feasibility of hot pepper management through biocontrol agents may need additional investigation to come up with conclusive results.

Key words: Fusarium wilt, Biological control, Pathogenicity, Trichoderma spp

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INTRODUCTION

Hot pepper (Capsicum annuum L.) is an important vegetable crop in tropical areas worldwide. It belongs to the Solanaceae family and in the genus Capsicum (Dias et al., 2013). Capsicum is the second most important genus in family Solanaceae next to Solanum (Berhanu et al., 2011). Hot pepper is a high-value spice crop, which is consumed as vegetable, spice, or condiment in its fresh, dried, or processed forms. Hot pepper is very important vegetable and spice crop in Ethiopia, but its production is constrained by many diseases including Fusarium wilt (Fusarium oxysporium f.sp. capsici) (Assefa et al., 2015). Despite its importance as commercial spice and vegetable crop, the hot pepper acreage, production, and productivity are falling (Fekadu and Dendena, 2006). Hot pepper is attacked by several bacterial, viral and fungal diseases as well as insect pests. Among the known hot pepper diseases, those

caused by Fusarium spp., Phytophthora spp., Leveillula taurica, Verticilllum spp., Rhizoctonia solani, Colletotrichum spp. and Cercospora capsici (Mekonen and Chala, 2014); bacterial diseases caused by Xanthomonas campestris pv. visicatoria, Ralstonia solanacearum and Erwinia carotovora Kassahun et al., 2016); and viral disease such as pepper mottle virus (Tameru et al., 2003) have been reported in Ethiopia.

Fusariums wilt disease, caused by the soil-borne fungus Fusarium oxysporum f.sp. capsici, is the most serious disease affecting pepper plants, reducing growth, fruit yield, and pepper quality (Wongpia and Lomthaisong 2010). Fusarium wilt of pepper has become increasingly common on open-field-grown peppers in recent years. The fungus can infiltrate a plant with its sporangial germ tube or mycelium via penetrating plant roots, through wounds, or straight through the root tip or at the point of lateral root development (Koste and Bart, 2013).

Fusarium wilt is a disease that affects hot peppers all around the world. Ethiopia is one of a few African countries that produce capsaicin and oleoresin for the export market from locally selected materials that contributed substantially to the national economy. Among biotic factors that affect pepper production in Ethiopia, Fusarium wilt that is caused by Fusarium oxysporium is one of the most economically important diseases, and it accounts for yield losses of up to 80% (Aklilu et al., 2007). In recent years, the importance of the disease has been increasing and is given considerable attention by hot pepper producers and other stakeholders. Fusarium wilt from untreated Marako-fana resulted in a relative yield reduction of 68-71% in Ethiopia (Teshome et al., 2012).
Fusarium wilt symptoms begin with modest vein clearing on the outer portion of the younger leaves, followed by epinasty (downward drooping) of the older lower leaves. The disease is distinguished by a slight yellowing of the lower leaves and withering of the upper leaves, followed by permanent drooping of the entire plant and browning of the vascular tissue (Black and Rivelli, 1990). The most prevalent symptoms of F. oxysporium infection were leaf chlorosis, vascular discolouration and plant wilting. Fusarium oxysporium f.sp. capsici is a soil borne fungal pathogen and thrives in conditions with high soil moisture (20%) and temperature (25-30°C) (Sekhon and Singh, 2007).
Fusarium wilt disease is soil-borne in nature, making the application of fungicides impractical due to their environmental and health hazards to applicators and consumers, as well as their non-selective nature that also affects beneficial organisms. As a sustainable and ecofriendly alternative, biological control using biocontrol agents such as species from the genus Trichoderma has been recognized (Sabalpara et al., 2009) as a promising approach for managing soil-borne plant pathogens. These Trichoderma species suppress pathogen populations either by directly parasitizing them or by competing for essential nutrients, thereby limiting their growth. Biological control strategies are considered environmentally safe, economical, and effective. They operate through various mechanisms, including competition for space and nutrients, stimulating host plant defenses to induce tolerance or resistance, and producing antibiosis through the synthesis of harmful fungal chemicals or enzymes (Matar et al., 2009). Overall, Trichoderma-based biocontrol agents offer a targeted and sustainable method for managing fungal plant diseases in agricultural systems (Abada and Eid, 2014). In Integrated disease management program, biological control is crucial because the Fusarium pathogen is soil-borne and can survive for years, making single control methods (like crop rotation or some fungicides) less effective on their own. BCAs provide long-term, consistent disease control by establishing a beneficial microbial balance in the plant root system (rhizosphere).

Furthermore, due to the pathogen's nature (it survives in the soil) control measures such as fungicide use are limited. Yet, biological control agents with an eco-friendly approach are increasingly required in crop protection strategies to combat the indiscriminate use of chemicals. Many investigations on biocontrol agents have revealed antifungal activity against Fusarium oxysporium f.sp. capsici (Heydari & Pessarakli, 2010; Endriyas et al., 2020). As there is a lack of knowledge on the potential of biological control agents for pepper wilt disease management in Southern Ethiopia, evaluation of different biocontrol agents is important. Therefore, this study

was designed with the objectives of analyzing the pathogenic diversity of Fusarium oxysporium f.sp. capsici isolates and to evaluate the biocontrol potential of some antagonists for the management of the disease.

MATERIALS AND METHODS

Characterization and Pathogenicity Test of the Fusarium oxysporium f.sp. capsici Isolates

Diseased Sample Collection, Isolation and Identification

Disease plant samples were collected from three administrative zones (Gurage, Silte and Halaba) and five pepper growing districts (Abeshge, Meskan Mareko, Lanfro and Wera) of the southern region of Ethiopia during 2022 main cropping season. Stem/root samples from diseased pepper plants showing the typical symptoms of Fusarium wilt (lesions on the stem and root, brown vascular discoloration, leaf epinasty and yellowing, wilting and plant death) were taken from each field. The samples were placed in plastic bags and labeled with relevant information and brought to Hawassa University Plant Protection Laboratory and preserved at 4°C until isolation and identification of the pathogen.

The infected root/stem samples were cut into smaller pieces using sterile scissors and washed under running tap water in the laboratory. The segments were surface sterilized with 2% sodium hypochlorite solution for 3 min, washed three times with sterile distilled water, and dried on sterile blotting paper. Five patches of the plant specimens were then plated onto potato dextrose agar (PDA) medium supplemented with Antibiotics (streptomycin 30 µg/l, chlortetracycline 10mg/lt), to avoid bacterial contaminations and incubated at 25±1°C for 7 days. Seven days after incubation, the colonies were transferred and sub-cultured on to fresh PDA plates to obtain pure cultures. Then spores of the fungi were subsequently identified according to their cultural and morphological features (Leslie and Summerell, 2006; Booth, 1971).

Cultural and Morphological Characterization

The cultural (macroscopic) and morphological (microscopic) features of *F.oxysporum* isolates were characterized according to Leslie and Summerell (2006) and Booth (1971). Accordingly, the colony characters: color (upper and reverse), colony shape, margin, elevation and radial growth, days to pigmentation and colony surface texture were visually examined in 7 days old pure cultures. Fungal colonies (single spore) were transferred into fresh SNA media (Spezieller Nährstoffarmer Agar) (1L of distilled water, 1g KH_2PO_4, 1g KNO_3, 0.5g $MgSO_4 \cdot 7H_2O$, 0.5g KCl, 0.2g Glucose, 0.2g Sucrose, 20g Agar) medium (Leslie and Summerell 2006) to get conidia. Conidia characteristics: presence or absence of microconidia and chlamydospore, number of septa in macroconidia, shape of macroconidia, and shape of microconidia were examined.

