# SCREENING OF MICROBIAL ANTAGONISTS AND PLANT EXTRACTS AGAINST SELECTED TOMATO PATHOGENS AND THEIR POTENTIAL IN THE MANAGEMENT OF BACTERIAL WILT

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## DECLARATION

This thesis is my original work and has not been submitted for the award of a degree in any other university

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## DEDICATION

I dedicate this thesis to my family and friends who supported and encouraged me throughout the study period.

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**CHAPTER ONE**

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## ABBREVIATIONS AND ACRONYMS

AEZ-Agro ecological Zone

APPPC- Asian and Pacific Plant Protection Commission

AUDPC-Area Under Disease Progress Curve

CABI- Centre for Agriculture and Bioscience International

CFU-Colony Forming Unit

DAI-Days after inoculation

EPPO- European and Mediterranean Plant Protection Organization

FAO- Food and Agricultural Organization of the United Nations

FAOSTAT- Food and Agricultural Organization Corporate Statistical Database

GOK-Government of Kenya

IAPSC- Inter African Phytosanitary Council

KALRO-Kenya Agricultural and Livestock Research Organization

KARI-Kenya Agricultural Research Institute

KBS-Kenya Bureau Services

KHCP- Kenya Horticulture Competitiveness Project

LH-Lower highland

LM-Lower highland

NARL-National Research Laboratories

TSA-Tryptone Soya Agar

UH-Upper Highlands

UM-Upper midland

USAID- United States Agency for International Development

## GENERAL ABSTRACT

Tomato production is greatly affected by major bacterial and fungal phytopathogens such as *Ralstonia* solanacearum, *Fusarium oxysporum* f. sp. *lycopersici* and *Alternaria solani*. Bacterial wilt, caused by *Ralstonia solanacearum*, can result in up to 100% yield losses. The disease has no easily affordable and accessible effective management method and therefore farmers are forced to abandon production once the pathogen is established in the greenhouse or field. The main objective of the study was to evaluate the efficacy of local microbial isolates and plant extracts in managing bacterial wilt under field conditions.

Microbial antagonists were isolated from soils collected in five counties. Plant extracts of 19 different plants were prepared in ethanol. Screening for activity of the antagonists was conducted using dual culture techniques while the activity of plant extract was assessed using the paper disk diffusion and food poisoned techniques. Degree of activity was measured as the diameter of zone of inhibition for bacteria and diameter of colonies for fungi. The most active antagonists and plant extracts against *R. solanacearum in vitro* were evaluated for their efficacy under field conditions. The microbial antagonists evaluated included *Trichoderma hamatum, T. atroviride, T. harzianum, Bacillus subtilis, Acinetobacter* spp., *Serratia* spp. The plant extracts were from *Curcuma longa, Rosmarinum officinallis* and *Tagetes minuta*. Commercial formulations of *T. viride* (Bio Cure F®) and *Pseudomonas fluoresce* (Bio Cure B®) were included as standard checks. The products were applied as drenches every two weeks commencing at transplanting until the tenth week after transplanting.

Approximately 59.6% of the microorganisms isolated from soil samples were fungi while 40.4% were bacteria. Agroecological zone (AEZ) LH1-UM1 in Meru had the most diverse microorganisms and the highest number of antagonists while LM 3 in Kirinyaga had the most abundant fungal and bacterial isolates. All the microbial antagonists and plant extracts tested showed significant ($P \leq 0.05$) variations in antagonistic activity against the test pathogens. Among the fungal isolates, *Trichoderma* spp. were the most frequently isolated antagonists with 28 isolates exhibiting inhibition. They had the highest inhibitory activity producing zones of inhibition ranging from 45.0 mm to 26.2 mm on *R. solanacearum* and colony growth reduction on *Fusarium oxysporum* ranging from 79.6% to 62.9%. The most active bacterial antagonists were *Bacillus* spp. and *Paenibacillus* spp. However, *Serratia* spp. (Sia 5Q) produced the largest inhibition zone on *R.*

*solanacearum* while *Bacillus* spp. (Abog Z30) exhibited the highest colony growth reduction on *F. oxysporum*. In the case of plant extracts, *Tagetes minuta* exhibited the largest inhibition zone on *R. solanacearum* while *Curcuma longa* showed the highest inhibition on *Alternaria solani and F. oxysporum*.

Under field conditions, the effectiveness of microbial antagonists and plant extract significantly ($P \leq 0.05$) differed between the two experiments. *Trichoderma hamatum* was the most effective against bacterial wilt in experiment 1, reducing crop mortality, incidence and area under disease progress curve (AUDPC) by up to 51.7, 49.3 and 58.2%, respectively. It also exhibited the highest percentage yield increase by up to 196.4% compared to control. In contrast, *B. subtilis* (Abo 20 Z7) showed superior bacterial wilt suppression in experiment 2, with a reduction of 44.6, 48.5 and 51.0% on crop mortality, incidence and AUDPC, respectively. It also increased top biomass by approximately 62% in both experiments. It was surpassed by *Serratia* spp. which increased yield by 233.0% compared to control in experiment 2. However, contrasting results were observed for plant extracts. *Tagetes minuta* showed the highest reduction in bacterial wilt incidence and yield increase in experiment 1 by up to 17.8 and 110.5% but exhibited the poorest results in experiment 2. Similarly, *C. longa* exhibited superior activity in experiment 2, increasing yield by 180.4% but performed very poorly in experiment 1. These results demonstrate that local microbial antagonists and plants confer antibacterial and antifungal activity against plant pathogens and can be exploited for biocontrol of plant diseases.

Key words: Biocontrol, Microbial pesticides, Botanical pesticides, Bacterial wilt.

# CHAPTER ONE : INTRODUCTION

## 1.1 Background

Tomato (*Solanum lycopersicum*) is a member of the Solanaceae family primarily produced for human consumption as beverage base, fruit and vegetables but is also an essential source of traditional medicine (CABI, 2020). It is native to South and Central Americas where it was first domesticated but was introduced in Africa in 1604 by Portuguese explorers in their territories around South Africa (Naika *et al.*, 2005; Kelley and Boyhan, 2014; Infornet Biovision; 2016). Tomato is currently produced worldwide and remains one of the most popular vegetables (Starke Ayre, 2014; Kelley and Boyhan, 2014; Infonet Biovision, 2016).

In Kenya, it is the second most grown vegetable crop after potatoes (Maerere *et al.*, 2006), produced both under field and greenhouse conditions by small and large scale farmers (Monsanto, 2013). Tomatoes are critical in meeting domestic and nutritional food requirements, creation of employment and generation of income as well as foreign exchange earnings (Sigei *et al.*, 2014). It has significant nutritional value providing a healthy and well-balanced diet. It contains lycopene, an anti-carcinogen along with vitamins A, B, C and other minerals such as potassium, phosphorus, calcium and iron (Naika *et al.*, 2005; Kelley and Boyhan, 2014). Despite its economic and nutritional importance, its value chain is faced with numerous constraints. These include agronomic problems like biotic and abiotic diseases, inadequate post-harvest technologies and disorganized market infrastructure, which enables unpredictable price fluctuations (Sigei *et al*., 2014).

*Ralstonia solanacearum* is an endemic soil pathogen (Kinyua *et al.*, 2014) attacking more than 200 plant species from 50 different families (Champoiseau, 2008). It usually penetrates the crop through the root system resulting in irreversible wilting and finally plants death (Kinyua *et al.*, 2014). Bacterial wilt of tomatoes is caused by either Race 1 or Race 3 of *R. solanacearum*. Race 1 is limited to the tropics, subtropics, and warm temperate regions with an extensive host range. In contrast, Race 3 has a very narrow host range, initially described as pathogenic to potatoes and tomatoes, but also infects and exhibit symptoms in other solanaceous crops. Race 3 biovar 2 has been reported in highlands of tropics, subtropics, and all temperate regions around the world except North America (Hayward 1991; Champoiseau *et al.*, 2009).

Bacterial wilt has been reported to be the most damaging pathogen of tomato and related solanaceous crops, affecting 77% of farms in Kenya (Kaguong'o *et al.,* 2010). Disease prevalence and incidences as high as 90% and 99% have been observed in Embu county (Kago *et al.,* 2016) and Marakwet district (Kwambai *et al.,* 2011), respectively. It has also been reported as the cause of desertion of greenhouse tomato production due to unbearable losses incurred by farmers countrywide (GoK, 2015).

### 1.2 Problem Statement

Bacterial wilt is a soil-borne phytopathogen with a broad host range and wide geographical distribution. It is excessively aggressive with longtime persistence in the soil (Hayward 1991; Champoiseau, 2008; Kaguong'o *et al.,* 2010; Potato South Africa, 2015). Elphinstone, (2005) and Champoiseau *et al*., (2010) reported that bacterial wilt devastation affected more than three million families in 80 countries, causing an annual worldwide loss exceeding USD 950 million. Numbers that have since increased. The damage is more pronounced in the tropics and subtropics (Hayward, 1991). It has been reported as the second most destructive plant disease after late blight in the production of crops from the Solanaceae family (Champoiseau *et al.,* 2010).

In Kenya, it has been cited by farmers as the most problematic tomato disease to manage (Kago *et al.,* 2016). Many farmers have been forced to abandon previous productive greenhouses and fields once the pathogen is established (Hayward., 1991; Ji *et al*., 2005; GoK, 2015; Aloyce *et al*., 2017). The problem is further worsened by the dimininshing productive land sizes and the high costs associated with establishment of precision agricultural structures such as greenhouses.

Several methods, including cultural, biological, chemical, quarantine and host resistance, have been proposed for the management of bacterial wilt (EPPO, 2004; Champoiseau *et al*., 2010; Kinyua *et al.,* 2014). However, cultural practices such as crop rotation and intercropping are impractical because the disease have a wide host range and the pathogen can survive in the soil for long periods (Kaguongo *et al.,* 2008). Although plant resistance has been reported as the most promising approach (Kaguongo *et al.,* 2008; Muthoni *et al.,* 2012; Potato South Africa, 2015), breeding for resistance is very difficult where no dominant genes are known (Bawa, 2016). In most cases, tomato varieties that have been developed for resistance against bacterial wilt are not widely adopted by farmers and consumers because of lower fruit quality and yield (Yuliar *et al*., 2015).

Moreover, most of the varieties with partial resistance still get lantently infected and act as sources of pathoen transmission (Muthoni *et al.*, 2012).

Methyl bromide, which used to be effective soil fumigant for management of bacterial wilt, is expensive and has been banned for agricultural use in most countries (Champoiseau *et al.,* 2010; Muthoni *et al.,* 2012). Furthermore, adverse health and environmental effects associated with chemicals have raised a lot of concern on their use in agricultural production (Nicolopoulou-Stamati *et al*., 2016). Generally, there is no practical and effective chemical control or any single method with 100% efficacy for the management of bacterial wilt (Xue *et al.,* 2009; Champoiseau *et al.,* 2010).

### 1.3 Justification

The integration of biopesticides in the management of phytopathogens has gained a lot of attention lately because of the negative health and environmental effects associated with synthetic pesticides (Junaid *et al*., 2013). Currently, there is a lot of pressure on reducing the use of synthetic pesticides as a result of increased consumer awareness, demand for organically produced food and political pressure (Heydari and Pessarakli, 2010; Suprapta, 2012; van Lenteren *et al*., 2018). As a result, some of the previously most used synthetic pesticides have been banned or restricted in agricultural use (Ownley *et al*., 2010; O'Brien, 2017). In Kenya, a total of 35 products have banned for agricultural use, while five products have been allowed for restricted use only (PCPB, 2020).

Biopesticides offer a viable alternative to synthetic pesticides (Heydari and Pessarakli, 2010; Pe´rez-Garcı´a *et al*., 2011; Naher *et al*., 2014; Din *et al*., 2016) given that they can surmount the problems associated with the use of synthetic pesticides (Anjarwalla *et al*., 2016). Specifically, biopesticides are eco-friendly, sustainable, economical, possess very low mammalian toxicity, readily biodegradable, has minimal risk of resistance development and reduced effects on non-target organisms compared to synthetics (Shrisha *et al*., 2011; Carmona-Hernandez *et al*., 2019; Kohl *et al*., 2019). Moreover, their efficacy is comparable to those of synthetics (O'Brien, 2017).

However, the potential of microbial biocontrol agents is yet to be fully exploited (Junaid *et al*., 2013; Van Lenteren *et al*., 2018), given that the market share of biopesticides is less than three percent of the total pesticides industry (Suprapta, 2012). For example, only 92 biocontrol products are registered for biocontrol of crop insect pests and diseases in Kenya and five products for

biocontrol of soil-borne fungal pathogens, the majority of which are imported (PCPB, 2020). Producing biocontrol products from locally isolated microorganisms and plants will reduce over-reliance on imported products, minimize the challenge of adaptability and efficiency and create job opportunities (Anjarwalla *et al*., 2016).

Previous studies have shown that the local environment harbor numerous microbial antagonists (Fulano *et al*., 2016) and plants with antimicrobial properties (Muthomi *et al*., 2017) which can be commercially availed to farmers for management of pests and diseases if harnessed and formulated using appropriate techniques (Muthomi *et al*., 2017). These biopesticides will assist local farmers who are exporting their products to Europe to comply with Europeans union pesticide maximum residue limits while providing quality and healthy products (Anjarwalla *et al*., 2016). Moreover, farmers can boost their income while providing sustainable and eco-friendly management options by cultivating and selling these pesticidal plants (Anjarwalla *et al*., 2016).

### 1.4 Objectives

The broad objective was to minimize tomato losses due to *Ralstonia solanacearum*, *Fusarium oxysporum,* and *Alternaria solani* through exploration, development, and use of local microbial antagonists and plant extracts.

The specific objectives of this study were:

i. To screen microbial antagonists and plant extracts for activity against major pathogens of tomato
ii. To evaluate the efficacy of microbial antagonists and plant extracts in managing bacterial wilt of tomato under field condition.

### 1.5 Hypotheses

i. Isolated microorganisms and crude plant extracts possess antimicrobial properties with activity against major tomato pathogens
ii. Microbial antagonists and plant extracts can effectively manage bacterial wilt of tomato

# CHAPTER TWO : LITERATURE REVIEW

## 2.1 Tomato production in Kenya

Tomato *(Lycopersicon esculentum)* is native to South and Central America (Naika *et al*., 2005; Starke Ayres, 2014; Infonet-Biovision, 2019) and was introduced to Africa by Spanish and Portuguese explorers (Naika *et al*., 2005; Kelley and Boyhan, 2014; Starke Ayres, 2014). As of 2017, Asia produced more than half of total world tomato output, followed by Europe, the Americas, and Africa. China is the leading producer of tomato, followed by India and the USA (FAOSTAT, 2019).

This crop is the second most widely grown vegetable in Kenya and East Africa after potatoes (Maerere *et al.,* 2006). It has been ranked first as a priority commodity in the vegetable crop value chain in Kenya (KARI, 2011). Tomatoes are mainly grown by small scale farmers with a few large scale producers (Monsanto, 2013). They are primarily produced under mixed farming (Musebe *et al.,* 2006; Wiersinga *et al.,* 2008), initially, solely under field production until the adoption of greenhouse technology (Mbaka *et al.,* 2013). While potato presents the most significant share by volume, tomatoes have the biggest share by value (USAID-KHCP, 2012). Rio Grande is the most grown variety favored by 32% of tomato farmers, followed by Cal J at 16%, then Kilele F1 at 11% (Ochilo *et al.,* 2018).

It has gained a lot of importance as an income-generating commodity in high potential and peri-urban areas in the past decade (Mbaka *et al.,* 2013). In Mwea west sub county, 71.6% of the farmers indicated that tomato was the most important income earner in the region (Mwangi *et al*., 2015). It contains lycopene, an antioxidant with ant carcinogenic properties, as well as essential vitamins A, B, and C and minerals, including potassium, phosphorus, iron, and calcium (Naika *et al*., 2005; Kelley and Boyhan, 2014).

## 2.2 Constraints of tomato production in Kenya

Tomato production in many developing countries in Africa is unprofitable mainly because of challenges associated with the production, post-harvest handling, marketing, or a combination of any of them (Arah, 2015). Production constraints include poor agronomic practices on nutrient management, pest and disease management, irrigation, weed management, and harvesting (Sigei *et al.,* 2014).

Pests and diseases remain the most problematic challenge in tomato production (Monsanto, 2013). Tomato farmers from 14 counties in Kenya reported insects (34%), fungi (23%), bacteria (13%), nutrient deficiencies (12%), mites (8%), viruses (3%), nematodes (2%), and water molds (2%) as the major constraints of tomato production (Ochillo *et al*., 2018). Incidences of insect pests increased from 26% in 2013 to 36% in 2017, while those of diseases decreased over the same period. Bacterial incidences reduced from 12% to 11%, fungal from 27% to 22%, nematodes from 3% to 1% and viruses from 5% to 2% (Ochillo *et al*., 2018). In another study, Mwangi *et al*. (2015) found that 53.8 % of tomato farmers in Mwea West sub-county considered early and late blights as the most important diseases. Wilt diseases followed at 32.1%, nutritional diseases at 6.6%, nematodes, and pest at 3.8% each. The majority of these farmers also indicated that management of the blight diseases was easily achieved through regular spraying of fungicides.

Market penetration is hindered by poor road infrastructures, high transportation costs, unstable market prices, poor storage facilities, and market cartels. Similarly, the remoteness of tomato processing facilities and the non-availability of local packaging industries all affect production costs and commercialization of tomatoes (Sigei *et al.,* 2014).

### 2.3 Bacterial wilt of tomato

#### 2.3.1 Importance of bacterial wilt

Bacterial wilt is the second most important production constraint of solanaceous crops in tropical and subtropical after late blight (Priou, 1999; Champoiseau *et al*., 2010). It is considered one of the most damaging pathogens affecting more than 200 plant species from 50 different families (Champoiseau and Momol, 2008). This wide host range makes it difficult to have generalized economic damage. Therefore, losses are commonly expressed on a crop-by-crop basis, which can vary from small crop losses to severe economic injuries (Kinyua *et al*., 2014). Serious economic losses are caused by crop death, tuber decays, or phytosanitary destruction of entire produce where the disease is suspected in quarantine areas (Elphinstone, 2005; Kinyua *et al*., 2014). It is a quarantine pest in EPPO, APPPC, and IAPSC (EPPO, 2014), and trade restrictions have been imposed on all host commodities exported to these regions. Besides, the management of this disease is very costly and contributes heavily to economic losses (Kinyua *et al*., 2014).

The occurrence of bacterial wilt has been reported in approximately 80 countries and is estimated to affect 3.75 million acres with annual global damage exceeding $950 a year (Champoiseau *et*

*al*., 2010). However, direct yield losses vary widely according to host, climate, pathogen strain, cultivar, soil type, and cropping practices (Kinyua *et al*., 2014). The most significant economic losses have been documented for potatoes, tomatoes, and tobacco in south-eastern USA, Brazil, Indonesia, Colombia, and South Africa (EPPO, 2014). In India, some studies reported between 10% and 100% mortality of tomato crops and yield losses between 0-91percent (Elphinstone, 2005). More recently, bacterial wilt has been associated with the desertion of greenhouse tomato production by many farmers in Kenya due to continuous losses (GoK, 2015).

### 2.3.2 Distribution and host range of bacterial wilt

Bacterial wilt is primarily soil and waterborne disease, which spread through infested soils, contaminated irrigation or surface water, workers' tools, and through the use of infected planting material. Soil organisms that injure the roots like nematodes and insects also aid in the dispersal of the pathogen from one crop to the other (Champoiseau *et al*., 2010; Kinyua *et al*., 2014; Potato South Africa, 2015). The movement of infected vegetative material has mostly contributed to the long-range spread, especially importation and planting of latently infected potato tuber seeds. Weed hosts in tropical lowland multiply the bacterium population in the soil all year round and particularly aid in distribution by offering shelter sites for the pathogen in addition to hosts in-between seasons in the absence of appropriate hosts (Hayward, 1991).

Bacterial wilt has a worldwide distribution but is notably more damaging in the tropics, subtropics, and warm temperate regions (Hayward, 1991). It has been reported in Australia, southeastern United States, all potato producing countries in Latin America except Ecuador, and was a severe constraint in Europe, particularly in Belgium, England, France, The Netherlands, Spain, Italy, and Portugal. In Africa, bacterial wilt occurs throughout central and southern Africa and has been a serious production setback in many countries, including Kenya, Uganda, and Ethiopia. In Asia, this pest has been reported in India, Pakistan, Nepal and Bhutan, Indonesia, the Philippines, southern Vietnam, Laos, Japan, and south China (Priou *et al*., 1999).

Bacterial wilt is one of the most damaging plant diseases and has been reported to affect more than 200 plant species from 50 different families, including crop plants, weeds, and ornamentals (Champoiseau, 2008). The most susceptible crops include tomatoes, potatoes, eggplant, pepper, banana, and groundnuts (Priou *et al*., 1999).

### 2.3.3 Biology, classification and identification of the causal agent; *Ralstonia solanacearum*

*Ralstonia solanacerum* is a Gram-negative, rod-shaped, strictly aerobic bacterium with a single, polar flagellum. The bacterium measures 0.5 x 0.7 x 1.5-2.0 µm in size. It flourishes at temperatures between 27˚C and 32˚C and always exhibit different morphological properties in general media, depending on the virulence of the isolates observed (CABI, 2020). Virulent isolates appear pearly cream white, irregular, flat, and fluidal colonies with centers whorled while avirulent type colonies are small, round, butryous colonies that are entirely cream-white on general media (Champoiseau, 2008; CABI, 2020). A semi-selective modified SMSA media has been developed for the detection of this bacterium in water, soil, and plant extracts. The distinction between avirulent and virulent isolates can be observed in Tetrazolium Chloride (TZC) media (Champoiseau, 2008).

*Ralstonia solanacearum* is a species complex with multiple variability at the species level (Champoiseau, 2008; Meng, 2013). It has been classified into five races and five biovars based on the pathogen's host range and its ability to utilize several carbohydrate substrates and metabolize different alcohols, respectively (Champoiseau, 2008). Race 1 has a vast host range attacking crops from multiple families while race 2 is limited to Musaceous species only. Race 3 primarily attacks potatoes and tomatoes, and race 4 is specific to ginger, while race 5 infects mulberry and is currently limited to China (He *et al*., 1983). Race 1 and 3 are present in all the five continents, race 2 predominates tropical areas in South America, and the Philippines, race 4 occurs in Asia while race 5 is limited to China (Elphinstone, 2005).

Biovar classification was developed primarily on the ability of the pathogen to utilize and oxidize disaccharides such as cellobiose, lactose, and maltose and hexose alcohols, including dulcitol, mannitol, and sorbitol). Biovar 1 strains uses none. Biovar 2 solely utilizes disaccharides, and Biovar 3 uses all while Biovar 4 strains utilize hexose only. Biovar 5 metabolizes all except dulcitol and sorbitol (Hayward, 1964; He *et al*., 1983). There is no correlation between biovars and races except for biovar 2-A, which often corresponds to race 3 and biovar 5 considered identical to race 5 (Meng, 2013). Biovars of *R. solanacearum* differ significantly in geographical distribution, which is suggestive of separate evolutionary origins (Hayward, 1991). Biovars 1 and 3 are reported to predominate Americas and Asia respectively, 2 and 4 occur in Australia and China (Pitkethley,

1981), while 5 is found in India and Indonesia (Tahat and Sijam, 2010). Biovar 3 is the most predominant isolate in lowland regions (Hayward, 1991).

A new phylogenetic classification scheme based on DNA sequence variation has been described for *R. solanacearum* (Champoiseau, 2008). The new classification scheme was developed due to poor definition and unsatisfactory taxonomic classification of *R. solanacearum* races (Meng, 2013). This classification has described four phylotypes that correlate with the geographical origin of these strains. Phylotypes 1,2,3,4 originated from Asia, America, Africa, and Indonesia, respectively (Fegan and Prior, 2005; Champoiseau, 2008). These phylotypes are further divided into sequivars, which are clusters of isolates with highly conserved DNA sequences (Champoiseau, 2008; Popoola *et al*., 2015).

**Detection and Diagnosis**

Symptomatology is the foremost step indicative of *R. solanacearum* infection used in detection and diagnostics A battery of complementary microbiological and molecular experiments is required for accurate detection, diagnosis and identification of the pathogen from asymptomatic and symptomatic plants (Champoiseau and Momol, 2008). Initial screening for detection of bacterial wilt in plants, water and soil involve tests such as bacterial streaming, isolation and culture in semi selective media, species specific PCR and immunodiagnostic, and pathogenicity test (Champoiseau and Momol, 2008; Kinyua *et al*., 2014). The vascular flow test is considered a rapid field diagnostic technique for initial distinction of bacterial infection from other wilts (Priou *et al*., 1999; Potato South Africa, 2015), which can then be further confirmed through potassium hydroxide test to distinguish *Ralstonia solanacearum* infection from *Clavibacter michiganensis* subsp. *Sepedonicus* whose symptoms in the tuber could be confused for bacterial wilt. The latter will not form a thread while the former will (Priou *et al*., 1999). These screening methods cannot identify the pathogen to race and biovar level and most are only sensitive to higher pathogen population (Champoiseau and Momol, 2008; Champoiseau *et al*., 2010).

Phylotypes and biovars identification can be done through DNA based methods and biovar test (Champoiseau *et al.,* 2010; CABI, 2020). DNA based employ PCR and Real-Time PCR assays with phylotype and biovar specific primers and DNA probe hybridization. Phylotypes can further be sub-classified into sequivars through PCR amplification and sequence analysis of

endoglucanase (*egl*) gene (Fegan and Prior, 2005). Different biovars can be identified based on their utilization of disaccharides (cellobiose, lactose and maltose) and oxidation of alcohol (dulcitol, mannitol and sorbitol). Race determination is impossible because the pathogens' strains lack race-cultivar specificity and phylogenetic unity (Priou *et al*., 1999; Champoiseau and Momol, 2008; Champoiseau *et al.,* 2009; Champoiseau *et al.*, 2010; Kinyua *et al*., 2014).

### 2.3.4 Symptoms of bacterial wilt on tomato

Above-ground symptoms are exhibited through wilting, stunting, and foliage chlorosis (Priou *et al*., 1999), which are similar in both potato and tomato and are indistinguishable for the different strains of the pathogen (Champoiseau *et al.*, 2009). The initial stage of disease development is manifested as wilting of the youngest leaves, usually one or half of the leaflets, during the hottest period of the day, and the plant appears to recover at night when temperatures cool down. Rapid wilting and desiccation of the leaves set in as the disease progress under favorable conditions. It leads to overall wilting and yellowing of foliage before eventual plant death even though the desiccated leaves remain green (Champoiseau and Momol, 2008).

Sometimes wilting happens so fast without leaf yellowing where severe infection sets in a conducive environment (Priou *et al*., 1999; Potato South Africa, 2015) and may kill plants in four to seven days following the appearance of the first symptom (Champoiseau *et al*., 2010). The vascular bundle characteristically turns brown at advanced stages of disease development (Priou *et al*., 1999; Potato South Africa, 2015). Stunting can appear at any stage of plant growth, but an infected tomato may only show this symptom when it's just about to ripen because of rapid fruit expansion. The stems of extremely susceptible varieties usually collapse with visible grey-white bacterial ooze on the surface (Champoiseau and Momol, 2008; Champoiseau *et al.*, 2009).

High temperatures between 24˚C to 30˚C favor symptom expression. Latency occurs when the plants remain asymptomatic in conditions that are ideal for pathogen development (Champoiseau and Momol, 2008; Kinyua *et al*., 2014). Additionally, symptom expression and rate of disease development greatly vary with host susceptibility and pathogen strain aggressiveness (Alvarez *et al.* 2010).

### 2.3.5 Epidemiology of bacterial wilt

Bacterial wilt is a soil and waterborne disease that can survive for years in infested water, debris, plant material, and weed host, wet and deep soil layers as an inoculum reservoir (Hayward, 1991; Champoiseau and Momol, 2008; Champoiseau *et al*., 2009). Sheltered survival sites also exist in rhizospheres of resistant, symptomless and weed hosts. These sites, together with and epiphytic phase of the pathogen, have been described to be of importance in the survival of the pathogen (Hayward, 1991). Though the epiphytic phase is considered of minor significance in disease epidemiology (Champoiseau and Momol, 2008), it is a very important source for renewal of soil inoculum population (Hayward, 1991). Inoculum survival in aquatic environment heavily relie on the soil pH, presence of resident competing, antagonistic or parasitic microorganisms, salt levels and particuae matter. Other factors that can also affect pathogen survival include soil type, soil moisture content and soil organic matter (Champoiseau, 2008).

Dispersal and spread of primary inoculum occurs through plant to plant, movement of plant materials, dissemination through handling and soil transfer by machinery, irrigation with contaminated water, insect transmission and dissemination through waterways (Hayward, 1991; Champoiseau and Momol, 2008; CABI, 2020). Vegetative propagation is the most important in long range spread, especially latent infection, which has been associated with extensive local and international spread (Hayward, 1991; Champoiseau *et al*., 2009).

Generally, bacterial wilt development is optimal in rainy seasons that avail high soil moisture levels ideal for survival, multiplication and spread of the inoculum (CABI, 2020). The disease is also favoured by high temperatures, ranging from 24°C to 35°C (CABI, 2020; Champoiseau, 2008). Even though the specific temperature optima for disease development vary from one pathogen races and biovars to the other (CABI, 2020), the most destructive starins for tomatoes, Race3 biovar 2, causes optimal destruction at 27°C (Champoiseau, 2008). However, bacterial wilt development, virulence and expression is highly suppressed in the absence of sufficient water (CABI, 2020) and at temperatures exceeding 35°C or below 10°C (CABI, 2020; Champoiseau, 2008). Such cnditions that do not favour disease expression can also conceal extensive infection in latncy (CABI, 2020)

The pathogen primarily gains entry into the plant through wounding of the roots caused by crop handling practices, soil organisms or emergence of lateral roots. It can also gain entry through the

stem injuries as a result of insect feeding or handling damage (Hayward, 1991; Potato South Africa, 2015). Once inside, the bacterium colonizes the surrounding small cells before moving into the xylem in vascular bundle (Hayward, 1991; Potato South Africa, 2015), where it causes wilting through interfering with water transport (Priou *et al*., 1999)

### 2.4 Biological control of soil borne phytopathogens

#### 2.4.1 Biological control of soil borne pathogens using microbial antagonists

Soil borne plant pathogens are among the most complicated to manage (Wang *et al*., 2018) partially because they have a wide host range and form resistant resting structures that persists for long periods in the soil (Amin *et al*., 2010). Biological control (Amin *et al*., 2010), specifically the use of fungal and bacterial antagonists in management of soil borne pathogens seems the most viable alternative to chemical pesticides (Heydari and Pessarakli, 2010; Naher *et al*., 2014). The use of other microbial products containing viruses, algae and protozoa besides fungi and bacteria as active ingredients have also been reported (Gupta and Dikshit, 2010; Kachhawa, 2017; O'Brien, 2017). Disease suppression is achieved through direct antagonism involving competition, production of toxic metabolites, parasitism or indirect interaction resulting into induced host plant resistance (Whipps, 1997; Kohl *et al*., 2019).

Successful colonization of specific infection sites is a prerequisite for an antagonist which suppresses the pathogen through competition for resources and space (Pal and Gardener, 2006; Singh, 2015). Antibiosis is mediated through production of secondary metabolites which can be highly deleterious to growth and metabolic activities of other organisms in very low quantities (Stirling and Stirling, 1997; Raaijmakers *et al*., 2002; Pal and Gardener, 2006). They can either be nonvolatile or volatile (Haas and Defago, 2005) and single microbial antagonist may produce different individual antibiotics with either specific or multiple suppression, though many antibiotics have a broad spectrum of activity (Raaijmakers *et al*., 2002; Pal and Gardener, 2006; Alabourette *et al*., 2006).

