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Biocontrol effects of endophytic and rhizospheric bacteria against the root pathogen *Fusarium oxysporum* of hot pepper (*Capsicum annum* L.)

Gezahign Fentahun*, Mulugeta Kibret, Baye Stotaw and Atrsaw Asrat

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Abstract

Plant disease needs to be controlled to keep the quality of products and the abundance of food produced by farmers all over the world. Hot pepper production in Ethiopia has been reduced from time to time. This is mainly due to the outbreak of different diseases especially fusarium wilt in the hot pepper growing areas. The objective of this study was to isolate and characterize Plant Growth Promoting Bacteria from the hot pepper rhizosphere and internal tissues with potential biocontrol activity against *Fusarium oxysporum*. Thirty healthy and vigorous hot pepper plants with intact roots and soil from the rhizospheric region were collected randomly from hot pepper growing areas of Yimali Kebele. A total of 23 endophytic and rhizospheric bacteria were isolated from the hot pepper root and rhizospheric soil. The combined endophytic and rhizospheric bacteria showed a significant effect on the growth of *F. oxysporum* than single isolates (P d"0.05). The bacteria isolated from Markofana hot pepper varieties of black clay soil showed a better antagonistic effect on *F. oxysporum* than bacteria isolated from local hot pepper varieties of reddish sandy soil. A significant percentage of inhibition between combined endophytic and rhizospheric bacteria [endophytic (GE) + rhizospheric (GR) isolates] compared to GE+GE and GR+GR (P =0.002) was obtained. Antagonistic bacterial isolates were able to produce different hydrolytic enzymes such as chitinase, protease, and cellulase. *Bacillus* species showed better antagonistic performance and decreased radial growth of *F. oxysporum* than *Pseudomonas* species.

Keywords: Antagonistic bacteria, Biocontrol, *Capsicum,* Endophytic, *Fusarium oxysporum,* Rhizospheric

Department of Biology, College of Science, Bahir Dar University, P.O. Box 79, Bahir Dar, Ethiopia
*Corresponding author, Email: gezaf2187@gmail.com

Introduction

Hot pepper (*Capsicum annum* L.) has been recognized since 7500 BC during the starting of civilization in the Western Hemisphere (MacNeish 1964). Pepper is grown in many countries of the world and its production for culinary and vegetable uses has been increased from time to time. It is the world's most important vegetable after tomato and used as fresh, dried, or processed products, as vegetables and spices or condiments (Ninfali et al. 2005). Hot pepper is one of the major income-generating crops for most lowland and mid altitudes and it plays a great role in food security in Ethiopia (Roukens 2005). According to the Ethiopian, Export Promotion Agency (EEPA 2003), the major pepper-producing regions in Ethiopia include Amhara, Southern Nations and nationalities region (SNNR), and Oromia. In those regions, hot pepper generated an income of 122.80 million birrs for farmers in 2000/01. Moreover, in 2004/05, this value was increased to 509.44 million birrs for smallholder farmers. This indicates that hot pepper serves as one of the important income sources to the lowland and mid-altitude smallholder farmers and as an exchange-earning item of trade in the country (Abraham Alemu et al. 2016). In the 2015 production year, hot pepper for green and dry fruit production accounted for 78% of the total area covered under vegetables (CSA 2015). However, the national yield is very low as compared to other countries which were 855,477 tones and 3,165,614 tones with an average of 8.8 and 2.1 tones/hectare green and dry pepper yield, respectively (CSA 2015; EIAR 2016). As a result, *Capsicum* productivity in Ethiopia is far below the world average that strongly demands immediate productivity improvement. The reason for the reduction of hot pepper production is also attributed to poor cultural practices, the prevalence of fungal (blights, wilt, damping-off), and bacterial as well as viral diseases (Fekadu Mariame and Dandena Gelmesa 2006).

Fusarium wilt caused by *Fusarium oxysporum* is one of

the most important fungal diseases of hot pepper that reduces its productivity in almost all pepper-growing countries all over the world. *F. oxysporum* plays the role of a silent killer. Besides, *F. oxysporum* is not discriminating; it can cause disease in nearly all agriculturally important plants. *F. oxysporum* has been known for its efficiency in establishing suppressiveness in the soil than other species of *Fusarium* and can infect plant roots without apparent effect (Alabouvette et al. 2009). *Fusarium oxysporum* pathogen undergoes asexual reproduction by producing three different types of spores; such as macro-conidia, chlamydospores, and micro-conidia. Chlamydospores serve as the primary inoculum for the disease occurrence. Inoculum populations and susceptibility of cultivar drive prevalence and severity of the disease. Initially, fusarium wilt symptoms will appear on upper leaves, flowers, and twigs. The disease can occur in almost all stages of plant growth and diseased plants may be found in groups or patches across the fields (Jimenez-Diaz and Jimenez-Gasco 2011). Inoculum population primarily drives fusarium wilt development. Therefore, the disease can be controlled by exclusion, eradication, and reduction in efficiency of inoculum. The key management options of fusarium wilt include various agronomic practices (i.e. delayed planting, intercropping, deep ploughing, and avoidance of dense planting), use of chemicals (seed dressing with fungicides), biological control (Plant growth-promoting rhizobacteria (PGPR) may be effectively exploited as control agents) and sowing of certified wilt resistant cultivars (Jimenez-Diaz and Jimenez-Gasco 2011).