Pathogenicity Test

Pathogenicity test of 29 isolates was conducted in Southern Agricultural Research Institute (SARI) under greenhouse conditions at a temperature of 28°C using the susceptible variety Mareko Fana (Kassahun et al., 2016). Inoculum of the individual isolates was prepared on PDA. From 10 days old cultures, conidia were harvested to 15ml beaker by adding 10 ml of sterile distilled water (SDW) in each Petri plate. To remove mycelial masses, the suspension was filtered through sterile cheese cloth. From the filtered culture, conidia were resuspended in SDW and the final conidial concentration was adjusted to $1x10^6$ spore/ml using a haemocytometer.

Inoculation was performed following the standard cut-root dip inoculation technique (Herman and Perl-Treves, 2007; Karimi et al., 2010). Accordingly, roots of 4 weeks old seedling of hot pepper, raised in the greenhouse at 28°C in a pot filled with oven sterilized (at 65°C for 72 hours) soil, compost and sand mixture in the ratio of 1:0.5:0.5, respectively, were trimmed with a sterile scissor and submerged into tubes containing 9 ml of the spore suspension for 30 minute.

Roots of control plants were similarly cut and dipped in SDW for the same period of time. Then, inoculated seedlings were transplanted into 3litre capacity pots filled with oven sterilized soil (at 65°C for 72 hours) , compost and sand in the ratio of 1:0.5:0.5, respectively (Demissie et al., 2021) arranged in a Completely Randomized Design

(CRD) and grown in the greenhouse under conditions described above. For each isolate, a total of 9 seedlings (3 seedlings/pot with 3 replications) were used. Similar numbers of plants per replication were used as control. Koch's postulate was tested by the isolation of the pathogen from diseased plants and re-inoculating the isolated *F. oxysporum* isolates onto new and healthy plants. Observation of similar symptom and re-isolation and characterization of morphological features were done.

Evaluation of Biocontrol Agents Against the Pathogenic Isolates

***In Vitro* Test**

Trichoderma spp. i.e. *Trichoderma harzianum, T. viride, T. longibrachiatum, T. hamatum, T. asperellum* and *T.atroviride*), and a bacterium (*Bacillus subtilis)* obtained from the stock culture of Ambo Plant Protection Research Center were tested for their antagonistic activity against *Fusarium oxysporium* f.sp. *capsici* by dual culture technique.

From five days old culture with the help of sterilized corkborer, 5mm mycelial discs of antagonist as well as pathogenic isolates was taken from the tip of young hyphal growth both for antagonist as well as pathogenic isolates and placed on Potato dextrose agar (PDA) at the opposite side of each other (dual culture) incubated at 28°C for one week. A distance of 5cm between the inoculation point of *Fusarium oxysporium* isolates and the antagonists (*Trichoderma* spp.) was kept for formation of inhibition zone. In case of evaluation of bacterial antagonist, 24 hr. old culture of *Bacillus subtilis* was streaked with inoculating loop at one end of the Petri plates and mycelial disc (5mm) of the test fungus (*Fusarium oxysporum* f.sp. *capsici*) was placed at the other end.

For each BCA-FOC combination, three replicates were used and arranged in CRD. Mycelial growth inhibition of FOC due to the BCAs was measured as radius of FOC colony towards the BCA colony and in the corresponding control Petri dishes starting from three days post incubation (DPI) and continued until seven DPI. For control, three replicates were used and arranged in CRD, then the radius of the colony was measured from center to the edge of the plate. Finally, percent radial mycelial growth inhibition was calculated by comparing FOC colony growth in BCA plates with that of the control plates and expressed in percent using the formula described below (Rini and Sulochana, 2006).

$$I = \frac{C-T}{C} \; x\,100$$

Where, I= Percent of inhibition in growth of the *Foc*

C= Radial growth of the *Foc* (cm) in control plate

T= Radial growth of the *Foc* (cm) in the antagonists.

In Vivo Test

Mass Production and Preparation of Biocontrol Agents and Fusarium Inoculum

Mass productions of *Trichoderma* spp. were prepared by using sorghum grain. In each pot 3kg of soil was added and each antagonist was applied in three replication. 3kg soil in each pot was inoculated with 60g of inocula of each antagonist (thus a total of 180g substrate was used for three replicates in each antagonist (Rini and Sulochana, 2006). The samples of moistened substrates were transferred to 500 ml sized conical flask and autoclaved twice at 15 psi at 121°C for 30 minutes. The flasks were allowed to cool down under room temperature before inoculation of antagonists. The culture of *Trichoderma* spp. were prepared by harvesting the spore from one week old culture of *Trichoderma* spp. and a concentration of 10^6 spore/ml was injected into the autoclaved flask with sterile water and incubated at 25±2°C for two weeks (Singleton et al., 1992). Finally after two weeks the antagonists were grown in the substrate and air dried at room temperature before applied to the soil. For *Bacillus subtilis*, inoculum was prepared according to Zhang et al. (2009) (with minor modification). Bacterial culture was grown in nutrient agar for 72 h and with shaking at 150 rpm and adjusted to a concentration of 10^8 CFU ml^{-1} for soil treatments and seedling root dip method (Zhang et al., 2009).

For soil treatments, peat (compost) was used as the bacterial carrier or delivery substrate in soil treatments. Sterile peat was drenched with bacterial broth cultures at the rate of 50 ml of bacterial broth

to 50g of peat. Peat was air dried at room temperature for 48 h and then processed into a powder form (Zhang et al., 2009). However, the controls were dipped in sterilized water and transplanted as the same as other treatments.

The isolate identified as the most aggressive was used for the evaluation of biocontrol agents. Mass production of Fusarium inoculum was prepared by using sand-barley (1:1, w: w and 40% water) mixture. The samples of moistened mixture of sand-barley with water (substrates) were transferred to 500 ml sized conical flask and autoclaved twice at 15 psi at 121°C for 30 minutes. The flasks were allowed to cool down under room temperature before inoculation. The autoclaved medium was then inoculated with a 5-mm disk of more aggressive *Fusarium oxysporium* f.sp. *capsici* test pathogen and incubated at 25±2°C for two weeks (Singleton et al., 1992). After two weeks *Fusarium oxysporium f*.sp. *capsici* inoculum was grown in the substrate and air dried at room temperature before it was applied to the soil.

Inoculation Methods

Soil application (SA), antagonists were applied to the soil at the rate of 20g/kg of soil a week prior to transplanting, and also Fusarium inoculum was mixed to the soil at the rate of 20g/kg in to the pot one day before sowing (Rini and Sulochana, 2006). The control treatment was inoculated only with Fusarium inoculum one day before sowing. Seedling root dip application, *Fusarium oxysporium* f.sp. *capsici* inoculum were mixed to the soil at the rate of 20g/kg in to the pot one day before sowing and solutions of all Trichoderma used as a treatment were prepared at the concentration of $1x10^6$ spore/ml. Hot pepper seedlings (Mareko fana) were carefully uprooted and their roots were washed with sterilized water and dipped in a solution of biocontrol agent for 30 minute. Tween-20 was used as sticker during seedling root dip (SRD) after which the seedlings were transplanted into prepared pots in green house.

Experimental Treatments and Design

The experiment was laid out in a CRD. Fifteen (15) treatments were used for the experiment with three replications and a total of 45 pots were used (Table 1). In each pot three plants were used, thus a total of 135 plants.