Parasitism and predation involves recognition of target pathogen and consequential production lytic enzymes which facilitate degradation of cell wall and penetration into the target pathogen (Alabourette *et al*., 2006). Mycoparasitism has been described for antagonists through morphological disturbance or penetration of hyphae, sclerotia or spores into the pathogen (Whipps,

1997; Stirling and Stirling, 1997). Microbial predation of pathogens is non-specific with unpredictable level of disease control while some like *Trichoderma* spp. exhibit predation only under nutrient limited conditions (Pal and Gardener, 2006).

Host induced resistance stimulated by antagonists can either be systemic acquired resistance (SAR) or induced systemic resistance (ISR). While the former is mediated by salicyclic acid (SA) produced as a result of pathogen infection, ISR is mediate by jasmonic acid and/or ethylene produced as a result of recognition of non-pathogenic rhizobacteria (Pal and Gardener, 2006). Given that there is no direct interaction between the pathogen and the antagonist, induction of host defense mechanism can only be effective when the inducing agent is applied before the crop is challenged with the target pathogen (Pal and Gardener, 2006).

Microbial antagonists are widespread across many genera and quite a number have shown successful disease control through manipulation of soil microbial community (Weller, 1988; Kabeil *et al.,* 2008). Usually, the microbes growing in the host plant rhizosphere provide ideal biological control agents since this environment provide frontline defense against major and minor primary pathogen infection or secondary spread (Weller, 1988; Kabeil *et al.,* 2008). Efficiency of microbial antagonists to control diseases depend on their inherent ability to adapt to a range of environmental and nutritional conditions and faster rate of multiplication than the pathogen (Sharma *et al*., 2009).

Several studies have demonstrated successful suppression of soil borne fungal and bacterial pathogens using antagonistic bacteria. Sang *et al*., (2008) reported successful suppression of *Phytophthora capsici* in pepper under artificial and natural field infestation by *Pseudomonas corrugate*, *Flavobacterium* spp., and *Lysobacter enzymogenes*. Similarly, diverse strains of *Paenibacillus* spp. aggressively suppressed Fusarium crown and root rot of tomato in the greenhouse (Xu and Kim, 2014). In addition, Liu *et al*., (2014) observed that application of a consortium of two strains of *B. subtilis* and *Chryseobacterium* spp. provided higher biocontrol efficacy compared to single strain application against individual and a complex of Ralstonia wilt, Phytophthora blight and Meloidogyne spp. on bell pepper. Among the bacterial biocontrol products, *Bacillus* spp., are the most commercially registered for control of foliar and soil borne pathogens as well as Lepidopteran caterpillars (Van Lenteren *et al*., 2018). Other than disease suppression abilities, most of these antagonists also double up as plant growth promotion

rhizobacteria (Sang *et al*., 2008; Xu and Kim, 2014; Labuschagne *et al*., 2010) commonly applied as biofertilizer for growth and yield enhancement (Labuschagne *et al*., 2010).

Similarly, numerous reports of successful inhibition of soil borne fungal and bacterial pathogens by fungal antagonists have been reported. Species of genera *Trichoderma* ssp., *Paecilomyces* spp., *Verticillium* spp., *Aspergillus* spp., *Gliocladium* spp., and *Fusarium* spp., among other have been developed into commercial products that are used worldwide for management of diverse soil borne and foliar plant pathogens (Van Lenteren *et al*., 2018). The genus *Trichoderma* was first described in 1930s as a host of versatile potential biocontrol agents (Ha, 2010; Naher *et al*., 2014) and the first inhibitory activity of *Trichoderma lingnorum* (viride) on *Rhizoctonia solani* was first demonstrated by Weindling (1932). To date, *Trichoderma* is still the most aggressive and widely commercialized biological control product for soil borne diseases worldwide (Van Lenteren *et al*., 2018). *Trichoderma* spp., has shown significant suppression of soil borne fungal pathogens including *Phytophthora* spp., *Pythium* spp., (Naher *et al*., 2014; O'Brien, 2017), *Rhizoctonia* spp., (Mahmoud, 2016), *Fusarium* spp., (Babychan and Simon, 2017) and even bacterial pathogens such as *R. solanacearum* in diverse crops (Konappa *et al*., 2018; Yendyo *et al*., 2018; Nahar *et al*., 2019). Furthermore, this fungus is ubiquitous in nature and has even shown superior disease suppression and yield increment compared to chemical pesticides (Ha, 2010).

The use of biological control products has been increasing annually and an estimate of 30 million hectares were applied with biocontrol products in 2015 alone. North America and Europe are the largest producers and consumers of biocontrol agents. However, Latin America followed by Asia have shown a tremendous increase in utilization of BCAs while Africa records the least consumption (Van Lenteren *et al*., 2018). Globally, only 209 strains from 94 species of microbial biocontrol agents are available commercially, produced by about 200 small to medium scale companies with a few multinational agrochemical producers only venturing into the sector recently (Van Lenteren *et al*., 2018). However, the future looks bright for production and consumption of biocontrol products as the market demand has been steadily increasing by an estimate of 15% yearly compared to chemical pesticides which is stuck at 5% to 6% annual increase. Furthermore, consumer awareness and increased demand for organically produced food stuff has compelled many retail supermarkets to imply even more stringent measures on pesticide use than the government in developed countries (Van Lenteren *et al*., 2018).

### 2.4.2 Management of soil borne pathogens using plant products

Pesticidal plants represents a vast source of untapped antimicrobials (Agrawal *et al*., 2016) yet their use is as ancient as the beginning of mankind (Dubey *et al.*, 2010; Azmir *et al*., 2013; Narasimha Murthy *et al.*, 2013). Initially, plants were only considered as sources of food but became useful as a natural cure of human diseases and improvement of health after their medicinal properties were discovered (Azmir *et al*., 2013). In the recent decades, most investigations have concentrated on exploitation of plant products to treat human diseases while exploration on efficacy against phytopathogens has received little attention (Prakash and Karmegan, 2012). Despite the neglect, it has been reported that pesticidal plants were widely used in commercial production for millennia until 1940s when chemical pesticides were developed (Anjarwalla *et al*., 2016).

Integrating plant based natural products in management of plant diseases is one of the most effective alternatives to chemical pesticides (Din *et al*., 2016). Pesticidal plants produce secondary metabolite with antimicrobial activity (Verma *et al*., 2012; Gurjar *et al.*, 2012) for protecting themselves against a variety of pathogens (Gurjar *et al.*, 2012; Sen and Batra, 2012). These active compounds are valuable sources of new and active molecules (Das *et al*., 2010) that when tapped using appropriate procedure and formulated into products that can be used by farmers in suitable concentrations to manage plants pests and diseases (Muthomi *et al*., 2017).

Soil borne disease inhibition through incorporation of plant products (Gamliel *et al*., 2000; Agrios, 2005; FAO, 2016; Shafique *et al*., 2016; Mihajlovic *et al*., 2017), plant extracts and essential oil has been reported (Mihajlovic *et al*., 2017). Successful suppression of soil borne pathogens through bio fumigation with members of Brassicaceae (Agrios, 2005; Nega,2014; FAO, 2016; Shafique *et al*., 2016; Mihajlovic *et al*., 2017) and Alliacae (Nega, 2014; FAO, 2016; Mihajlovic *et al*., 2017) families incorporated as soil amendments has been demonstrated. Plant from Brassicaceae release huge amounts of bio-toxic isothiocianates which are broken down from Sulphur compounds produced from the plants (Nega, 2014; FAO, 2016; Shafique *et al*., 2016; Mihajlovic *et al*., 2017). Analogously, members of Alliacae such as onions and garlic also produce compounds with broad spectra of activity on microorganisms such as thiosulfinates and disulphides when crushed (Mihajlovic *et al*., 2017). Despite the efficacy of most of these crops, the use organic amendments in managing soil borne diseases are not widely used due concerns on

their side effects including non-selectivity, scale practicality and cost effectiveness (Mihajlovic *et al*., 2017).

Plant extracts and essential oils from diverse sources of medicinal plants have been reported to possess antimicrobial activity against a wide range of plant pathogens (Mihajlovic *et al*., 2017). *In vitro* bioassays indicated active growth inhibition of *F. oxysporum* and *R. solani* by crude extracts from different herbal plants (Muthomi *et al*., 2017). Similarly, antibacterial activity against diverse strains of *R. solanacearum* with different plant species has been reported *in vitro* (Murthy and Srinivas 2012; Narasimha-Murthy *et al*., 2013). However, relatively fewer reports of *in vivo* activity of plant extracts and essential oils against soil borne pathogens are available (Mihajlovic *et al*., 2017). Correspondingly, Hanaa *et al*., (2011) observed that treating tomato seedlings with aqueous extracts of neem and willow successfully reduced incidences of Fusarium wilt at six weeks after infection in controlled pot experiments. Equally, greenhouse experiments have shown successful reduction in bacterial wilt incidences when artificially infected tomato plants are drenched with aqueous extracts of *Allium fistulosum* (Deberdt *et al*., 2012).

Plants with pesticidal properties are widely available and cost effective perspective to sustainable bio and organic farming (Rodino *et al*., 2014; Anjarwalla *et al*., 2016). They are eco-friendly, possess very low mammalian toxicity, easily biodegradable, possess low risk of resistance development and hazards on non-target organisms. They also possess no adverse effects on seed viability, plant growth and food quality (Shrisha *et al*., 2011).

**2.5 Management of Fusarium wilt**

*Fusarium oxysporum* f. sp. *lycopersici*, the causal agent of Fusarium wilt of tomato was first described in England by Masse G. E in 1895 (Bawa, 2016). The disease has affected production of tomato worldwide, especially in warmer regions (Bawa, 2016) and was ranked fifth most problematic fungal plant pathogen internationally (Raza *et al*., 2017). Several methods including use of chemicals, host plant resistance, cultural, physical, biological and use of natural products have been proposed for its management (Fravel *et al*., 2003; Bawa, 2016).

Chemical pesticides and plant resistance are the main management methods used by many farmers against the pathogen (Fravel *et al*., 2003; Bawa, 2016). While chemicals are mostly favored because they are fast acting, they are disastrous to non-target organisms, human health and less

viable for large scale application (Bawa, 2016). In the case of plant resistance, developing resistant varieties can be problematic in cases where no dominant gene is known. Similarly, resistance is likely to breakdown in case of high pathogen population or emergence of a new pathogen races that are more virulent (Fravel *et al*., 2003; Bawa, 2016).

The problems associated with these management methods have led to increased search for better biological options (Fravel *et al*., 2003; Raza *et al*, 2017). Several strains and isolates of fungi and bacteria have been exploited for management of *F. oxysporum* f. sp. *lycopersici*, either alone or incombination (McGovern, 2015). Successful *in vitro* inhibition of mycelial growth of the pathogen has been reported. Mwangi *et al*., (2019) observed that *T. harzianum* and *Purpureocillium lilacinus* effectively inhibited the colony growth of *F. oxysporum* f. sp. *lycopersici* by up to 51.9% and 44%, respectively. Similar findings were observed by Samaras *et al*., (2016) who showed that the *B. amyloliqufaciens* reduced mycelial growth of the pathogen by up to 24.1 % *in vitro*. In 2012, Alwathnani and Perveen reported high inhibition of mycelial growth of *F. oxysporum* by fungal antagonists including *T. harzianum, A. niger* and *Penicillium* spp. and methanolic extracts from two cyanobacteria; *Phormidium autumnale* and *Nostoc linckia*. The authors also observed that the fungal and cyanobacteria antagonists reduced disease incidence on tomato plants inoculated with the pathogen under greenhouse conditions.

Internationally, the highest number of biocontrol studies on Fusarium wilt of tomato since the year 2000 have exploited *Pseudomonas* spp., followed by *Trichoderma* spp. and *Penicillium* spp. However, 65% of the studies reported that *Bacillus* spp.was the most effective, followed by non-pathogenic Fusarium (60%) and *Trichoderma* spp. (57%) (Raza *et al*., (2017). Ghazalibiglar *et al*., (2016) found that diverse isolates of *T. atroviride* suppressed Fusarium wilt by up to 69% under glasshouse conditions while in 2005, Akkopru and Demir reported that single and dual treatments involving *Glomus intraradices* and three rhizobacteria; *P. putida*, *P. fluorescence* and *Enterobacter cloacae*, exhibited between 8.6 and 58.6% reduction in disease severity under greenhouse condition. In most cases, disease reduction is usually accompanied by plant growth promotion (Akkopru and Demir, 2005; Chandel *et al*., 2010; Alwathnani and Perveen, 2012; Razza *et al*., 2017) and the efficacy can even surpass performance of chemical fungicides (McGovern, 2015). Disease reduction is coffered through suppression of spore production and germination and

is mediated through competition, antibiosis and induction of host plant resistance (Fravel *et al*., 2003; McGovern, 2015).

Generally, biocontrol of plant pathogenic fungi using botanicals has not been extensively studied despite the fact that they are biodegradable and pose low risk to environmental and human health (Lecomte *et al*., 2016). Essential oils and plant extract from *Salvia officinalis, S. tomentosa* ***and*** *S. cryptantha* exhibited strong antifungal activity against *F. oxysporum*, and a colony growth reduction of up to 65.3% was observed for water extracts of *S. officinalis* (Yılar and Kadıoğlu, 2016). Similarly, La Torre *et al*., (2016) also observed complete inhibition of *F. oxysporum* conidial growth at 24 and 48 hours after inoculation from essential oils of clove and thyme. In that experiment, clove also exhibited superior disease management under greenhouse conditions, reducing Fusarium wilt severity by 42.4% compared to untreated control. Several studies have illustrated that the efficacy of plant extracts and essential oils in suppression of *F. oxysporum* is dependent on the concentration and varies from species to species (de Rodriguez *et al*., 2015; Nasrin *et al*., 2018).

### 2.6 Management of Early blight

*Alternaria solani* is one of the most damaging pathogens of tomato affecting tomato leaves, fruits and stems (Gondal *et al*., 2012). This pathogen is soil inhabiting as well as airborne (Ghazanfar *et al*., 2016) and can cause huge losses through premature dropping of fruits (Gondal *et al*., 2012). Though it has spread to all major tomato and potato production regions (CABI, 2020), it is more devastating in warm parts of the world, especially the tropics and subtropics (Gondal *et al*., 2012; Ghazanfar *et al*., 2016). The main management practices for reducing losses associated with Early blight include cultural practices which aim at minimizing the extensive exposure to leaf wetness, resistance and synthetic pesticides (Gondal *et al*., 2012). However, not all farmers and consumers preferred varieties are resistant and therefore application of chemical fungicides has been widely adopted by farmers (Ghazanfar *et al*., 2016; Joseph *et al*., 2017). The misuse and negative effects of these chemicals to human and environment health has pushed for intensive research to come up with better management options for Early blight. The use of biological control and natural products from plants offers an economically and environmentally viable option.

Previous studies have identified some potential natural products through conducting a series of *in vitro* antagonistic bioassays. Meena *et al*., (2017) reported that *T. harzianum* and *T. viride* produced volatile and non-volatile substances that significantly inhibited the mycelial growth of *Alternaria solani* by up to 75.8 and 78.8%, respectively. The authors also reported that the antagonistic fungi produced volatile compounds which reduced the mycelia growth by 31.0% and 27.6%, respectively. Similar findings were observed by Paramanadham *et al*., (2017) which showed that two isolates of *P. aeruginosa* successfully inhibited the mycelial growth of *A. solani* by 72.1 and 74.5% *in vitro*. Dual application of antagonistic isolates of *B. subtilis* and *P. fluorescence* exhibited synergistically superior colony growth reduction of up to 31.5% while single applications only produced colony growth reductions of 19.5% and 12.5%, respectively (Sundaramoorthy and Batabaskar, 2012).

The active isolates *in vitro* also showed significant disease suppression under the greenhouse conditions. The synergic effect of combining antagonistic *B. subtilis* and *P. fluorescence* translated to superior disease suppression in the greenhouse compared to single application of ether isolates (Sundaramoorthy and Batabaskar, 2012). Similarly, *P. aeruginosa* isolates previously found active *in vitro*, reduced the disease severity under greenhouse conditions by 73.2 and 84.6%, respectively (Paramanadham *et al*., 2017). Further evaluation into mode of action of the isolates with *in vitro* antagonism revealed that *T. harzianum* and *T. viride* produced glacial acetic acid and propyl-benzene volatiles with antifungal effects (Meena *et al*., 2017). The antagonists also produced non-volatiles such as D-glucose, 17-octadecynoic acid and 6-O-a-D-galactopyranosyl, which also possess antifungal properties.

In the case of plant extracts, a study by Raza *et al*., (2016) showed that different plant species including, *Azadarichta indica*, *Allium sativum*, *Parthenium hysterophorus*, *Datura stramonium* and *Eucalyptus camaldulensis* inhibited the colony growth of *A. solani* by up to 69.7, 66.2, 59.4, 49.5 and 49.3%, respectively. Similarly, Derbalah *et al*., (2011) showed that *Bauhinia purpurea* inhibited colony growth of *A. solani* by up to 79.4%, and reduced disease severity under the greenhouse by up to 69.6%. The authors also analyzed the extract toxicity by examining the histological changes in organs of treated rats and found that *B. purpurea* extracts had low toxicity compared to untreated control. Similar to activity against other pathogens, the

effectiveness of plants extracts showed significant ($P \leq 0.05$) variation depending on the concentration.

### 2.7 Management of bacterial wilt

Field management of bacterial wilt is very difficult because of its wide host range and geographical distribution, long soil survivability and wide biological variation (Martin and French, 1985; Champoiseau and Momol 2008). There is no single method with 100% efficacy in controlling this disease but integration of preventive, phytosanitary, cultural, chemical and biological methods has been reported to offer some level of protection against this pathogen (EPPO, 2014; Champoiseau *et al*., 2010; Kinyua *et al.,* 2014).

Prevention and quarantine are most appropriate where the pathogen is present but is not established everywhere or is not known to occur (Champoiseau *et al*., 2009). Critical observation of cultural sanitation in regions where the bacterium is endemic is very important to keep non infested areas clean. Such practices include: planting certified disease-free seeds and plantlets, disinfection of farm implements, controlling surface runoff and avoidance of surface water for irrigation, bio-fumigation with mustard and radish, crop rotation with members of non solanaceae family and restriction of movement within the farm to avoid risk of redistributing the pathogen (Champoiseau *et al*., 2009; Champoiseau *et al*., 2010; Kinyua *et al.,* 2014). Siting greenhouses away from the field production sites, use of pathogen free planting media, frequent disinfection of tools, trays and frames, limiting plant handling, weed management and avoidance of sub-irrigation water are critical preventive measures for greenhouse disease management (Champoiseau *et al*., 2009). Quarantine on the other hand involves reinforcement of regulations, sanitation measures, protocols and inspection to prevent introduction of the pathogen (Champoiseau *et al*., 2009).

Cultural management of bacterial wilt through soil ammendments using diverse maerials have shown varied success. Numerous studies have reported succesull suppression of bacterial wilt through application of plant residues from diverse plant species such as chili, Chinese gall, cloves, lemin grass, etc. (Yuliar *et al*., 2015). Contrastingly, fewer studies hav shown successful supprision of bacterial wilt using animal wastes (Yuliar *et al*., 2015). Islam and Toyota, (2004) reported that application of farmyard manure and poultry manure suppressed bacterial wilt through increasing soil microbial activity and populationof culturable bacteria and fungi. In contrast, addition of

preplanting inorganic calcium oxide and urea has also showed inconsistent results in reducing R. solanacearum population and bacterial wilt incidence under field conditions (Michel *et al*., 1997).

Similarly, previous research on intercropping and crop rotation have yielded inconsistent results on suppression of bacterial wilt. Crop rotation reduce the multiplication and accumulation of *R. solanacearum* that is associated with continous cultivation of a single species of susceptible varieties (Yuliar *et al*., 2015). Adebayo *et al*., (2009) observed that rotating and intercropping tomato with pure and mixed strands of *Manihot esculenta*, *Mucuna puriens* and *Crotalaria juncea* siginificantly reduced *R. solanacearum* population and bacterial wilt incidence compared to control plots. Accordingly, the effectiveness of either techniques was highly dependent on the plant species used (Michel *et al*., 1997; Adebayo *et al*., 2009). In contrast, Michel *et al*, (1997) observed that intercropping tomato with cowpea, soybeans and welsh onion significantly reduced bacterial wilt incidence compared to control plots but had no effect on population of soil *R. solanacearum*.

Plant resistance is one of the most effective, economical, and environmentally friendly method in management of bacterial wilt (Boshou, 2005; Elphinstone, 2005; Muthoni *et al.*, 2012). It has been the most exploited control strategy for this disease (Wang and Lin, 2005; Ahmed *et al*., 2013), with breeding programs concentrating around economically important crops (Boshou, 2005; Elphinstone, 2005). However, most of the existing resistant varieties have undesirable qualities such as small fruit sizes (Champoiseau and Momol, 2008; Champeseau *et al.*, 2010; Yuliar *et al.*, 2015), which is a major hindrance to release and adoption of these varieties by farmers and consumers (Yuliar *et al*., 2015). Furthermore, most of these moderately resistant cultivars have been associated with latent infection (Priou *et al*., 1999; Muthoni *et al.*, 2012) and quiete expensive for majority of small scale farmers to afford. It is important to note that effectiveness of plant host resistance is highly dependent on geographical location because of variability in pathogen strains and density, temperature, soil moisture, and presence of root-knot nematode (Hanson *et al*., 1996; Wang *et al*., 1998; Wang and Lin, 2005; Champoiseau and Momol, 2008; Ahmed *et al*., 2013). Future breeding is expected to improve on crop yield through genetic enhancement for bacterial wilt resistance by adopting biotechnological approaches (Yuliar *et al*., 2015).

Chemical control substances such as Actigard (acibenzolar-S-methyl) have been reported to have some efficacy in greenhouse management of bacterial wilt but to a lesser extent under field

conditions in small scale trials (Pradhanang *et al.,* 2005; Ji *et al.,* 2007). Use of bactericides (copper) and antibiotics (streptomycin, ampicillin, tetracycline and penicillin) and soil fumigation with vapam, methyl bromide, or chloropicrin has shown inconsistent results in suppression of bacterial wilt under field conditions as well (Champoiseau and Momol, 2009). Fumigation is very expensive, tedious and impractical to large scale application while Methyl bromide has already been phased out for agricultural use in Kenya (Muthoni *et al.,* 2012). Other compounds like chlorine or peracetic can be used to treat irrigation water (Champoiseau *et al.,* 2010), while sodium hypochlorite are more appropriate for spot treatment after rogueing wilted plants or general field sanitation though expensive and tedious to apply (Kaguongo *et al.,* 2008). Chemical application should be integrated with other methods to minimize selection pressure for pathogen resistance (Champoiseau and Momol, 2009).

# CHAPTER THREE : EFFICACY OF MICROBIAL ANTAGONISTS AND PLANT EXTRACTS AGAINST TOMATO PATHOGENS *IN VITRO*

## 3.1 Abstract

This study was conducted to isolate and screen local microbial antagonists and plant extracts for activity against *R. solanacearum*, *F. oxysporum* and *A. solani in vitro*. Isolation of microbial antagonists was done through serial dilution and screening for activity through dual culture techniques. Plant extracts were prepared in ethanol and screened through poisoned food and paper disc diffusion techniques. Antifungal activity was measured as a reduction of colony diameter while antibacterial activity was measured as the zone of inhibition. All the microbial antagonists and plant extracts tested showed significant ($P \leq 0.05$) differences in level of antagonistic activity against the test pathogens. Approximately 59.6% of total microbial isolates were fungi while 40.4% were bacteria. Twenty percent of the fungal isolates, predominantly *Trichoderma* spp., exhibited maximum growth inhibition against the three pathogens. The biocontrol agents produced inhibition zone ranging from 45.0 to 26.2 mm on *R. solanacearum* and from 79.6 to 62.9% colony growth reduction on *F. oxysporum*. *Bacillus* spp. and *Paenibacillus* spp., comprised the highest proportion of the antagonistic bacterial isolates. Approximately 35.7% and 26.7% showed inhibitory activity against *R. solanaceaum* and *F. oxysporum*, respectively. *Serratia* spp. (Sia 5Q) was the most active against *R. solanacearum* producing an inhibition zone of up to 34.5 mm while *Bacillus* spp. (Abo Z30) exhibited the highest percentage colony growth reduction on *F. oxysporum* by up to 52.6%. In the case of plant extracts, *Tagetes minuta* produced the largest inhibition zone on *R. solanacearum* of up to 11.5 mm while *Curcuma longa* had the highest percentage colony growth reduction on *A. solani* of up to 65.0%.

Key words: Biocontrol, microbial pesticides, botanical pesticides, phytopathogens

### 3.2 Introduction

Plant diseases is a major production constraint in both agricultural and horticultural systems (O'Brien, 2017; Junaid *et al*., 2013) causing up to 25% annual productivity reduction worldwide (Lugtenberg, 2015; O'Brien, 2017) and in response, farmers have resorted to heavy use of agrochemicals to mitigate the losses (Pe´rez-Garcı´a *et al*., 2011). Although agrochemicals have contributed significantly to spectacular improvement in crop productivity and quality in the past century, a lot of concern has been raised on their extensive application in production (Chandrashekara and Manivannan, 2012).

Chemical pesticides have been associated with environmental pollution and toxicity (Heydari and Pessarakli, 2010; Chandrashekara and Manivannan, 2012; Suprapta, 2012; O'Brien, 2017), contamination of food through residual chemicals that pose health risk to humans and animals (Ownley *et al*., 2010; Naher *et al*., 2014) as well as other non-target living organisms (Rehma *et al*., 2017). Furthermore, exclusive use of chemicals is a precursor to resistance development owing to high selection pressure exerted by single molecules-single site mode of action (Kohl *et al*., 2019). As a result, a lot of people have developed a change in attitude towards pesticide use and political pressure on removal of hazardous chemicals from the market has escalated (Heydari and Pessarakli, 2010; Suprapta, 2012), leading to deregistration of a number of synthetic pesticides from the market (Ownley *et al*., 2010; O'Brien, 2017).

Consequently, the drawbacks associated with chemical pesticides has led to a strong demand for more sustainable alternatives or supplements for pests and disease management (Pe´rez-Garcı´a *et al*., 2011; Rehman *et al*., 2017). Among these, the use of biological control agents (Junaid *et al*., 2013) and botanicals (Bhagwat and Datar, 2014) offers an interesting alternative (Pe´rez-Garcı´a *et al*., 2011) and is gaining a lot of importance (Junaid *et al*.,2013).

Microbial biocontrol products including antagonistic bacteria, fungi and viruses or a mixture of either in management of plant diseases (O'Brien, 2017), are ecofriendly, safe and economical compared to chemical pesticides (Carmona-Hernandez *et al*., 2019). Most biocontrol agents are pathogen specific and harmless to non-target species (O'Brien, 2017). Moreover, the ability of biocontrol agents to employ multiple modes of action reduces the probability of resistance development (Ownley *et al*., 2010) and their degree of disease suppression is comparable to that of chemical pesticides in the long run (O'Brien, 2017). Furthermore, evidence has been presented

indicating that root and leaf associated microbes can also increase plant growth and improve yield output (Ab Rahman *et al*., 2018).

Similarly, the use of locally sourced plants materials to manage crop pests and diseases is an ancient technique and so has remained a common practice in food production to date (Dubey *et al.,* 2010; Narasimha Murthy *et al.,* 2013). Plants with pesticidal properties produce compounds and substances that are toxic to phytopathogens when applied on infected crops (Castillo *et al*., 2012; Gurjar *et al.,* 2012; Bhagwat and Datar, 2014). They have evolved to develop vast diversity of chemicals with pesticidal effect due to acute and intense selection pressure exerted by the pathogens (Dubey *et al.,* 2010). The most important subclass of secondary metabolites with antimicrobial properties are phenols and their oxygen derivatives which serve as plant defense substances and include phenols, phenolic acids, quinones, flavones, flavonoids, flavonols, tannins and coumarins (Gurjar *et al.,* 2012). Furthermore, botanicals are also eco-friendly, safe at any concentration of application and easily degradable by natural soil microbes (Yang *et al.,* 2010; Dubey *et al.,* 2010; Gurjar *et al.,* 2012).

Successful suppression of phytopathogens by plant extracts (Shrisha *et al.,* 2011; Bhagwat and Datar, 2014) and antagonistic microorganisms (Koley *et al*., 2015; Topo and Naik, 2015) has been reported. In line with these reports, the current study was carried out determine antimicrobial activity of local microbial antagonists and plant extracts against major tomato pathogens *in vitro*.

### 3.3 Materials and Methods

#### 3.3.1 Collection of soil samples

Soil samples for isolation of microbial isolates were collected through purposive sampling to capture wilt infected farms in ten Agro Ecological Zones (AEZs). The counties and AEZs were selected because they are the main tomato and potato production regions in the country (KBS, 2014; GOK, 2015) and also suffer huge losses due to high prevalence and incidences of bacterial wilt (Kago *et al.,* 2016). Soil sample were collected from different regions as illustrated in table 3.1.

Twenty farms were sampled from each AEZ and in each farm soil was collected from the top 15 cm rhizosphere in five spots located 10 meters apart. The samples were thoroughly mixed and approximately 200 grams drawn for microbial isolation (Deberdt *et al.,* 2012; Yang *et al.,* 2012; Biratu *et al.,* 2013). In addition, three healthy tomato or potato plants together with their

surrounding rhizosphere soil was collected from bacterial wilt infested fields. The collected plants and soil were placed in plastic bags, transported in cool boxes to the laboratory and stored in the refrigerator at 4°C before use (Yang *et al.*, 2012; Biratu *et al.*, 2013). Isolation of microorganisms from the soil was done within 2 days after soil collection.

Some of the samples collected from the different AEZs were taken to NARL- KALRO Kabete for analysis of physicochemical properties. Analysis of physicochemical properties was conducted following diverse techniques described by Hinga *et al*., (1980). Consequently, the soil chemical properties analysed included soil pH, total nitrogen, total organic carbon, potassium, phosphorus, calcium, magnesium, manganese, copper, zinc, sodium and iron. Similarly, analysis of soil physical properties concentrated on the proportions of clay, sand and silt in the soil. The soil properties were used to explain variations in soil microbial properties.

Table 3.1: Soil sampling regions and Agro Ecological Zones located in different Counties

| County | Region/Area | AEZs | Number of collected soil samples |
|---|---|---|---|
| Embu | Siakago | LM 3 | 20 |
| Kirinyaga | Mwea-Kimbimbi | LM 3 | 20 |
| | Mwea-Wanguru | LM 4 | 20 |
| Meru | Abogatuchi West | LH1-UM1 | 20 |
| | Timau | LH3-LH4 | 20 |
| Murang'a | Kigumo | UM 3 | 20 |
| | Makuyu | UM 4 | 20 |
| Nyandarua | Njabini | UH1-UH2 | 20 |
| | Tulaga | UH2 | 20 |
| | Ndunyu Njeru | UH2-UH3 | 20 |

LM – Lower Midlands, LH – Lower Highlands, UM – Upper Midlands, UH – Upper Highlands

### 3.3.2 Isolation, quantification and identification of microbial antagonists

Isolation of microorganisms from the soil was done through plate dilution technique as described by Noveriza and Quimio, (2004), Xu and Kim, (2014), Srivastava *et al.,* (2014) and Mohammad (2015). Ten grams of soil was weighed and mixed in 90 ml of sterile distilled water (SDW). The

mixture was transferred into 250 ml conical flask and vigorously shaken for five minutes before allowing it to settle 15 minutes. One milliliter was aseptically siphoned with a micropipette and transferred to nine milliliters SDW in universal bottles. This process was repeated until a dilution of $10^{-5}$ was obtained. For fungi isolation, molten Potato Dextrose Agar (PDA) at 45 ˚C was amended with streptomycin 250 mg/l and 20 ml dispensed into petri dishes that had been treated with one milliliter of $10^{-4}$ soil suspension dilution. For bacteria isolation, one milliliter of $10^{-5}$ soil suspension dilution was poured into sterile petri dishes then 20 ml of molten nutrient agar (NA) was added. The petri dishes were swirled gently to evenly mix the soil suspension and the media then set to stand for 30 minutes to cool and solidify. The experiment was replicated three times and incubated at 24± 2˚C for three and five days for bacteria and fungi, respectively, then sealed with parafilm.