Biological control of *Fusarium oxysporum* of hot pepper causing fusarium wilt diseases by using bacteria is becoming necessary. Since the disease is recognized as more limiting factors in the production of many crops and management with chemicals is economically not viable and unsafe for the environment. *Fusarium oxysporum* is able to survive in the soil for long periods and thus plant susceptible genotypes cannot be grown in an infested field for up to 30 years (Ploetz 2000). Although many studies have been done on biocontrol of *Fusarium oxysporum* by using endophytic or rhizospheric bacterial isolates of hot pepper, the documented information on biocontrol of *Fusarium oxysporum* by using both endophytic and rhizospheric bacteria of hot pepper (*Capsicum annum* L.) in Ethiopia is limited. Several researchers have reported that using mixtures of bio-control agents (BCAs) have increased the consistency of bio-control across sites with different conditions. In studies on the infection of potato by *Phytophthora capsici* greater disease control was achieved using a mixture of three bacterial BCAs compared to using the single strains (Kim et al. 2008). The objectives of this study were to isolate and characterize rhizospheric and endophytic bacteria from the hot pepper, to evaluate the *in vitro* antagonistic activities of bacterial isolates on *F. oxysporum* and to test the mechanisms by which the bacteria inhibit the growth of the *F. oxysporum*.

Materials and Methods

Description of the Study Area: Hot pepper plants with intact roots and rhizospheric soil were collected from hot pepper growing areas of Yimali kebele, Guangua Wereda, Awi zone, Amhara National Regional State. Yimali is located in the Guangua wereda, Agew Awi Zone of the Amhara Region (Figure 1). Yimali kebele is one of the highly productive kebeles in the Guangua wereda. It is known for producing hot pepper, maize, teff, sorghum, and other cash crops. However, hot pepper is a highly produced cash crop. The hot pepper varieties that are being cultivated in this study area is Markofana hot pepper variety (>90% of hot pepper cultivated) and local variety (<5%). The hot pepper cultivation pattern has been following mono-cropping and crop rotation. The color of the soil in this kebele is majorly black clay and reddish. This kebele has a longitude and latitude of 10Ú57'N 36Ú30'E and an elevation of 1583 meters above sea level. Yimali has a tropical climate. The rainy seasons here have a good deal of rainfall, while the winters have very little. The average annual temperature of Yimali is 20 ÚC. It has an annual average rainfall of 1747 mm.

Study Design: The study was an experimental study design that was aimed to isolate and characterize endophytic and rhizospheric bacteria from hot pepper. In addition, it was designed to assess endophytic and rhizospheric bacteria on their antagonistic efficiency to the disease of hot pepper caused by a fungus (*Fusarium oxysporum*) with laboratory (*in vitro*) based investigation.

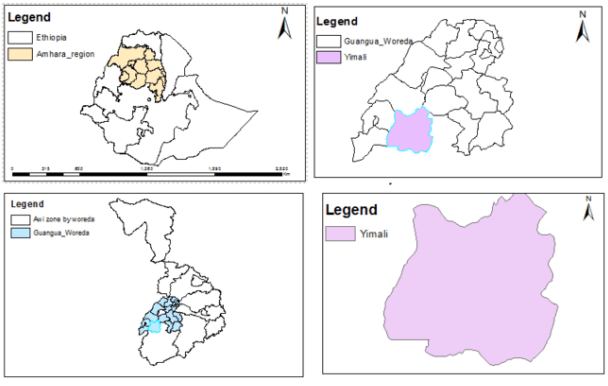

Figure 1: The map of the study area.

Sample Collection: Individual healthy and vigorous hot pepper plants with intact roots and soil from rhizospheric region were collected randomly from hot pepper growing areas of Yimali kebele, Guangua Wereda, Awi Zone, Amhara National Regional State. Yimali kebele was selected purposively since this kebele is a major hot pepper producing area than other kebeles in the Guangua wereda. Stratified sampling procedure was used to select farmers' field from the study area. This sampling technique was done based on the soil color (black and reddish soil). In each field, five healthy hot pepper plants and soil samples (10 cm around the root and 15cm depth) were collected and selected randomly. Furthermore, 30 samples of healthy hot pepper plants and soil samples were collected from each of black clay and reddish sandy soils. From 30 samples of each soil types 20 samples were Markofana and 10 samples were local hot pepper varieties. Sampling was done during flowering and fruiting sage of hot pepper. For this study, samples of rhizospheric soil (50 g for each sample) and hot pepper with intact roots were collected in to 5 kg capacity plastic bags disinfected by alcohol. It was taken to the Microbiology Laboratory, Department of Biology, Bahir Dar University, and stored at 4 ÚC for 48 hours until laboratory analysis was performed.

Source of fungal pathogens: For the purpose of this study, *Fusarium oxysporum* was obtained from the University of Gondar, Mycology laboratory. The obtained culture of *Fusarium oxysporum* was refreshed on the Potato dextrose agar (PDA) (Sigma-Aldrich, Germany) and the purity of the culture was checked by morphological characteristics of the colony.

Isolation of endophytic bacteria: The roots were surface disinfected with 95% alcohol (for 15 minute) and 5% NaOCl (for 5 minute) and rinsed several times with sterile water and blot dried on sterile filter paper. Both ends of each root was burnt with a flame and fragmented into 1cm segments. The success of surface disinfection was checked by rolling the root pieces on the surface of nutrient agar (Sigma-Aldrich, Germany). Checking for microbial growth on the nutrient agar after three days of incubation was done at 27 ± 2 ÚC (Yingwu et al. 2009). Then, one gram of hot pepper roots was aseptically weighed and each piece of root was macerated and crushed into normal saline solution in a sterile mortar to form a suspension. Aliquots of 50 ìL from a serial dilution from 10^{-3} to 10^{-5} were plated on nutrient agar in triplicate. The plates were incubated at 27 ± 2 ÚC for 24 to 72 hours. Bacterial colonies were purified on nutrient agar by streak plating method (Perez et al. 2010).