Table 1. Treatments used for the *in vivo* experiment

Treat. No	Treatment
1	*Trichoderma viride* (SA) + *Foc* (SA)
2	*Trichoderma harzianum* (SA) + *Foc* (SA)
3	*Trichoderma asperellum* (SA) + *Foc* (SA)
4	*Trichoderma atroviride* (SA) + *Foc* (SA)
5	*Trichoderma longibrachiatum* (SA) + *Foc* (SA)
6	*Trichoderma hamatum* (SA) + *Foc* (SA)
7	*Bacillus subtilis* (SA) + *Foc* (SA)
8	*Trichoderma viride* (SRD) + *Foc* (SA)
9	*Trichoderma harzianum* (SRD) + *Foc* (SA)
10	*Trichoderma asperellum* (SRD) + Foc (SA)
11	*Trichoderma atroviride* (SRD) + Foc (SA)
12	*Trichoderma longibrachiatum* (SRD) + Foc (SA)
13	*Trichoderma hamatum* (SRD) + Foc (SA)
14	*Bacillus subtilis* (SRD) + Foc (SA)
15	Control + Foc (SA)

Where, SA =Soil application, SRD = seedling root dip

Disease Assessment

Disease data (severity) were collected at 7, 14, 21, 28, 35, 42 and 49 days after inoculation (Wongpia and Lomthaisong 2010; Demissie et al., 2021). To determine the disease severity 0-5 scale was used based on the percentage of affected plant part (Table 2). Disease severity index (DSI) was calculated following (McKinney, 1923).

$$DSI = \frac{PxQ}{MxN} \text{ x } 100$$

Where **P** = Number of plants in each disease severity class

Q = Numerical value of each severity class

M = Maximum disease score (the highest possible rating)

N = Total number of plants assessed

The area under the disease progress curve (AUDPC) was also calculated as follows.

$$\%-AUDPC = \sum_{n=1}^{n} \left(\frac{Yi+1+yi)}{2} (\text{ti}+1 - \text{ti} \right)$$

Where n=total number of observations, Yi = initial DSI at the i^{th} observation, ti= time of the i^{th} assessment in days, and n= total number of observation, t=day after inoculation, the unit for y in the sample data is % of development stage unit. The value of DSI and AUDPC were used to classify the level of pathogenicity amongst the different Fusarium isolates (Demissie et al., 2021). Moreover, plant fresh and dry weight was taken.

Table 2. Assessment scale for pepper Fusarium wilt severity.

Scale(0-5)	Description
0	No symptoms
1	Initial symptoms or 1–10% chlorosis of leaves
2	10–20% chlorosis of leaves
3	20–50% chlorosis of leaves
4	>50% chlorosis of leaves and initial symptoms flaccidity of the top leave
5	Completely or the major part of the plant wilted or death

To evaluate the *in vitro* efficacy of antagonists, diameter of inhibition zone (mm) was measured. Diameter of inhibition zone (mm) was measured by taking the average diameter of inhibition zone observed in three petridish for each antagonist and calculated using the following formula (Rini and Sulochana, 2007).

$\frac{C-T}{C}$ x 100 Where, I= Percent of inhibition in growth of the *Foc*

C= Radial growth of the FOC (cm) in control plate

T= Radial growth of the FOC (cm) in biological control in *Trichoderma* spp.

To evaluate *in vivo* efficacy of antagonists, wilt incidence, plant height, number of leaves, root length, fresh and dry weight of shoot and root was taken.

Data Analysis

All experimental data such as disease and plant data were subjected to analysis of variance using one way ANOVA by SAS software version 9.3 (SAS, 2017). Least Significant Difference (LSD) at 0.05 probability level was used for mean separation.

RESULTS AND DISCUSSION

Characterization and pathogenicity test of Fusarium oxysporium f.sp. capsici isolates

Macroscopic characterization

In this study from eighty samples, twenty nine isolates were identified as *Fusarium oxysporium* f.sp. *capsici* (Appendix 1). Seventeen (58.6%) of the isolates had pink colony color while nine isolates (31%) had white colony color and the remaining three isolates (10.4%) had creamy white colors on PDA plates (Fig. 1 and Appendix 1. Regarding the colony pigmentation, seven isolates (24%) had light reddish purple pigmentation, 13 isolates (45%) had intense reddish purple and the remaining nine isolates (31%) had pale yellow (Dull white) pigmentation.

Thirteen isolates (44.8%) had irregular shapes, whereas 14 isolates (48.3%) had circular and two

isolates (6.9%) had filamentous shapes. The elevations of the colony were flat type in 19 (65.5%) isolates and raised in 10 (34.5%) isolates. The colony margins of the isolates were entire in 17 (58.6%) isolates undulate in 10 (34.5%) isolates and Filliform in two isolates (6.9 %). The colony growth, 18 (62%) isolates had fluffy growth, 10 (34.5%) isolates had suppressed growth and only one isolate (3.5%) had scanty fibrous growth (Fig 1 and Appendix 1). Regarding the colony surface texture, 16 (55.2%) isolates had smooth, eight (27.6%) isolates had rough and five isolates (17.2%) had rugose colony surface texture. Considering the radial growth of isolates, isolates were grouped as slow growing (2.4-2.9 cm), medium growing (3-3.5 cm), and fast growing (3.6-4.5 cm) according to Gabrekiristos *et al.* (2020). Accordingly, four (13.8%) isolates were slow growing, eight (27.6%) isolates were medium growing and the remaining 17 (58.6%) isolates were fast growing. The current results agree with the finding of Kaushal (2016) who reported colony color variation (from pink to white) in FOC isolates of hot pepper and also similar result were reported by Gabrekiristos *et al.* (2020). Jaywant (2016) also reported the production of white, cream white and creamy-pink to pink colony colors by *Fusarium oxysporum* isolates. The differential colour of the *Fusarium oxysporum* isolates may be due to the presence of specific pigment produced as by-product and involved in the enzymatic activities.