Quantification of microorganisms was done after incubation as describe by Agadagba, (2014), Srivastava *et al.,* (2014) and Okumu *et al.,* (2018). The microbial colonies appearing after incubation were carefully examined under the microscope to detect diversity then counted and grouped according to cultural and morphological similarity. Identification of the isolated microorganisms was conducted as described under section 3.3.7. The number of colony forming units was calculated by the formula:

$$\text{CFU/g of soil} = \frac{\text{Average of colonies counted x Dilution factor}}{\text{volume plated}}$$

Pure fungal isolates were sub cultured on PDA and incubated at 24± 2˚C for seven days then stored in the refrigerator at 4˚C until use. In the case of bacterial isolates, sub culturing was done on NA and incubated at 27 ˚C for three days then stored at 4˚C.

### 3.3.3 Isolation and identification of tomato pathogens

*Ralstonia solanacearum* and two fungal (*Fusarium oxysporum* f. sp. *lycopersici*; *Altenaria solani*) were isolated from diseased tomato plants collected from farmers' field in Mwea. For isolation of *R. solanacearum*, stems of infected tomato plants were sterilized in 1.5% of sodium hypochlorite solution for three minutes and consecutively rinsed in three changes of sterile distilled water (Narasimha-Murthy and Srinivas, 2012). The stems were chopped into pieces measuring 10 cm from the collar region and dipped in universal bottles containing 10 ml of SDW for oozing of

bacterial cells (Kinyua *et al.,* 2014). A small amount (0.1 ml) of the suspension was spread-plated in triplicate on pre-dried surfaces of Triphenyl Tetrazolium Chloride (TTC (0.1% dextrose, 0.1% peptone, 0.01% casamino acid and 0.18% agar with five ml of a 1% stock solution of 2-3-5 triphenyl tetrazolium chloride) agar media and incubated at 28˚C for 48 hours (Biratu *et al.,* 2013). Isolated colonies with characteristic features of *R. solanacearum,* that is, white or cream colored, irregularly shaped, highly fluidal, and opaque (Champoiseau *et al.,* 2010) were further purified on TTC media through streak plating to obtain pure cultures and incubated in inverted positions (Biratu *et al.,* 2013).

Fungal pathogens were isolated as described by Ansari *et al.,* (2012). *Fusarium oxysporum* f. sp. *lycopersici* was isolated from the roots and collar region of infected tomato plant while *Altenaria solani* was isolated from infected leaves. The infected parts were cut into pieces of five millimeters each then surface sterilized as described above. The sterilized parts were transferred onto petri dishes containing sterile PDA media and incubated at 24± 2˚C for 15 days.

Pathogen slide cultures were prepared as described by Wijedasa and Liyanapathiran (2012) for identification of fungal pathogens. Consequently, a sterile, bent glass rod was placed inside a sterile petri dish containing a sterile filter paper. A sterile glass slide was placed ontop of the bent glass rod then an aliquot of sterile molten PDA media dropped on its surface. The molten media was set for five minutes to solidify then inoculated with a small pathogen mycelia picked from a pure pathogen culture using sterile inoculating needles and covered with a sterile cover slip. The filter paper was moistened to maintain the required humidity. The petri dish lids were replaced then incubated at 24± 2˚C for 14 and 10 days for *F. oxysporum* and *A. solani*, respectively.

Identification of *F. oxysporum* f. sp. *lycopersici* was done as described by Leslie and Summerell, (2006), respectively. Accordingly, isolates with the following features were selected for further invsetigations. Slightly curved, 3-septate macroconidia arranged in a sporodochia, Tapered and curved apical cells and basal cells with foot shaped to pointed structures. Eliptical to kidney shaped microconidia hosted in short monophialides and false heads. Chlamydospores formation either terminal or intercalary in single, paired or chains.

Similarly, *A. solani* isolates were identified as described by Alhussaen (2012). Consequently, isolates possessing the following characteristics were selected. Conidiophores formed either singly

or in groups of olivaceous to flexous brown. Conidia with either 2-7 transverse septa and 1-4 longitudinal septa. Conidial shape was either slightly flexous or solitary straight, muriform to ellipsoidal, tapering to beak and pale in colour, sometimes branched.

### 3.3.4 Pathogenicity test on tomato seedlings

Pathogenicity test to assess virulence of *R. solanacearum* was conducted following a slight modification of the procedure described by Chandrashekara *et al*., (2012). Consequently, Rio Grande tomato seedlings were raised in seedling trays filled with sterilized soil for three weeks under typical screen house conditions. Plant nutrition was supplied through application foliar spray and watering was conducted twice daily, in the morning and early afternoon.

Potting media was prepared through mixing soil and sand in the ratio of 1:2. The potting media was autoclaved at 121°C for 30 minutes, set to cool then filled in pots measuring 18 cm in height and 30×15 cm in width. Three tomato seedlings were carefully picked from the seedling trays and root attached soil washed using sterile tap water. A sterile scissor was used to clip off a few tertiary roots then dipped in *R. solanacearum* suspension, standardized to $1\times 10^8$ CFUs/ml for 20 minutes. Additional 20 ml of the pathogen inoculum was added to the root region during transplanting. *Ralstonia solanacearum* inoculum was prepared as described under section 3.3.5. The seedlings were transplanted on moistened potted media and observed daily for symptom expression. Plants showing characteristic bacterial wilt symptoms were selected and used for pathogen isolation.

Fungal pathogens inoculum was prepared as described by Nirmaladevi and Srinivas, (2012). *Fusarium oxysporum* f. sp. *lycopersici* and *A. solani* were isolated and identified as described under section 3.3.3. Mass multiplication of the pathogen was conducted on sorghum grains. Briefly, 200 grams of sorghum was soaked in water for 12 hours, washed and autoclaved at 121°C for 30 minutes. The sorghum was set to cool then transferred on sterile polythene bags. Five pathogen disc picked from young and fresh cultures were transferred onto the sorghum and each pathogen culture was set separately. The inoculated sorghum was incubated at 24±2˚C for 14 days. Approximately, 50 grams of colonized sorghum was aseptically drawn and flooded with sterile distilled water to extract the conidia. The suspension was filtered through a sterile double layer cheese cloth and conidia counted using a hemocytometer. The inoculum suspension was diluted appropriately to achieve $1 \times 10^7$ conidia/ml.

In the case of *F. oxysporum* f. sp. *lycopersici* pathogenicity test, 20-day old Rio Grande tomato seedlings were prepared as described above. Similarly, a sterile scissor was used to clip off some roots then seedling dipped in the pathogen suspension for 20 minutes. Three seedlings were transplanted in potting media prepared as described above. The experiment was replicated three times and incubated in a greenhouse in completely randomized design. Plant watering was conducted as required (Nirmaladevi and Srinivas, (2012). Plants showing characteristic Fusarium wilt symptoms were selected and used for pathogen isolation.

*Alternaria solani* pathogenicity test was conducted as described by Nashwa and Abo-Elyousr, (2012). For this purpose, 20 day-old Rio Grande tomato seedlings were transplanted in pots prepared as described above. The seedlings were set to grow for one week before pathogenicity test was conducted. Consequently, *A. solani* inoculum prepared as described above was sprayed on the seedling's leaves then covered with polythene bags for 48 hours to maintain high humidity required for infection to occur. The experiment was replicated five times and pots were arranged in the completely randomized block design in the screen house. Disease severity was assessed after two weeks and symptomatic plants were selected for isolation of pathogen for further studies.

### 3.3.5 Standardization of *Ralstonia solanacearum* inoculum

Standardization of *Ralstonia solanacearum* inoculum was done to determine appropriate pathogen density for *in vitro* bioassay. The inoculum was standardized to $1.0 \times 10^8$ CFU/ml by serial dilution as described by Benson (2002) and Seleim *et al.*, (2011). Pure pathogen streaks prepared in fresh NA were washed with 10 ml of sterile distilled water into a sterile beaker and one ml of the suspension was siphoned and serially diluted to $10^{-12}$ dilution. One milliliter of the suspension was drawn from $10^{11}$ and $10^{12}$ dilutions using a micropipette and separately poured into sterile petri dishes. Approximately 20 milliliters of molten NA media were added to the inoculum, swirled and set to stand until solidification. The experiment was replicated three times and incubated in completely randomized design (CRD) at 28˚C for three days in inverted position. Isolated colonies growing on the media were counted on plates that had between 30 to 300 colonies. The formula below was used to work out the number of colony forming units per ml of the inoculum and the corresponding quantity of dilution water require to achieve $1.0 \times 10^8$ CFU/ml of inoculum suspension.

$$\text{CFU/ml} = \frac{\text{Average number of colonies counted}}{\text{volume plated}} \times \text{Dilution factor}$$

### 3.3.6 Screening of bacterial isolates for efficacy against tomato pathogens *in vitro*

Isolated bacterial isolates were screened for activity against *R. solanacearum* and *F. oxysporum* f.sp. *lycopersici* through dual plating methods. Isolates with potential antagonistic activity against *F. oxysporum* f sp. *lycopersici* were further tested for efficacy against *A. solani.* Antagonistic bioassay against *R. solanacearum* was carried out through agar spot inoculation as described by Almoneafy *et al.,* (2012) and Rado *et al.,* (2015). Approximately 200 µL *R. solanacearum* ($1.0 \times 10^8$ CFU/ml) prepared from a 48-hour old culture was aseptically plated on pre-dried surfaces of Tryptone Soya Agar (TSA) media using a micropipette and evenly spread using sterile L-shaped glass rods. Isolated colonies of freshly prepared bacteria antagonists from a 48-hour old culture were picked using a blunt sterile inoculating needle. They were point inoculated at 4 equidistant points on the surface of the pre inoculated petri dishes so that each point of inoculation was 3 cm from the center of the petri dish. The petri dishes were incubated in CRD at 24± 2˚C for 48 hours and the diameter of inhibition zone was measured every 24 hours.

For screening bacterial isolates against fungal pathogens, a technique described by Landa *et al.,* (1997), Toppo and Naik, (2015) and Mahmoud, (2016) was used. Five millimeter mycelia agar plugs were cut from the edges of an actively growing seven-day old pathogen culture using a sterile cork borer. A single plug was picked using a pair of sterile forceps and aseptically placed at the center of the petri dishes containing sterile PDA media. Pure colonies of bacterial isolates from 48-hour old cultures were picked with a sterile wire loop and streaked at four equidistant points, approximately three centimeters from the center of the petri dish. The plates were incubated at 24± 2˚C for eight days in CRD and diameter of the pathogen colony was measured every 48 hours after inoculation. Percentage reduction in colony diameter of the pathogen was calculated using the formula:

$$\text{Percentage inhibition} = \frac{R1-R2}{R1} \times 100$$

Where, R1= radial growth of pathogen colony in control

R2 = radial growth of pathogen colony in dual inoculation

### 3.3.7 Screening of fungal isolates for efficacy against tomato pathogens *in vitro*

Dual culture technique was used to screen fungal isolates for activity against *R. solanacearum*, and *F. oxysporum* f. sp. *lycopersici* as described by Devi and Chhetry, (2012) and Marcellano *et al.,* (2017). The active isolates against *F. oxysporum* f. sp. *lycopersici* were further tested for activity against *A. solani*. For bioassays against *R. solanacearum*, five mm mycelial disks were cut from the edge of a seven-day old fungal isolate culture. These plugs were seeded at the center of petri dishes containing PDA media that had been pre inoculated with *R. solanacearum* through spread plate method as described under bioassay for bacterial activity against *R. solanacearum* (Powthong *et al.,* 2013; Sibero *et al.,* 2017; Marcellano *et al.,* 2017). Three replicates were maintained for each treatment. The petri dishes incubated at 24± 2˚C for 48 hours in CRD. Data was taken by measuring the diameter of inhibition zone around the fungal plugs every 24 hours after inoculation.

In testing for activity against fungal pathogens, molten PDA media was amended with 250 mg of streptomycin/liter then approximately 20 ml poured into sterile petri dishes. Five millimeter mycelia agar plugs were cut and transferred to the center of the petri dishes as described above. Similarly, five mm mycelia agar plugs from fungal isolates were cut and placed on the petri dish at four equidistant points from the pathogen plug. petri dishes seeded only with pathogen plugs were maintained as controls (Baral *et al.,* 2011; Devi and Chhetry, 2012). The experiment was replicated thrice and plates incubated at 24± 2˚C for ten days in CRD. Measurements on growth of the pathogen was taken through measuring diagonal diameters of the pathogen every 48 hours. The percentage reduction in colony diameter of the pathogen was calculated by the formula described under the section on screening for activity of bacteria isolates against fungal pathogens.

### 3.3.8 Identification of active bacterial and fungal antagonists

The most active bacterial antagonists were identified through a series of standard physiological and biochemical tests as described by Holt *et al*., (1994), Schleifer, (2009) and Vos *et al*., (2011). These tests included gram staining and morphology, salt tolerance at 2% and 5%, growth in broth, motility, cellulose hydrolysis, lipase, catalase, hydrogen sulphide production, urease test, indole production, urease, nitrate reduction, oxidative and fermentative and utization of sugars tests.

In the case of fungi, identification was done based on cultural and morphological characteristics as described by Gams and Bisset, (2002), Watanabe, (2002), Samuels *et al*., (2012), and Sharma

and Sigh, (2014). Isolates were grown on PDA and cultural characteristics such as aggregation of conidiophore into fascicles, pustules or in effuse form, production of diffusible pigments, colony surface texture, margin, pattern, pigments, color and tint of surface and reverse, growth rates, smell and fragrance, quality of aerial hyphae were considered. Microscopic including conidiophore morphology and branching pattern, conidial morphology and topology, phialides characteristics, presence and characteristics of chlamydospores or sporangium and characteristics of the mycelia were observed and recorded for identification (Figure 3.1).

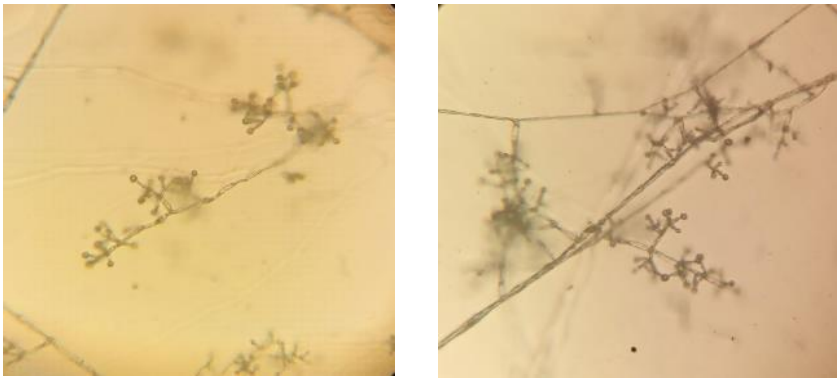

Figure 3.1: Spore bearing structures of *Trichoderma* spp. (×40 magnification)

### 3.3.9 Preparation of botanical crude extracts

Leaves, rhizomes, bulbs and stems from 19 plants were collected from Kenyatta University medicinal plants garden, University of Nairobi field station and others purchased from Ngara market in Nairobi for antimicrobial bioassays (Table 3.2).

Plant materials were washed in running tap water to remove dirt then air dried in shade to constant weight (Mahesh and Satish, 2008; Sen and Batra, 2012). One hundred grams of each of the dry material was separately weighed and blended in 500 ml of 90 % ethanol. The mixture was set to stand in the laboratory at room temperature for 48 hours with periodical agitation. It was filtered through double layer cheese and Whatman No. 1 filter paper then concentrated in a vacuum evaporator at 50˚C (Al-Samarrai *et al* 2012; Rodino *et al.*, 2014; Mostafa *et al*., 2018). The crude extracts obtained were further placed in an oven set at 60˚C to vaporize any remaining ethanol. They were used immediately for bioassays and surplus stored in the refrigerator at 4˚C until further use.

Table 3.2: Different plant parts of selected plants collected from different source for antimicrobial bioassays tests.

| Plant parts used | Common name | Scientific name | Source |
|---|---|---|---|
| Leaves | Moringa | *Moringa oleifera* | Kenyatta University |
| | Uganda greenheart | *Warbugia ugandensis* | Kenyatta University |
| | Small fruit teclea | *Teclea nobilis* | Kenyatta University |
| | Neem | *Azadarachta indica* | Kenyatta University |
| | Mint | *Mentha piperita* | Ngara Market Nairobi |
| | Basil | *Ocimum basilicum* | Ngara Market Nairobi |
| | Candle bush | *Senna alata* | Kenyatta University |
| | Croton | *Croton megalocarpus* | Kenyatta University |
| | Lemon grass | *Cymbopogon citrate* | Kenyatta University |
| Leaves and Stem | Mexican marigold | *Tagetes minuta* | University of Nairobi |
| | Rosemary | *Rosmarinum officinalis* | Ngara Market Nairobi |
| | Goat weed | *Ageratum conyzoides* | University of Nairobi |
| | Sage | *Salvia officinalis* | Ngara Market Nairobi |
| | Chenopodium | *chenopodium* spp. | Kenyatta University |
| | Thyme | *Thymus vulgaris* | Ngara Market Nairobi |
| | fleabane | *Conyza sumantresis* | University of Nairobi |
| Rhizomes | Turmeric | *Curcuma longa* | Ngara Market Nairobi |
| | Garlic | *Allium sativum* | Ngara Market Nairobi |
| | Ginger | *Zingiber officinale* | Ngara Market Nairobi |

### 3.3.10 Screening of plant extracts activity against tomato pathogens *in vitro*

Antibacterial and antifungal activity of alcoholic extracts from 19 selected plants were screened for activity against *Ralstonia solanacearum*, *Fusarium oxysporum* f.sp. *lycopersici* and *Altenaria solani*. Screening for crude extracts activity against *R. solanacearum* was conducted through paper disc diffusion technique as described by Owoseni and Sangoyomi (2014) and Biswal (2015). Approximately 200µL of the standardized pathogen suspension was drawn using a micropipette and inoculated on pre dried surfaces of TSA media. The suspension was then spread evenly using a sterile L-shaped glass rod and set to stand for 30 minutes for the liquid to be absorbed into the media. Sterilized Whitman No. 1 filter paper disk (6mm diameter) were soaked in crude extracts

for 30 minute then transferred onto the surface of the pre-inoculated media using a pair of sterile forceps. A set of four discs were used for each petri dish, placed at four equidistant points and one cm from the edges. Control plates were set with paper disks dipped in sterile distilled water. The experiment was conducted in triplicate for each extract and plates incubated at 28˚C for 48 hours in CRD. The zone of inhibition was examined as the clear halo surrounding the paper disk and measured after incubation.

Antifungal activity was carried out through poisoned food technique as described by Rodino *et al.,* (2014) and Muthomi *et al.,* (2017). Approximately two milliliters of selected plant extracts were separately siphoned using a micropipette and added to 100 ml of sterile, molten PDA media in a 250 conical flask at 45˚C then swirled gently to mix. Twenty milliliters of the treated media were poured into sterile petri dishes and set to cool and solidify. Five millimeter mycelial disks were cut using a sterile cork borer from the edges of seven-day old pathogen cultures and seeded at the center of the petri dishes. Control plates were set without adding any material to growth media. The experiment was replicated three times and incubated at 24± 2˚C for eight days in CRD. The colony diameter of the pathogen was measured every 48 hours after plating. Percentage reduction of colony diameter growth was worked out through the formula given under the section of activity of fungal isolates against fungal pathogens.

### 3.3.11 Data analysis

Data on microbial counts, antagonistic activity of both bacteria and fungi isolates and effects of plant extracts on the three pathogens were processed in Ms. Excel version 2016 before analysis. Microbial counts were calculated to CFU/g of soil and percentage colony growth reduction on fungal pathogens were worked out relative to controls. All data were analyzed by Genstat® 15th Edition software. Mean differences on microbial counts relative to AEZs, activity of bacterial and fungal antagonists and effects of plant extracts on the test pathogens were subjected to Analysis of Variance (ANOVA) and means separated using Fisher's protected Least Significant Difference ($P \leq 0.05$).

### 3.4 Results

#### 3.4.1 Physicochemical properties of soil collected from different agro ecological zones

Soil chemical and physical properties varied between the agro ecological zones in relation to soil pH, proportions of organic carbon and other elements (Table 3.3).

Table 3.3: Physicochemical properties of soil samples collected from different agro ecological zones

| Soil parameters | Kirinyaga LM 3 | | Nyandarua UH1-UH2 | | Meru LH 1-UM 1 | | Embu LM 3 | | Murang'a UM 3 | |
|---|---|---|---|---|---|---|---|---|---|---|
| Fertility results | value | comment | value | comment | value | comment | value | comment | value | comment |
| Soil pH | 5.92 | Am | 4 | Ae | 5.79 | Am | 6.25 | As | 5.14 | Am |
| Total Nitrogen % | 0.13 | L | 0.14 | L | 0.13 | L | 0.11 | L | 0.14 | L |
| Total Org. Carbon % | 1.16 | L | 1.28 | L | 1.36 | M | 0.95 | L | 1.12 | L |
| Phosphorus ppm | 55.0 | Ad | 60.0 | A | 90.0 | H | 50.0 | Ad | 45 | Ad |
| Potassium me% | 0.98 | Ad | 0.3 | A | 2.07 | H | 0.83 | Ad | 0.4 | Ad |
| Calcium me% | 1.2 | L | 1.6 | L | 5.8 | Ad | 6.2 | Ad | 1.8 | L |
| Magnesium me% | 2.46 | Ad | 1.5 | Ad | 4.04 | H | 1.1 | Ad | 1.3 | Ad |
| Manganese me% | 1.05 | Ad | 1 | Ad | 1.03 | Ad | 0.57 | Ad | 0.42 | Ad |
| Copper ppm | 1.06 | Ad | 2.75 | Ad | 5.79 | Ad | 2.6 | Ad | 5.67 | Ad |
| Iron ppm | 36.5 | Ad | 95.3 | Ad | 49 | Ad | 19.3 | Ad | 22 | Ad |
| Zinc ppm | 9.64 | Ad | 3.8 | L | 30.5 | Ad | 3.8 | L | 6.4 | Ad |
| Sodium me% | 0.22 | Ad | 0.44 | Ad | 0.94 | Ad | 0.4 | Ad | 0.4 | Ad |
| % Clay | 52 | | 24 | | 32 | | 38 | | | |
| % Sand | 36 | | 56 | | 52 | | 50 | | | |
| % Silt | 12 | | 20 | | 16 | | 12 | | | |
| Textural class | C | | SCL | | SCL | | SC | | | |

Key: As-Slightly Acidic, Am-Medium acidic, Ae-Extremely acidic, L-Low, A-Adequate, M-Moderate, H-High, C-Clay, SCL-Sandy Clay Loam, SCL-Sandy Clay Loam, SC-Sandy Clay, ppm- Parts per million.

The soil pH ranged from extreme acidity of 4.0 in UH2/UH3 in Nyandarua county to slightly acidic (6.15) in LM3 Embu county. The percentages organic carbon was generally low except for LH1-UM1 in Meru county where the highest proportion of 1.36% was detected and classified as moderate. Potassium, Phosphorus and Magnesium ratios were adequate in Kirinyaga, Embu and Nyandarua counties but high in Agro Ecological Zone (AEZ) LH1-UM1 in Meru while total nitrogen was generally low in all the regions ranging from 0.11 to 0.14. Calcium was low in Kirinyaga and Nyandarua counties while zinc was low in soil samples from Nyandarua and Embu counties. Manganese, Copper, iron and Sodium were adequate in all the tested soils.

### 3.4.2 Microbial antagonists isolated from different Agro ecological Zones

One hundred and twelve bacterial isolates and one hundred and sixty-five fungal isolates were isolated from soil samples collected in 10 Agro Ecological Zones (AEZs) distributed in five counties (Figure 3.2). The microbial isolates were categorized into 54 bacterial types and 80 fungal types based on similarity of their cultural and macroscopic characteristics (Appendix 3, Appendix 4).

Population of soil bacteria and fungi significantly ($P \leq 0.05$) differed within the AEZs (Table 3.4, Appendix 3, Appendix 4). Whitish cream, punctiform and glistening bacterial colonies were the most prevalent and frequently isolated with levels of up to $2.4 \times 10^7$ CFU/g of soil. Agro ecological zones Lower LH 1-UM 1 in Meru county had the most diverse bacterial types isolated, that is, 61.1% of the total bacterial types isolated. In contrast, Agro ecological zone LM 3 in Kirinyaga county had the least bacterial types detected, that is only 35.2% of the total bacterial types isolated. Conversely, soils collected from LM 3 in Kirinyaga county had the highest number of total bacteria population whereas those collected from LH3-LH4 in Meru county had the least number. Generally, only 25.9% of isolated bacterial types were detected in all AEZs. Bacteria population in AEZs of the same county were not significantly ($P \leq 0.05$) different (Appendix 3).

In the case of fungi, only 2.5% of the isolated types were distributed across all sampled AEZs. White, cottony, small, concave and circular fungi identified as *Penicillium* spp. was the most prevalent and frequently isolated fungi in all AEZs. However, White, cottony, concave and circular fungi with yellow to dark brown spots in the center identified as *Aspergillus* spp. had the highest levels isolated from a single AEZ with up to $8.0 \times 10^4$ CFU/g of soil in LM 3 in Kirinyaga county (Appendix 4). Agro ecological zone LH 1-UM 1 in Meru county also had the highest number of phenotypically different fungal types isolated (45%), while AEZ UH 2 in Nyandarua county had the least (18.8%). Lower Midlands 3 in Kirinyaga county had the highest number of total fungal population detected and was the only county where the number of total isolated fungi was significantly ($P \leq 0.05$) different between the AEZs (Table 3.4, Appendix 4).

Other important fungi identified were: *Trichoderma* spp. (White, flat, granulated with pale green dots, spreading and white, floccose, spread all over, irregular); *Fusarium* spp. (Purplish white, cottony or woolly and pinkish white, cottony, raised, large, irregular, spreading) and *Altenaria* spp.

(Dark gray to black, flat to raised, varicose, irregular and medium to large) which were also isolated in all the AEZs (Appendix 4).

Table 3.4: Microbial population, types and number of antagonistic fungal and bacterial isolates from different agro ecological zones (AEZs) of five counties against *Ralstonia solanacearum* (RS) and *Fusarium oxysporum* f. sp. *lycopersici* (FOL) *in vitro*

| County | AEZ | Mean microbial population | | Number of total types isolated | | Number of antagonists | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Bacteria ($1\times10^4$ CFU/g) | Fungi ($1\times10^3$ CFU/g) | Bacterial | Fungal | Bacterial antagonists | | Fungal antagonists | |
| | | | | | | RS | FOL | RS | FOL |
| Embu | LM 3 | 14.0bc | 0.8cd | 27.0 | 12.0 | 6.0 | 4.0 | 2.0 | 6.0 |
| Kirinyaga | LM 3 | 40.8a | 2.6a | 19.0 | 27.0 | 3.0. | 3.0 | 1.0 | 13.0 |
| Kirinyaga | LM 4 | 39.8a | 1.7b | 23.0 | 25.0 | 3.0 | 3.0 | 2.0 | 4.0 |
| Meru | LH1-UM1 | 19.1bc | 1.7b | 33.0 | 36.0 | 9.0 | 40.0 | 13.0 | 23.0 |
| Meru | LH3-LH4 | 9.6c | 1.6bc | 26.0 | 33.0 | 4.0 | 3.0 | 3.0 | 7.0 |
| Muranga | UM 3 | 23.9b | 1.3bcd | 21.0 | 26.0 | 4.0 | 2.0 | 1.0 | 4.0 |
| Muranga | UM 4 | 15.6bc | 1.2bcd | 30.0 | 21.0 | 1.0 | 1.0 | 1.0 | 5.0 |
| Nyandarua | UHI-UH2 | 16.3bc | 1.1bcd | 24.0 | 17.0 | 4.0 | 5.0 | 2.0 | 4.0 |
| Nyandarua | UH2 | 11.3c | 0.8d | 19.0 | 17.0 | 3.0 | 3.0 | 1.0 | 1.0 |
| Nyandarua | UH2-UH3 | 10.7c | 0.8d | 21.0 | 14.0 | 1.0 | 2.0 | 5.0 | 5.0 |
| Means | | 20.1 | 1.4 | | | | | | |
| LSD | | 8.83 | 0.47 | | | | | | |
| CV% | | 466.6 | 437.8 | | | | | | |

Means accompanied by different letters within a column are significantly different according to Fishers Protected LSD test ($P \leq 0.05$)

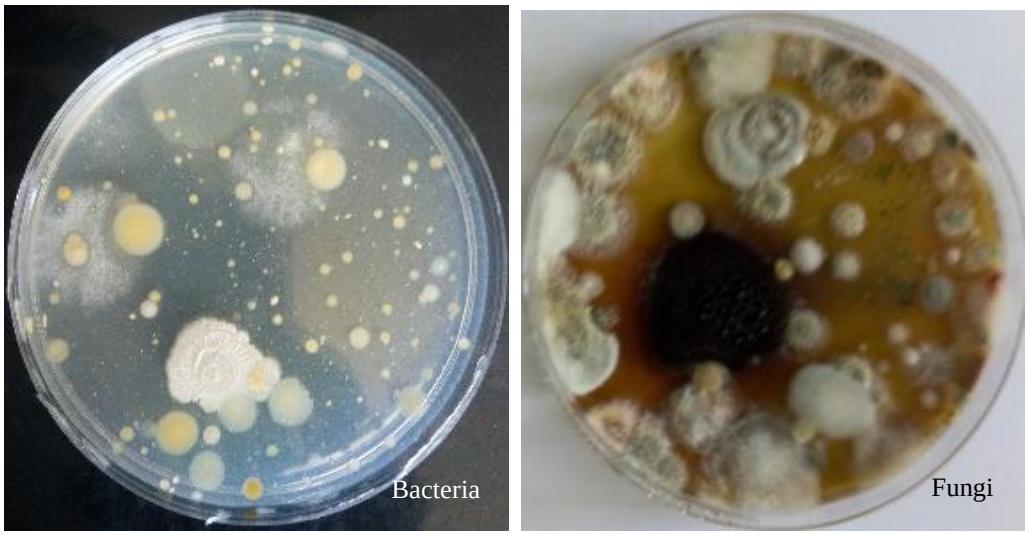


Figure 3.2: Colony forming units in bacterial and fungal isolation plates at three and five days respectively

The highest number of bacterial and fungal antagonists were isolated from AEZ LH1-UM1 in Meru county with the exception of bacterial antagonists against *F. oxysporum* which was highest in UH1-UH2 in Nyandarua county. Contrastingly, the least number of bacterial and fungal antagonists were isolated from UH2-UH3 and UH2 in Nyandarua county, respectively (Table 3.4).

### 3.4.3 Antagonistic activity of fungal isolates against tomato pathogens

One hundred and sixty-five fungal isolates were isolated from rhizosphere soils of tomato and potato crops in ten AEZs spread across five counties. The fungal isolates were subjected to antifungal activity against *F. oxysporum* and *A. solani* and antibacterial activity against *R. solanacearum in vitro* (Figure 3.3). Antagonistic effect of the isolates was rated based on means of percentage colony growth reduction for fungal pathogens and means of diameter of inhibition zones for bacterial pathogen on the tenth and second day respectively.