Isolation of rhizospheric bacteria: Ten gram of the sieved soil sample was mixed separately in 90 mL of distilled water in 150 mL flask. The flask was shaken on orbital shaker for 30 minutes at 120 rpm. Then, 0.1 mL of the suspension from serial dilutions 10^{-3}, 10^{-4} and 10^{-5} was transferred to nutrient agar plates and spread uniformly using bent glass rod. Finally, the plates were incubated at 30 ± 2 ÚC for 48 hrs (Kafrawi et al. 2014).

Characterization of rhizospheric and endophytic bacteria: Colony morphology such as colony size, consistency, shape, and color of all the bacterial isolates were recorded. Biochemical tests such as Grams staining, Indole-3-acetic acid (IAA), Nitrate Reduction Test, Citrate Utilization Test, Catalase Test, Methyl Red Test (MR), and Indole Production were conducted to characterize the isolated bacteria.

Gram staining: Gram staining is important to differentiate different group of bacteria as gram negative or gram positive. First, crystal violet stain was added to the smear on the microscopic slide and wait for one minute. Then it was washed under tap water and Gram's iodine was added and waits for one minute. Again, after washing under tap water, it was exposed to ethanol alcohol for decolorization and then washed immediately with tap water. Finally, safranin was added as counter stain and washed after 30 seconds. After drying, microscopic examination was done under oil immersion objective to saw gram reaction. Gram positive and gram negative bacteria were indicated by purple and pink color respectively.

Indole-3-acetic acid (IAA): IAA was determined by the method of Patten and Glick (2002). The endophytic bacteria were grown in Luria broth (Sigma-Aldrich, Germany) supplemented with L-tryptophan Sigma-Aldrich, Germany) (1ìg/ mL) for 72 hours. At the end of the incubation, cultures were centrifuged at 10,000g for 10 minutes and the supernatants were collected. 1 mL of this culture filtrates were allowed to react with 2 mL of Salkowski's reagent (Sigma-Aldrich, Germany) (2 mL of 0.5 M $FeCl_3$, 49 mL of distilled water and 49 mL of 70% $HClO_4$) at 28 ± 20C for 30 minutes. The rhizospheric bacteria were also grown on nutrient agar (NA) supplemented with L-tryptophan (0.1g/L). At the end of the incubation, pink color development was indicated the presence of IAA (Cappuccino and Sherman 2002).

Nitrate reduction test: In this test the enzyme nitrate reductase is produced which reduces nitrates to nitrites or water and free nitrogen gas. The nitrite produced combines with sulphanilic acid and alpha-naphthylamine to form a diazo red dye. Nitrate reduction was tested

by inoculation of substrates into a nitrate broth medium (Sigma-Aldrich, Germany), which contains 1% KNO3 and then incubating them for 72 hours at 30 ÚC. After incubation, drops of sulphanilic acid and alpha-naphthylamine were added. Nitrate reduction was observed by adding 0.2 mL naphthylamine and 0.2 mL sulphanilic acid reagent to each sample tube. A positive test was resulted in a red color while negative reactions remained yellow (Cappuccino and Sherman 2002).

Citrate utilization test: In the determination of the capability of the bacterial isolates to use citrate as their energy (carbon) source, Simmons' Citrate agar (NEOGEN, USA) slants were used (Harold 2002). The isolates were inoculated under aseptic conditions into sterile Simmon's Citrate agar slants by using a sterile wire loop and then it was incubated in a clean incubator at 30 °C for 24 hours. Observation was made on color change (Cappuccino and Sherman 2002). The isolates were inoculated in a medium having sodium citrate and a pH indicator bromothymol blue. Inorganic ammonium salts were also present in the medium, which was utilized as a sole nitrogen source. Utilization of sodium citrate was produced Na_2CO_3 which results in alkaline pH that changes the color of the medium from green to blue.

Catalase test: This test detects the enzyme catalase that is present in the majority of cytochrome containing aerobic bacteria that forms hydrogen peroxide, the oxidative end result of aerobic sugar breakdown. The isolates were inoculated aseptically into the sterile Tryptic Soy agar (Sigma-Aldrich, Germany) by using a sterile wire loop and placed in a clean incubator for incubation at 30 °C for 24 hours. The catalase activity was determined by adding 3% hydrogen peroxide to the cultures. A positive result was indicated by forming bubbles (Cappuccino and Sherman 2002).

Methyl red (MR test): The MR test was carried out to determine the isolates ability to oxidize glucose meanwhile stabilizing and producing high acid concentration end products. The isolates were inoculated aseptically into sterile MR-VP broth (Oxoid, UK) by using a sterile wire loop and incubated inside a clean incubator at 30 °C for 24 hours. 1mL of every culture were picked and mixed with methyl red indicator (MR test) and observations were done depending on the color change from yellow to red (Cappuccino and Sherman 2002).

Indole production: The test identifies isolates with the ability to produce the enzymes tryptophanase that removes the amino group from tryptophan to form indole, pyruvic acid and ammonia, and cysteine desulfurase, that produces pyruvate, ammonia and hydrogen sulfide from sulfur containing amino acids. Indole reacts with the Kovac's reagent (p-dimethylamino-benzaldehyde) (Harold 2002), to form a deep red color. Kovac's reagent (Sigma-Aldrich, Germany) was added to each test tube of the 48 hour culture, according to the protocol of Harold (2002). The presence of a cherry red layer in the media was indicated a positive result for indole production while negative results were indicated by color remaining brown (Cappuccino and Sherman 2002).