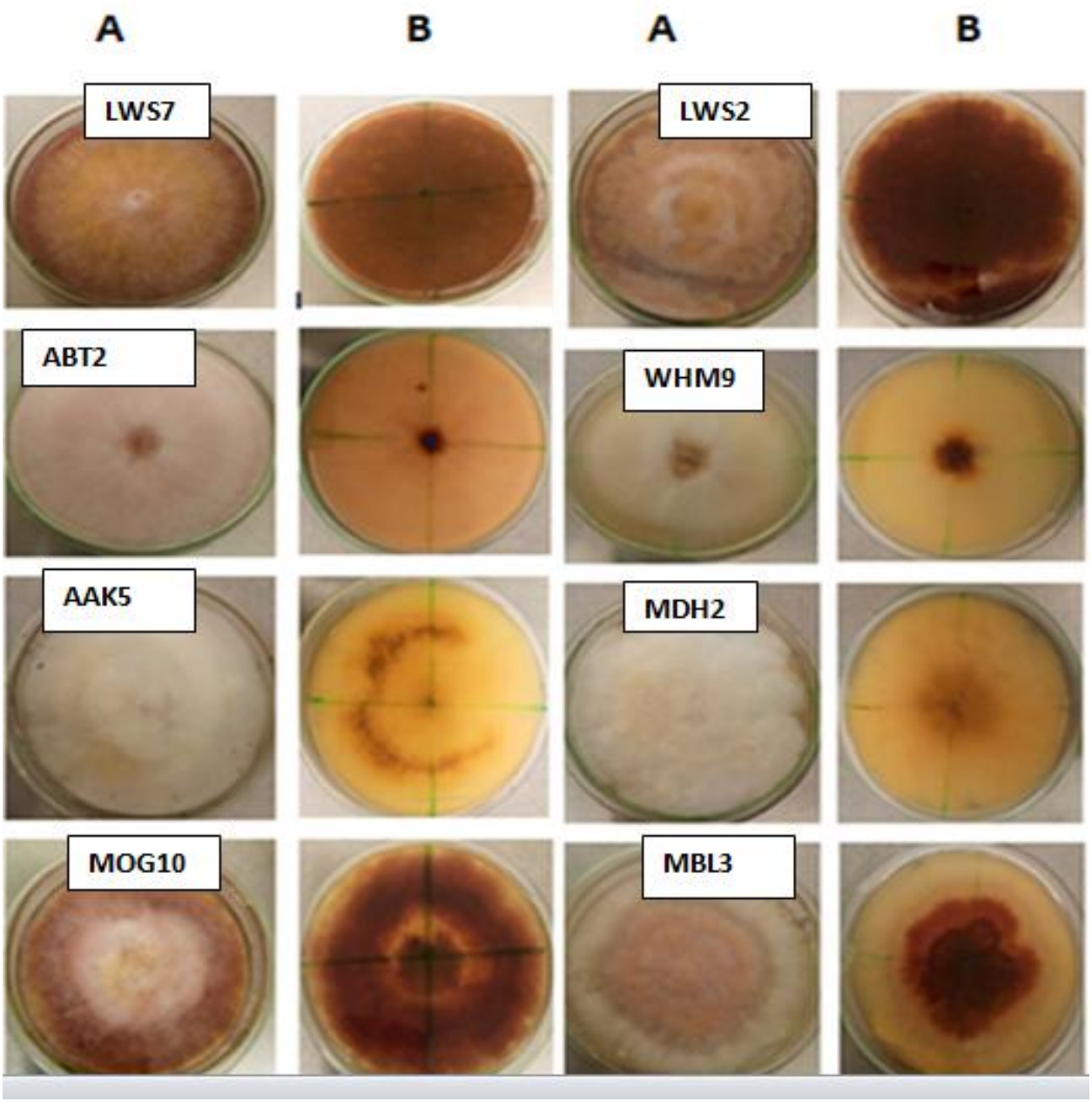

Figure 1. Pure cultures of *Fusarium oxysporium* f.sp. *capsici* isolates surface view (A) and reverse view (B) on PDA plates across a column respectively.
Note: AAKS and **ABT2** are isolates from Abeshge, **LWS2** and **LWS7** from Lanfro, **MOG10** and **MBL3** from Meskan, **WHM9** from Wera and **MDH2** from Mareko district.

Microscopic Characterization

Among 29 FOC isolates, sixteen of these isolates had straight shape, and 13 isolates had sickle shaped macro-conidia Fig 2 and Table 3. Variations in the number of septations in the macroconidia were also observed. The most frequent macroconidia septations were in the range of 2 to 3 and less frequently in between 2 and 5 conidial

septations were observed. Twenty six isolates had microconidia without septation; among them 15 isolates had oval shape, and 11 isolates had eliptical shape and the other three isolates had no microconidia at all. All the 29 isolates also produced chlamydospore in single and in pairs (Fig 2 and Table 3). Results from the present work agree with those from Jaywant (2016), Ferniah *et al*. (2014) and Kaushal (2016) that reported the ability of the pathogen to produce macroconidia, microconidia, hyphae with septation and chlamydospores. In the current study, three isolates namely MOG10, WAT6 and LST6 did not produce microconidia, however the macroconidia characteristics along with the colony feature were sufficient to identify them as isolate of *Fusarium oxysporium* in this regards.

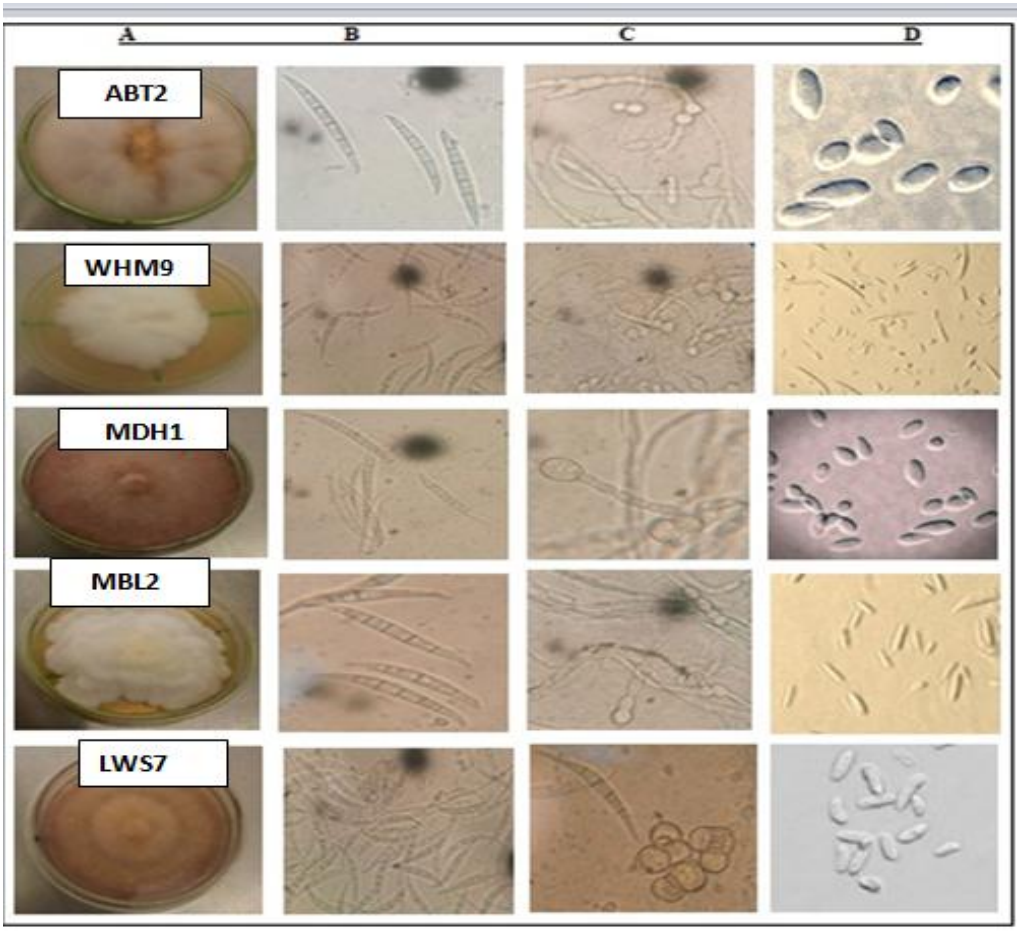

Figure 2. Pure cultures of *Fusarium oxysporium f.sp. capsici* isolate growing on PDA plates and their corresponding conidia observed under microscope (40x).

Where, A= Pure culture of the isolate of *Fusarium oxysporium f.sp. capsici* , B= Macroconidia, C= Chlamydospore, D=Microconidia across a column respectively.