Approximately 25.5% of the isolates showed significant ($P \leq 0.05$) variation in levels of antagonistic activity against *R. solanacearum*, producing zones of inhibition (ZOI) ranging from 45.0 mm to 9.5 mm. Ninety percent of the most active isolates producing more than 25.0 mm ZOI were identified as *Trichoderma* species. *Trichoderma hamatum* (Abo 5) produced the largest inhibition zone of up to 47.5 mm while the least inhibition zone was produced by *Trichoderma harzianum* (Wang 10). Similar trend was observed in the repeat experiment of the best 12 isolates (Table 3.5).

Similarly, the efficacy of fungal antagonists against fungal pathogens significantly ($P \leq 0.05$) differed. Approximately 42.4% of the isolates exhibited varying levels of antagonistic activity against *F. oxysporum*. The means of percentage colony growth inhibition ranged from 20.4% to 78.3%. Approximately 18.2% of the total isolates assayed had more than 60% growth reduction and were further tested against *A. solani* and repeated against *F. oxysporum*. *Trichoderma harzianum* (Abo 11) exhibited the highest percentage colony diameter growth reduction of up to 80.8% and 78.8% on *F. oxysporum* and *A. solani*, respectively. Majority of the antagonists showed the highest level of growth reduction on the tenth day for both *F. oxysporum* and *A. solani*. Generally, *F. oxysporum* was more susceptible to the activity of antagonist compared to *A. solani* (Tables 3.6, Table 3.7).

Table 3.5: Diameter of inhibition zones on *Ralstonia solanacearum* by antagonistic fungi for experiments 1 and 2

| | | | Inhibition Zones (mm) | |
|---|---|---|---|---|
| Treatment | Isolate code | Isolate origin | Experiment 1 | Experiment 2 |
| *T. hamatum* | Abog 5 | Abogatuchi | 45.0 | 38.8 |
| *T. atroviride* | Abog 14 A | Abogatuchi | 44.0 | 39.0 |
| *T. citroviride* | Kimb 2 | Kimbimbi | 43.0 | 38.7 |
| *Trichoderma spp* | Nduny 24 C | Ndunyu njeru | 42.5 | 31.7 |
| *T. harzianum* | Mak 4 | Makuyu | 42.3 | 38.2 |
| *Trichoderma spp* | Abog 14 Y | Abogatuchi | 41.7 | 29.0 |
| | Tim 10 A | Timau | 41.2 | 38.5 |
| *T. harzianum* | Abog 11 | Abogatuchi | 40.0 | 30.7 |
| | Abog 2 A | Abogatuchi | 39.8 | 34.2 |
| *T. aereoviride* | Abog 1 D | Abogatuchi | 39.7 | 29.0 |
| *T. atroviride* | Wang 15 | Wang'uru | 39.0 | 32.0 |
| | Tim 2 | Timau | 38.7 | 28.3 |
| *T. konigii* | Abog 1 C | Abogatuchi | 38.2 | * |
| *Trichoderma spp* | Kigu 20 | Kigumo | 37.8 | * |
| *Trichoderma spp* | Abog 1 A | Abogatuchi | 36.8 | * |
| *Trichoderma spp* | Abog 1 | Abogatuchi | 36.5 | * |
| *Hypocrea vinosa* | Abog 15 C | Abogatuchi | 36.0 | * |
| *Trichoderma spp* | Abog 6 | Abogatuchi | 36.0 | * |
| *Trichoderma spp* | Nduny 24 A | Ndunyu njeru | 36.0 | * |
| *Trichoderma spp* | Nduny 24 E | Ndunyu njeru | 35.8 | * |
| *Trichoderma spp* | Tim 10 | Timau | 35.5 | * |
| *T. harzianum* | Abog 12 | Abogatuchi | 34.5 | * |
| *T. viride* | Abog 1 F | Abogatuchi | 34.3 | * |
| *Trichoderma spp* | Njab 2 | Njabini | 33.7 | * |
| *T. pseudokonigii* | Sia 7 | Siakago | 33.3 | * |
| *T. virens* | Sia 2 | Siakago | 33.0 | * |
| | Tula 16 | Tulaga | 32.2 | * |
| *T. citroviride* | Tula 4 | Njabini | 32.2 | * |
| *T. pseudokonigii* | Nduny 24 D | Ndunyu njeru | 32.0 | * |
| *Trichoderma spp* | Nduny 24 B | Ndunyu njeru | 28.5 | * |
| *T. harziunum* | Wang 10 | Wang'uru | 26.2 | * |
| Control | | | 0.0 | 0.0s |
| Means | | | 35.8 | 34 |
| LSD (P≤0.05) | | | 5.5 | 3.9 |
| CV % | | | 9.5 | 7 |

Key: Abog- Abogatuchi, Tim-Timau, Sia-Siakago, Kimb-Kimbimbi, Wang-Wang'uru, Kigu-Kigumo, Mak-Makuyu, Nja-Njabini, Tul-Tulaga, Nduny-Ndunyu Njeru, CV-Coefficient of Variation, LSD- Least Significant Difference. Data fields marked with ***** are for isolates that were not considered in the repeat experiment.

Table 3.6: Percentage colony diameter reduction on *Fusarium oxysporum* f.sp. *lycopersici* by different species of antagonistic fungi

| | | | Days after inoculation | | | | |
|---|---|---|---|---|---|---|---|
| Species | Isolate code | Origin | 2 | 4 | 6 | 8 | 10 |
| *T. harzianum* | Abog 11 | Abogatuchi | 34.1 | 60.8 | 73.4 | 77.9 | 79.6 |
| *T. hamatum* | Abog 5 | Abogatuchi | 24.0 | 55.3 | 70.0 | 74.3 | 76.8 |
| *T. harzianum* | Abog 12 | Abogatuchi | 21.7 | 54.4 | 69.0 | 74.5 | 76.8 |
| *T. atroviride* | Wang 15 | Wang'uru | 28.7 | 56.1 | 69.7 | 73.3 | 75.9 |
| *Trichoderma spp* | Tim 10 | Timau | 19.4 | 53.2 | 67.9 | 73.3 | 75.3 |
| | Abog 2A | Abogatuchi | 30.2 | 54.9 | 68.8 | 72.4 | 74.8 |
| | Tul 16 | Tulaga | 17.0 | 54.4 | 68.2 | 73.1 | 74.8 |
| *T. aereoviride* | Abog 1D | Abogatuchi | 24.8 | 58.2 | 71.4 | 74.3 | 74.7 |
| *T. viride* | Abog 1F | Abogatuchi | 24.1 | 56.1 | 67.1 | 72.4 | 74.2 |
| *T. pseudokonigii* | Nduny 24D | Ndunyu njeru | 18.7 | 51.1 | 66.2 | 71.9 | 74.2 |
| *T. citroviride* | Kimb 2 | Kimbimbi | 13.2 | 50.2 | 66.2 | 71.6 | 74.2 |
| *Hypocrea vinosa* | Abog 15C | Abogatuchi | 32.6 | 61.7 | 70.5 | 74.5 | 74.0 |
| *Trichoderma spp* | Nduny 24B | Ndunyu njeru | 20.2 | 50.6 | 65.9 | 69.5 | 73.5 |
| *Trichoderma spp* | Kigu 20 | Kigumo | 18.6 | 50.2 | 65.3 | 70.9 | 73.5 |
| *T. konigii* | Abog 1C | Abogatuchi | 28.7 | 53.3 | 66.8 | 68.5 | 73.1 |
| *T. citroviride* | Njab 4 | Njabini | 14.7 | 49.4 | 64.2 | 70.0 | 72.9 |
| | Tim 2 | Timau | 31.8 | 55.3 | 63.9 | 69.5 | 72.7 |
| *Trichoderma spp* | Nduny 24E | Ndunyu njeru | 27.1 | 55.7 | 67.7 | 69.3 | 72.7 |
| *T. harziunum* | Wang 10 | Wang'uru | 14.0 | 50.2 | 63.3 | 69.5 | 72.7 |
| *T. pseudokonigii* | Sia 7 | Siakago | 11.6 | 51.5 | 66.2 | 70.9 | 72.7 |
| *Trichoderma spp* | Abog 6 | Abogatuchi | 28.7 | 53.2 | 66.2 | 69.2 | 71.8 |
| *T. virens* | Sia 2 | Siakago | 9.3 | 50.6 | 64.8 | 71.2 | 71.6 |
| *T. harzianum* | Mak 4 | Makuyu | 24.8 | 51.1 | 64.2 | 68.8 | 71.4 |
| *Trichoderma spp* | Tul 2 | Tulagi | 24.0 | 57.3 | 63.4 | 67.6 | 70.5 |
| *Trichoderma spp* | Abog 1 | Abogatuchi | 24.9 | 54.1 | 66.0 | 69.7 | 70.3 |
| | Tim 10A | Timau | 18.6 | 54.8 | 68.0 | 69.3 | 70.3 |
| *T. atroviride* | Abog 14A | Abogatuchi | 20.2 | 51.1 | 62.8 | 63.7 | 67.5 |
| *Trichoderma spp* | Nduny 24C | Ndunyu njeru | 8.5 | 45.2 | 61.2 | 67.3 | 67.5 |
| *Trichoderma spp* | Abog 14Y | Abogatuchi | 19.4 | 49.4 | 63.4 | 64.4 | 65.9 |
| *Trichoderma spp* | Abog 1A | Abogatuchi | 18.6 | 52.4 | 66.2 | 70.7 | 65.5 |
| *Trichoderma spp* | Nduny 24A | Ndunyu njeru | 13.2 | 50.2 | 62.9 | 71.7 | 62.9 |
| Control | | | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| Means | | | 20.8 | 51.6 | 64.4 | 68.6 | 70.1 |
| LSD(P≤0.05) | | | 2.8 | 2.6 | 2.6 | 2.4 | 2.9 |
| CV % | | | 8.2 | 3.1 | 2.5 | 2.1 | 2.5 |

Key: Abog- Abogatuchi, Tim-Timau, Sia-Siakago, Kimb-Kimbimbi, Wang-Wang'uru, Kigu-Kigumo, Mak-Makuyu, Nja-Njabini, Tul-Tulaga, Nduny-Ndunyu Njeru, CV-Coefficient of Variation, LSD- Least Significant Difference.

Table 3.7: Percentage colony diameter reduction of mycelia growth of *Altenaria solani* by diverse species of antagonistic fungi

| | | | Days after inoculation | | | | |
|---|---|---|---|---|---|---|---|
| Treatment | Isolate code | Isolate origin | 2 | 4 | 6 | 8 | 10 |
| *T. harzianum* | Abog 11 | Abogatuchi | 27.7 | 44.9 | 58.4 | 71.6 | 77.5 |
| *Trichoderma spp.* | Tim 10 | Timau | 25.5 | 47.8 | 60.0 | 70.4 | 76.6 |
| *Trichoderma spp.* | Abog 5 | Abogatuchi | 21.4 | 42.0 | 56.9 | 70.0 | 76.3 |
| *Trichoderma spp.* | Abog 14B | Abogatuchi | 26.5 | 41.4 | 56.3 | 69.2 | 75.6 |
| *T. viride* | Abog 1F | Abogatuchi | 25.6 | 44.3 | 58.5 | 69.6 | 75.3 |
| *Trichoderma spp* | Abog 1 | Abogatuchi | 17.6 | 45.8 | 58.5 | 69.6 | 75.3 |
| *T. konigii* | Abog 1C | Abogatuchi | 23.5 | 42.8 | 58.4 | 68.8 | 75.0 |
| *Trichoderma spp.* | Mak 4 | Makuyu | 22.5 | 41.3 | 56.9 | 67.6 | 75.0 |
| *T. harzianum* | Wang 10 | Wang'uru | 18.5 | 40.6 | 57.9 | 68.4 | 74.7 |
| *T. harzianum* | Abog 12 | Abogatuchi | 17.5 | 41.4 | 53.2 | 67.7 | 74.7 |
| | Abog 2A | Abogatuchi | 27.6 | 46.4 | 59.0 | 68.8 | 74.1 |
| *Trichoderma spp.* | Abog 14A | Abogatuchi | 24.5 | 47.1 | 58.5 | 67.6 | 74.1 |
| *T. atroviride* | Wang 15 | Wang'uru | 22.4 | 42.0 | 56.4 | 66.8 | 73.7 |
| *T. aereoviride* | Abog 1D | Abogatuchi | 23.5 | 40.6 | 58.4 | 68.8 | 73.4 |
| *T. citroviride* | Njab 4 | Njabini | 21.6 | 35.6 | 53.2 | 66.0 | 73.4 |
| *Hypocrea vinosa* | Abog 15C | Abogatuchi | 19.4 | 40.6 | 57.9 | 68.0 | 73.4 |
| *Trichoderma spp.* | Tim 2 | Timau | 24.6 | 43.5 | 57.4 | 67.2 | 73.1 |
| *T. citroviride* | Kimb 2 | Kimbimbi | 14.4 | 38.5 | 54.3 | 66.4 | 72.8 |
| *Trichoderma spp* | Kigu 20 | Kigumo | 16.3 | 36.2 | 53.7 | 65.2 | 72.2 |
| | Njab 2 | Njabini | 16.3 | 37.7 | 52.7 | 64.0 | 71.2 |
| *T. virens* | Sia 2 | Siakago | 13.4 | 34.9 | 52.2 | 64.5 | 71.2 |
| *Trichoderma spp.* | Abog 6 | Abogatuchi | 16.3 | 37.0 | 52.1 | 63.3 | 70.6 |
| | Tul 16 | Tulagi | 13.2 | 36.2 | 53.7 | 64.8 | 70.6 |
| *Trichoderma spp* | Nduny 24B | Ndunyu njeru | 14.3 | 33.4 | 51.1 | 62.5 | 70.3 |
| *Trichoderma spp.* | Abog 1A | Abagatuchi | 2.0 | 29.0 | 48.4 | 61.7 | 69.3 |
| | Tim 10A | Timau | 19.4 | 41.3 | 51.6 | 63.6 | 69.0 |
| *T. pseudokonigii* | Nduny 24D | Ndunyu njeru | 14.5 | 28.3 | 46.3 | 60.5 | 68.7 |
| *T. pseudokonigii* | Sia 7 | Siakago | 13.2 | 31.9 | 50.0 | 62.9 | 68.7 |
| *Trichoderma spp* | Nduny 24E | Ndunyu njeru | 23.6 | 24.0 | 46.4 | 58.2 | 65.8 |
| *Trichoderma spp.* | Nduny 24C | Ndunyu njeru | 20.3 | 21.8 | 44.8 | 56.6 | 65.5 |
| *Trichoderma spp.* | Nduny 24A | Ndunyu njeru | 19.4 | 20.3 | 42.7 | 56.5 | 64.9 |
| Control | | | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| Means | | | 19.0 | 36.8 | 52.4 | 63.7 | 70.1 |
| LSD(P≤0.05) | | | 5.9 | 6.1 | 4.1 | 3.4 | 2.8 |
| CV% | | | 19.0 | 10.1 | 4.8 | 3.3 | 2.5 |

Key: Abog- Abogatuchi, Tim-Timau, Sia-Siakago, Kimb-Kimbimbi, Wang-Wang'uru, Kigu-Kigumo; Mak-Makuyu, Nja-Njabini, Tul-Tulaga, Nduny-Ndunyu Njeru, CV-Coefficient of Variation, LSD- Least Significant Difference.

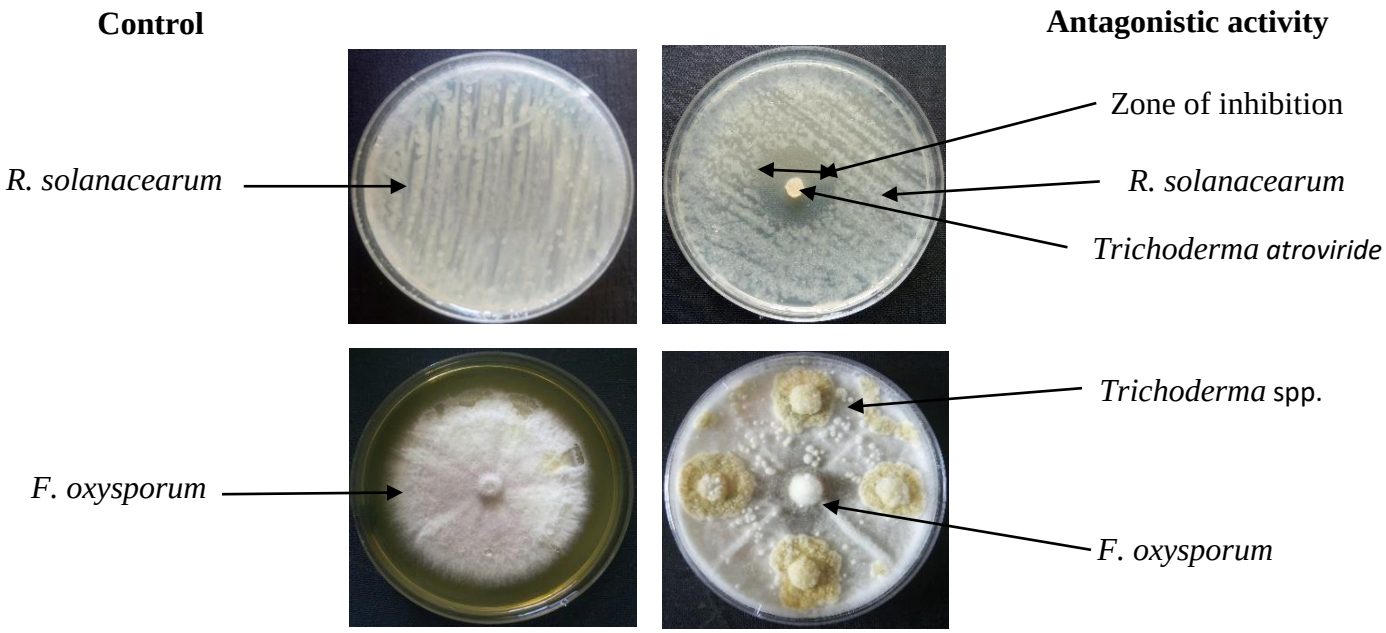


Figure 3.3: *In vitro* activity of some antagonistic fungal isolate against bacterial and fungal test pathogens at one and eight days after inoculation, respectively**.**

### 3.4.4 Antagonistic activity of bacterial isolates against tomato pathogens

A total of 112 bacterial isolates were isolated from rhizosphere soils of tomato and potato across ten AEZs in five counties. They were screened and tested for activity against *R. solanacearum*, *F. oxysporum* and *A. solani* as previously described (Figure 3.4). Antagonistic activity of the isolates was rated based on means of diameter of the inhibition zones on bacterial pathogen and percentage colony growth reduction of fungal pathogens. Among the bacterial isolates recovered, 35.7% significantly (P≤0.05) differed on levels of antagonistic activity on *R. solanacearum*, thus producing zones of inhibition ranging from 30.1 mm to 5.0 mm. *Serratia* spp. (Sia 5Q) was the most effective in inhibiting growth of *R. solanacearum*, producing an inhibition zone of up to 34.5 mm (Table 3.8).

Similarly, significant (P≤0.05) variations was observed for bacterial antagonists against the fungal pathogens. Approximately 26.8% of the isolates screened for efficacy against *F. oxysporum* exhibited more than 10% colony diameter growth reduction on the eighth day after inoculation. *Bacillus* spp. (Tim Z30) had the highest percentage colony growth reduction of up to 52.6% on *F. oxysporum* compared to untreated control. In the repeat experiment, a similar trend was observed and the isolates exhibited higher activity compared to the first experiment (Tables 3.9).

Table 3.8: Diameter of inhibition zones on *Ralstonia solanacearum* by antagonistic bacteria for experiment 1 and 2

| | | Zones of inhibition (mm) | |
|---|---|---|---|
| Treatment | Isolate codes | Experiment 1 | Experiment 2 |
| *Serratia* spp. | Sia 5Q | 30.1 | 27.7 |
| *Paenibacillus* spp | Abog 14Y | 25.3 | 22.1 |
| *Bacilllus* spp. | Abog 20 Z7 | 24.8 | 20.1 |
| *Bacillus* spp. | Kimb 3O | 23.8 | 25.2 |
| Unidentified | Tim 10 Z5 | 19.3 | 19.5 |
| *Paenibacillus* spp. | Wang 16Ki | 19.2 | 19.0 |
| *B. cereus* | Abog 8D | 18.5 | 15.7 |
| *Paenibacillus* spp. | Kimb 9L | 17.5 | 17.1 |
| *Arthrobacter* spp. | Sia 3E | 17.2 | 15.6 |
| *B. subtilis* | Abog 20 Z21 | 16.6 | 13.5 |
| *Listeria spp.* | Sia 3 | 16.6 | 15.6 |
| Unidentified | Mak 6 | 16.5 | 14.4 |
| *Pseudomonas* spp. | Sia 6H | 15.7 | 12.4 |
| *B. licheniformis* | Abog 14B | 15.7 | 16.7 |
| *B. cereus* | Wang 17 | 15.1 | 14.4 |
| *Acinetobacter spp.* | Tul 15R | 15.0 | 18.3 |
| Unidentified | Nduny 27R | 14.7 | 14.0 |
| *B. mycoides* | Tul 18R | 14.4 | 14.8 |
| *Pseudomonas* spp. | Abog 4A | 14.0 | 11.0 |
| *B. circulans* | Tul 12 | 14.0 | 12.7 |
| Unidentified | Njab 1B | 13.0 | 10.3 |
| *B. cereus* | Kigu 23R | 12.9 | 15.1 |
| *B. subtilis* | Tim 3 | 12.3 | 10.4 |
| Unidentified | Njab 1A | 12.1 | 9.9 |
| Unidentified | Njab 7R | 12.1 | 7.8 |
| *Pseudomonas spp.* | Wang 16Kii | 11.3 | 10.6 |
| Unidentified | Mak 6B | 11.0 | 8.2 |
| *Bacillus spp.* | Tim 3 Z31 | 9.8 | 8.7 |
| *Bacillus spp.* | Tim 3 Z30 | 8.9 | 9.6 |
| *Paenibacillus spp.* | Mak 7 | 8.5 | 4.5 |
| *Bacillus spp.* | Njab 9 C10 | 8.1 | 5.4 |
| *B. subtilis* | Njab 9C | 7.3 | 9.1 |
| Control | | 0.0 | 0.0 |
| Means | | 13.5 | 14.2 |
| LSD (P≤0.05) | | 4.5 | 2.9 |
| CV % | | 20.2 | 12.4 |

Key: Abog- Abogatuchi, Tim-Timau, Sia-Siakago, Kimb-Kimbimbi, Wang-Wang'uru, Kigu-Kigumo, Mak-Makuyu, Nja-Njabini, Tul-Tulaga, Nduny-Ndunyu Njeru, CV-Coefficient of Variation, LSD- Least Significant Difference.

Table 3.9: Percentage colony diameter reduction of mycelia growth of *Fusarium oxysporum* f. sp. *lycopersici* by diverse species of antagonistic bacteria for experiment 1 and 2.

| | | Experiment 1 | | | | Experiment 2 | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Days after inoculation | | | | | | | |
| Antagonists | Isolate codes | 2 | 4 | 6 | 8 | 2 | 4 | 6 | 8 |
| *Bacillus* spp. | Tim 3 Z30 | 16.5 | 33.3 | 43.7 | 49.8 | 10.8 | 40.1 | 52.0 | 62.6 |
| *Bacillus* spp. | Tim 3 Z31 | 18.7 | 29.9 | 40.3 | 47.9 | 12.3 | 39.0 | 48.4 | 58.8 |
| *Paenbacillus* spp. | Wang 16Ki | 18.9 | 34.4 | 45.4 | 45.9 | 12.3 | 36.6 | 49.5 | 60.6 |
| *Bacillus* spp. | Kimb 3O | 17.2 | 26.7 | 37.2 | 41.9 | 6.9 | 31.9 | 42.8 | 53.5 |
| *B. subtilis* | Njab 9C | 15.1 | 24.5 | 34.9 | 41.3 | 6.9 | 26.4 | 37.6 | 46.8 |
| *B. circulans* | Tul 12B | 16.5 | 23.5 | 35.9 | 40.8 | 12.3 | 33.4 | 41.4 | 48.1 |
| *Arthrobacter* spp. | Sia 3E | 16.5 | 27.8 | 35.7 | 40.4 | 11.5 | 33.4 | 46.5 | 57.3 |
| *B. subtils* | Abog 20 Z7 | 13.6 | 23.1 | 23.0 | 34.7 | 7.7 | 13.5 | 12.9 | 28.1 |
| *Paenibacillus* spp | Kimb 9L | 16.0 | 34.1 | 36.6 | 33.9 | 10.8 | 15.1 | 17.0 | 29.8 |
| *Bacillus spp.* | Njab 7R | 15.2 | 26.4 | 35.8 | 33.3 | 10.0 | 15.1 | 15.5 | 29.8 |
| *Bacillus* spp. | Njab 9C 10 | 5.8 | 15.6 | 19.3 | 33.1 | * | * | * | * |
| *Acinetobacter* spp. | Tul 15R | 14.5 | 15.3 | 24.5 | 32.8 | * | * | * | * |
| *B. mycoides* | Tul 18R | 14.5 | 16.7 | 27.2 | 31.6 | * | * | * | * |
| *Pseudomonas* spp. | Wang 16Kii | 15.2 | 27.1 | 30.2 | 31.4 | * | * | * | * |
| *Paenibacillus spp.* | Mak 7 | 9.4 | 9.0 | 18.0 | 29.7 | * | * | * | * |
| *Pseudomonas* spp. | Sia 6Hii | 9.4 | 13.8 | 23.9 | 28.8 | * | * | * | * |
| *B. cereus* | Kigu 23R | 4.4 | 16.7 | 23.8 | 27.7 | * | * | * | * |
| *B. licheniformis* | Abog 14B | 13.8 | 19.5 | 24.5 | 26.8 | * | * | * | * |
| *B. cereus* | Wang 17 | 16.0 | 17.7 | 22.9 | 26.6 | * | * | * | * |
| *B. mycoides* | Nduny 23R | 12.9 | 10.8 | 16.8 | 21.4 | * | * | * | * |
| *Paenibacillus* spp. | Abog 14Y | 13.7 | 7.5 | 18.6 | 17.8 | * | * | * | * |
| *B. subtilis* | Tim 3 | 5.8 | 4.3 | 14.5 | 17.8 | * | * | * | * |
| *Serratia* spp. | Sia 5Q | 10.9 | 4.4 | 18.2 | 13.0 | * | * | * | * |
| *P. fluorescence* | Kimb 6A | 5.7 | 9.4 | 11.9 | 12.8 | * | * | * | * |
| *Pseudomonas* spp. | Abog 4A | 14.4 | 6.5 | 15.5 | 12.5 | * | * | * | * |
| *B. cereus* | Abog 8D | 5.8 | 12.9 | 21.5 | 6.5 | * | * | * | * |
| *Listeria* spp. | Sia 3 | 7.3 | 6.6 | 14.7 | 3.8 | * | * | * | * |
| Unidentified | Njab 1A | 4.4 | 7.6 | 15.7 | 15.1 | * | * | * | * |
| Unidentified | Mak 6 | 5.0 | 11.9 | 15.2 | 14.4 | * | * | * | * |
| Unidentified | Njab 1B | 12.3 | 17.7 | 20.7 | 14.3 | * | * | * | * |
| Unidentified | Nduny 27R | 11.5 | 8.7 | 13.2 | 14.0 | * | * | * | * |
| Unidentified | Tim 10 Z5 | 11.6 | 5.6 | 15.2 | 2.1 | * | * | * | * |
| Control | | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| Means | | 10.6 | 14.7 | 21.8 | 21.8 | 9.2 | 25.9 | 33.1 | 43.2 |
| LSD (P≤0.05) | | 2.4 | 3.6 | 3.5 | 3.6 | 3.2 | 3.6 | 3.2 | 3.7 |
| CV % | | 14.1 | 14.9 | 10 | 10.1 | 20.6 | 8.2 | 5.7 | 5.1 |

Key: Abog- Abogatuchi, Tim-Timau, Sia-Siakago, Kimb-Kimbimbi, Wang-Wang'uru, Kigu-Kigumo, Mak-Makuyu, Nja-Njabini, Tul-Tulaga, Nduny-Ndunyu Njeru, CV-Coefficient of Variation, LSD- Least Significant Difference. Data fields marked with ***** are for isolates that were not considered in the repeat experiment.

Table 3.10: Percentage colony diameter reduction of mycelia growth of *Altenaria solani* by antagonistic bacteria

| Antagonists | Isolate codes | Days after inoculation | | | |
|---|---|---|---|---|---|
| | | 2 | 4 | 6 | 8 |
| *Bacillus* spp. | Tim Z30 | 29.7a | 39.3a | 44.0a | 47.4a |
| *Paenibacillus* spp. | Wang 16Ki | 30.4a | 37.4ab | 40.4ab | 43.1ab |
| *Bacillus spp.* | Tim Z31 | 27.2ab | 35.4ab | 39.2b | 43.5ab |
| *Bacillus* spp. | Kimb 3O | 28.0ab | 34.0bc | 37.6b | 40.5bc |
| *B. circulans* | Tul 12B | 27.2ab | 35.4ab | 36.4b | 37.9cd |
| *Arthrobacter spp.* | Sia 3E | 20.0c | 26.7de | 31.7c | 36.9cd |
| *B. subtilis* | Njab 9C | 23.4bc | 29.6cd | 31.6c | 34.2d |
| *Bacillus* spp. | Njab 7R | 28.9a | 29.7cd | 23.7d | 23.4e |
| *Pseudomonas* spp. | Wang 16Kii | 28.8a | 33.0bc | 29.6c | 21.4e |
| *Paenibacillus* spp. | Kimb 9L | 22.4c | 24.8e | 17.6e | 20.5e |
| Control | | 0.0d | 0.0f | 0.0f | 0.0f |
| Means | | 24.2 | 29.6 | 30.2 | 31.7 |
| LSD (P≤0.05) | | 4.7 | 4.5 | 4.5 | 4.6 |
| CV% | | 11.5 | 8.8 | 8.7 | 8.6 |

Means accompanied by different letters within a column are significantly different according to Fishers Protected LSD test (P ≤0.05)

*Alternaria solani* was less susceptible to inhibitory effects of antagonistic bacteria compared to *F. oxysporum* at 8 DAI. The efficacy of bacterial antagonists also increased with time and the most active isolates exhibited maximum growth reduction on the 8$^{th}$ DAI. (Tables 3.9, Table 3.10). Generally, the most effective isolates in inhibiting growth of *F. oxysporum* were also the most active against *A. solani* but were poor against *R. solanacearum* and vice versa. Specifically, only three isolates, *Paenibacillus* spp. (Wang 16Ki), *Paenibacillus* spp. (Kimb 3O), and *Bacillus* spp. (Kimb 9L) existed among the ten best isolates inhibiting growth of all the three pathogens (Table 3.8, Table 3.9, Table 3.10).