In vitro antagonistic activity: All rhizospheric and endophytic bacterial isolates were screened for their antagonism in a dual culture assays. *Fusarium oxysporum* with active mycelia of seven days old were placed at the center of a Petri-dish containing Potato Dextrose Agar (PDA). Then, a sample of each bacterium was placed in direction of the cardinal points and incubated at 28 ÚC for 3 days as described by Hernandez et al. (2014). The Petri-dish inoculated with pathogen alone in the absence of antagonist served as control and the experiment was done in triplicates. The radial growth of fungal mycelium on each plate was measured and the percent inhibition of growth over control was determined using the formula of Mehetre and Kale (2011).

$$\text{Percentage of inhibition of mycelia} = \frac{R_1 - R_2}{R_1} * 100$$

Where, R_1 = radial growth of mycelium (control), R_2 = radial growth of mycelium (treatment)

Determination of Mechanisms of inhibition: For determination of chitinase production, each isolate was inoculated onto a PDA plate and incubated at 28 ± 2 ºC in the dark until zones of chitin clearing were seen around the colonies and used to indicate the chitinase activity of each isolates (Yuan and Crawford 1995). For determining protease production a loop full of bacterial culture was streaked on skimmed milk agar plate. After 48 hours of incubation at 28^0C, the development of clear zone around the streak was considered as a positive result for protease production. In order to determine cellulase production, carboxymethyl cellulose (CMC) (Sigma-Aldrich, Germany) were used a basal medium ($NaNO_3$ 1g, KCl 1g, K_2HPO_4 1g, $MgSO_4$ 3g, yeast extract 0.5, agar 15g, distilled water 1000 mL). The bacteria were streaked on the medium and incubated at 28^0C for 3 days. The plates were flooded with 0.01% Congo red solution (Sigma-Aldrich, Germany) for 15 min and destained using 1% NaCl solution for 5 min. The formation of clear zone indicated the degradation of CMC and this was considered as positive for cellulase production (Cappuccino and Sherman 1992).

Data Analysis: One way ANOA followed by Tukey Honestly Significant Difference (Tukey HSD) test was used to compare the percentage of inhibition of all combined isolates. Independent T test was used to compare the means of single and combined isolates of endophytic and rhizospheric bacteria ($P \leq 0.05$, at $\alpha = 0.05$), correlation between zone of inhibition, radial growth of mycelium (R2) and percentage of inhibition (PI) ($\alpha = 0.01$) by using SPSS 23 Version.

Results and Discussion

Characterization of rhizospheric and endophytic bacteria: A total of 23 endophytic and rhizospheric bacteria were isolated from the roots of hot pepper and rhizospheric soil. Out of these isolates 14 were isolated from roots of hot pepper (endophytic bacteria) and the remaining 9 isolates were isolated from rhizospheric soil (Table 1). From those endophytic isolates 7 of them were isolated from samples black clay soil (GE1, GE2, GE3, GE4, GE5, GE7 and GE9) and the remaining 7 endophytes were isolated from reddish soil (GE6, GE8, GE10, GE11, GE12, GE13 and GE14). In addition, five of rhizospheric isolates were isolated (GR2, GR4, GR5, GR6 and GR8) from black clay soil and four isolates were isolated (GR1, GR3, GR7 and GR9) from reddish sandy soil samples. All of the isolates were characterized on the basis of different colony morphologies like color, shape, size, and consistency. Based on morphological (Table 1) and biochemical characteristics (Table 2), the bacterial isolates were assigned to two genera of bacteria i.e. *Bacillus* (GE5, GE7, GE8, GE9, GE10, GE11, GE14, GR1, GR2, GR4, GR5 and GR6) and *Pseudomonas* (GE1, GE2, GE3, GE4, GE6, GE12, GE13, GR3, GR7, GR8 and GR9) (Table 2). On the basis of colony morphology and biochemical characteristics (Table 2), isolates were tentatively identified as *Bacillus* sp. followed by *Pseudomonas* sp. (Tabel 1). The occurrence of *Bacillus* sp. is in agreement with the previous reports where *Bacillus* sp. has been frequently isolated from tomato (Banerjee et al. 2010; Abhilipsa and Sukantibala 2017; Seema et al. 2018). The preponderance of *Bacillus* is may be due to its ability to efficiently use the nutrients provided by plants through exudates, including that root exudates exert a selective pressure on the proliferation of specific group of bacteria (Ajilogba et al. 2013).

In vitro antagonistic activity: Ten bacterial isolates showed antagonistic activity against *F. oxysporum*. Out of these antagonistic bacterial isolates, 7 of them were endophytic (GE1, GE3, GE6, GE7, GE9, GE10 and GE12) and 3 were rhizospheric (GR1, GR4 and GR9) bacteria. *Bacillus* and *Pseudomonas* species are well known bacterial antagonists that have ability to suppress the growth of fungal phytopathogens including *Fusarium oxysporum* (Sivasakthi et al. 2014). It has been found

Table 1: Morphological characterization of endophytic and rhizospheric bacteria

Farmlands	Name of Isolates	Colony characteristics			
		Size	Shape	Color	Consistency
$MSBF_2$	GE1	Large	Irregular	Cream	Sticky
$MSBF_1$	GE2	Large	Circular	White	Rough
$MSBF_2$	GE3	Large	Irregular	Greenish	Sticky
$LSBF_{ii}$	GE4	Large	Circular	Greenish	Sticky
$MSBF_3$	GE5	Medium	Circular	White	Sticky
$MSRF_c$	GE6	Large	Irregular	Greenish	Sticky
$LSBF_{ii}$	GE7	Small	Circular	White	Sticky
$MSRF_a$	GE8	Medium	Irregular	Whitish	Rough
$MSBF_4$	GE9	Medium	Circular	Whitish	Sticky
$MSRF_b$	GE10	Medium	Circular	Whitish	Rough
$MSRF_b$	GE11	Small	Circular	Whitish	Rough
$MSRF_c$	GE12	Large	Circular	Cream	Sticky
$LSRF_A$	GE13	Large	Circular	Cream	Sticky
$LSRF_B$	GE14	Small	Irregular	Whitish	Rough
$MSRF_d$	GR1	Small	Irregular	Whitish	Rough
$LSBF_i$	GR2	Small	Irregular	Whitish	Rough
$LSRF_B$	GR3	Large	Circular	Whitish	Sticky
$MSBF_1$	GR4	Medium	Circular	White	Sticky
$MSBF_3$	GR5	Small	Irregular	Cream	Rough
$LSBF_i$	GR6	Medium	Circular	Whitish	Sticky
$LSRF_A$	GR7	Large	Circular	Greenish	Sticky
$MSBF_4$	GR8	Large	Circular	Greenish	Rough
$MSRF_d$	GR9	Large	Circular	Greenish	Sticky