Note: **LW57** is isolate from Lanfro, **ABT2** from Abeshge, **MDH1** from Mareko, and **MBL2** from Meskan and **WHM9** from Wera district

Table 3. Microscopic features of *Fusarium oxysporium* f.sp. *capsici* isolates from major pepper growing districts of southern Ethiopia during 2022 main cropping season

SN.	Isolate code	Conidia				
		Shape of macro conidia	Number of septa.	Micro conidia	Shape of microconidia	Chlamydospore (After 15 days)
1	**AAK1**	Straight	3-4	+	Oval	+
2	**AAK2**	Sickle shaped	3-5	+	Eliptical	+
5	**AAK5**	Sickle shaped	3-5	+	Oval	+
7	**AAK7**	Straight	3-5	+	Oval	+
9	**ABT2**	Straight	4-5	+	Oval	+
12	**ABT5**	Sickle shaped	2-3	+	Oval	+
15	**ABT8**	Straight	2-4	+	Eliptical	+
19	**MOG3**	Sickle shaped	3-5	+	Oval	+
25	**MOG9**	Sickle shaped	2-4	+	Eliptical	+
26	**MOG10**	Straight	2-4	-	-	+
28	**MBL2**	Sickle shaped	2-5	+	Eliptical	+
29	**MBL3**	Straight	2-4	+	Oval	+
32	**MDH1**	Straight	2-3	+	Oval	+
33	**MDH2**	Sickle shaped	2-4	+	Oval	+
39	**MDH8**	Sickle shaped	2-5	+	Eliptical	+
40	**MDH9**	Straight	3-5	+	Eliptical	+
42	**MDM2**	Straight	3-4	+	Oval	+
43	**MDM3**	Straight	2-4	+	Oval	+
51	**WHM3**	Straight	4-5	+	Oval	+
55	**WHM7**	Sickle shaped	3-4	+	Eliptical	+
57	**WHM9**	Sickle shaped	2-5	+	Oval	+
62	**WAT5**	Straight	2-3	+	Eliptical	+
63	**WAT6**	Straight	2-3	-	-	+
65	**LST1**	Straight	2-3	+	Eliptical	+
68	**LST4**	Sickle shaped	2-3	+	Oval	+
70	**LST6**	Sickle shaped	2-5	-	-	+
73	**LST9**	Sickle shaped	2-5	+	Oval	+
75	**LWS2**	Straight	3-5	+	Eliptical	+
80	**LWS7**	Straight	2-5	+	Eliptical	+

Where, "+" sign indicates presence and "-" sign indicates absence.

Pathogenicity Test

The results of pathogenicity test indicated that Fusarium isolates from the current study were able to infect pepper plants leading to symptoms such as stunted growth, leaf shading and yellowing, curling, wilting, flaccidity and or plant death while no symptoms were found on control plants. Symptoms were observed starting from the second week of post-inoculation whilst no symptoms were found on control plants (Fig 3). The specific symptoms and disease severity varied depending on the virulence level of the specific isolate of *Fusarium oxysporum* f.sp. *capsici*. Furthermore, re-isolation of the pathogen from diseased plants and re-inoculating the isolated fungi onto new and healthy plants was done and all the 29 isolates were pathogenic to pepper. Therefore, Koch's postulates were fulfilled.

Fusarium oxysporium f.sp. *capsici* isolates were grouped into three based on DSI value (disease severity index). Isolate with DSI value between 41–100% was considered as highly pathogenic: between 21–40% moderately pathogenic and between 1-20% weak pathogen) according to Demissie et al. (2021). In the current study, one isolate was identified as highly pathogenic, 27 isolates were moderately pathogenic, and one isolate was weakly pathogenic (Table 4). The highest (116.7) and the lowest (23.7) AUDPC values were recorded in isolates LWS7 and AAK2, respectively, and this confirms highly significant variability between the isolates. Moreover, fresh and dry weight of root and stem were measured and results also revealed significance variability among the isolates (Table 4).

Based on current results, isolate LWS7 was found as the most pathogenic isolate that induced disease and was used as inoculum for the studies involving biocontrol agents. The difference in virulence among the isolates may be due to differences in genes responsible for pathogenicity and virulence

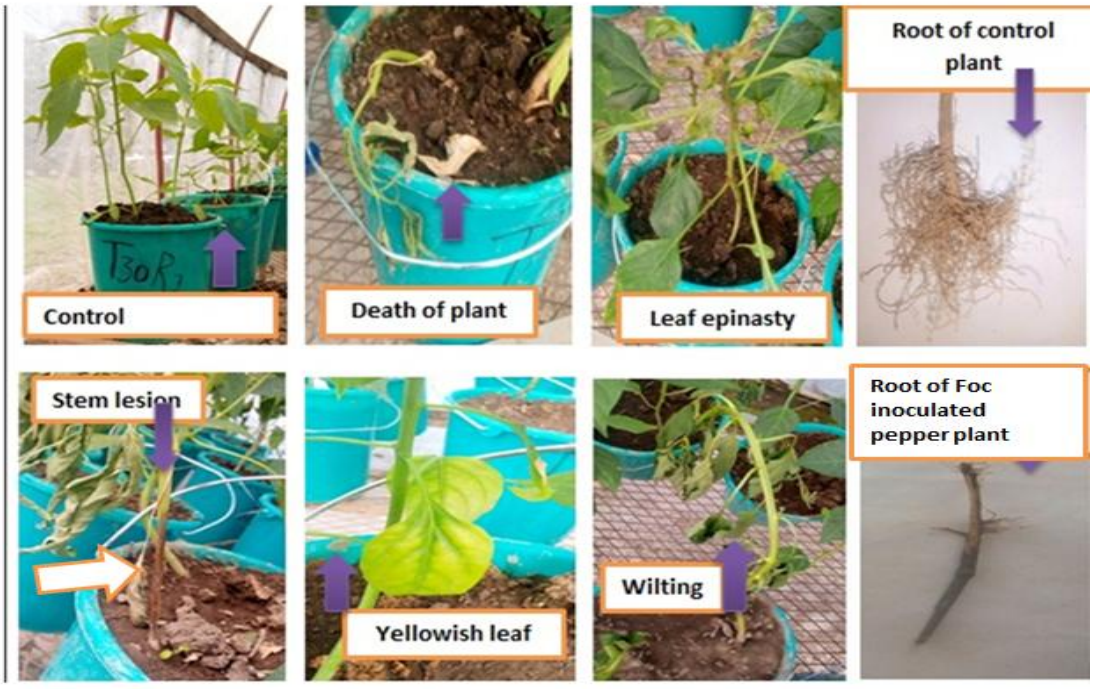

Figure 3. Picture of different symptoms on Foc treated (Inoculated) and untreated (Control) pepper plants)

Table 4. Pathogenicity of *Fusarium oxysporium* f.sp. *capsici* isolates on susceptible pepper variety Mareko fana

Isolates	AUDPC (%-day)	DSI (%)	RFW (g)	SFW (g)	RDW (g)	SDW (g)	Pathogenicity
LWS7	116.70	44.79	8.80	24.80	1.23	4.63	Highly Pathogenic
MDH1	67.30	37.49	9.33	36.60	2.10	17.16	Moderately pathogenic
MDH8	68.89	33.68	9.30	35.50	2.63	17.40	Moderately pathogenic
MDH2	69.65	33.00	9.67	44.00	2.56	22.50	Moderately pathogenic
LST9	39.00	29.50	9.66	48.56	2.30	23.00	Moderately pathogenic
LWS2	44.00	28.30	10.3	47.00	3.20	18.66	Moderately pathogenic
LST1	46.70	27.90	9.66	33.30	1.60	8.66	Moderately pathogenic
MOG3	37.74	27.00	9.66	47.90	2.40	15.26	Moderately pathogenic
ABT8	36.95	26.67	9.83	46.60	2.90	24.80	Moderately pathogenic
ABT5	37.74	26.00	9.50	59.16	2.80	24.50	Moderately pathogenic
AAK7	24.46	26.00	9.33	59.56	1.50	29.00	Moderately pathogenic
LST4	26.00	25.70	9.67	60.00	1.67	13.56	Moderately pathogenic
WHM9	51.80	25.40	9.50	54.70	3.00	21.66	Moderately pathogenic
MOG10	43.90	25.00	10.33	58.90	2.96	23.70	Moderately pathogenic
ABT2	34.95	25.00	9.66	51.70	2.16	20.20	Moderately pathogenic
MDH9	29.20	24.40	9.50	46.10	1.50	15.00	Moderately pathogenic
AAK5	68.50	24.00	9.66	35.60	1.40	12.30	Moderately pathogenic
WHM3	50.97	24.00	9.30	35.80	2.03	15.76	Moderately pathogenic
AAK1	42.37	24.00	9.30	41.80	1.76	15.50	Moderately pathogenic
AAK2	23.70	24.00	9.30	52.56	2.60	21.80	Moderately pathogenic
MDM2	64.97	23.80	9.67	36.80	1.56	18.00	Moderately pathogenic
MDM3	49.00	23.50	9.33	36.60	1.70	12.00	Moderately pathogenic
WHM7	43.94	23.50	9.30	39.30	2.90	19.43	Moderately pathogenic
MOG9	32.26	23.50	10.33	60.90	3.10	20.30	Moderately pathogenic
WAT6	29.17	22.86	9.33	35.90	2.96	15.43	Moderately pathogenic
MBL3	29.00	22.00	10.33	43.90	3.20	16.90	Moderately pathogenic
MBL2	46.26	21.58	10.00	45.40	3.50	17.30	Moderately pathogenic
LST6	28.00	21.30	9.16	60.26	2.90	22.86	Moderately pathogenic
WAT5	35.37	19.68	9.50	40.90	2.26	21.00	Weakly pathogenic
Control	—	-—	12.16	88.30	3.23	46.33	-—
CV (%)	6	6.6	6.4	1.7	5	4.68	-—
P value	<0.0001	<0.0001	0.0003	<.0001	<.0001	<.0001	-—