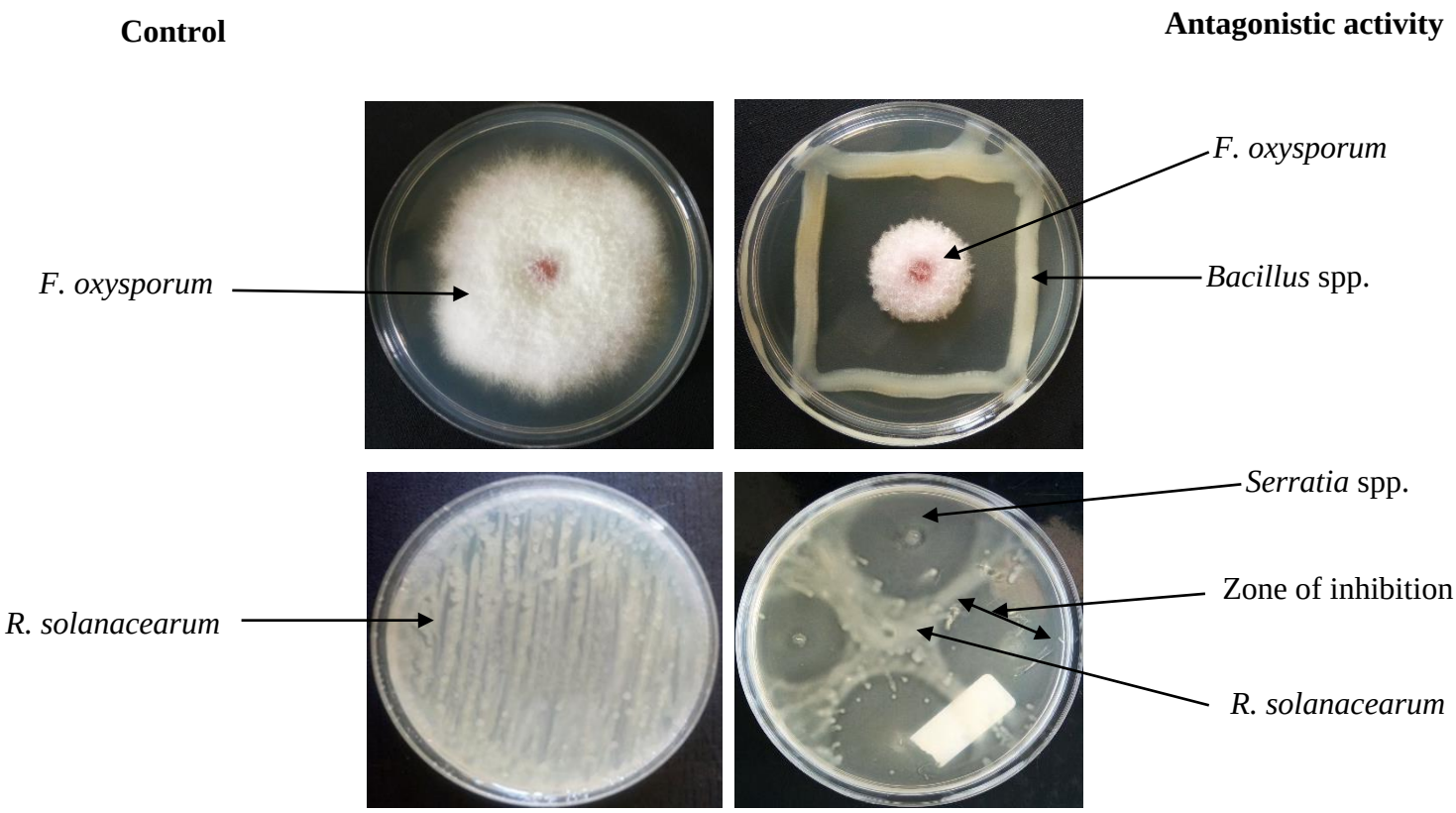


Figure 3.4: *In vitro* activity of some antagonistic bacterial isolates against fungal and bacterial test pathogens at eight and one day after inoculation, respectively.

Table 3.11: Morphological, biochemical and growth characteristics of antagonistic bacterial isolates

| Isolate codes | Growth on broth | Morphology | Grams staining | Motility | Salt tolerance (5% | Cellulose utilization | Starch hydrolysis | Catalase | H2S Production | Indole production | Lipase | urease production | O/F test | Sugar utilization | | | | Proposed name |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | | | | | Sucrose | Glucose | Mannitol | Lactose | |
| Sia 3 | Turbid | Bacilli | + | - | + | + | - | + | + | + | - | - | F | + | + | + | + | *Listeria* spp. |
| Sia 3E | sediment | Bacilli | + | - | + | - | - | + | + | + | + | + | - | - | - | - | - | *Arthrobacter* spp. |
| Sia 5 Z34 | Turbid | Coccus | + | - | + | - | - | + | + | + | + | - | - | - | - | - | - | *Staphylococcus* spp. |
| Sia 5Q | Turbid | Bacilli | - | - | + | - | + | + | + | + | - | - | O | - | + | - | - | *Serratia* spp. |
| Wang 16Ki | sediment | Bacilli | + | - | + | - | + | + | + | + | - | + | - | - | - | - | - | *Paenibacillus* spp. |
| Wang 16Kii | Turbid | | - | + | + | - | - | + | - | + | - | - | | + | + | + | - | *Bacillus* spp. |
| Wang 17 | sediment | Streptobacilli | + | + | + | + | + | + | | | - | - | F | + | + | - | - | *B. cereus* |
| Wang 17 Z1 | sediment | Streptobacilli | + | + | + | + | + | + | - | + | - | + | F | - | - | - | - | *Bacillus* spp. |
| Kimb 9L | sediment | Bacilli | + | - | + | - | + | + | + | + | - | + | - | - | - | - | + | *Paenibacillus* spp. |
| Kimb 6A | Turbid | Streptobacilli | - | + | + | - | + | + | - | + | - | - | F | - | + | - | - | *P. flourescence* |
| Abog 1Y | Turbid | Bacilli | - | + | + | - | + | + | - | + | - | + | F | + | + | + | + | *Pseudomonas* spp. |
| Abog 1X | Flocculent | Bacilli | + | + | + | - | | - | - | + | - | - | F | + | + | - | + | unidentified |
| Abog 14 Z20 | Turbid | Bacilli | + | - | + | + | + | - | + | - | + | + | - | + | - | - | - | *Paenibacillus* spp. |
| Abog 14Q | Turbid | Bacilli | + | - | + | + | | + | + | + | - | - | F | + | + | + | + | *Paenibacillus* spp. |
| Abog 14B | Flocculent | Bacilli | + | + | + | + | + | + | + | + | - | + | F | + | + | + | - | *B. licheniformis* |
| Abog 17B | sediment | Streptobacilli | + | + | + | - | + | - | - | + | + | + | F | + | + | + | + | *B.mycoides* |
| Abog 20 Z21 | Flocculent | Bacilli | + | + | + | + | + | - | + | + | - | - | F | + | + | + | - | *B. subtulis* |
| Abog 20 Z7 | sediment | Bacilli | - | + | + | - | + | + | + | + | + | - | - | - | - | - | - | *B. subtilis* |
| Abog 4 | sediment | Bacilli | + | - | + | + | + | + | - | - | - | - | F | + | + | + | - | *B. subtilis* |
| Abog 4A | Turbid | Bacilli | - | + | + | + | + | + | - | + | - | - | F | - | + | + | - | *Pseudomonas* spp. |
| Abog 8D | sediment | Streptobacilli | + | + | + | + | + | + | - | + | + | - | F | + | + | - | - | *B. cereus* |
| Tim 10 Z5 | Turbid | Bacilli | + | - | + | + | - | + | + | - | + | - | | | | | | unidentified |
| Tim 3 | sediment | Bacilli | + | + | + | + | + | + | - | + | - | - | F | - | + | - | - | *B. subtilis* |

**Continued**

| Isolate codes | Growth on broth | Morphology | Grams staining | Motility | Salt tolerance (5% | Cellulose utilization | Starch hydrolysis | Catalase | H2S Production | Indole production | Lipase | urease production | O/F test | Utilization of sugar | | | | Proposed name |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | | | | | Sucrose | Glucose | Mannitol | Lactose | |
| Tim 3 Z30 | sediment | Bacilli | + | - | + | - | - | + | + | + | - | + | - | - | - | - | - | *Bacillus* spp. |
| Tim 3 Z31 | sediment | Bacilli | + | - | + | - | - | + | + | + | - | + | - | - | - | - | - | *Bacillus* spp. |
| Kigu 23 | sediment | Streptobacilli | + | + | + | | + | + | - | + | - | + | F | + | + | - | - | *B. cereus* |
| Mak 6 | Turbid | Streptobacilli | + | + | + | + | + | - | - | + | - | - | F | + | + | + | - | unidentified |
| Mak 4 | sediment | Coccus | + | - | + | - | - | + | + | - | - | + | - | - | - | - | - | *Staphylococcus* spp. |
| Mak 7 | Turbid | Bacilli | + | - | + | - | + | + | - | + | - | - | F | + | + | + | - | *Paenibacillus* spp. |
| Mak 9B | Turbid | Bacillus | + | + | + | + | + | + | - | + | - | - | F | + | + | + | - | *B. subtilis* |
| Njab 1A | Turbid | Streptobacilli | + | + | + | - | + | - | + | + | + | - | - | - | - | + | + | unidentified |
| Tul 12B | Turbid | Bacilli | - | + | + | - | + | + | + | + | - | - | F | + | + | + | + | *B. circulans* |
| Tul 15R | Turbid | Bacilli | - | - | + | - | + | + | | + | - | + | - | - | - | + | + | *Acinetobacter* spp. |
| Nduny 27R | Flocculent | Streptobacilli | + | + | + | + | + | - | - | + | - | - | F | + | + | - | - | unidentified |
| Njab 9C | sediment | Bacilli | + | - | + | + | + | - | + | - | - | - | - | - | - | - | - | *Bacillus spp.* |
| Nduny 23r | Turbid | Streptobacilli | + | - | + | - | + | + | | + | - | - | F | + | + | - | - | *B. mycoides* |

### 3.4.5 Activity of plant extract against tomato pathogens

A total of 19 plants were tested for antimicrobial activity against bacterial and fungal test pathogens through paper disc diffusion and poisoned food techniques, respectively (Figure 3.5). All the crude plant extracts tested significantly ($P \leq 0.05$) differed in their level of growth inhibition on *R. solanacearum, F. oxysporum* f.sp. *lycopersici* and *A. solani*.

Approximately 36.8% of the total plants assayed produced inhibition zones greater than nine millimeters on *R. solanacearum*. These comprised of two weed plants, two trees and three spices. *Tagetes minuta* produced the largest inhibition zone of up to 11.5 mm on *R. solanacearum*. It was closely followed in activity by *R. officinalis* and *C. longa* while *C. citrate* had the least effect. Similar trends were observed in repeat experiments with *T. minuta* exhibiting the largest inhibition zone and *T. nobilis* producing the smallest zone of inhibition (Tables 3.12). *Curcuma longa, R. officinalis* and *S. officinalis* exhibited strong antifungal and antibacterial activity whereas some like *T. minuta* only had good antibacterial activity but poor antifungal activity (Table 3.12, Table 3.13, Table 3.14).

For antifungal screening, only 15.8% including two spices and one tree had more than 50 % colony diameter growth reduction on *F. oxysporum*. More than half (52.6%) of the screened plants gave less than 30% colony diameter growth reduction on *F. oxysporum* (Table 3.13). *Curcuma longa* exhibited the highest percentage reduction in colony diameter growth of *F. oxysporum* by up to 55.7 %. It was closely followed in activity by *S. officinalis* and *T. nobilis* while *O. basilicum* had the least antifungal activity among the tested plant species. In the repeat experiment, crude extract of *C. longa* remained the most active, exhibiting percentage reduction in colony diameter growth of *A. solani* and *F. oxysporum* by up to 65.0% and 59.2%, respectively at eight DAI (Table 3.13, Table 3.14).

Generally, there was a reduction in antifungal activity of crude extracts over time and most extracts achieved peak inhibition on the second day after inoculation except for *C. longa, S. officinalis* and *A. conyzoides.* Contrast to findings of microbial antagonists' activity, *A. solani* was more susceptible to effects of crude plant extracts compared to *F. oxysporum* f.sp. *lycopersici* (Table 3.13, Table 3.14).

Table 3.12: Diameter of inhibition zones on *Ralstonia solanacearum* by crude plant extracts for experiment 1 and 2.

| | Inhibition Zones in millimeter | |
|---|---|---|
| Source of Extract | Experiment 1 | Experiment 2 |
| Mexican marigold | 11.3 a | 10.9a |
| Rosemary | 10.3 b | 9.0bc |
| Turmeric | 10.0 bc | 9.4b |
| Moringa | 9.6 bcd | 8.8bcd |
| Uganda greenheart | 9.3 cde | 8.0def |
| Garlic | 9.3 cde | 7.9defg |
| Goat weed | 9.1 def | 8.1cde |
| Sage | 8.5 efg | 7.1fg |
| Small fruit teclea | 8.3 fgh | 7.0g |
| Chenopodium | 8.3 fgh | 7.3efg |
| Thyme | 8.1 ghi | 7.4efg |
| Ginger | 8.1 ghi | 9.1b |
| Neem | 7.9 ghi | * |
| Mint | 7.8 ghi | * |
| Fleabane | 7.8 ghi | * |
| Basil | 7.8 ghi | * |
| Candle bush | 7.7 ghi | * |
| Croton | 7.6 hi | * |
| Lemon grass | 7.3 i | * |
| Control | 6.0 j | 6.0 h |
| Means | 8.6 | 8.2 |
| LSD(P≤0.05) | 0.9 | 0.9 |
| CV % | 6.1 | 6.9 |

Means accompanied by different letters within a column are significantly different according to Fishers Protected LSD test (P ≤0.05). Data fields marked with ***** are for isolates that were not considered in the repeat experiment.

Table 3.13: Percentage colony diameter reduction of mycelia growth of *Fusarium oxysporum* f. sp. *lycopersici* by crude plant extracts in experiment 1 and 2

| | Experiment 1 | | | | Experiment 2 | | | |
|---|---|---|---|---|---|---|---|---|
| | Days after inoculation | | | | | | | |
| Source of extract | 2 | 4 | 6 | 8 | 2 | 4 | 6 | 8 |
| Turmeric | 55.5a | 53.9 ab | 54.6 a | 55.2 a | 60.8a | 57.9a | 56.9a | 58.9a |
| Sage | 45.8 d | 50.0 cd | 48.8 b | 52.3 ab | 47.2c | 48.3b | 46.1b | 46.9b |
| Small fruit teclea | 51.8 bc | 57.0 a | 51.5 ab | 50.7 bc | 48.7bc | 40.0cd | 37.9c | 37.8c |
| Rosemary | 53.0 ab | 51.5 bc | 48.2 b | 47.0 c | 41.5de | 41.7c | 39.1c | 39.4c |
| Goat weed | 51.8 bc | 47.3 d | 40.9 c | 42.1 d | 46.7c | 41.2cd | 38.5c | 31.4e |
| Ugandan greenheart | 36.8 e | 34.5 f | 35.9 de | 38.6 de | 50.4b | 34.2e | 30.6d | 27.0f |
| Thyme | 47.2 d | 39.9 e | 38.2 cd | 35.1 ef | 47.2c | 38.9d | 38.2c | 35.4d |
| Mexican marigold | 48.2 cd | 41.5 e | 32.2 f | 32.4 f | 28.8g | 24.1g | 21.6f | 21.7g |
| Chenopodium | 30.1 fg | 31.4 fg | 33.7 ef | 32.4 f | 38.5e | 36.4de | 30.3d | 28.4ef |
| Croton | 30.7 fg | 28.6 gh | 23.3 gh | 26.9 g | 32.8f | 30.0f | 26.5e | 26.3f |
| Mint | 21.7 h | 25.5 hi | 26.7 g | 26.4 g | 43.8d | 40.8cd | 38.7c | 31.2e |
| Garlic | 27.7 g | 19.7 j | 19.4 ij | 23.9 gh | 39.2e | 35.8e | 32.4d | 30.8e |
| Ginger | 38.6 e | 33.7 f | 22.4 hi | 19.9 i | 44.0d | 39.7cd | 30.6d | 29.4e |
| Neem | 31.3 f | 24.7 i | 24.8 gh | 21.4 hi | * | * | * | * |
| Candle bush | 18.1 i | 14.7 k | 15.9 jk | 19.4 ij | * | * | * | * |
| Moringa | 4.2 k | 10.9 l | 14.7 k | 15.9 jk | * | * | * | * |
| Fleabane | 7.2 jk | 6.9 m | 9.5 l | 15.4 k | * | * | * | * |
| Lemon grass | 6.0 k | 0.3 n | 4.3 n | 2.7 l | * | * | * | * |
| Basil | 9.6 j | 11.2 l | 1.5 mn | 1.7 m | * | * | * | * |
| Control | 0.0 l | 0.0 n | 0.0 m | 0.0 lm | 0.0h | 0.0h | 0.0g | 0.0h |
| Mean | 30.8 | 29.2 | 26.8 | 27.8 | 40.6 | 36 | 33.2 | 32.1 |
| LSD | 3.6 | 3.4 | 3.5 | 3.8 | 2.5 | 2.6 | 1.8 | 2.4 |
| CV % | 7.1 | 7 | 7.9 | 8.3 | 3.7 | 4.3 | 3.2 | 4.4 |

Means accompanied by different letters within a column are significantly different according to Fishers Protected LSD test ($P \leq 0.05$). Data fields marked with ***** are for isolates that were not considered in the repeat experiment.

Table 3.14: Percentage colony diameter reduction in mycelia growth of *Altenaria solani* by crude plant extracts from selected plant species

| Source of extract | Days after inoculation | | | |
|---|---|---|---|---|
| | 2 | 4 | 6 | 8 |
| Turmeric | 61.6 a | 64.1 a | 65.5 a | 64.3 a |
| Ginger | 48.8 b | 42.2 bc | 44.7 b | 44.3 b |
| Small-fruited teclea | 48.8 b | 41.3 bc | 44.4 b | 43.5 b |
| Thyme | 49.6 b | 42.3 bc | 43.6 b | 42.3 b |
| Sage | 37.5 d | 41.8 bc | 41.9 bc | 42.3 b |
| Rosemary | 40.0 cd | 39.9 cd | 39.7 cd | 40.0 c |
| Goat weed | 40.7 c | 43.7 b | 36.9 de | 37.2 d |
| Ugandan greenheart | 42.5 c | 36.9 de | 36.5 de | 36.5 d |
| Garlic | 40.9 c | 34.4 e | 33.7 ef | 30.5 e |
| Mexican marigold | 40.8 c | 35.9 e | 30.7 f | 29.5 e |
| Croton | 33.6 e | 27.7 f | 23.9 g | 24.9 f |
| Control | 0.0 f | 0.0 g | 0.0 h | 0.0 g |
| Means | 40.4 | 37.5 | 36.8 | 36.3 |
| LSD (P≤0.05) | 2.9 | 3.0 | 3.6 | 2.3 |
| CV % | 4.3 | 4.8 | 5.8 | 3.8 |

Means accompanied by different letters within a column are significantly different according to Fishers Protected LSD test (P ≤0.05)

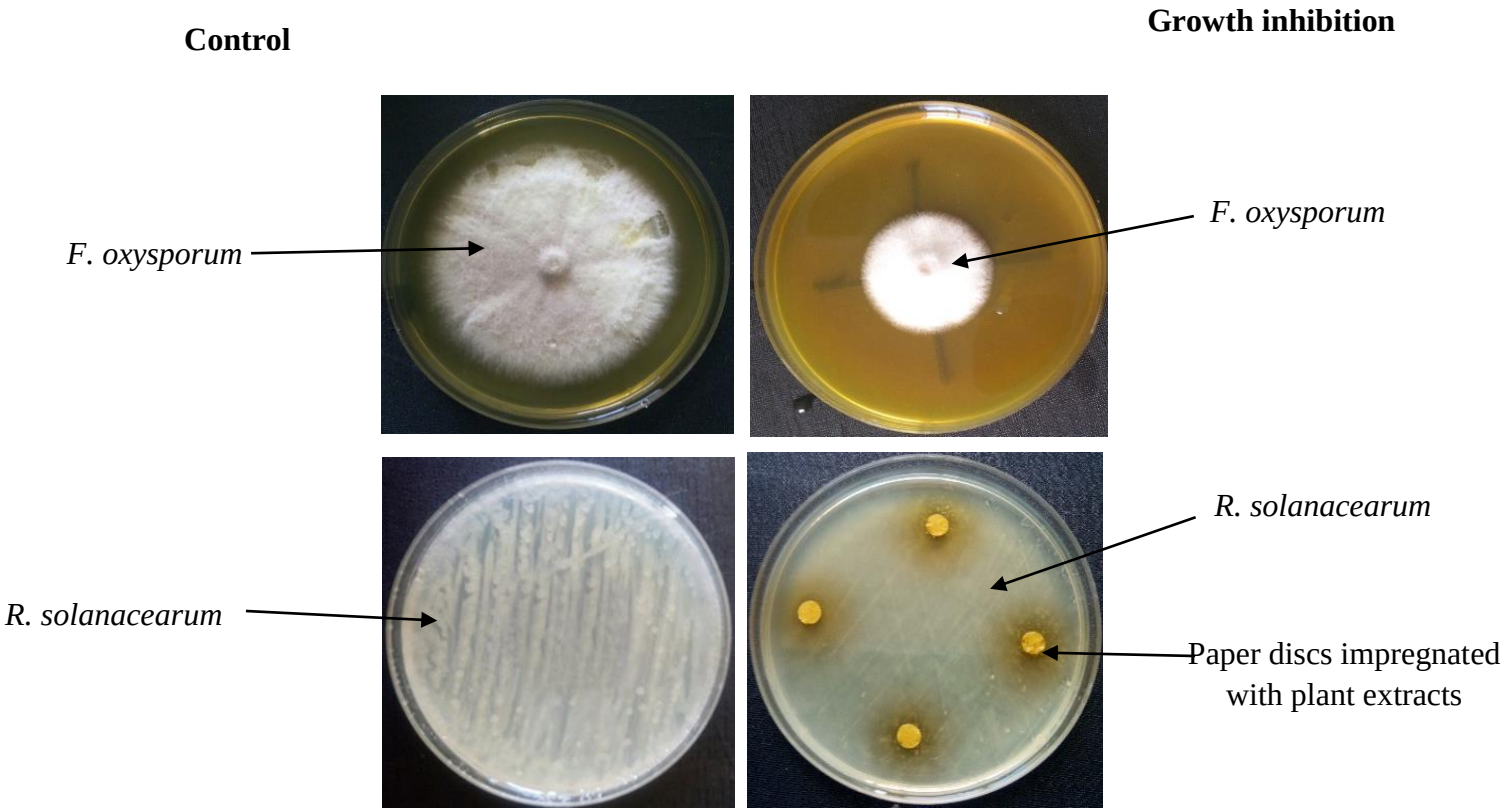


Figure 3.5: *In vitro* activity of *Curcuma longa* against *F. oxysporum* and *R. solanacearum* at eight and one day after inoculation, respectively

## 3.5 Discussion

### 3.5.1 Microbial antagonists isolated from different Agro ecological Zones

Abundance and diversity of soil fungi and bacteria greatly varied within and between the different AEZs. While the highest microbial population was isolated in LM 3 in Kirinyaga county, LH1-UM1 AEZ in Meru county had the highest microbial diversity. Generally, bacteria were more abundant and evenly distributed while fungi were poorly distributed with more types detected. Analysis of soil samples from different AEZs indicated that the soils were quite similar in mineral content except for organic carbon, phosphorus, potassium and magnesium which all peaked in LH1-UM1 in Meru county. Similarly, soil pH varied from highly acid in Nyandarua county to near neutral in LM 3 in Embu county. Soils from LM 3 in Kirinyaga county and LH1-UM1 in Meru county were both moderately acidic.

Numerous studies have cited different factors as determinants of soil microbial abundance, diversity and distribution. While Tederso *et al*., (2014) observed that climatic factors, followed by edaphic factors and spattertial patterning were the primary determinants of global fungal community composition and richness, several studies have cited edaphic factors as the primary influencers of microbial community structure and composition (Okoth *et al*., 2007; Maina *et al*., 2015; Mohammad, 2015; Pino *et al*., 2016). In other studies, land use and management (Okoth *et al*., 2007; Kamaa *et al*., 2011; Srivastava *et al*., 2014; Maina *et al*., 2015) and vegetation or crop cover (Okoth *et al*., 2007; Tedersoo *et al*., 2014; Li *et al* 2017) have also been identified as some of the key factors affecting microbial community composition.

In the current study, bacteria and fungi abundance, diversity and distribution assumed independent patterns, and no single determinants could fully explain the variations observed within and between the AEZs of the two groups together. Similarly, Pino *et al*., (2016) observed that microbial abundance, diversity and richness followed different patterns given that they responded to different soil attributes. Among the soil properties, soil pH has been indicated as the key factor influencing microbial community structure (Wang *et al*., 2017). Conversely, it has been reported that bacteria are more sensitive to slight differences in pH compared to fungi (Rousk *et al*., 2010) which make them exhibit drastic changes in community composition within a narrow pH range (Fernandez-Calvino and Baath 2010; Rousk *et al*., 2010). In contrast, fungi are rarely affected by pH since they have wider pH optima, usually 5 to 9 pH units (Rousk *et al*., 2010).

It has also been reported that neutral pH favors bacterial growth in the soil while acidic conditions favor fungal dominance (Rousk *et al*., 2009). These relationships between soil pH and microbial abundance and diversity partially explains why acidic soils in Nyandarua county AEZs had relatively low microbial populations and fewer bacterial types compared to other AEZs. Moreover, studies have indicated that the decrease in microbial biomass in acidic soils is associated with aluminum toxicity at low pH (Pietri and Brookes, 2008; Rousk *et al*., 2009).

Similarly, it also partly rationalizes why AEZ LM 3 in Kirinyaga county had the highest bacterial and fungal abundance while LH1-UM1 in Meru county had the most diverse microbial community. In both cases, the soil pH was slightly acidic and thus favored high abundance of bacteria and diversity of both bacteria and fungi. Previous studies have reported positive linear relationship between soil pH and bacteria abundance (Higashida and Takao 1986; Rousk *et al*., 2010). Rousk *et al*., (2010) reported that soil pH had a strong influence on bacterial community composition while Higashida and Takao (1986) observed that fungal abundance was not affected in anyway by the soil pH since it is a group of organisms which are highly tolerant to changes in pH. The abundance of bacteria is highly favored by near neutral pH (Fernandez-Calvino and Baath 2010; Wang *et al*., 2017) and previous research on effects of pH on growth of soil bacterial community showed that bacterial growth peaked at near neutral pH but was lowest in acidic soils (Fernandez-Calvino and Baath, 2010). However, diversity of both fungi and bacteria are positively correlated with soil pH and usually increase with a rise in pH (Rousk *et al*., 2010). Furthermore, Fierer and Jackson (2006) demonstrated that soils with higher pH of about 5.5, which is just approximately 0.3 pH units below that of LH1-UM1 in Meru, were about 26% richer in species composition compared to soils with acidic pH of 4.1.

However, the findings herein disagree with observations of Lauber *et al*., (2009), Bartram *et al*., (2014) and Qi *et al*., (2018) which reported that soil pH was the primary and most important predictor of bacterial community structure. For instance, if considering pH alone, then soils collected in LM 3 in Embu county should have harbored the highest abundance and diversity of bacteria since they had the highest pH. In contrast, these soils had the least number of bacterial types detected while the abundance was multiple fold below those isolated from LM 3 in Kirinyaga county. This weak relationship between soil pH and microbial community patterns observed herein indicate that other factors other than pH could be responsible for differences in microbial

abundance and diversity between the different AEZs. Moreover, previous research has shown that the effects of soil pH on microorganisms is greatly confounded by other factors such as different soil types, climate and vegetation (Pietri and Brookes, 2008).

High population of bacterial and fungal isolates detected in LM 3 in Kirinyaga county could also have been caused by soil physical properties, climatic conditions or land use and farming practices. Examination of soil textural classes revealed that soils in Kirinyaga county had the highest clay content compared to soils collected from other counties. Similarly, it has been previously reported that this AEZ is predominated with vertisols which are high in clay content (FAO, 2001; Jaetzold *et al*., 2010). Soils with high clay content have been shown to harbor higher population of microorganisms compared to loamy and sandy soils (Hamarashid *et al*., 2010; Mohammad, 2015) because they have high organic content, better water holding capacity and nutrient availability (Grayston *et al*., 2004; cookson *et al*., 2005). Furthermore, the small size particles protect microorganisms from predators through spore size exclusion (Sessitsch *et al*., 2001; Zang *et al*., 2007).

Analysis of soil components classified soils from LH1-UM1 in Meru county as sandy clay loam with approximately 32% clay content. The agro ecological zone is dominated with humic nitisols and andosols (Jaetzold *et al*., 2010) that are high in percentage organic matter (FAO,2001; Jaetzold *et al*., 2010) and composed of 30% clay material (FAO,2001). Previous studies have shown that fine size soils with high nutrient content are associated with high microbial diversity (Sessitsch *et al*., 2001; Hamarashid *et al*., 2010; Mohammad, 2015). Soil organic matter improves nitrogen content, aeration and water holding capacity which have been shown to positively correlate with microbial activities (Mohammad *et al*., 2015), and has been associated with perceived immense biological biomass and activity in the top soil (Talbot *et al*., 2014).

This AEZ also had the highest proportions of organic carbon, phosphorus, potassium and magnesium compared to soils collected from other regions. Conversely, Qi *et al*., (2018) observed that soil organic matter, total and available nitrogen (N), phosphorus (P) and potassium (K) showed correlation with bacterial community composition. Soils collected from forest lands and napier farms were found to favor abundance and diversity of *Trichoderma* spp. due to high amounts of C, N, P and K while those sampled from coffee farms recorded the poorest proportions of the fungus due to low proportions of these macronutrients (Okoth *et al*., 2007). Despite LM 3 in Embu

having the least diverse bacterial types, it had the second highest fungal types isolated after LH1-UM1 in Meru (Table 4). Coincidentally, these two were the only AEZs with adequate proportions of calcium (C). The concentrations of calcium in the soil and annual precipitation was found to positively correlate with total fungal diversity and was a strong predictors of global fungal diversity by Tederso *et al*., (2014). Similarly, a strong positive correlation between community-level fungal diversity and concentration of calcium, magnesium and manganese ions was observed by He *et al*., (2017) while assessing the fungal diversity and community composition in Chinese zonal forests.

The transverse agro ecological zone LH1-UM1 also receives the highest mean annual rainfall of between 1400 mm to 2600 mm (Jaetzold *et al*., 2010) of the ten AEZs. Furthermore, most farmers in the region irrigate their fields off season (Gildermacher *et al*., 2009). Farming practices such as minimum tillage, mulching, soil conservation (GOK, 2014) and incorporation of farm yard manure in the fields (Gildermacher *et al*., 2009; Muriithi and Yu, 2015) are also commonly exercised by farmers. Numerous studies in soil fungi have demonstrated that fungal diversity is positively correlated with climatic factors that avail moisture such as precipitation and evapotranspiration (Tedersoo *et al*., 2014; Peay *et al*., 2016). Similarly, reduced tillage has been associated with high activity and functional diversity of microorganisms compared to full tillage (Anna *et al*., 2017). Equally, addition of manure in the field was found to increase fungal and bacterial diversity in the soil through generation of organic matter and nutrients for the microorganisms (Okoth *et al*. 2007; Kamma *et al*., 2008).