Where GE= endophytic isolates and GR= rhizospheric isolates, MSBF= Markofana hot pepper samples of black soil from different farmlands, LSBF= Local hot pepper variety samples of black clay soil from farmlands, MSRF= Markofana hot pepper samples of reddish soil from farmlands, LSRF= Local hot pepper variety samples of reddish sandy soil from different farmlands. Subscript 1,2,3,4, i, ii, a, b, c, d, A, B, C, D are the number of fields.

Table 2: Biochemical characterization of both endophytic and rhizospheric bacterial isolates

Isolates	Biochemical tests							
	Gram reaction	MR	Indole	Citrate	Catalase	IAA	Nitrate Reduction	Probable identification
GE1	-	+	+	+	+	-	+	*Pseudomonas* sp.
GE2	-	+	-	-	+	-	+	*Pseudomonas* sp.
GE3	-	+	-	+	+	+	+	*Pseudomonas* sp.
GE4	-	+	-	+	+	-	+	*Pseudomonas* sp.
GE5	+	-	+	+	+	-	+	*Bacillus* sp.
GE6	-	+	-	+	+	+	+	*Pseudomonas* sp.
GE7	+	+	-	+	+	-	-	*Bacillus* sp.
GE8	+	-	-	+	+	-	-	*Bacillus* sp.
GE9	+	-	-	+	+	-	+	*Bacillus* sp.
GE10	+	+	-	+	+	-	+	*Bacillus* sp.
GE11	+	+	-	+	+	-	+	*Bacillus* sp.
GE12	-	+	-	+	+	-	+	*Pseudomonas* sp.
GE13	-	-	+	+	+	-	+	*Pseudomonas* sp.
GE14	+	+	-	+	+	+	+	*Bacillus* sp.
GR1	+	+	-	+	+	-	+	*Bacillus* sp.
GR2	+	+	-	+	+	-	-	*Bacillus* sp.
GR3	-	-	-	+	+	-	+	*Pseudomonas* sp.
GR4	+	-	-	-	+	-	+	*Bacillus* sp.
GR5	+	+	-	+	+	-	+	*Bacillus* sp.
GR6	+	-	-	+	+	-	+	*Bacillus* sp.
GR7	-	+	-	+	+	-	+	*Pseudomonas* sp.
GR8	-	-	-	+	+	-	+	*Pseudomonas* sp.
GR9	-	+	-	+	+	-	+	*Pseudomonas* sp.

Where + stands for positive and - stands for negative

that all the antagonistic bacterial isolates caused significant reduction to the radial growth of mycelium compared with the controlled ones that contains only *F. oxysporum*. This reduction might be due to multiple modes of actions of the tested bacterial antagonists against the fungal pathogen (Amaresan et al. 2014). Among the *Bacillus* species, isolate GE9 showed the best performance on antagonism where the radial growth of the pathogenic fungus *F.oxysporum* was 8mm as compared to 60mm (control) (Table 3). *Pseudomonas* and *Bacillus* species are well known for biological control of fusarium wilt of pepper caused by *Fusarium oxysporum* (Suryanto et al. 2010; Abada and Ahmed 2014). In case of endophytes, the highest percentage of inhibition (PI) against *Fusarium oxysporum* was obtained by GE9 (86.7%). GE9 was isolated from black clay soil and Markofana hot pepper variety. But the lowest PI was shown by GE10 (61.7%) which was isolated from reddish sandy soil and Markofana hot pepper variety. This shows that local varieties might be more susceptible to fungal diseases than other hot pepper varieties such as Markofana (Aklilu et al. 2018). GE9 isolate (*Bacillus* species) is followed by GE1 isolate (*Pseudomonas* sp.) in performance and decreased radial growth of *F. oxysporum*.

Zone of inhibition had direct relation with percentage of inhibition for both endophytic and rhizospheric antagonistic bacteria against radial growth of *Fusarium oxysporum* in dual culture *in vitro*. There is very strong positive correlation between zone of inhibition and percentage of inhibition for both endophytic (P = 0.0001, r =0.98) and rhizospheric bacteria (P = 0.0001, r =1). This result indicated that increase in zone of inhibition had effect on percentage of inhibition of mycelial growth. However, there is very strong negative correlation between zone of inhibition and radial growth of mycelium in both endophytic (P = 0.0001, r = -0.98) and rhizospheric bacteria (P = 0.0001, r = -1). This indicated that increase in zone of inhibition had negative effect on radial growth of mycelium. In addition, radial growth of mycelium had very strong negative correlation with percentage of inhibition (P = 0.0001, r = -1). This is shows that as radial growth of mycelium get increase; percentage of inhibition would be decreased (Djordjevic et al. 2011). The strongest antagonism was exhibited by the isolate GR4 (*Bacillus* sp.) with percentage of inhibition value of 70% (Table 4**)**. This isolate was also isolated from black clay soil and Markofana hot pepper variety. The lowest percentage of inhibition value (65.0%) was found in GR9 (*Pseudomonas* sp.). Isolate GR4 that showed the highest value of percentage of inhibition in this research was the most effective in the growth inhibition of mycelium *F. oxysporum* (Djordjevic et al. 2011).