Where, RFW=Root fresh weight, RDW= Root dry weight, SFW=Shoot fresh weight, SDW=Shoot dry weight. Highly Pathogenic: DSI =41–100%, moderately pathogenic: DSI =21–40%, weak: DSI = 1–20%. (Demissie *et al.,* 2021), (Aklilu *et al*., 2018) Where, DSI= Disease severity index. % - AUDPC values were also used to classify the level of pathogenicity among the different Fusarium isolates (Demissie et *al*., 2021).

Evaluation of Biocontrol agents against the pathogenic isolates

***In vitro* test**

All seven biocontrol agents (BCAs) significantly ($P < 0.0001$) reduced the radial growth of *Fusarium oxysporum* f.sp. *capsici in vitro* compared to the control treatment. The highest (81.8%) and the lowest (48%) mycelial growth inhibition were obtained from *Trichoderma harzianum* and *Trichoderma hamatum*, respectively, (Fig 4 and Table 5). The significant reduction in mycelia growth by biocontrol agents might be the result of the antagonistic organisms action adopting numerous types of mechanisms such as antibiosis, parasitism, induced resistance, and competition for nutrients and lytic enzymes to inhibit the growth of *Fusarium oxysporium* f.sp.

capsici (Suprapta, 2012; Segarra et al., 2013). Endriyas et al. (2020) reported that the fungal BCAs, the *Trichoderma* spp. were the most effective and provided up to 85.2% mycelial growth inhibition which agrees with results of the current study. *T. viride* and *T. harzianum* as the most significant antagonistic organisms due to secretion of extracellular lytic enzymes and other compounds like harzianien and viridian which enhance their antagonistic activity against Fusarium wilt of pepper (Ozbay and Newman, 2004). *Trichoderma* spp. hinder pathogenic invasion through the release of organic metabolite such as chitinase, pachybasin and volatile inhibitory compounds *i.e.* acetaldehyde (Bunker and Kusum, 2001).

Bacillus subtilis caused a very highly significant (P<.0001) reduction in the radial growth of *Fusarium oxysporium* f.sp. *capsici* compared with control treatment. The efficacy of *Bacillus subtilis* was significantly higher than two of *Trichoderma* species (*T.atroviride and T.hamatum)* and significantly lowers than the other four species of *Trichoderma*. The current results were in agreement with the findings of Abada and Ahmed (2014), and Endriyas et al. (2020) who reported significant reduction to the radial growth of *Fusarium oxysporium* f.sp. *capsici* due to *Bacillus subtilis*.

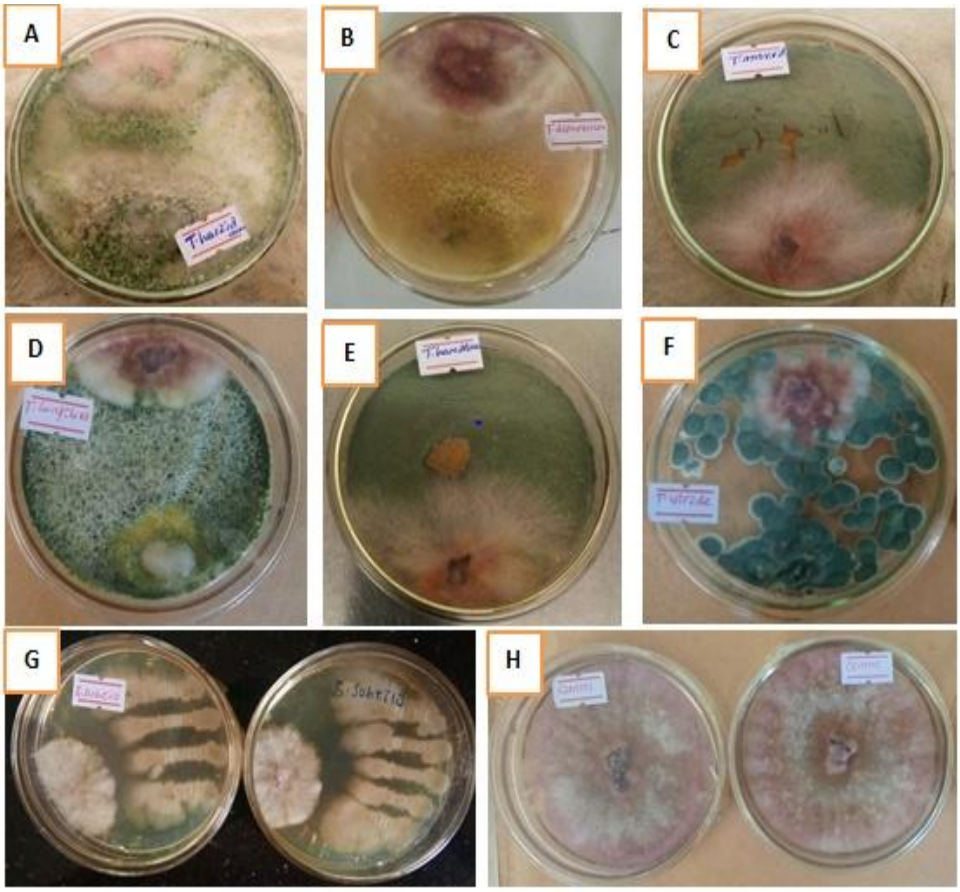

Figure 4. Dual culture assay on potato dextrose agar showing mycelial growth inhibition of *Fusarium oxysporium* f.sp.*capsici* (LWS7). Where, A=*T.harzianum,* B=*T.asperellum,* C=*T.atroviride,* D=*T.longibrachiatum,* E=*T.hamatum,* F= *T.viride,* G=*Bacillus subtilis,* H=*Control*

Table 5. Effect of biocontrol agents on *Fusarium oxysporium* f.sp. *capsici* mycelium growth

Antagonists	Mean radial growth of Foc (mm) ±SE	Inhibition (%)	Scale of antagonistic activity
Trichoderma harzianum	16.33±0.27	81.8[a]	****
Trichoderma viride	25.00±0.27	72.2[b]	***
Trichoderma asperellum	26.33±0.47	70.7[b]	***
Trichoderma longibrachiatum	27.00±0.47	70.0[b]	***
Bacillus subtilis	34.00±0.47	62.0[c]	***
Trichoderma atroviride	45.67±0.54	49.3[d]	*
Trichoderma hamatum	46.67±2.72	48.0[d]	*
Control (Without antagonist)	90.00	0.0[e]	
LSD(0.05)	4.55		
CV (%)	4		
P-value	**<.0001**		

Means with the same letter in a column are not significantly different at P≤0.05.
*The effectiveness of Trichoderma isolates was determined using the following inhibitory scale (Soytong, 1988)
**** = Very high antagonistic activity (above 75%)
*** = High antagonistic activity (61-75%)
** = Moderate antagonistic activity (51-60%)
* = Low antagonistic activity (<50%)
- = No antagonistic activity

In Vivo test

Effect of biocontrol agents on Fusarium wilt disease.