Mwea region which is located in LM 3 and LM 4 in Kirinyaga county has been known for intensive tomato and rice production both on rainy season and off season through irrigation. Mwangi *et al*., (2015) and Nguetti *et al*., (2018) reported that tomato farmers in this region applied excessive chemical pesticides on their farms due to misconception, poor knowledge and lack of skills. For instance, in Mwea west sub county, almost half of the farmers applied pesticides in the soil, even when the target pest was a foliage feeding pests **(**Mwangi *et al*., 2015). This poor chemical use practice could have caused the low microbial diversity observed in LM 3 in Kirinyaga through extinction of susceptible species, leaving the pesticide tolerant types to proliferate without competition for resources hence the high populations but low diversity. Moreover, intensified agricultural production and direct application of mineral fertilizers, chemical pesticides and

herbicides into the soil have been shown to reduce above and below ground biodiversity (Wolinska, 2019). Similarly, Okoth *et al*., (2007) observed that land intensification through frequent cultivation and input application had a negative effect on occurrence and diversity of *Trichoderma* spp. under different land use and management systems in Embu, Kenya.

It was observed that more bacterial types were shared among the ten AEZs compared to fungi which had more than double the number of isolate types occurring only in a single AEZ compared to bacteria. Conversely, bacterial abundance was several folds higher compared to that of fungi. These findings are in tandem with reports of berg *et al*., (2006), Pino, (2016) and Li *et al*., (2017) which showed that species composition of soil microbial community vary between experimental site and AEZs with different conditions. Peay *et al.,* (2016) reported that bacteria and fungi widely differed in their diversity pattern and community dynamics with respect to soil pH, climate and habitat, that is, terrestrial or marine habitat. Alike the findings herein, the authors reported that fungi were more heterogeneous than bacteria even at closely related environments with large differences in community composition and diversity across samples from the same habitat.

According to Pino, (2016), fungi were more common or dominant while bacteria and archaea were more uniformly distributed across the sampling sites. Similarly, when Lauber *et al*., (2009) observed that most soils shared only a small proportion of the bacterial phylotypes, with majority of the phylotypes only existing in single soil, in a survey employing pyrosequencing technique to characterize bacterial communities in north and south America.Moreover, it has also been reported that bacteria are the most abundant microorganisms in the soil (Srivastava *et al*., 2014; Toppo and Naik, 2015; Wolinska, 2019) because they are adapted to wide variations in soil properties compared to other soil microorganisms (Papiernik *et al.,* 2007).

In this study, *Aspergillus* spp. were the most abundantly isolated genus in LM 3 in Kirinyaga county while *Penicillium*, *Fusarium* and *Trichoderma* species were the most prevalent and frequently isolated in all the ten AEZs. These observations are in agreement with reports of Pino, (2016) which found that *Fusarium* spp. and *Penicillium* spp. were the most abundant genera of the soil fungi inhabiting New South Wales, Australia. Equally, Okumu *et al.,* (2018) reported that members of genera *Aspergillus*, *Trichoderma* and *Penicillium* dominated acidic soils collected from Koibem and Kapkerer experimental sites in Kenya while assessing the effects of lablab green manure on population of soil microorganisms and establishment of common beans.

Genera *Aspergillu*s, *Fusarium* and *Penicillium* are known to comprise important human, animal and plant pathogens, food spoilage microbes as well as producers of mycotoxins (Suanthie *et al*., 2009; Egbuta *et al*., 2017). In contrast, some species of these saprophytes are prolific producers of beneficial metabolites and have been exploited as biocontrol agents to manage plant diseases (Mishra *et al*., 2015). Genus *Trichoderma* also consist of novel species with ability to suppress multiple plant disease (Waghunde *et al.,* 2016). For instance, *T. harzianum* and *T. polysporum* marketed as Trianum P and Binab T, respectively, have been developed into commercial products for management of soil borne and root pathogens (Koul, 2011; Mishra *et al*., 2015).

The findings in this study indicate that population, diversity and distribution of microbial isolates greatly vary among AEZs. It also suggests that a rich diversity of microorganism with antagonistic effect are well established in regions with diverse agro ecological condition.

### 3.5.2 Activity of fungal antagonists against tomato pathogens *in vitro*

Fungi isolated from soils collected in diverse regions conferred varying antagonistic effect against both fungal and bacterial pathogens including *F. oxysporum, A. solani* and *R. solanacearum. Trichoderma* spp. isolates were superior in pathogen inhibition. The antagonists significantly differed in degree of antifungal and antibacterial activity and the pathogens responded differently to their effects with dissimilar levels of sensitivity. These observations are in agreement with reports of Popiel *et al*., (2008) and Fulano *et al*., (2016) which reported that isolates of *Trichoderma* spp. were more effective in inhibiting growth of phytopathogens *in vitro* than other fungal genera. Similarly, an array of previous studies has demonstrated that antagonistic fungi conferred different levels of inhibition against dissimilar pathogens or diverse strains of the same pathogen (Naglot *et al*., 2015; Fulano *et al*., 2016 and Rai *et al*., 2016).

Different species of *Trichoderma* showed varied levels in inhibition on colony growth of fungal pathogens. These results are in line with the findings of Popiel *et al*., (2008), Meena *et al*., (2017) and Redda *et al*., (2018). These authors observed that species of *Trichoderma* vary in ability to inhibit growth of fungal phytopathogens. In this study, *Trichoderma harzianum* (Abog 11) was the most effective in inhibiting colony growth of *F. oxysporum* f. sp. *lycopersici* and *A. solani.* Supremacy of *T. harzianum* over other *Trichoderma* spp. in inhibition of soil and foliar fungal pathogens *in vitro* has been reported in several studies (Maheshwari and Vidhya, 2016; Fulano *et al*., 2016; Cherkupally *et al*., 2017; Meena *et al*., 2017). Fulano *et al*., (2016) observed superior

activity of *T. harzianum* against *A. solani*, *F. solani*, *C. lindemuthianum* and *R. solani* while Cherkupally *et al*., (2017) found that *T. harzianum* isolated from brinjal field in India inhibited colony growth of *F. oxysporum* by more than 80%.

Diverse isolates of *T. harzianum* showed different potency levels in inhibiting the growth of the fungal and bacterial pathogens. Different strains or isolates of same species of an antagonist has been shown to exhibit varying degree of antagonistic activity against the same or different pathogens (Abbas *et al*., 2017). Similar findings from previous studies have demonstrated that different strains and isolates of *T. harzianum* conferred varying levels of antagonistic activity against the same pathogen (Redda *et al*., 2018). Alike the findings in this study, antagonistic potential of other species of *Trichoderma* such as *T. viride* (Perveen and Bokhari, 2012; Meena *et al*., 2017), *T. citrinoviride, T. atroviride, T. pseudokonigii, T. konigii, T. roseei. T. virens and T. koniggii* (Popiel *et al*., 2008) against diverse phytopathogenic fungi are well documented.

In this study, *Fusarium oxysporum* showed high susceptibility to the activity of fungal antagonists than *A. solani.* High sensitivity of *F. oxysporum* to activity of antagonists compared to other pathogens has been reported. Maheshwari and Vidhya, (2015) observed higher vulnerability of *F. oxysporum* to antagonistic effect of *Trichoderma* spp. than *Colletotrichum capsici* and *Botryodiplodia theobromae*. Similarly, Fulano *et al.,* (2016) observed that *A. solani* was the least sensitive to activity of antagonists compared to *C. lindemuthianum, F. solani* and *R. solani*. However, Meena *et al*., (2017) reported that *Alternaria alternata* was more susceptible to compounds produced by *Trichoderma* spp. than *Fusarium* spp. The variations in response to activity of *Trichoderma* metabolites by different phytopathogens **(**Naglot *et al.,* 2015) could be due to differences in cell wall integrity exhibited by the test pathogens (Fulano *et al*., 2016).

Antagonistic activity against fungal pathogens was conferred through competition, mycoparasitism and antibiosis. Antibiosis was manifested through development of inhibition zones between the pathogen and antagonist colonies. Occurrence of mycoparasitism and competition for space was observed as the restriction of pathogen colony and overgrowth of the antagonists over the pathogen. Concurrently, numerous studies have demonstrated that antagonistic fungi suppress growth of fungal phytopathogens *in vitro* through mycoparasitism, antibiosis and competition for nutrients and space (Perveen and Bokhari, 2012; Abbas *et al*., 2017).

Colonies of the antagonistic isolates exhibited faster growth than those of *F. oxysporum* and *A. solani* in this study. This rapid growth of antagonist's colony enables it to outcompete the pathogen for nutrients and space hence overwhelming it in the proce**ss (**Rai *et al*., 2016; Cherkupally *et al*., 2017). It is also important to note that synergism between different modes of action is required for efficient biocontrol of pathogens (Perveen and Bokhari, 2012; Naglot *et al*.2015).

Similarly, the fungal antagonists conferred varying levels of activity against *R. solanacearum. Trichoderma hamatum* was the most potent in inhibiting growth of the bacterium. Effectiveness of *Trichoderma* spp. in growth inhibition of diverse strains of *R. solanacearum* has been reported (Narasimha-Murthy and Srinivas, 2012; Cheng *et al*., 2015). Narasimha-Murthy and Srinivas (2012) found that isolates of *T. asperellum* dorminated over other species of *Trichoderma* in inhibiting growth of *R. solanacearum.* Similarly, Cheng *et al*., (2015) reported that *T. hamatum* exhibited superior inhibition of *R. solanacearum* growth compared to *T. virens* and *T. asperellum*.

The clear zone of inhibition produced in the dual culture plates inoculated with *R. solanacearum* and fungal antagonists suggest that the antagonistic fungi produced substances with antibacterial properties into the growth media. Similar observations have been made in other studies in which production of halo zones were associated with excretion of antibiotics and enzymes with antibacterial activity into the growth media by the antagonists (Narasimha-Murthy and Srinivas 2012; Cheng *et al*., 2015).

### 3.5.3 Activity of bacterial antagonists against tomato pathogens *in vitro*

Antagonistic bacteria isolated from different AEZs conferred diverse levels of antagonism against *R. solanacearum*, *F. oxysporum* f.sp. *lycopersic*i and *A. solani*. Effectiveness of antagonists against bacterial and fungal pathogens differed. Whereas some isolates showed broad spectra of activity against both bacterium and fungal pathogens, others only inhibited growth of either the bacterium or fungal pathogens. Among the fungal pathogens, *F. oxysporum* was more sensitive to the activity of bacterial biocontrol than *A. solani*.

These findings corroborate the reports by Anith *et al*., (2003, 2004), Koley *et al*., (2015), Xu and Kim (2014) and Marroni, (2015) which revealed that diverse species and strains of bacterial antagonists conferred varying levels of inhibition against different strains and species of phytopathogens. Anith *et al*., (2003) observed that bacterial antagonists conferred varying degree of antagonism from poor to very high inhibition levels against *Phytophthora capsici* in dual culture

bioassays. Similarly, antagonistic strains of *Bradyrhizobium japonica*, *Bacillus* spp. and *Pseudomonas* spp. exhibited broad spectrum of activity against *Botrytis* spp. *Fusarium* spp., *Phytophthora* spp., *Rhizoctonia* spp., and *Schlerotina* spp. (Imran *et al*., 2012).

Antifungal properties of soil borne bacteria has been documented in numerous studies. Koley *et al*., (2015) reported strong colony growth inhibition of *A. solani* by *Pseudomonas fluorescens* and *Bacillus subtilis*. Marroni, (2015) found that diverse strains of *Bacillus* spp. exhibited effective growth and sporulation reduction on *Macrophomina phaseolina* while Abaidoo *et al*., (2011) observed successful inhibition of root/soil borne fungal pathogens of cowpea caused by *Fusarium oxysporum, F. verticillodes, F. equiseti, F. solani* and *R. solani* by antagonistic *B. subtilis*. Diverse strains of *Paenibacillus* spp. were demonstrated to confer different levels of antagonistic activity against *Fusarium oxysporum* f. sp. *radicis-lycopersici*, the causal agent of *Fusarium* crown and root rot in dual culture assay (Xu and Kim, 2014). Similarly, Minaeva *et al*. (2008) reported that *Fusarium oxysporum* was more sensitive to antifungal properties of *Pseudomona*s spp. than *Bipolaris* spp.

Similarly, several reports on inhibition of *R. solanacearum* growth by antagonistic bacteria has been reported. Numerous studies have reported effectiveness of different species and strains of bacterial antagonists against *R. solanacearum in vitro* (Rado *et al*., 2015; Tahir *et al*., 2016; Yendyo *et al*., 2018; Sakthivel *et al*., 2019; Subedi *et al*., 2019). In this study, *Serratia* spp. produced the largest zone of inhibition followed by *B. subtilis* while *Acinetobacter* spp. exhibited the clearest zone of inhibition. These findings agree with reports by Xue *et al*., (2012) which revealed that *Serratia* spp. was superior to other antagonists in inhibiting growth of ten genetically different strains of *R. solanacearum*. Similarly, the findings herein also concur with findings of Sakthivel *et al*., (2019) which revealed that six strains of *Bacillus* spp. exhibited the highest growth inhibition against *R. solanacearum* among the 65 isolates tested for antagonism.

However, the findings in this study differ with reports of Jangir *et al*., (2018) and Yendyo *et al*., (2018) which found that *P. fluorescens* showed higher inhibitory activity than *B. subtilis* against *R. solanacearum* isolated from soy beans and tomato plants, respectively. These variations could be due to differences in susceptibility of the test strains of *R. solanacearum* to activity of the antagonists or aggressiveness of antagonist towards the pathogen. Previous research on biocontrol of bacterial wilt has indicated that different strains of *R. solanacearum* respond differently to the

activity of bacterial antagonists *in vitro* (Rado *et al*, 2015; Subedi *et al*., 2019). Moreover, it has also been reported that the biocontrol ability of antagonists is isolate specific and not species dependant (Redda *et al*., 2018) and hence identical species can confer different levels of growth inhibition against same isolate of a pathogen.

Although the modes of action were not determined in this experiment, visual observations indicated that antagonism against *R. solanacearum* occurred through antibiosis while inhibition of fungal colonies was conferred through antibiosis and lysis of cell wall. Antibiosis against *R. solanaceraum* was manifested as production of halo zones at points where the antagonists were inoculated. Similarly, antibiosis against fungal pathogens was discerned as restriction of fungal colony growth and occurrence of a clear zones between the bacterial and fungal colonies. In contrast, lysis of fungal cell wall was indicated by deformation and collapse of fungal colony margins adjacent to antagonistic bacterial colonies.

Correspondingly, a lot of studies have demonstrated that inhibition through antibiosis is usually indicated by presence of halo zones as a result of production of diffusible antibiotics into the growth media (Abaidoo *et al*., 2011; Xue and Kim, 2014; Marroni, 2015; Hassan *et al*., 2018). Some isolates of antagonistic bacteria also produce enzymes which enable them to feed on cell wall components of fungi, mainly glucan, protein and chitin, which constitute the cell wall (Spadaro and Droby, 2016). The feeding results into breakdown and collapse of the cell wall that can be visually manifested as hyphae abnormalities like lysis, degradation and deformation **(**Won *et al*., 2019).

### 3.5.4 Activity of plant extract against tomato pathogens

Zones of inhibition produced against *R. solanacearum* and colony growth reduction of *F. oxysporum* and *A. solani* observed in the current study suggest that crude plant extracts prepared in ethanol possess antibacterial and antifungal. Crude extract of turmeric (*Curcuma longa*) was the most active in inhibiting growth of *F. oxysporum* and *A. solani* while extracts of marigold (*Tagetes minuta*) was the most effective against *R. solanacearum.*

Effectiveness of crude plant extracts in inhibition of phytopathogens has been reported. Our findings concur with reports of Muthomi *et al.,* (2017) which revealed that *C. longa* extracts were superior in inhibiting growth of *A. solani, P. ultimum, R. solani and F. oxysporum.* The findings also agree with observations of Chen *et al*., (2018) which reported that crude extracts of *C. longa*

possess broad spectra of activity and exhibited varying levels of growth inhibition against 11 phytopathogenic fungi spread across 7 genera, among them *F. oxysporum* and *A. alternata*.

However, our findings differ with those of Pattaratanawadee *et al.,* (2006) which reported that crude extracts of *C. longa* were ineffective against *F. oxysporum* and *Aspergillus* spp. The differences in antimicrobial activities could be due to extraction methods (Alkhail, 2005), extraction solvents ( Gurjar *et al.,* 2012), mode of extraction, nature of extracts, growth stage of the plant when harvested for extraction, concentration of active extract components (Nieto *et al.,* 2018), nature of test pathogen (Din *et al.,* 2016), climatic conditions and growth conditions of the plant (Webster, 2008) which are all known to affects stability and effectiveness of microbial properties of crude plant extracts.

In line with the findings of this study, numerous studies have also reported broad spectra antibacterial activity of crude plant extracts sourced from different plant species. Mekbib, (2016) and Din *et al*., (2016) reported that extracts of *T. patula* and *T. minuta* effectively inhibited growth of *R. solanacearum in vitro* while Kwamboka *et al.,* (2016) observed that aqueous extracts from *T. minuta* exhibited strong *in vitro* growth inhibition against *Pectobacterium carotovorum* the causal agent of soft rot and vascular wilt of vegetables. Similar observations were made by Murthy and Srinivas (2012) and Narasimha-Murthy *et al*., (2013) which reported that crude extract of *C. longa* was effective in inhibiting growth of *R. solanacearum.* Effective *in vitro* growth inhibition of phytopathogenic *P. carotovorum* and *R. solanacearum* by crude extracts of *R. officinalis* has also been reported by Jarrar *et al.*, (2010) and Alamshahi and Nezhad, (2015), respectively. Although reports on activity of *T. nobilis* on phytopathogens are rare, the current study show strong antifungal and moderate antibacterial activity.

Comparison of activity of crude plant extracts on physiologically different tomato pathogens in this study indicated that some plants possess both antifungal and antibacterial activities while others only possessed one of the two properties. For instance, strong antifungal and antibacterial activity exhibited by *C. longa* and *T. vulgaris* has also been reported in previous studies (Alavijeh *et al*., 2012; Gonelimali *et al*., 2018). Thus these plants have higher comparative advantage in spectra of activity over others like *T. minuta* and *T. nobilis* which only possessed either strong antibacterial or antifungal activities.

The crude extracts assayed were prepared from plants belonging to different families. Din *et al*., (2016) suggested that variation in activity of crude plant extracts against phytopathogens is a result of higher quantities of secondary metabolites with antimicrobial properties in some plants than others. Plants in zingiberaceae family such as *C. longa* and *Z. officinale* are rich in substances of phytochemical interest such as curcuminoids which greatly vary in physico-chemical characteristic, chemical structures and functional properties (Revathy *et al.,* 2011). Curcumin has been identified as the most important compound in curcuminoid responsible for antimicrobial properties in *C. longa* (Alavijeh *et al*., 2012; Gurjar *et al*., 2012).

Antibacterial activity exhibited by *T. minuta* has been associated with presence of flavonoids, saponins, alkaloids, and tannins in the crude extracts (Opinde *et al.,* 2016) while antimicrobial properties of extracts of *R. officinalis* are attributed to presence of phenolics (Nieto *et al.,* 2018), specifically rosmarinic acid, carnosic acid (Ahmed *et al,* 2011; Nieto *et al.,* 2018) and caffeic acid (Nieto *et al.,* 2018). Similarly, a number of phenolic compounds comprising of rosmarinic acid (Okamura *et al*., 1994; Cuvelier *et al*., 1996; Martins *et al.,* 2015), caffeic acid and quercetin (Abdelkader *et al*., 2014), with antimicrobial properties have been isolated from crude extracts of *S. officinalis*. In contrast, crude extracts of *Teclea* spp. contain alkaloids, terpenoids (Kuete *et al*., 2008; Onyancha *et al.,* 2014) tannins, flavonoids, steroids and saponins (Nuru *et al*., 2018).

These bioactive compounds individually or synergistically, affects the life processes of microorganisms through acting as chelating agents, binding protein molecules, changing production of structural components, destroying or weakening permeability barriers of the cell membrane and interfering with the cell physiology (Rongai *et al.,* 2015). This study shows that different parts of spices, trees and weeds have compounds that possess antibacterial and antifungal properties against major plant pathogens. These plants can be exploited and applied as bio pesticides which are less harmful to human health and environment compared to chemical pesticides.

# CHAPTER FOUR : EFFICACY OF MICROBIAL ANTAGONISTS AND PLANT EXTRACTS IN MANAGEMENT OF BACTERIAL WILT OF FIELD GROWN TOMATO

## 4.1 Abstract

Bacterial wilt (*Ralstonia solanacearum*) causes up to 100% yield losses in tomatoes and has compelled many farmers to abandon previous productive farms. Consequently, the absence of an effective management method demands an intensive such for a functional management option. The study was carried out to evaluate the efficacy of microbial antagonists and plant extracts in managing bacterial wilt under natural infestation. Antagonistic *Trichoderma hamatum*, *T. atroviride*, *T. harzianum*, *Bacillus subtilis*, *Serratia* spp., *Acinetobacter* spp. and plant extracts from *Curcuma longa*, *Rosmarinum officinallis* and *Tagetes minuta* were evaluated. Commercial formulations of *T. viride* (Bio Cure F®) and *Pseudomonas fluorescence* (Bio Cure B®) were included as standard checks. The products were applied as soil drenches every two weeks commencing at transplanting until the tenth week after transplanting. Data was collected on plant stand count, disease incidence, disease severity, disease distribution, plant height, plant biomass and fruit weight. *Trichoderma hamatum* was the most effective in experiment 1, reducing crop mortality, incidence and AUDPC by up to 51.7, 49.3 and 58.2%, respectively. It also exhibited the highest percentage yield increase by up to 196.4% compared to control. In contrast, *B. subtilis* showed superior disease suppression in experiment 2, reducing crop mortality, incidence and AUDPC by up to 44.6, 48.5 and 51.0%, respectively. It also increased biomass by approximately 62.0% in both experiments. It was closely followed by *Serratia* spp., which also gave the highest yield output in experiment 2 of up to 233.0%. However, contrasting results were observed for plant extracts where *T. minuta* showed the highest disease reduction and yield increment in experiment 1, but performed poorest in experiment 2. Similarly, *C. longa* exhibited superior activity in the second experiment but performed very poorly in experiment 1. These findings suggest that the local environment is a rich harbor of potential biocontrol agents that can be sourced for management of bacterial wilt. The most active antagonistic isolates should be formulated into biocontrol products for management of bacterial wilt of tomato.

Key words: *Bacillus subtulis*, *Trichoderma* spp., Botanical pesticides, Bacterial wilt, Tomato

## 4.2 Introduction

Biological control of plant disease is the application of microbial biocontrol products including antagonistic bacteria, fungi and viruses or a mixture of either in management of plant diseases (O'Brien, 2017). Within the past few decades, the use of synthetic pesticides has been questioned owing to numerous health and environmental hazards associated with them (Heydari and Pessarakli, 2010; Suprapta, 2012; O'Brien, 2017) and as a result, a lot of consumer preference has seen a rise in demand for organically produced foods with minimal pesticide use (Suprapta, 2012; Van Lenteren *et al*., 2018).

However, high disease pressure in farmers' fields have forced farmers to resort to heavy use of agrochemicals to mitigate the losses (Pe´rez-Garcı´a *et al*., 2011). The heavy and misappropriated use of these chemicals has resulted into highest pesticide related mortalities in sub-Sahara Africa despite the fact that they are the least consumers of these pesticides (Anjarwalla *et al*., 2016)! Additionally, many African farmers still have limited access to synthetic chemicals owing to their cost and restricted distribution networks (Anjarwalla *et al*., 2016). Moreover, lack of knowledge on use and misuse of chemicals (Mwangi *et al*., 2015) have led to resistance development and restrictions on export markets abroad (Anjarwalla *et al*., 2016). For instance, farmers willing to export there produce to European countries have to comply with maximum residue limits standards set by the importing countries, which is still a challenge to many local farmers (Anjarwalla *et al*., 2016).

Although a number of different methods have been proposed for management of bacterial wilt (Jiang *et al*., 2017), they are rarely helpful once the soil is infested with the pathogen (Kressin, 2014). Soil fumigation with chemical pesticides have been reported as the most effective method to lower the inoculum density in contaminated soils. However, fumigation is very expensive, environmentally destructive, difficult to apply (Wang and Lin, 2005; Champoiseau *et al*., 2010), impractical to small scale farmers (Wang and Lin, 2005; Ramesh, 2008) and most soil fumigants such as methyl bromide has already been banned in many countries including Kenya (Muthoni *et al*., 2012). Furthermore, efficacy of chemicals is greatly reduced due to endophytic sheltering of the pathogen inside plant's xylem (Aloyce *et al*., 2017).

Other proposed management methods for the disease are rarely helpful once the pathogen is established in the field (Kressin, 2014). Crop rotation is impractical due to wide host range and

long period of time required (Hayward, 1991; Muthoni *et al*., 2012; Aloyce *et al*., 2017) while breeding efforts have not developed varieties with total resistance and the available varieties are less desirable to the consumers (Champeseau *et al.,* 2010; Yuliar *et al.,* 2015).

Biological control is a promising alternative for management of bacterial wilt (Jiang *et al*., 2017) as they are safer, ecofriendly, productively sustainable and possess minimal risk of resistance build up by the pathogen (Kohl *et al*., 2019). Furthermore, their efficacy is comparable to that of chemical pesticides (O'Brien, 2017). To this regard, numerous studies focusing on biological control has been initiated (Hyakumachi, 2013; Toppo and Naik, 2015) and research on biocontrol of bacterial wilt has tremendously increased from 10% in 2005 to 54% in 2014 relative to total number of research done on bacterial wilt. Almost 90% of all biocontrol trials have used bacterial antagonists while only 10% have targeted fungi (Yuliar *et al*., 2015). Consequently, bacteria belonging to genera *Pseudomonas* (Rai *et al*., 2017), *Acinetobacter*, *Serratia* (Xue *et al*., (2012), *Bacillus* (Singh *et al*., 2016) *Streptomyces* (Rad *et al*., 2015) etc. have shown successful suppression of the disease. Similarly, different species of *Trichoderma* have also shown strong disease suppression under greenhouse and field conditions (Konnapa *et al*., 2018; Nahar *et al*., 2019).

Despite all the benefits, the potential of biocontrol in disease suppression is yet to be fully exploited (Junaid *et al*., 2013) given that the market share of bio pesticides is less than three percent of the total pesticides industry (Suprapta, 2012). Even though numerous studies have identified a large number of potential biocontrol products against a great number of pathogens, only a few products have been commercially developed (O'Brien, 2017). In Kenya for example, only five microbial biocontrol products are registered for control of root and soil borne fungal pathogens but none for soil bacterial pathogens. Interestingly, nearly all of the registered biocontrol products in Kenya are imported (PCPB, 2020).

## 4.3 Materials and Methods

### 4.3.1 Description of the study area

The field experiment was carried out in a bacterial wilt hotspot farm in Mwea division at geographical coordinates -0.747941°S, 37.414035°N (Figure 4.1). The experimental region is in agro ecological zone Lower Midland (LM 4). It is located at altitudes between 1090 m to 1220m above sea level with a mean annual rainfall between 800mm and 950 mm that is distributed across

two seasons. The first rainy season start at the end of March and produce an average of 400 mm while the second rainy season commence in mid to end October with an average of 300 mm per season. The temperature ranges from 13.7°C to 30.4°C with an annual mean of 22°C. The area is predominated with deep, dark grey to black firm vertisols. These soils are imperfectly drained, infertile and fertilizer application is recommended for improved yield. Tomato production ranks second after maize-beans intercrop production (Jaetzold *et al.*, 2010). The irrigation schemes and rivers supply water that enable farmers to produce tomatoes all year round (Jaetzold *et al.*, 2006).

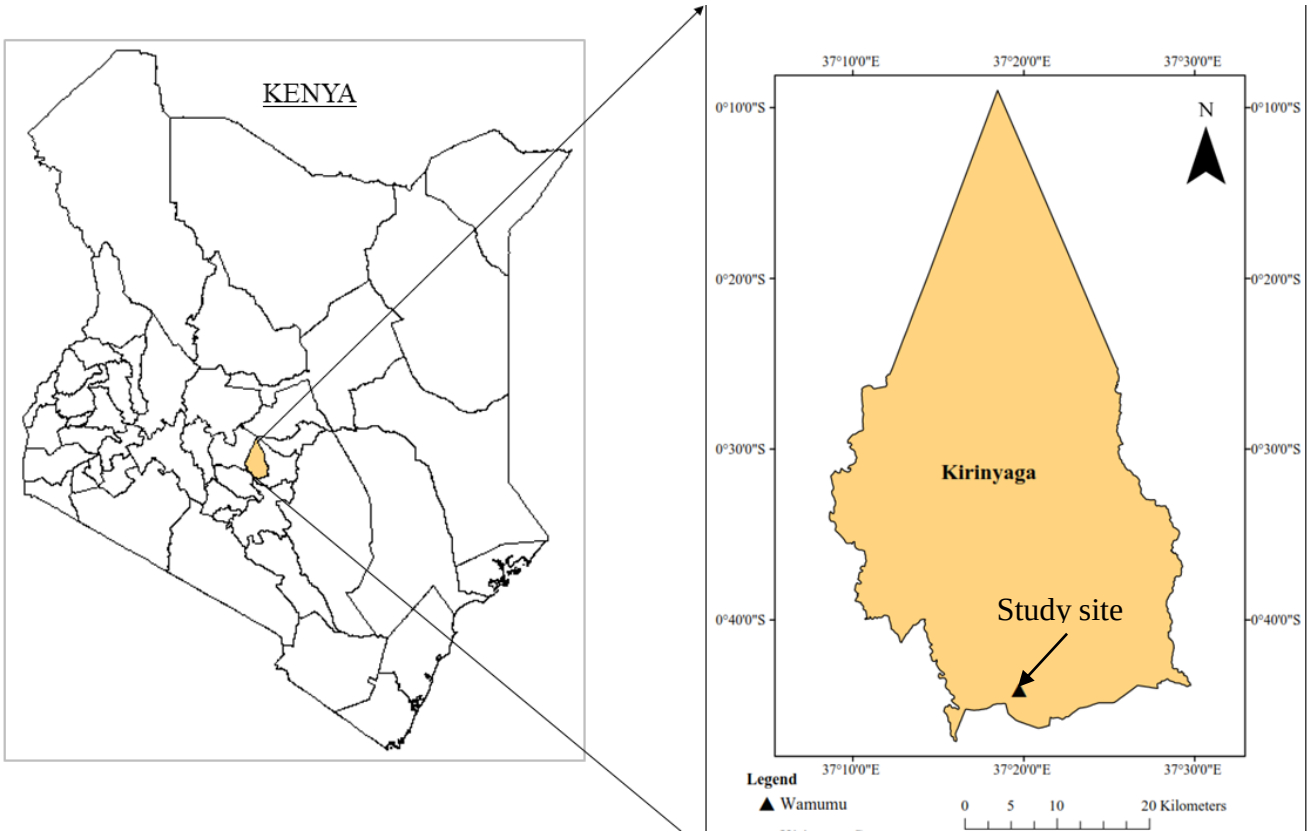


Figure 4.1: A map of Kirinyaga County showing the study site

### 4.3.2 Multiplication and formulation of fungal antagonists

Multiplication of fungal antagonists on sorghum was carried out as described by Mishra *et al.*, (2011), Bhattacharje and Utpa, (2014) and Kumar *et al*., (2014). Two hundred grams of sorghum was soaked in water for 12 hours then filled in 500 ml beaker and covered using aluminium foil. It was autoclaved at 121°C for 30 minutes and cooled to room temperature. The sterile sorghum was transferred into sterile polythene bags and inoculated with seven antagonists' mycelial agar

plugs cut from the edges of 5 day old cultures with a 7 mm diameter cork borer. The polythene bags were incubated at 24± 2˚C for 21 days (Figure 4.2a). The bags were periodically opened under a laminar flow hood for aeration and shaken to disperse mycelia and spores. The colonized sorghum was aseptically air dried at 27°C (Figure 4.2b, c), then crushed to powder using a blender. Talcum powder was used as carrier material in the formulation. The powder was sterilized and cooled to room temperature then mixed with the sorghum powder in the ratio of 1:1 w/w and thoroughly homogenized. The population of fungal antagonist per gram of the formulation was determined through serial dilution and pour plate method (Niranjana *et al.,* 2009; Mishra *et al.,* 2011; Sallam *et al.,* 2013). Depending on the population density determined for each antagonist, the talcum formulations was diluted appropriate to obtain $1 \times 10^8$ CFU/ml and applied as soil drenches.