In vitro test for combined effects of bacterial isolates: All antagonistic endophytic bacteria were

combined each other to see the effect on the radial growth of mycelium. The combined effects of endophytic bacteria (Table 5) had shown significant effect on the growth of *Fusarium oxysporum* than single isolates (Table 3). As independent sample t-test shown that, there is significant percentage of average inhibition differences between single isolate antagonism and the combined effects against *Fusarium oxysporum* (P = 0.05). This might be due to multiple ways of inhibition mechanisms that combined isolates brought together against *F. oxysporum*. Furthermore, due to the combination the weakness of one isolate might be covered by another. This result is in line with the findings of Sundaramoorthy and Balabaskar (2013). The highest percentage of inhibition was shown by the combination of GE1+GE6 and GE6+GE9 (90%). However, the lowest percentage of inhibition was also shown by GE3+GE10 (68.3%). The combined rhizospheric antagonistic bacteria had shown significant percentage of inhibition difference on radial growth of mycelium (Table 6) than single isolates (Table 4). As independent sample t-test shown that, there is significant percentage of average inhibition differences between single and combined rhizospheric bacteria against *Fusarium oxysporum* (P =0.009). The highest percentage of inhibition was shown by the combination of GR1+GR9 (80%). However, the lowest percentage of inhibition was also shown by GR4+GR9 (75%).

During this study, the combined effects of endophytic and rhizospheric bacteria isolated from hot pepper grown in Yimali Kebele shown that there is significant percentage of inhibition on radial growth of mycelium (Table 7) than individually combined isolates of both endophytic and rhizospheric bacteria (Table 5 and Table 6). In general, analysis of variance shown that, there is significant percentage of average inhibition difference between combined effects of endophytic and rhizospheric bacteria (GE+GR) compared to GE+GE and GR+GR (P =0.002). In addition, statistical analysis of Tukey Honestly Significant Difference (HSD) shown that, there is significant percentage of average inhibition difference between combined effects of endophytic and rhizospheric bacteria (GE+GR) and individually combined isolates of endophytic bacteria (GE+GE) against *Fusarium oxysporum* (P = 0.004). The combined GE+GR shown the greater mean difference when it is compared to the mean of GE+GE (i.e. the mean of

Table 3: Effect of endophytic bacteria on the growth of *Fusarium oxysporum*

Name of isolate	Zone of inhibitions (mm)	Radial growth of mycelium treatment (mm)	Radial growth of mycelium control (mm)	Percentage of inhibition
GE1	18±.9	9±1.8	60	85.0
GE3	5±1.8	22±1.3	60	63.3
GE6	15±.5	13±1.3	60	78.3
GE7	10±1.8	20±.5	60	66.7
GE9	19±.5	8±.9	60	86.7
GE10	5±1.3	23±.5	60	61.7
GE12	11±1.3	15±.9	60	75.0

Where, Radial growth of mycelium and inhibition zone is the mean± standard deviation of each isolates in triplicate.

Table 4: Inhibition of *Fusarium oxysporum* by rhizospheric bacteria

Name of isolate	Zone of inhibitions (mm)	Radial growth of mycelium treatment (mm)	Radial growth of mycelium control (mm)	Percentage of inhibition
GR1	10±1.8	19±1.8	60	68.3
GR4	11±1.3	18±.9	60	70.0
GR9	8±.9	21±.5	60	65.0

Where, Radial growth of mycelium and inhibition zone is the mean± standard deviation of each isolates in triplicate.

Table 5: Inhibition of *Fusarium oxysporum* by combined endophytic bacteria

Combined isolates	Zone of inhibitions (mm)	Radial growth of mycelium treatment (mm)	Radial growth of mycelium control (mm)	Percentage of inhibition
GE1 + GE3	19±.5	8±.9	60	86.7
GE1+ GE6	20±1.3	6±1.8	60	90.0
GE1+ GE7	19±1.8	10±1.3	60	83.3
GE1+ GE9	18±.9	10±1.3	60	83.3
GE1+ GE10	18±.9	9±1.8	60	85.0
GE1+ GE12	17±.5	12±.5	60	80.0
GE3+ GE6	16±.5	12±.5	60	80.0
GE3+ GE7	12±.5	15±1.8	60	75.0
GE3+ GE9	18±.9	11±1.3	60	81.7
GE3+ GE10	6±1.8	19±.5	60	68.3
GE3+ GE12	8±.9	18±.9	60	70.0
GE6+ GE7	16±.5	13±.5	60	78.3
GE6+ GE9	20±1.3	6±1.8	60	90.0
GE6+ G10	17±.5	12±.5	60	80.0
GE6+ GE12	16±.5	13±.5	60	78.3
GE7+ GE9	19±.5	8±.9	60	86.7
GE7+ GE10	11±1.3	17±.5	60	71.7
GE7+ GE12	12±.5	16±.5	60	73.3
GE9+ GE10	19±.5	8±.9	60	86.7
GE9+ GE12	20±1.3	7±.5	60	88.3
GE10+GE12	13±.5	15±.5	60	75.0

Where, Radial growth of mycelium and inhibition zone is the mean± standard deviation of each combined isolates in triplicate

Table 6: Inhibition of *Fusarium oxysporum* by combined rhizospheric bacteria

Combined isolates	Zone of inhibitions (mm)	Radial growth of mycelium treatment (mm)	Radial growth of mycelium control (mm)	Percentage of inhibition
GR1+ GR4	17±.5	12±.5	60	80.0
GR1+ GR9	16±.5	13±.5	60	78.3
GR4+ GR9	14±.5	15±.9	60	75.0

Where, Radial growth of mycelium and inhibition zone is the mean± standard deviation of each combined isolates in triplicate.