Soil application (SA)

All the seven biocontrol agents tested in the current experiment significantly decreased disease incidence as compared to the control treatment. The lowest (37.8 %) and the highest (53.3%) disease incidence were recorded with *Trichoderma harzianum* and *Trichoderma hamatum* treatment respectively (Table 6). In the current study, *Trichoderma viride* controlled the disease up to 60%. Results are in line with Anjum et al. (2020), where *Trichoderma* treatments applied in soil significantly decreased fusarium wilt chili disease. Sharma et al. (2004) also reported that soil application of *Trichoderma viride* controlled root rot of *Prosopis cineraria* caused by *Fusarium solani* by up to 56%.

The reduced disease incidence may be due to *Trichoderma* spp. acting as biocontrol agents through mycoparasitism, disrupting pathogen cell walls and absorbing nutrients and by enhancing plant resistance via root architecture improvement during pathogen interaction (Yao et al., 2023).

The saprophytic nature, and fast mycelial growth and strong environmental adaptability of *Trichoderma*, seizes the invasive part of the pathogenic fungi in the root of a plant, thus hindering the invasion of the pathogen fungi. Additionally, it might be possible that *Trichoderma* rapidly absorb the nutrients required for the growth of the pathogenic fungi, resulting in nutrient deficiency and inhibiting the growth and reproduction of the pathogenic fungi (Halifu et al., 2020; Bazghaleh et al., 2020).

A wilt incidence of 46.7% was recorded in soil inoculated with *Bacillus subtilis*, while the maximum disease incidence (86.7%) was recorded in the control treatment. Zhang et al. (2009) reported that soil treatments of *Bacillus subtilis* provided better protection against Fusarium root rot of Soybean caused by *Fusarium oxysporum* and *Fusarium graminearum* pathogens. Ghonim (1999) and Amer and Utkhede (2000) reported *Bacillus subtilis* protected tomato plants against *Fusarium oxysporum* f.sp. *lycopersici* and cucumber and lettuce from root rot against *Pythium aphanidermatum* diseases. This might be

due to the fungistatic and fungitoxic modes of *Bacillus subtilis* to disrupt of hyphae following contact with the fungal pathogen at the leaf surface. Moreover colonization of plant roots by *Bacillus subtilis* can induce a distinct broad spectrum resistance response in both below and above ground parts of the plant.

Seedling Root Dip (SRD)

The current result revealed that all the inoculated biocontrol agents significantly decreased disease incidence in comparison with control treatment in a seedling root dip test. Results are more or less in line with that of the soil inoculation test. The lowest (31%) and highest (46.67%) disease incidence were recorded with *Trichoderma viride* and *Trichoderma hamatum,* respectively,(Table 7). Forty percent disease incidence was recorded in seedlings inoculated with *Bacillus subtilis* while the maximum disease incidence (86.7%) was recorded in the control treatment.

The current results are in agreement with those of Ankita et al. (2018) and Nazneen et al. (2021), where seedling treatment with *Trichoderma viride* and *Trichoderma harzianum* were found to have highly significant effect in managing wilt disease and exhibit lesser incidence in hot pepper under field and glass house conditions. This might be due to increasing of sporulation of Trichoderma around the hyphae of Fusarium that decreases the pathogen population (Sharma et al. 2011). Ahmed et al. (2003) also reported that *Trichoderma harzianum* was effective against root rot of Pepper caused by *Ralstonia solani* when they were used as suspensions for root drenching. This might be due to the ability of Trichoderma to quickly adsorb to the roots of crops for propagation, and the hyphae quickly wrap the roots of crops to form a protective layer, protect the roots of crops from the invasion of pathogens, and kill the nearby pathogens (Halifu et al. 2020).

Kloepper et al. (2004) and Szczech and Shoda (2006) reported application of *Bacillus subtilis* to seedlings has been found effective for suppressing soil borne diseases and has successfully induced systemic resistance in the treated plants. Additionally, Ahmed et al. (2003) and Lee et al. (2008) reported the effectiveness of *Bacillus subtilis* against *Ralstonia solani* and *Phytophthora capsici* in pepper plants. This might be due to *Bacillus subtilis* rapidly grows, outcompeting pathogens for space and preventing spore infection (Cazorla et al. 2007).

Table 6. Effect of biocontrol agents on the Fusarium wilt disease under greenhouse condition

Mode of treatment application	Treatment	Incidence (%) Mean±SE
Soil application (SA)	*T.harzianum*	37.78±0.36^{efg}
	T.viride	40.00±0.31^{def}
	T.asperellum	44.44±0.92^{cde}
	T.longibrachiatum	46.67±0.54^{bcd}
	T.atroviride	51.00±0.92^{bc}
	T.hamatum	53.33±0.72^{b}
	B.subtilis	46.67±0.54^{bcd}
Seedling root dip (SRD)	*T.harzianum*	33.33±0.27^{fg}
	T.viride	31.00±0.90^{g}
	T.asperellum	37.78±0.64^{efg}
	T.longibrachiatum	40.00±0.47^{def}
	T.atroviride	42.20±0.55^{de}
	T.hamatum	46.67±0.54^{bcd}
	B.subtilis	40.00±0.47^{def}
	Control	**86.67±0.27^{a}**
CV (%)		**10**
LSD (0.05)		**7.59**

Means with the same letter in a column are not significantly different at P≤0.05.

Effect of Biocontrol Agents on Hot Pepper Growth

The biocontrol treatments had significant ($p<0.05$) impact on pepper growth parameters including plant height, number of leaves, root length, and fresh and dry weight of roots and shoots of hot pepper (Table 7).

The height of pepper plants in the current experiment varied from 32cm in the negative control plants to 63cm in plants treated with *Trichoderma harzianum*. The number of pepper leaves ranged from 17 in control plants to 47 in *Trichoderma viride* treated plants (SA).

The maximum (10.83cm) root length was recorded with *Trichoderma viride* (SRD) and minimum (6cm) root length were recorded with soil application of *Trichoderma atroviride* and *Trichoderma hamatum* followed by *Trichoderma asperellum* (SA) and *Trichoderma hamatum* (SRD) that resulted in 7cm root length. The lowest root length (4.8cm) was obtained in control plants. The maximum (45.67g) and minimum (36.67g) shoot fresh weight were recorded with *Trichoderma harzianum* (SRD) and *Trichoderma hamatum* (SA) respectively; however, the lowest shoot fresh weight (24.33g) was obtained in control plants. The maximum (23.76g) and minimum (17.76g) shoot dry weight were recorded with *Trichoderma harzianum* (SRD) and *Trichoderma hamatum* (SA) respectively; however, the lowest shoot dry weight (8g) was obtained in control plants. The maximum (8.47g) and minimum (6.16g) root fresh weight were recorded with *Trichoderma harzianum* (SRD) and *Trichoderma hamatum* (SA) respectively, however; the lowest root fresh weight (3.13g) was obtained in control plants. The maximum (3.97g) and minimum (2.6g) root dry weight were recorded with *Trichoderma harzianum* (SRD) and *Trichoderma atroviride* (SA) respectively, however the lowest root dry weight (1.63g) was obtained in control plants.