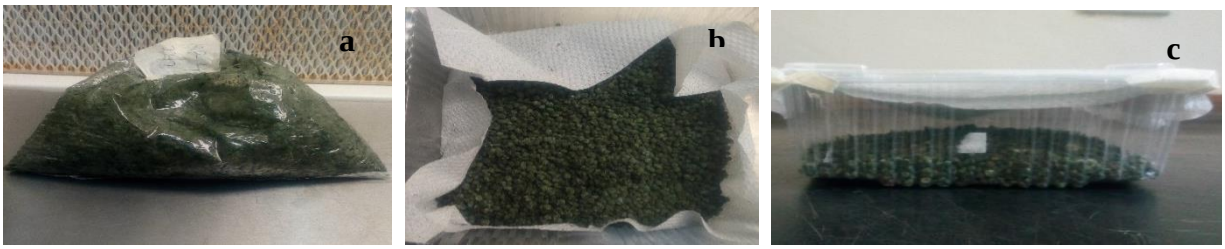

Figure 4.2: Preparation of fungal antagonists for field application

### 4.3.3 Multiplication and formulation of bacterial antagonists

Multiplication of bacterial antagonists was done on nutrient broth (10g peptone, 10g beef extract, 5g sodium chloride, final pH 7.3 ± 0.1 in one liter of sterile distilled water) and formulated with talcum powder as described by Niranjana *et al*., (2009), Mishra *et al.,* (2011) and Sallam *et al.,* (2013). Approximate 100 ml of sterile nutrient broth was prepared in a 250 ml conical flask and autoclaved at 121°C for 20 minutes then cooled to room temperature. Isolated colony of the antagonist from a 2day-old culture was picked using a sterile wire loop and aseptically inoculated into the broth and incubated in a shaker at 150 rpm for 72 hours at 24± 2˚C. The talcum powder was sterilized, cooled and mixed with the bacterial culture in ratio of ratio of 2:1 w/v. The mixture was aseptically dried in a sterile tray covered with absorbent paper towels at 27°C for five days. The powder obtained was aseptically homogenized in a blender and packed in polythene bags, stored at 4°C until use. The population of bacterial antagonists per gram of the formulation was determined through spread-plating 100 µL aliquots of serial dilutes from $10^8$ to $10^{12}$ on pre-dried

surfaces of nutrient agar. Depending on the population density determined for each antagonist, the talc formulations were diluted appropriate to obtain $10^8$ CFU/ml and applied as soil drench.

### 4.3.4 Field experiment layout and design

Field experiment was carried out in farmer's field in Mwea under natural infestation as described by Morsy *et al*., (2009). Natural infestation was visualised through inspection of symptom expression on tomato crops from the previous cropping season. Equally, soil samples and diseased plants were collected for further investigation. *Ralstonia solanacearum* isolation and identification was conducted as described under section 3.3.3 then virulence conducted through Koch postulates as described above.

The experiment was arranged in Randomized Complete Block Design and replicated four times. Treatment plots were set at 3M × 3M and one-meter alley was spaced between adjacent plots and blocks. The first experiment commenced in late July 2018 to October 2018 while the repeat experiment started in late December 2018 until April 2019, under irrigation. The experiments were laid down in the same field with the first and repeat experimental plots sited on different positions but adjacent to each other. In experirment one, the same untreated control plots were used to compare all the treatments but in experiment two, separate control plots were set for microbial antagonists and plant extracts. This was done due to variations in disease incidence and severity in field positions where the plots were sited as was indicated by the previous tomato cropping season.

Rio Grande variety, which is highly susceptible to bacterial wilt (Aslam *et al.,* 2017), was used in the experiment. Seedlings were raised according to standard farmers practice where a 1M-width bed was raised and soil thoroughly mixed with 5 kg of well decomposed manure. Seeds were sown on shallow farrows dug using a thin stick on top of the seedbed and firmly covered with soil and mulch. Watering was done as appropriate and mulch was removed upon germination of seeds. Transplanting was done when the seedlings were three and half week old at spacing of 60cm × 60cm. Di Ammonium Phosphate (DAP) fertilizer was applied at the rate of 200kg/Ha at transplanting and top dressing with CAN done at four weeks and eight weeks after transplanting at the rate of 100kg/Ha and 200kg/Ha, respectively. Weeding and irrigation was done as required.

Nine treatments including three fungal antagonists *T. hamatum, T. atroviride, T. harzianum,* three bacterial antagonists *B. subtilis*, *Serratia* spp. and *Acinetobacter* spp., three crude plant extracts

from turmeric (*C. longa*), rosemary (*R. officianalis*) and marigold (*T. minuta*) were assessed. Standard checks including commercial *T. viride* (Bio cure F®), commercial *P. fluorescence* (Bio cure B®) and a negative control without any treatment were also included in the experiment. Treatment application was initiated at transplanting and repeated every two weeks until the tenth week after transplanting.

Consequently, the treatments were appropriately diluted to achieve the desired antagonist population density and plant extract concentration prior to application. Antagonistic fungi and bacteria were diluted to achieve a population density of $1 \times 10^8$ CFU/ml while crude plant extract were mixed with water in the ratio of 1:50 v/v. One hundred milliliters of Bio Cure F® and ten milliliters of Bio Cure B® were each diluted with one liter of water, following the manufacturer's recommendation, to achieve a final population density of $10^8$ CFU/ml. The treatments were applied through drenching 50 ml of the suspension around the root zone of the plants on moist soil. Negative control plots were drenched with water only. Data was collected on disease incidence, severity, distribution, plant height biomass and fruit yield.

### 4.3.5 Assessment of bacterial wilt incidence and severity

Disease incidence, severity and distribution was determined on weekly basis commencing three weeks after transplanting (WAT) until harvesting. Disease incidence was determined as the number of plants with visible bacterial wilt symptoms out of the total plant population multiplied by 100 as described by Guo *et al.,* (2004). Disease severity was scored for all wilted plants in the plot and an average per plot computed. Severity was scored on a scale of 0-4 as described by Yang *et al.,* (2012), where 0 was considered for no visible wilt symptoms; 1 for up to 25% of foliage wilted; 2 for foliage wilting between 26% to 50%; 3 for foliage wilting between 51% to 75% and 4 for wilting greater than 75%.

Disease distribution was measured on scale of 0-2, where 0 was considered for plots with no visible bacterial wilt symptoms on plants, 1 for plots where disease occurred in spots and 2 where the disease was uniformly spread in the whole plot. Incidences was converted to proportions so that 0= no plant infected and 1=all plants infected (Geraldine, 2016; Muremi, 2016). Data obtained from disease incidence, severity and distribution was used to work out percentage disease index:

$$\text{Percentage disease incidence} = \frac{\text{Number of wilted plants per plot} \times 100}{\text{Total plant population per plot}}$$

$$\text{Percentage Disease Index} = \frac{\text{Incidence score} + \text{Severity score} + \text{Distribution score} \times 100}{\text{Maximum disease score (7)}}$$

Area under the disease progress curve (AUDPC) was calculated from percentage disease incidence recorded each week as described by Shanner and Finney (1977):

$$AUDPC = \sum_{1=n}^{n-1} \frac{[Y_i + Y_{i+1}]\,[X_{i+1} - X_i]}{2}$$

Where Y = incidence (per unit) at the ith observation, Xi = time (weeks) at the ith observation, and n = total number of observations.

### 4.3.6 Assessment of crop growth and yield

Plant height and shoot biomass were taken at flowering stage using a meter rule and digital spring balance respectively. For plant height, five plants were randomly sampled out of the remaining population in each plot from the central row and measured. Similarly, three plants were sampled from the central row, carefully uprooted together with the roots and fresh weight taken immediately. Dry weight was recorded after drying the plants to constant weight in an oven at 60°C. Tomato fruits harvesting commenced upon observation of maturity indices exhibited by orange to pink tinge. Harvesting was done twice per week from all the remaining plants in the plot separately for three weeks and weighed. Cumulative weight recorded from different plots were used to analyze mean differences for different treatments.

### 4.3.7 Data analysis

Data on disease incidence, severity, distribution, plant height, biomass and fruit yield was processed in Microsoft Excel then subjected to Analysis of Variance (ANOVA) to determine significant differences among the treatments. The means were separated by Fisher's protected least significant difference at 5% level of significance using GENSTAT®.

## 4.4 Results

### 4.4.1 Effect of microbial antagonists on bacterial wilt of tomato

All the microbial antagonists tested under field conditions significantly ($P \leq 0.05$) differed in efficacy of reducing crop mortality, disease incidence, severity, index and AUDPC compared to untreated control at ten weeks after transplanting. There was high significant ($P \leq 0.05$) differences in the effects of microbial antagonists on crop mortality. *Trichoderma hamatum* exhibited the highest efficacy in reducing plant mortality by up to 51.7% and was closely followed by *B. subtilis* at 44.4% in experiment 1. The least efficacy was showed by *T. harzianum* and *Acinetobacter spp*., which each reduced mortality by 20.9% (Figure 4.3, Figure 4.4).

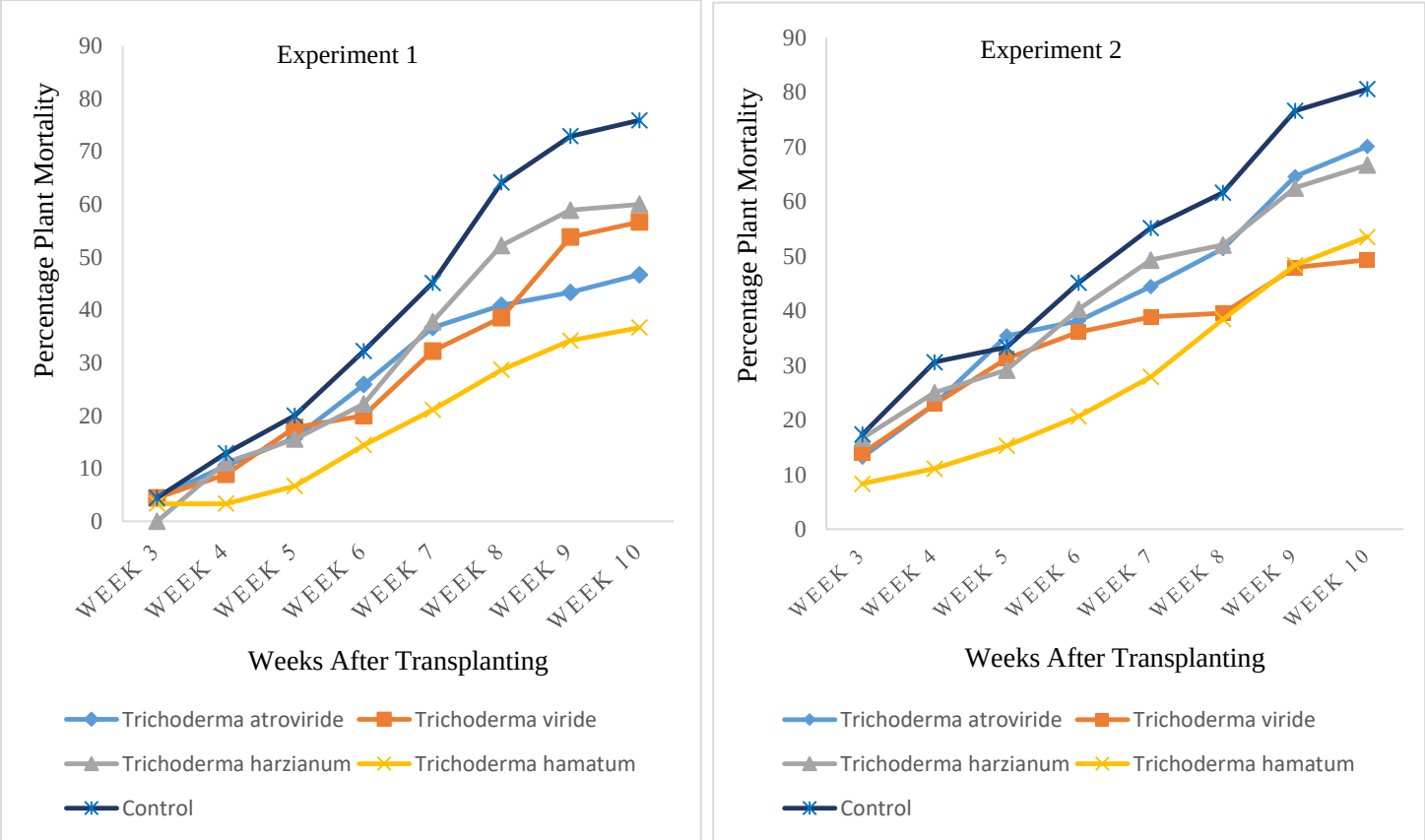


Figure 4.3: Percentage mortality of tomato plants drenched with different fungal antagonists in field experiement 1 and 2.

However, *Serratia* spp. and *T. viride* conferred superior reduction of crop mortality in experiment 2, by up to 38.8% for each antagonist at 10 WAT, respectively. *Bacillus* spp. followed closely at

34.5% while *T. atroviride* showed the least effectiveness in experiment 2 and was only capable of reducing mortality by 13.1% compared to untreated control (Figure 4.3, Figure 4.4).

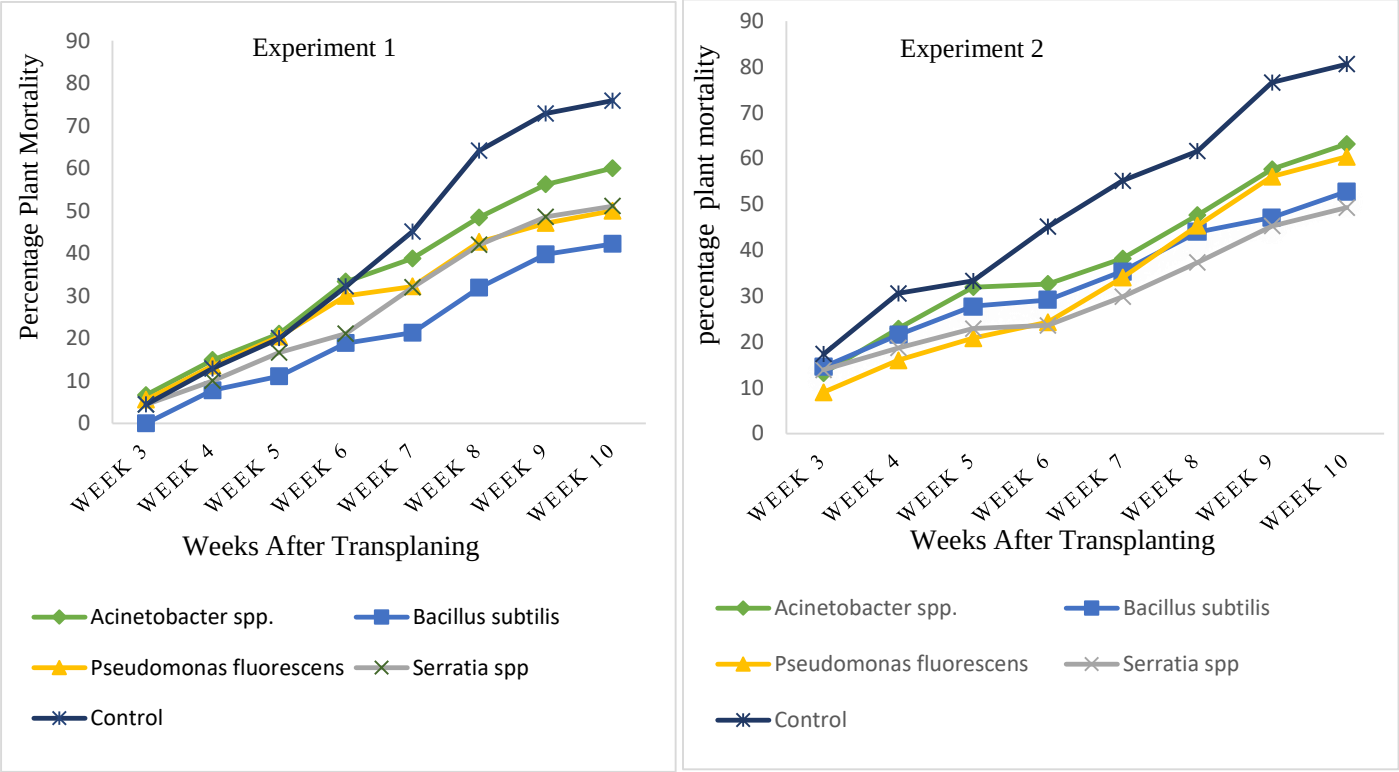


Figure 4.4: Percentage mortality of tomato plants drenched with different bacterial antagonists in field experiment 1 and 2, respectively.

A similar trend was observed for disease incidence and AUDPC. All the treatments significantly ($P \leq 0.05$) differed in reducing bacterial wilt incidence and AUDPC. *Trichoderma hamatum* remained the most effective in reducing disease incidence and AUDPC by up to 49.3% and 58.2%, respectively in experiment 1 (Figure 4.5, Table 4.1). In experiment 2, *Serratia* spp. and *B. subtilis* exhibited the highest reduction of wilt incidence by 46.2% and 48.5% and AUDPC by 51.9% and 51.0%, respectively (Figure 4.5, Tables 4.2).

Generally, isolated microbial antagonists *T. hamatum*, *B. subtilis and Serratia* spp. exhibited superior reduction of bacterial wilt incidence and crop mortality compared to standard controls, *T. viride* (Bio Cure F®) and *P. fluorescens* (Bio Cure B®) (Figure 4.3, Figure 4.4, Table 4.1, Table 4.2). Bacterial wilt incidences and plant mortality in control plots increased with time from 15.6 and 4.4% in the third week to 81.1 and 75.9%, respectively at week ten in experiment 1. A similar

trend was also observed for treated plots in both first and second experiments (Figure 4.3, Figure 4.4, Table 4.1, Table 4.2).

Table 4.1: Percentage mean disease incidence on tomato drenched with different microbial antagonists in field experiment 1

| | Weeks after transplanting | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Treatments | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| *T. hamatum* | 2.2 | 6.7 | 10.4 | 16.1 | 25.6 | 32.2 | 38.8 | 41.1 |
| *T.atroviride* | 12.2 | 17.0 | 20.3 | 32.2 | 33.3 | 40.0 | 48.8 | 51.1 |
| *T. harzianum* | 13.3 | 17.0 | 22.7 | 38.2 | 53.2 | 62.2 | 67.4 | 72.2 |
| *T. viride* | 5.6 | 13.7 | 17.0 | 33.3 | 38.7 | 50.7 | 58.8 | 62.2 |
| *B. subtilis* | 6.7 | 9.2 | 15.8 | 22.2 | 30.0 | 39.9 | 44.3 | 46.7 |
| *Serratia* spp. | 4.4 | 11.1 | 15.6 | 23.3 | 33.3 | 38.9 | 47.4 | 51.1 |
| *Acinetobacter* spp. | 8.9 | 15.1 | 24.0 | 30.4 | 36.1 | 48.9 | 56.7 | 60.0 |
| *P. fluorescens* | 11.1 | 15.0 | 21.4 | 28.7 | 33.1 | 42.6 | 49.6 | 53.3 |
| Control | 15.6 | 19.0 | 28.1 | 41.1 | 60.6 | 71.6 | 78.3 | 81.1 |
| Means | 8.9 | 13.8 | 19.5 | 29.5 | 38.2 | 47.4 | 54.5 | 57.7 |
| LSD | 5.5 | 5.8 | 9.4 | 11.3 | 14.7 | 16.5 | 12.9 | 11.5 |
| CV% | 35.9 | 24.2 | 27.9 | 22.2 | 22.3 | 20.1 | 13.7 | 11.5 |

Table 4.2: Percentage mean disease incidence on tomato plants drenched with different microbial antagonists in field experiment 2

| | Weeks after Transplanting | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Treatments | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| *T. hamatum* | 4.6 | 8.3 | 12.9 | 17.4 | 25.0 | 40.2 | 46.4 | 49.3 |
| *T. atroviride* | 10.2 | 20.4 | 27.5 | 35.6 | 47.7 | 59.9 | 66.9 | 68.5 |
| *T. harzianum* | 10.7 | 18.3 | 29.1 | 39.3 | 42.7 | 56.9 | 67.2 | 71.3 |
| *T. viride* | 11.3 | 21.0 | 24.2 | 27.4 | 29.9 | 36.3 | 44.7 | 49.2 |
| *B. subtilis* | 7.6 | 12.4 | 15.6 | 20.4 | 23.8 | 30.2 | 37.6 | 41.7 |
| *Serratia* spp. | 6.8 | 10.0 | 12.4 | 16.4 | 25.1 | 34.0 | 39.7 | 43.6 |
| *Acinetobacter* spp. | 11.2 | 19.1 | 21.5 | 26.1 | 30.8 | 40.3 | 49.7 | 55.4 |
| *P. fluorescens* | 6.7 | 11.2 | 17.3 | 26.4 | 34.8 | 45.4 | 53.2 | 57.5 |
| Control | 14.9 | 19.1 | 33.2 | 45.2 | 48.8 | 65.8 | 76.9 | 80.9 |
| Means | 9.3 | 15.5 | 21.5 | 28.2 | 34.3 | 45.4 | 53.6 | 57.5 |
| LSD | 8.3 | 9.1 | 11.5 | 14.0 | 17.8 | 18.4 | 20.7 | 20.0 |
| CV % | 61.0 | 40.2 | 36.7 | 34.1 | 35.6 | 27.3 | 26.5 | 23.8 |

Effectiveness of all bacterial antagonists in reducing bacterial wilt incidence increased in experiment 2 compared to experiment 1, with the exception of Bio Cure B® which decreased. Consuquently, *B. subtulis*, *Serretia* spp. and *Acinetobacter* spp. increased from 42.4, 36.9 and 31.5% in experiment one to 48.5, 46.2 and 31.7% in experiment two, respectively. Contrastingly, the efficacy of fungal antagonists reduced in experiment 2 relative to experiment 1, except for *T. harzianum* and Bio Cure F®. Consequently, *T. harzianum* and Bio Cure F® increased from 11.0 and 23.3% in experiment one to 11.8% and 39.2% in experiment two, respectively. *Trichoderma hamatum* and *T. atroviride* decreased from 49.3 and 37.0% in experiment one to 39.0 and 15.3% in experiment two, respectively (Table 4.2, Table 4.3).

Bacterial wilt severity sponteniously shifted between low and high levels from one week to the other for both experiments (Table 4.3, Table 4.4). This resulted in unclear disease progress patterns despite a number of treatments giving lower disease index compared to untreated control at 10 WAT (Table 4.5, Table 4.6).

Table 4.3: Mean severity of bacterial wilt on tomato plants drenched with different microbial antagonists in field experiment 1

| | Weeks After Transplanting | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Treatments | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| *T. hamatum* | 2.0 | 1.7 | 2.2 | 3.6 | 2.7 | 2.7 | 3.0 | 2.8 |
| *T. atroviride* | 3.2 | 2.5 | 2.2 | 2.0 | 1.5 | 1.6 | 3.4 | 2.5 |
| *T. harzianum* | 3.2 | 3.4 | 3.2 | 2.5 | 2.6 | 2.5 | 3.3 | 2.8 |
| *T. viride* | 1.8 | 2.8 | 3.1 | 2.7 | 2.5 | 2.9 | 3.3 | 3.0 |
| *B. subtilis* | 1.3 | 1.5 | 2.5 | 2.9 | 2.6 | 3.0 | 2.4 | 3.4 |
| *Serratia* spp. | 2.8 | 2.8 | 3.0 | 3.4 | 1.0 | 3.5 | 3.5 | 2.8 |
| *Acinetobacter* spp. | 1.5 | 1.2 | 2.3 | 1.3 | 3.7 | 2.8 | 2.4 | 2.2 |
| *P. flouresence* | 1.0 | 3.2 | 1.8 | 3.9 | 3.1 | 2.2 | 1.9 | 3.1 |
| Control | 1.8 | 2.9 | 3.3 | 3.0 | 3.3 | 3.6 | 2.7 | 3.6 |
| Means | 2.1 | 2.5 | 2.6 | 2.8 | 2.6 | 2.7 | 2.9 | 2.9 |
| LSD | 0.6 | 0.7 | 0.7 | 0.7 | 0.8 | 0.6 | 0.5 | 0.6 |
| CV% | 17.0 | 15.8 | 15.6 | 13.9 | 17.7 | 12.6 | 10.8 | 11.5 |

Table 4.4: Mean severity of bacterial wilt on tomato plants drenched with different microbial antagonists in field experiment 2

| | Weeks after Transplanting | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Treatments | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| *T. hamatum* | 0.0 | 1.8 | 2.8 | 2.8 | 3.0 | 3.1 | 3.4 | 1.7 |
| *T. atroviride* | 2.0 | 3.4 | 3.1 | 2.3 | 1.9 | 2.8 | 2.3 | 2.7 |
| *T. harzianum* | 2.6 | 2.3 | 2.9 | 3.0 | 3.2 | 3.1 | 2.8 | 3.4 |
| *T. viride* | 2.8 | 2.0 | 2.8 | 3.1 | 2.7 | 2.3 | 1.5 | 3.5 |
| *B. subtilis* | 1.6 | 1.5 | 1.0 | 1.5 | 3.4 | 2.5 | 2.2 | 2.0 |
| *Serratia* spp. | 2.0 | 2.1 | 2.8 | 1.7 | 1.2 | 2.2 | 3.1 | 2.3 |
| *Acinetobacter* spp. | 0.0 | 2.8 | 1.0 | 1.9 | 1.8 | 2.9 | 2.9 | 2.5 |
| *P. fluorescens* | 2.0 | 1.7 | 2.9 | 2.3 | 2.1 | 2.7 | 2.3 | 2.3 |
| Control | 2.5 | 2.1 | 3.4 | 0.0 | 2.9 | 3.3 | 1.9 | 2.9 |
| Means | 1.7 | 2.2 | 2.5 | 2.1 | 2.5 | 2.8 | 2.5 | 2.6 |
| LSD | 0.6 | 1.0 | 0.8 | 0.8 | 1.1 | 0.9 | 0.7 | 1.0 |
| CV% | 49.9 | 32.3 | 22.3 | 27 | 31.6 | 22.4 | 19.1 | 25.8 |

Table 4.5: Percentage mean disease index on tomato plants drenched with different microbial antagonists in field experiment 1

| | Weeks after transplanting | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Treatments | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| *T. hamatum* | 33.7 | 40.0 | 57.2 | 77.3 | 65.6 | 71.3 | 77.4 | 74.9 |
| *T. atroviride* | 75.6 | 62.4 | 49.1 | 62.4 | 54.8 | 57.1 | 84.1 | 71.9 |
| *T. harzianum* | 75.7 | 79.4 | 72.8 | 70.3 | 73.8 | 73.7 | 85.8 | 78.4 |
| *T. viride* | 47.5 | 71.0 | 70.3 | 71.8 | 69.8 | 77.2 | 83.6 | 80.3 |
| *B. subtilis* | 24.8 | 41.8 | 61.8 | 69.1 | 65.2 | 76.7 | 69.7 | 83.8 |
| *Serratia* spp. | 64.9 | 61.1 | 68.4 | 80.7 | 47.6 | 84.6 | 85.8 | 75.4 |
| *Acinetobacter* spp. | 41.8 | 38.8 | 60.6 | 50.9 | 86.1 | 75.1 | 71.4 | 68.1 |
| *P. fluorescens* | 34.9 | 66.4 | 48.3 | 88.2 | 77.6 | 65.6 | 63.3 | 80.0 |
| Control | 57.0 | 68.2 | 80.2 | 77.3 | 84.8 | 89.8 | 78.8 | 92.1 |
| Means | 50.6 | 58.8 | 63.2 | 72.0 | 69.5 | 74.6 | 77.8 | 78.3 |
| LSD | 18.2 | 17.1 | 22.8 | 14.0 | 13.6 | 9.3 | 7.6 | 7.9 |
| CV% | 20.8 | 16.8 | 20.8 | 11.2 | 11.3 | 7.2 | 5.6 | 5.9 |

Table 4.6: Percentage mean disease index of tomato plants drenched with different microbial antagonists in field experiment 2

| | Weeks After Transplanting | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Treatments | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| *T. hamatum* | 11.4 | 33.3 | 56.1 | 66.8 | 74.7 | 78.6 | 83.8 | 59.4 |
| *T. atroviride* | 47.9 | 72.6 | 77.0 | 62.9 | 61.8 | 77.5 | 70.3 | 76.8 |
| *T. harzianum* | 62.3 | 56.9 | 73.8 | 30.6 | 76.6 | 83.8 | 65.9 | 79.8 |
| *T. viride* | 66.6 | 38.7 | 57.0 | 70.0 | 71.2 | 66.6 | 55.7 | 86.0 |
| *B. subtilis* | 38.6 | 33.9 | 34.4 | 42.2 | 80.2 | 68.6 | 65.2 | 63.1 |
| *Serratia* spp. | 43.8 | 34.6 | 58.9 | 37.0 | 49.3 | 64.5 | 78.9 | 68.1 |
| *Acinetobacter* spp. | 30.2 | 56.3 | 31.6 | 52.3 | 58.0 | 75.0 | 77.5 | 72.2 |
| *P. fluorescens* | 51.0 | 36.2 | 61.4 | 62.0 | 63.2 | 74.0 | 68.3 | 68.9 |
| Control | 63.9 | 49.2 | 74.0 | 74.7 | 80.9 | 81.7 | 79.5 | 88.3 |
| Means | 46.2 | 45.8 | 58.3 | 55.4 | 68.4 | 74.5 | 71.7 | 73.6 |
| LSD | 24 | 20.2 | 17.3 | 19.5 | 16.2 | 14.6 | 10.8 | 13.5 |
| CV% | 35.6 | 30.3 | 20.3 | 24.1 | 16.2 | 13.4 | 10.3 | 12.6 |

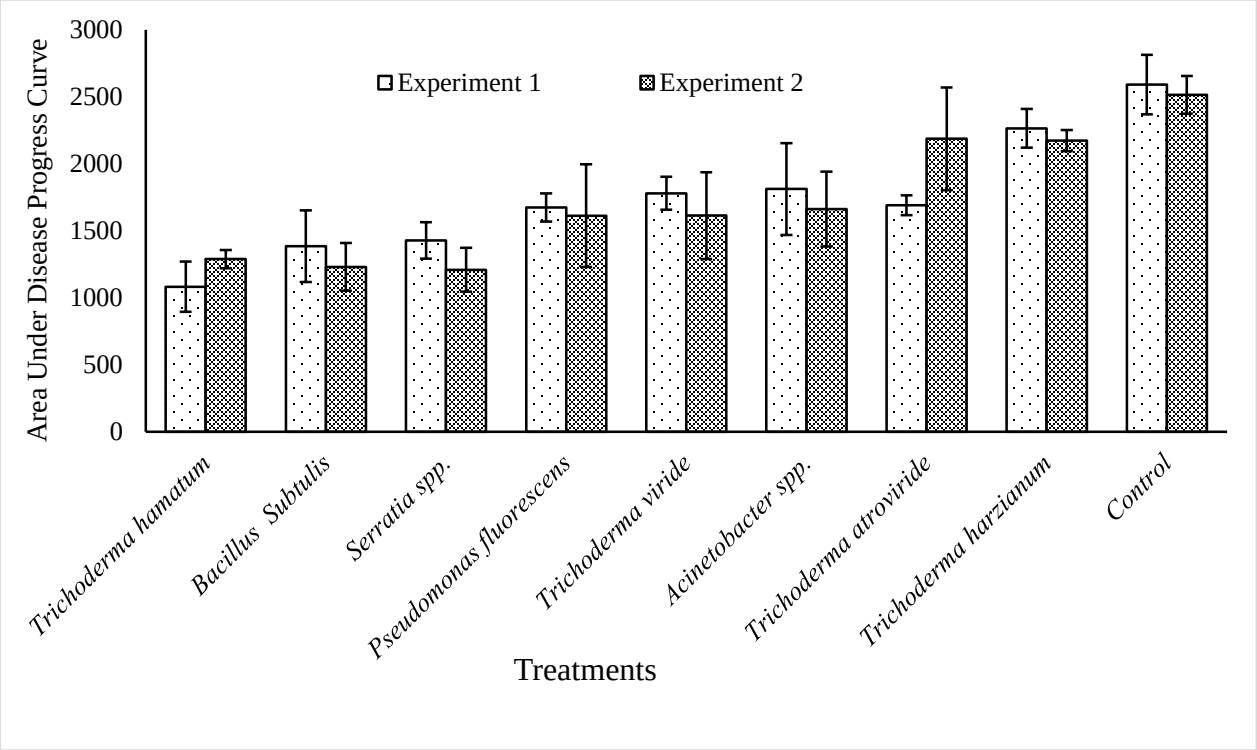


Figure 4.5: Area under disease progress curve of bacterial wilt on tomato drenched with different microbial antagonists for field experiments 1 and 2.