Table 7: Combined effect of endophytic and rhizospheric bacteria against *Fusarium oxysporum*

Combined isolates	Zone of inhibition (mm)	Radial growth of mycelium in treatment (mm)	Radial growth of mycelium in control (mm)	Percentage of inhibition
GE1 + GR1	19±.5	7±.5	60	88.3
GE1+ GR4	20±1.3	6±1.8	60	90.0
GE1+ GR9	19±1.8	7±.5	60	
GE3+ GR1	17±.5	8±.9	60	86.7
GE3+ GR4	17±.5	9±.5	60	85.0
GE3+ GR9	15±.9	10±1.3	60	83.3
GE6+ GR1	18±.5	8±.9	60	86.7
GE6+ GR4	22±.5	5±.5	60	91.7
GE6+ GR9	19±1.8	7±.5	60	88.3
GE7+ GR1	18±.5	9±.5	60	85.0
GE7+ GR4	15±.9	9±.5	60	85.0
GE7+ GR9	13±.5	6±1.8	60	90.0
GE9+ GR1	20±1.3	5±.5	60	91.7
GE9+ GR4	24±.5	3±.5	60	95.0
GE9+ GR9	21±.5	5±.5	60	91.7
GE10+GR1	11±1.3	15±.5	60	75.0
GE10+GR4	15±.9	12±.5	60	80.0
GE10+GR9	18±.5	7±.5	60	88.3
GE12+GR1	16±.5	8±.9	60	86.7
GE12+GR4	17±.5	9±.5	60	85.0
GE12+GR9	13±.5	14±.5	60	76.7

Where, Radial growth of mycelium and inhibition zone is the mean± standard deviation of each combined isolates in triplicate.

GE+GR > the mean of GE+GE by 6.03). There is also significant percentage of average inhibition differences between GE+GR and rhizospheric bacteria (GR+GR) against *Fusarium oxysporum* (P = 0.04). However, there is no significant percentage of average inhibition difference between combined isolates of endophytic (GE+GE) and rhizospheric bacteria (GR+GR) against *Fusarium oxysporum* (P = 0.708). This might be due to the mechanism they use for the inhibition of mycelium may be the same in both endophytic and rhizospheric bacteria against *Fusarium oxysporum*. Guetsky et al. (2002) and Xu et al. (2011) had also revealed that combination of antagonists can improve biological control of phytopathogens. The highest percentage of inhibition was shown by the combination of GE9+GR4 (95%). However, the lowest percentage of inhibition was also shown by GE10+GR1 (75%). In general, combination of isolated bacteria boosts the effect on the pathogen that is targeted to be inhibited. This better effect was resulted from combining different isolates, in case endophytic and rhizospheric bacteria together might be due to diverse mechanisms of inhibition against the pathogen. Furthermore, the combination of GE9 and GR4 that showed highest potential to inhibit growth of *Fusarium oxysporum* might be due to this reason. This finding agrees with the work of Sundaramoorthy and Balabaskar (2013). During this study, the combinations of *Bacillus* sp. of both endophytic and rhizospheric bacterial isolates were shown the highest percentage of inhibition than combined effects of *Bacillus* + *Pseudomonas* and *Pseudomonas* + *Pseudomonas* sp. against *F. oxysporum*. This result is in line with the finding of Abada and Ahmed (2014). This indicates that *Bacillus* sp. had better performance to inhibit radial growth of fungal pathogens.

Mechanisms of inhibition: Antagonistic microbes in rhizosphere protect the host plant by directly suppressing the growth and proliferation of phytopathogens (Hariprasad et al. 2014; Prasannakumar et al. 2015). Antagonistic bacteria, by their interactions with *F. oxysporum*, play a major role in microbial equilibrium and serve as powerful agents for biological disease control. In addition to antagonistic properties, these microbes are known to improve host health through several other mechanisms (Hariprasad et al. 2014). As it is shown in the table below, antagonistic bacteria were able to produce different enzymes that have antagonistic effect on the growth of *F. oxysporum*. In the present study, antagonistic bacterial isolates were tested for the production of different hydrolytic enzymes such as chitinase, protease, and cellulase. This result is in lined with the work of Srividya et al. (2012). In addition, productions of these enzymes by antagonistic bacteria were understood by observing clear zone around the bacterial colony on dual culture *in vitro*. Those enzymes were one of the mechanisms that antagonistic bacteria inhibited the growth of pathogenic *Fusarium oxysporum* causing fusarium wilt of hot pepper (Table 8). This result agrees with the work of Amaresan et al. (2014). However, some of antagonistic isolates were not positive for the production of all of those hydrolytic enzymes tested during this study. On the other hand, isolate GE7, GE9, GE10, and GR4 were positive for the production all of those enzymes.

This study has shown that antagonistic bacteria were not specific in their antagonistic activity (mechanism) against *F. oxysporum*. The antagonistic effect of isolated

Table 8: Mechanisms of inhibition by antagonistic bacterial isolates against *Fusarium oxysporum*

Name of isolates	Chitinase production	Protease production	Cellulase production
GE1	+	+	-
GE3	+	+	-
GE6	+	+	-
GE7	+	+	+
GE9	+	+	+
GE10	+	+	+
GE12	+	-	-
GR1	+	-	+
GR4	+	+	+
GR9	+	-	+