The current results are in agreement with those of Marra et al. (2006) and Anjum et al. (2020) reports, where *Trichoderma* spp. have been shown to improve the growth of lettuce, tomato, and pepper plants. It is also in line with the findings that *Trichoderma* spp. augmented plant growth in crops such as tomato, pepper, cucumber, cacao and beans (Macias et al., 2018). This may be related to the fact that *Trichoderma* spp. colonizes the plant roots and secretes chemical stimulants acting as endophytic symbionts beneficial for the plants (Harman et., al 2004). Similar results were also reported by those of Sharma et al. (2012), Sharma (2018), Stewart and Hill (2014) where increased growth of various crops with the use of antagonist *Trichoderma* spp. This indicates species of Trichoderma are unique groups of rhizospheric microorganisms associated with certain beneficial effects to enhance plant growth and development (Oszust et., al 2020, Panchalingam et al., 2022, Mohiddin et al., 2021).

Results of the current study also revealed that *Bacillus subtilis* may have promoted growth of hot pepper as compared with control treatment. This coincides with Xing et al. (2003) and Ryder (1998), who reported that *Bacillus subtilis* promoted plants. This might be due to that *Bacillus* grow very fast and occupies the court of infection and preventing pathogen spores to reach susceptible tissues in competition for spaces (Wolk and Sarkar, 1994). Additionally, Abada and Ahmed (2014) reported that plants grown in soil infested with Bacillus strains were of high values of plant height than that grown in the control (uninfected soil). The possible reason to this might be due to the fact that *Bacillus subtilis* is soil inhabitant and survive under a broad spectrum of environmental conditions.

Table 7. Effect of biocontrol agents on the growth parameters of hot pepper under greenhouse condition

Treatment application mode	Treatment	No. of Leaves Mean±SE	Plant height(cm) Mean±SE	Root length(cm) Mean±SE	Shoot fresh weight(g) Mean±SE	Shoot dry weight(g) Mean±SE	Root fresh weight(g) Mean±SE	Root dry weight(g) Mean±SE
Soil application	*T.harzianum*	45.7 ±1.2^{a}	57.7±1.2^{bc}	9.00±0.14^{c}	42.67±0.98^{b}	22.33±0.54^{ab}	7.33±0.13^{bcd}	3.53±0.05^{bc}
	T.viride	47.3 ±0.7^{a}	59.0±0.7^{b}	8.00±0.24^{de}	40.67±0.27^{cd}	20.33±0.72^{cde}	7.40±0.09^{bc}	3.67±0.05^{b}
	T.asperellum	35.7 ±1.2^{de}	40.0±0.7^{fg}	7.00±0.24^{fg}	39.67±0.72^{def}	19.23±0.05^{def}	6.67±0.27^{defg}	3.27±0.02^{de}
	T.longibrachiatum	36.0 ±1.1^{d}	42.7±1.2^{ef}	6.67±0.36^{gh}	38.00±0.47^{fg}	18.77±0.47^{ef}	6.46±0.25^{efg}	2.90±0.08^{f}
	T.atroviride	35.0 ±0.3^{def}	40.0±0.5^{fgh}	6.00±0.24^{h}	38.30±0.72^{efg}	17.83±0.13^{f}	6.30±0.16^{fg}	2.60±0.09^{g}
	T.hamatum	34.0±0.3^{defg}	39.0±0.5^{gh}	6.00±0.24^{h}	36.67±0.72^{g}	17.76±0.11^{f}	6.16±0.07^{g}	2.63±0.05^{g}
	B.subtilis	36.0±0.5^{de}	40.0±0.9^{fgh}	6.67±0.14^{gh}	39.33±0.72^{def}	18.80±0.36^{ef}	6.67±0.12^{defg}	3.06±0.11^{ef}
Seedling Root Dip	*T.harzianum*	42.0±0.5^{b}	63.0±0.5^{a}	9.67±0.27^{b}	45.67±0.54^{a}	23.76±0.35^{a}	8.47±0.28^{a}	3.97±0.09^{a}
	T.viride	39.0±0.7^{c}	56.0±0.9^{c}	10.83±0.27^{a}	43.06±0.05^{b}	22.00±0.73^{abc}	8.00±0.09^{ba}	3.77±0.02^{ab}
	T.asperellum	34.0±0.5^{defg}	46.7±0.5^{d}	8.33±0.27^{cd}	41.67±0.27^{bc}	20.90±0.63^{bcd}	7.40±0.32^{bc}	3.40±0.09^{cd}
	T.longibrachiatum	32.0 ±0.3^{g}	38.0±0.7^{gh}	7.33±0.27^{efg}	40.00±0.47^{cde}	19.86±0.51^{de}	6.90±0.23^{cdef}	3.23±0.02^{de}
	T.atroviride	33.7±0.3^{efg}	38.0±0.7^{gh}	7.33±0.27^{efg}	39.33±0.27^{def}	19.50±0.59^{def}	7.03±0.11^{cde}	3.17±0.07^{de}
	T.hamatum	32.0 ±0.3^{g}	37.0±0.9^{h}	7.00±0.50^{fg}	39.33±0.72^{def}	18.86±0.38^{ef}	6.67±0.11^{defg}	2.90±0.05^{f}
	B.subtilis	33.0 ±0.5^{fg}	45.0±0.5^{de}	7.67±0.3^{def}	40.33±0.27^{cd}	19.67±0.98^{def}	6.96±0.09^{cdef}	3.23±0.11^{de}
	Control	17.0±0.7^{h}	32.0±0.5^{i}	4.76±0.12^{i}	24.33±0.27^{h}	8.000±0.36^{g}	3.13±0.19^{h}	1.63±0.07^{h}
	LSD (0.05)	**2.4**	**2.7**	**0.9**	**1.97**	**1.9**	**0.67**	**0.26**
	CV	**4**	**3.6**	**7.5**	**3**	**5.97**	**5.97**	**5**

Means with the same letter in a column are not significantly different at P≤0.05.

CONCLUSIONS

Hot pepper (Capsicum annuum L.) is an important vegetable and spice crop in tropical areas worldwide including Ethiopia. Among the biotic factors that affect pepper production in Ethiopia, Fusarium wilt caused by Fusarium oxysporium f.sp capsici is one of the most economically important diseases as it accounts for yield losses of up to 80%. In the current work, twenty nine isolates of Fusarium oxysporium f.sp. capsici were identified from 80 samples. The pathogenicity of the 29 isolates on pepper was confirmed in a greenhouse experiment and isolate LWS7 was identified as the most virulent isolate. As a result, it was used to evaluate the efficacy of biocontrol agents. Results of the in vitro test revealed significant effect of the bioagents and the level of mycelial growth significantly varied among the antagonists tested with the highest (81.8%) and lowest (48%) inhibition caused by Trichoderma harzianum and Trichoderma hamatum, respectively. In addition to their significant effect on mycelia growth in-vitro, the biocontrol agents evaluated in the current experiment were also found to result in significant reduction in the disease and promote of the growth of hot pepper in soil application and seedling root dip tests.

From the current result it can be concluded that the Fusarium wilt of hot pepper can be managed by using biocontrol agents especially Trichoderma harzianum and Trichoderma viride. The present study also confirmed the variability of the Foc isolates and efficacy of biocontrol agents. Molecular identification Fusarium oxysporium f.sp. capsici of should be done to better characterize identified isolates in the country. The efficacy of more biocontrol agents against Fusarium wilt of pepper should be evaluated under greenhouse and field conditions on a large scale.

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DISCLOSURE STATEMENT

The authors declare that they have no conflicts of interest.

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