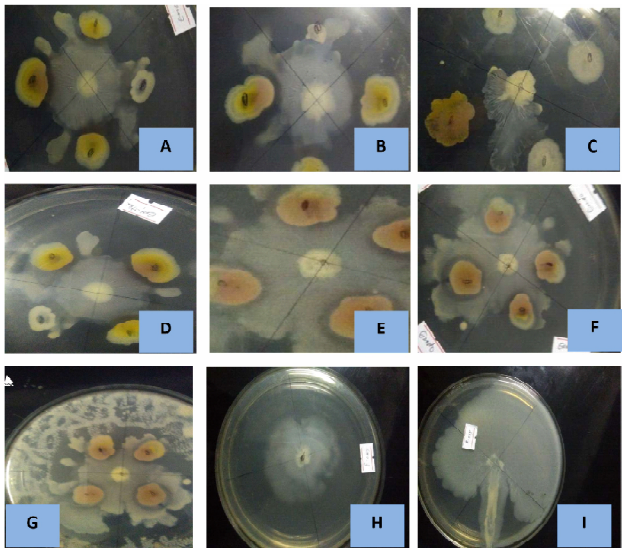

Figure 2: A, B, C and D) combined effects of endophytic and rhizospheric bacteria against *Fusarium oxysporum*; E) rhizospheric antagonists; F and G) endophytic antagonists; H, I and J were control group containing only *Fusarium oxysporum*.

bacterial species (*Pseudomonas* and *Bacillus* species) against *F. oxysporum* might be attributed to the production of â-1, 3-glucanase, siderophores, indole-3-acetic acid (IAA), and hydrogen cyanide (HCN) in addition to enzymes tested for their mechanisms during this study (Chen *et al*., 2010). GE1, GE3, GE6 and GE12 isolates were negative for the production of cellulase enzyme. In addition, GE12, GR1 and GR9 isolates were negative for the production of protease. However, GE7, GE9, GE10 and GR4 isolates (*Bacillus* species) were capable of producing all types of extracellular lytic enzymes tested for their mechanisms of inhibitions that are responsible for their antagonistic activity. Tolba and Soliman (2013) had also obtained the same result with this finding. From this study, it can be understood that *Bacillus* sp. had better effect on the *F. oxysporum* especially when they were combined to each other because of their ability to produce all kinds of enzymes that were tested during *in vitro* investigation. The findings of Srividya et al. (2012) support the current finding.

Conclusion

From the present study, it can be concluded that *Bacillus* and *Pseudomonas* species have the potential to suppress the colony growth of *F. oxysporum* which is the pathogen of wilt in hot pepper. The combinations of endophytic and rhizospheric bacteria have the better potential to suppress the radial growth of *F. oxysporum* especially combinations of *Bacillus* sp. had shown the highest percentage of inhibition. Antagonistic bacteria were able to produce hydrolytic enzymes i.e. chitinase, protease, and cellulose. During this study, most of isolates that had shown high antagonistic effect on *F. oxysporum* were isolated from Markofana hot pepper variety. Antagonistic bacteria that have ability to inhibit the growth of *Fusarium oxysporum in vitro* should be studied further.

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विश्व में किसानों द्वारा उत्पादित उत्पाद की गुणवत्ता बनाये रखने एवं खाद्य की प्रचुरता के लिये पौध रोगों को नियंत्रित करना आवश्यक है। समय–समय पर ईथियोपिया में तिखी मिर्च का उत्पादन घट गया। ऐसा केवल विभिन्न रोगों के प्रकोप विशेषतः तिखी मिर्च उत्पादक क्षेत्रों में फ्यूजेरियम उकठा रोग का होना है। इस अध्ययन का मुख्य उद्देश्य तिखी मिर्च के आन्तरिक उत्तकों एवं जड़ वाले क्षेत्रों से पौध विकास को प्रोत्साहन देने वाले जीवाणु को अलग करना व चरित्रीकरण करना है तथा *फ्यूजेरियम आक्सीस्पोरम* के प्रति प्रभावी जैव–नियंत्रक क्रियाओं के प्रभाविकता को ज्ञात करना है। कुल 30 स्वस्थ्य एवं ओजपूर्ण तीखे मिर्च के जड़ सहित पौधों व जड़ क्षेत्र के मृदा को तिखी मिर्च उत्पादन क्षेत्र (यीमाली केबेले) से एकत्रित किया गया। कुल 23 अंतः पादपी व जड़ क्षेत्रीय जीवाणुओं को तीखी मिर्च की जड़ व जड़ क्षेत्र अलग किया गया। संयुक्त रूप से अंतः पादपी व जड़ क्षेत्रीय जीवाणुओं ने एकल पृथक (आइसोलेट) (पी. ≤ 0.05) की तुलना में *फ्यूजेरियम आक्सीस्पोरम* की वृद्धि में सार्थक प्रभाव दिखाया। काली मृदा में उगायी जाने वाली मार्कोफाना तीखी मिर्च की किस्मों से पृथक किये गये जीवाणुओं ने लाल युक्त बलुई मृदा से पृथक किये गये स्थानीय तीखी मिर्च की किस्मों की तुलना में *फ्यूजेरियम आक्सीस्पोरम* के विरूद्ध उत्तम पाया गया। संयुक्त रूप से अन्तः पादपी व जड़ क्षेत्रीय जीवाणुओं [अन्तः पादपी (जी.ई.) + जड़ क्षेत्रीय (जी.आर.) ने जी.ई. + जी.ई. व जी.आर. + जी.आर. (पी. ≤ 0.002)] की तुलना में सार्थक निषेधक पाया गया। निषेधक जीवाणु आइसोलेट विभिन्न हाइड्रोलाइपिक एन्जाइमस् जैसे– चीटीनेस, प्रोटीऐज व सेलूलोज उत्पादन करने के योग्य थे। बैसलेस प्रजाति ने सबसे उत्तम निषेधक क्षमता प्रस्तुत किये और स्यूडोमोनस प्रजाति की तुलना में *फ्यूजेरियम आक्सीस्पोरम* की बहिः प्रकोष्ठीय विकास को कम किये।

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