### 4.4.2 Effects of plant extracts on bacterial wilt of tomato

There was no significant ($P \leq 0.05$) difference in the efficacy of plant extracts in reducing crop mortality, disease incidence, severity and disease index in both experiements at ten weeks after transplanting (Table 4.7, Table 4.8, Table 4.9, Table 4.10, Table 4.11, Table 4.12). In contrast, there was significant ($P \leq 0.05$) difference in AUDPC for the different treatments (Figure 4.7). Crude extracts of *Curcurma longa* and *Tagetes minuta* exhibited inconsistent results in both experiments. In the first experiment, *T. minuta* showed the highest reduction in crop mortality, disease incidence and AUDPC by up to 22.4, 17.8 and 20.2%, while *C. longa* exhibited the least reduction of 7.5, 6.8, and 17.4%, respectively (Figure 4.6, Figure 4.7, Table 4.7). In contrast, *C. longa* exhibited superior performance in experiment 2, reducing crop mortality, disease incidence and AUDPC by 33.3, 30.8 and 41.0%, respectively. *Rosmarinum officinalis* showed the least reduction in crop mortality, disease incidence and AUDPC by 9.8, 11.0 and 7.4%, respectively in experiment 2 (Figure 4.6, Figure 4.7, Table 4.8).

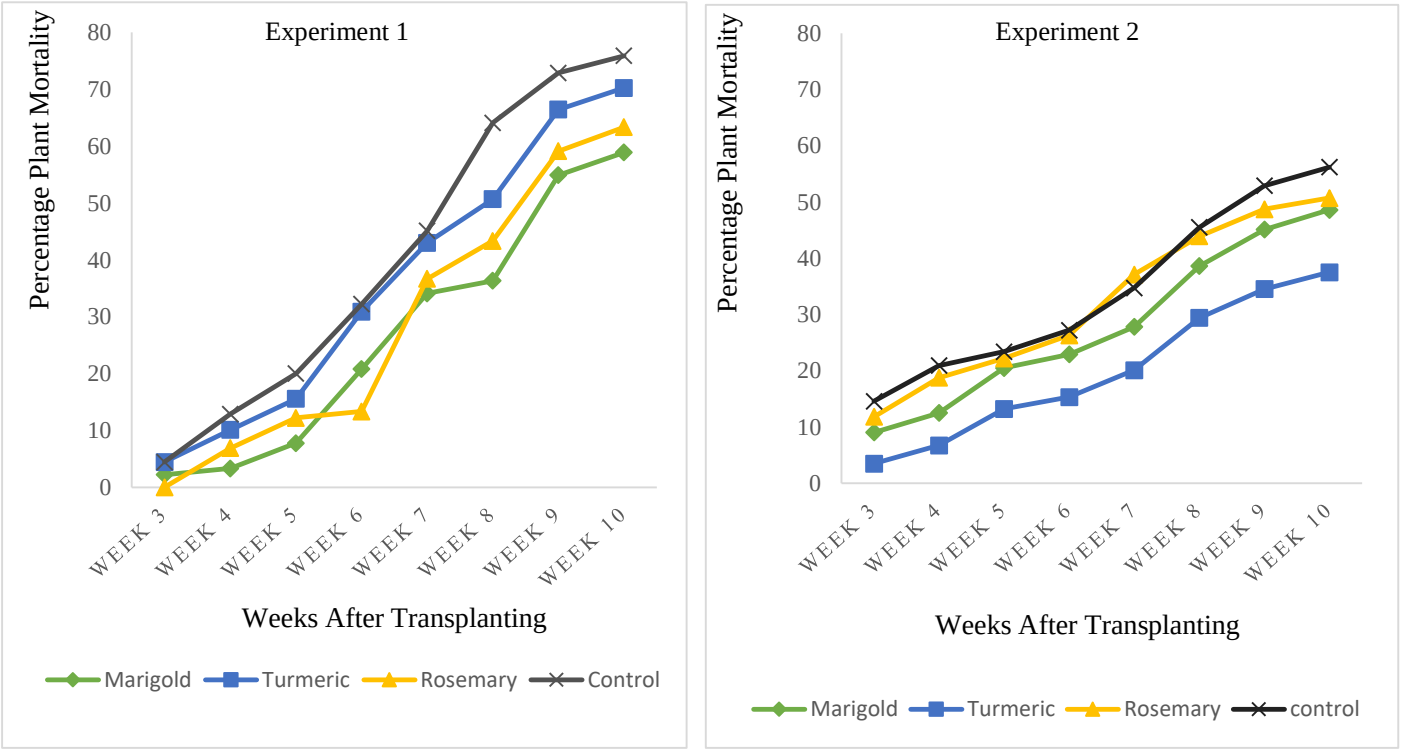


Figure 4.6: Percentage mortality of tomato plants drenched with crude extracts prepared from different plants in field experiment 1 and 2, respectively

Table 4.7: Percentage mean disease incidence on tomato plants drenched with different plant extracts in field experiment 1

| | Weeks after transplanting | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Treatments | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| *Tagetes minuta* | 5.6a | 14.7a | 28.2a | 42.2a | 48.9a | 54.1a | 63.1a | 66.7a |
| *Curcuma longa* | 8.9a | 11.2a | 20.3 ab | 37.8a | 45.6a | 62.8a | 72.8a | 75.6a |
| *Rosmarinum officinalis* | 7.8a | 15.9a | 23.6 ab | 41.1a | 46.7a | 57.8a | 66.9a | 70.0a |
| Control | 15.6a | 19.0a | 28.1 a | 41.1a | 60.6a | 71.6a | 78.3a | 81.1a |
| Means | 9.4 | 15.2 | 25.1 | 40.6 | 50.4 | 61.6 | 70.3 | 73.3 |
| LSD | 8.8 | 12.2 | 12 | 8.4 | 13.9 | 14.5 | 13 | 14.6 |
| CV% | 46.7 | 40.2 | 23.9 | 10.3 | 13.8 | 11.8 | 9.3 | 10.6 |

Means accompanied by different letters within a column are significantly different according to Fishers Protected LSD test ($P \leq 0.05$)

Table 4.8: Percentage mean disease incidence on tomato plants drenched with different plant extracts in field experiment 2

| | Weeks after Transplanting | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Treatments | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| *Tagetes minuta* | 7.9a | 12.0a | 15.1a | 18.1a | 22.0a | 35.6a | 41.3a | 44.4a |
| *Curcuma longa* | 5.7a | 6.4a | 9.3a | 13.6a | 15.0a | 20.1a | 32.3a | 35.9a |
| *Rosmarinum officinalis* | 8.9a | 13.0a | 17.9a | 23.6a | 28.5a | 31.9a | 42.2a | 46.2a |
| Control | 8.7a | 12.6a | 17.3a | 19.6a | 29.1a | 42.3a | 49.5a | 51.9a |
| Means | 7.8 | 110 | 14.9 | 18.7 | 23.7 | 32.5 | 41.3 | 44.6 |
| LSD | 7.1 | 10.1 | 11.5 | 11.3 | 14.2 | 17.1 | 21.2 | 22.6 |
| CV % | 57.0 | 57.2 | 48.4 | 37.6 | 37.5 | 33.0 | 32.0 | 31.7 |

Means accompanied by different letters within a column are significantly different according to Fishers Protected LSD test ($P \leq 0.05$)

There was no significant ($P \leq 0.05$) difference in severity and disease index of tomato plants drenched with crude extracts prepared from different plants at ten weeks after transplanting. Similarly, the mean severity for each treatment did not follow a linear disease progress curve and the the disease index sponteniuosly fluctuated between high and low levels from one week to the other (Table 4.9' Table 4.10, Table 4.11, Table 4.12).

Table 4.9: Mean severity of bacterial wilt on tomato plants drenched with different plant extracts in field experiment 1

| | Weeks After Transplanting | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Treatments | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| *Tagetes minuta* | 3.6a | 2.6a | 3.7a | 2.5a | 3.0a | 1.8b | 3.0a | 2.7a |
| *Curcuma longa* | 1.6c | 3.4 a | 2.3b | 2.4a | 3.7a | 3.1a | 2.3a | 2.9a |
| *Rosmarinum officinalis* | 2.5b | 3.0a | 2.4b | 2.8a | 2.9a | 3.2a | 2.3a | 3.0a |
| Control | 1.8bc | 2.9a | 3.3a | 3.0a | 3.3a | 3.6a | 2.7a | 3.6a |
| Means | 2.4 | 3 | 3 | 2.7 | 3.2 | 2.9 | 2.6 | 3.1 |
| LSD | 0.7 | 0.6 | 0.6 | 0.8 | 0.7 | 0.8 | 0.7 | 0.8 |
| CV% | 15.6 | 10.8 | 9.9 | 14.9 | 10.4 | 14.5 | 13.7 | 13.6 |

Means accompanied by different letters within a column are significantly different according to Fishers Protected LSD test ($P \leq 0.05$

Table 4.10: Mean severity of bacterial wilt on tomato plants drenched with different plant extracts in field experiment 2

| | Weeks after Transplanting | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| *Tagetes minuta* | 0.9b | 1.0a | 2.5a | 2.5a | 2.8a | 3.0a | 3.3a | 2.5a |
| *Curcuma longa* | 2.3a | 1.5a | 2.6a | 0.0b | 0.9a | 2.0b | 2.1b | 1.5a |
| *Rosmarinum officinalis* | 2.1a | 1.0a | 2.6a | 2.1a | 3.3a | 2.3b | 2.4b | 2.4a |
| Control | 2.8a | 1.9a | 3.0a | 2.4a | 1.6a | 3.3a | 4.0a | 1.9a |
| Means | 2.0 | 1.3 | 2.7 | 1.7 | 2.1 | 2.6 | 2.9 | 2.1 |
| LSD | 1.1 | 1.6 | 1.3 | 1.8 | 2.0 | 0.6 | 0.8 | 1.5 |
| CV% | 35.4 | 39.0 | 31.7 | 43.3 | 49.2 | 13.3 | 16.3 | 46.8 |

Means accompanied by different letters within a column are significantly different according to Fishers Protected LSD test ($P \leq 0.05$)

Table 4.11: Percentage mean disease index on tomato plants drenched with different plant extracts in field experiment 1

| | Weeks After Transplanting | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Treatments | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| *Tagetes minuta* | 76.2a | 57.8a | 80.7a | 70.3a | 78.4a | 62.0b | 80.9a | 76.7a |
| *Curcuma longa* | 47.2a | 73.5a | 64.8a | 68.5a | 88.4a | 81.8a | 72.3a | 80.7a |
| *Rosmarinum officinalis* | 55.9a | 74.1a | 66.7a | 74.2a | 76.7a | 83.0a | 71.0a | 81.9a |
| Control | 57.0a | 68.2a | 80.2 a | 77.3a | 84.8a | 89.8a | 78.8a | 92.1a |
| Means | 59.1 | 68.4 | 73.1 | 72.6 | 82.1 | 79.2 | 75.8 | 82.8 |
| LSD | 21.7 | 13.4 | 13.6 | 11.1 | 10.2 | 12.2 | 10.4 | 12.1 |
| CV% | 18.4 | 9.8 | 9.3 | 7.6 | 6.2 | 7.7 | 6.9 | 7.3 |

Means accompanied by different letters within a column are significantly different according to Fishers Protected LSD test ($P \leq 0.05$)

Table 4.12: Percentage mean disease index of tomato plants drenched with different plant extracts in field experiment 2

| | Weeks After Transplanting | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Treatmenst | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| *Tagetes minuta* | 27.9b | 23.1a | 52.2a | 59.0a | 71.0a | 76.2a | 81.8b | 70.6a |
| *Curcuma longa* | 54.4a | 25.9a | 56.0a | 19.8a | 44.1a | 60.0b | 62.7c | 67.6a |
| *Rosmarinum officinalis* | 60.2a | 23.3a | 57.2a | 48.0a | 79.1a | 65.3b | 68.5c | 56.6a |
| Control | 58.4a | 42.9a | 66.8a | 51.0a | 55.9a | 82.1a | 92.8a | 62.8a |
| Means | 50.2 | 28.8 | 58.0 | 44.5 | 62.5 | 70.9 | 76.4 | 64.4 |
| LSD | 24.7 | 20.7 | 24.8 | 36.6 | 29.3 | 7.6 | 10.7 | 22.2 |
| CV% | 30.7 | 44.8 | 26.7 | 51.4 | 29.3 | 6.7 | 8.7 | 21.5 |

Means accompanied by different letters within a column are significantly different according to Fishers Protected LSD test ($P \leq 0.05$)

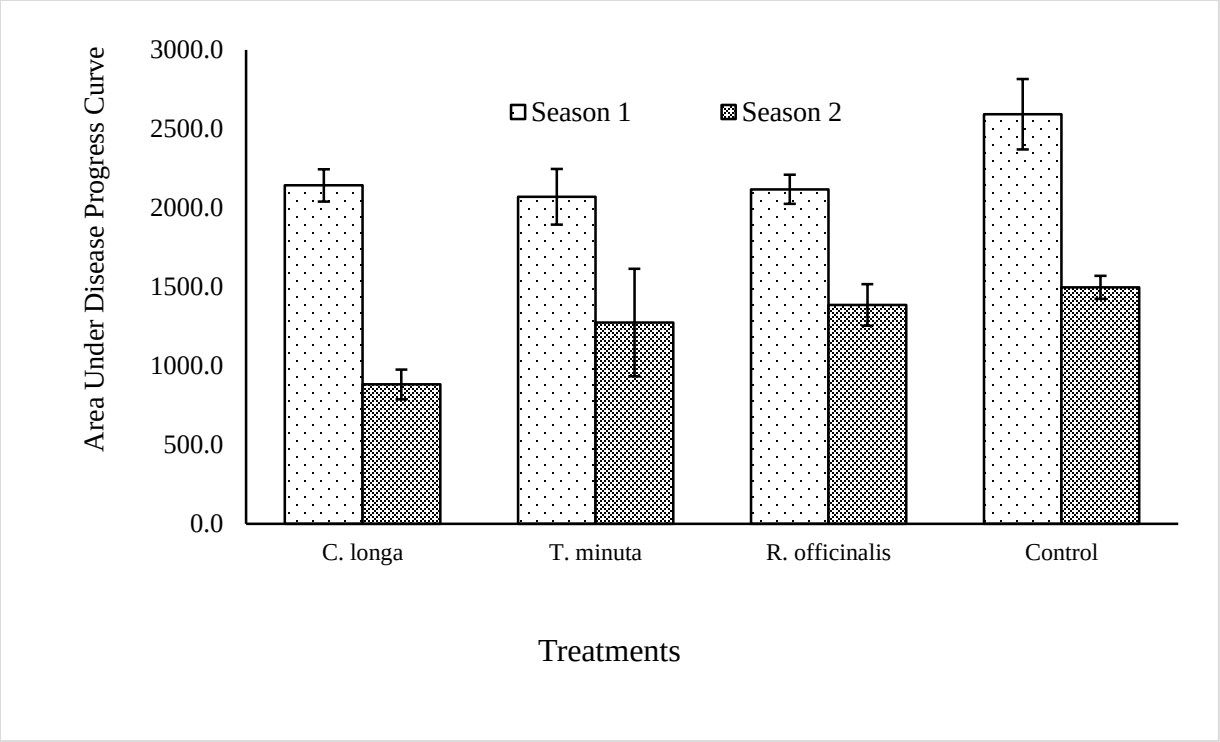


Figure 4.7: Area under disease progress curve of bacterial wilt on tomato drenched with different plant extracts for field experiments 1 and 2.

### 4.4.3 Effects of microbial antagonists on growth and yield of tomato

All microbial antagonists tested produced significantly (P≤0.05) different effects on plant growth and fruit yield. Generally, microbial antagonists were superior in increasing plant growth and yield compared to plant extracts (Table 4.13, Table 4.14, Table 4.15, Table 4.16). Although there was no significant (P≤0.05) difference in height of plants treated with different antagonists, *T. viride* and *T. hamatum* enhanced height by 12.7% and 8.4% in experiment 1 while *B. subtilis* and *Serratia* spp. each increased height by 6.4 % in experiment 2, respectively (Tables 4.13, Table 4.14).

Generally, bacterial antagonists were superior in biomass accumulation and resulted into plants with higher dry weight compared to fungal antagonists in both experiments. *Bacillus subtilis* treated plants had the highest biomass accumulation and increased dry weight by up to 62.0% and 62.3% in experiment 1 and 2, respectively. It was followed in activity by *Acinetobacter* spp. which increased dry weight by 32.8% in experiment 1, while in experiment 2, a comparable performance was observed for *T. hamatum* which enhanced dry weight by 51.3%. In contrast, a reduction of

2.2% and 0.9% was observed on dry weights of crops treated with *T. harzianum* in experiments 1 and 2, respectively (Table 4.13, Table 4.14).

Table 4.13: Means of height and weight of tomato plants and fruits drenched with microbial antagonists in field experiment 1

| Treatments | Plant height in Centimeters | Dry weight in Grams | Fruit weight in Kilograms/hectare |
|---|---|---|---|
| *T. hamatum* | 40.2 a | 33.3ab | 2297.0a |
| *T. atroviride* | 39.2 a | 28.2c | 1756.0bc |
| *T. harzianum* | 39.8 a | 25.6c | 1317.0d |
| *T. viride* | 41.8 a | 32.3b | 2128.0ab |
| *B. subtilis* | 38.9 a | 37.1a | 2011.0abc |
| *Serratia* spp. | 39.1 a | 32.5b | 1858.0bc |
| *Acinetobacter* spp. | 37.2 a | 34.8ab | 1606.0cd |
| *P. fluorescens* | 37.7 a | 34.4ab | 1203.0de |
| Control | 37.1 a | 26.2c | 775.0e |
| Means | 38.9 | 31.6 | 1661.0 |
| LSD | 3.8 | 4.1 | 438.1 |
| CV% | 6.6 | 8.9 | 18.1 |

Means accompanied by different letters within a column are significantly different according to Fishers Protected LSD test ($P \leq 0.05$)

Fruit yield varied widely among the treatments and there was high significant ($P \leq 0.05$) difference in weight of fruits. *Trichoderma hamatum* gave the highest yield increase of 196.4% followed by *T. viride* and *B. subtilis* at 174.6% and 159. 5%, respectively (Table 4.13). In contrast, *Serratia* spp. treated plants had the highest yield increament of upto 233.1% in experiment 2 and was not significantly($P \leq 0.05$) different to plant treated with *B. subtilis*, *T. viride*, *T. hamatum* and *Acinetobacte*r spp., which increased yield by 229.7%, 228.1%, 222.0% and 184.6%, respectively (Table 4.14).

Table 4.14: Means of height and weight of tomato plants and fruits drenched with microbial antagonists in field experiment 2

| Treatments | Plant height in Centimeters | Dry weight in Grams | Fruit weight in Kilograms/hactare |
|---|---|---|---|
| *T. hamatum* | 37.2 a | 35.7a | 1553.0a |
| *T. atroviride* | 35.8 a | 27.0bc | 769.0bc |
| *T. harzianum* | 36.0 a | 23.3c | 567.0c |
| *T. viride* | 38.1 a | 32.7ab | 1578.0a |
| *B. subtilis* | 37.8 a | 38.3a | 1586.0a |
| *Serratia* spp. | 38.1 a | 32.0ab | 1602.0a |
| *Acinetobacter* spp. | 36.5 a | 34.9a | 1369.0ab |
| *P. fluorescens* | 35.6 a | 33.9a | 828.0bc |
| Control | 35.8 a | 23.6c | 481.0c |
| Mean | 36.8 | 31.3 | 1148.0 |
| LSD | 4.9 | 6.5 | 616.2 |
| CV% | 9.1 | 14.2 | 36.8 |

Means accompanied by different letters within a column are significantly different according to Fishers Protected LSD test (P ≤0.05)

### 4.4.4 Effects of plant extracts on growth and yield of tomato

There was no significant (P≤0.05) difference in height of plants treated with different plant extracts in both experiment 1 and 2. Nevertheless, *C. longa* treated plants were the tallest and had up to 5.7 and 4.7% height increament compared to untreated control, in experiment 1 and 2, respectively. In experiment 1, *T. minuta* had the least effect and increased plant height by only 0.5% while in experiment 2, *R. officinalis* exhibited the lowest height increament of only 0.3% (Table 4.15, Table 4.16).

The different plant extracts exhibited significance (P≤0.05) difference in biomass accumulation only in experiment 2. *Curcuma longa* showed superior biomass accumulation in both experiments, increasing dry weight by 20.2 and 45.2% in experiment 1 and 2, respectively. However, *R. officinalis* showed the least percentage increase in dry weight of 15.3% in experiment 1 while *T. minuta* exhibited the lowest biomass increament of 21.8% in experiment 2 (Table 4.15, Table 4.16).

Table 4.15: Means of height and weight of tomato plants and fruits drenched with plant extracts in field experiment 1

| Treatments | Plant height in Centimeters | Dry weight in Grams | Fruit weigh in Kilograms/hactare |
|---|---|---|---|
| *Tagetes minuta* | 37.3a | 30.9a | 1631.0a |
| *Curcuma longa* | 39.2a | 31.5a | 1139.0b |
| *Rosmarinum officinalis* | 37.8a | 30.2a | 1193.0b |
| Control | 37.1a | 26.2a | 775.0c |
| Means | 37.9 | 29.7 | 1184.0 |
| LSD | 4.1 | 7.3 | 314.0 |
| CV% | 6.8 | 15.3 | 16.6 |

Means accompanied by different letters within a column are significantly different according to Fishers Protected LSD test (P ≤0.05)

Similarly, all the plant extracts tested showed significant (P≤0.05) variations in yield output for both experiment 1 and 2. However, *T. minuta* and *C. longa* exhibited contrasting performance in both experiments. Correspondingly, *T. minuta* showed the highest yield inreament in experiment one by 110.5% while *C. longa* had the least effect, increasing fruit weight by 47.0% in the same experiment. In experiment 2, *C. longa* exhibited superior performance, increasing yield by 180.4% while *T. minuta* showed the least increament of 62.9% (Table 4.15, Table 4.16).

Table 4.16: Means of height and weight of tomato plants and fruits drenched with plant extracts in field experiment 2

| Treatments | Plant height in Centimeters | Dry weight in Grams | Fruit weight in Kilograms/hactare |
|---|---|---|---|
| *Tagetes minuta* | 36.3a | 30.2ab | 1942.0b |
| *Curcuma longa* | 37.6a | 36.0a | 3342.0a |
| *Rosmarinum officinalis* | 36.0a | 35.7a | 2103.0b |
| Control | 35.9a | 24.8b | 1192.0b |
| Mean | 36.5 | 31.7 | 2144.8 |
| LSD | 4.5 | 6.8 | 1135.6 |
| CV% | 7.7 | 13.5 | 33.1 |

Means accompanied by different letters within a column are significantly different according to Fishers Protected LSD test (P ≤0.05)

## 4.5 Discussion

### 4.5.1 Efficacy of microbial antagonists on bacterial wilt of tomato

Soil drenching with antagonistic bacteria and fungi effectively reduced bacterial wilt incidence, index, AUDPC and crop mortality. The effectiveness of the antagonists varied with *T. hamatum* and *Bacillus subtilis* exhibitng superior perfomance. Variation in biocontrol effectiveness of diverse species of antagonistic bacteria and fungi on bacterial wilt has been reported by several authors (Guo *et al*., 2004; Nguyen and Ranamukhaarachchi, 2010; Abd-El-Khair and Seif El-Nasr, 2012; Subedi *et al*., 2019). Moreover, Abd-El-Khair and Seif El-Nasr, (2012) and Yendyo *et al*., (2018) observed that bacterial and fungal antagonists differed in their biocontrol effectiveness against bacterial wilt under field conditions.

Effectiveness of *Pseudomonas* spp., (Yendyo *et al*., 2018*), Acinetobacter* spp. (Xue *et al*., 2009), *Serratia* spp., (Guo *et al*., 2004; Xue *at al*., 2012) and *Bacillus* spp., (Yang *et al*., 2012; Singh *et al*., 2016) on management of bacterial wilt of diverse crops are well documented for both greenhouse and field experiments.

These findings corroborate results of several studies which reported superior activity of *Bacillus* spp., *Serratia* spp., and *Acinetobacter* spp. on management of bacterial wilt under greenhouse and field conditions. Almoneafy *et al*., (2014) and Bhai *et al*., (2019) reported that different species and strains of *Bacillus* spp. showed a superior efficacy in reducing bacterial wilt of tomato under greenhouse conditions and Yang *et al*., (2012) demonstrated that *B. subtilis* was superior to *Myroides odoratimimus, Bacillus amyloliquefaciens* and *Stenotrophomonas maltophilia* in suppressing this disease on ginger in the greenhouse. Hassan *et al*., (2018) also found that *B. subtilis* performed better than *Bacillus pseudomycoides, Streptomyces toxitricini* and *Stenotrophomonas maltophilia* in suppressing bacterial wilt of tomato in the greenhouse.

Under the field conditions, *Acinetobacter* spp. reduced wilt incidence by 58.4% in Huaian and by 64.8% Longyan, China. In both experiments, the biocontrol efficacy was higher than that conferred by streptomycin antibiotics and negative control (Xue *et al*., (2009). In 2004, Guo *et al*. reported that *Serratia* spp., fluorescent Pseudomonad and *Bacillus* spp. effectively reduced bacterial wilt incidence in four field trials in China where the biocontrol efficacy ranged from 63.6% to 94.1%. The authors found that fluorescent Pseudomonad and *Bacillus* spp. exhibited higher efficacy depending on climatic conditions whereas *Serratia* spp. always had the least efficacy except in a

single trial. Nevertheless, in 2012, Xue *et al*. showed that *Serratia* spp. exhibited between 19.5% to 70.3% biocontrol efficacy on seven different strains of *R. solanacearum* in the greenhouse and up to 70.2% under field conditions on tomato in China. However, the findings herein differ with findings of Seleim *et al*., (2011), Nawangsih *et al*., (2012) and Yendyo *et al*., (2018) which showed that *Pseudomonas fluorescence* exhibited better biocontrol efficacy on bacterial wilt than *Bacillus subtilis* under greenhouse and field conditions, respectively.

Biocontrol potential of bacterial antagonists in managing bacterial wilt depends on their ability to secrete inhibitory substances such as antibiotics, siderophores, cell wall degrading enzymes, competition for sites on root surface and induction of systemic resistance (Seleim *et al*., 2011). Antgonistic strains of *B. amyloliqefaciens* and *B. subtilis* have been shown to produce siderophores (Singh *et al*., 2016), that were identified by Yang *et al*., (2012) as the key biochemical used for biocontrol of *R. solanacearum* (Yang *et al*., 2012). However, Chen *et al*., (2013) found that surfactin was the most important antibiotic in exerting inhibition against *R. solanacearum* by *B. subtilis* strains. Additionally, efficient biocontrol potential of *B. subtilis* was found to strongly depend on formation of robust root-associated biofilms which enhanced cell colonization efficiency and increased concentration of antibiotics around the roots (Chen *et al*., 2013). Effective disease suppression by bacterial antagonists is contingent to application of multiple modes of action against the phytopathogens (Chen *et al*., 2013; Rai *et al*., 2017).

Several studies on application of fungal antagonists as biocontrol agents are documented. Correspondingly, studies on integration of *Trichoderma* spp. in farming practices such as soil amendments, BCAs or bio fertilizers for crop development and management of phytopathogens are have been reported (Konappa *et al*., 2018). These findings are in line with the report by Abd-El-Khair and Seif El-Nasr, (2012) which revealed that effectiveness of *T. hamatum* and *B. subtilis* in managing bacterial wilt of potato significantly differed both under greenhouse and three field experiments. Though *B. subtilis* exhibited the highest disease reduction generally followed by *T. hamatum*, the latter provided complete protection against potato brown rot in the third field experiment and was superior to *B. subtilis* (Abd-El-Khair and Seif El-Nasr, 2012). Similarly, Nahar *et al*., (2019) demonstrated that crop loss due to bacterial wilt of eggplant was significantly reduced under improved nursery management through application of *T. harzianum* and consequent transplanting into field treated with the same biocontrol agent.

Mycoparasitism, antibiosis, competition for space and nutrients have been reported as some of the biocontrol mechanisms exerted by *Trichoderma* spp. in suppression of phytopathogens (Abbas *et al*., 2017; Redda *et al*., 2018). Also of equal importance is induction of host plant resistance by antagonistic fungi. Konappa *et al*., (2018) observed an increase in activity of defense related enzymes after treating tomato plants with antagonistic isolates of *Trichoderma* spp.

### 4.5.2 Effects of microbial antagonists on growth and yield of tomato

All the microbial antagonists screened for bio efficacy against bacterial wilt exhibited varied levels of plant growth promotion and yield increament. These findings corroborate reports by Nawangsih *et al*., (2012) which showed no significance difference in plant heights treated with *Bacillus* spp. and *Pseudomonas* spp. The findings also concur with reports by Singh *et al*., (2012), Almoneafy *et al*., (2014) and Singh *et al*., (2016) which revealed that diverse species of *Bacillus* and strains of *B. subtilis* increased plant height, biomass and yield compare to untreated control. Xue *et al*., (2009; 2012) found that tomato and pepper plants treated with *Acinetobacter* spp. and *Serratia* spp. under bacterial wilt pressure had higher biomass and yield relative to untreated control while Sharma and Kumar (2009) reported that application of *T. viride* on tomato as a biocontrol agent against bacterial wilt resulted into yield increase by up to 142.1% and out performing *Azotobacter chroococcum*, plant extracts and chemicals.

Although the mechanisms through which the microbial antagonists increased plant biomass and yield were not assessed, previous studies indicate that beneficial microorganisms promote plant growth either directly through secretion of growth regulators (cytokinins, auxins, gibberellins) and facilitation of nutrient uptake (phosphate solubilization, nitrogen fixation) or indirectly through prevention and reduction of harmful effects of plant pathogens (Seleim *et al*., 2011; Stewart and Hill, 2014). Conversely, Stewart and Hill, (2014) suggested that *Trichoderma* spp. promote plant growth through production of phytohormones, secretion of vitamins, solubilization of nutrients, increasing uptake and translocation of nutrients, enhancing metabolism of carbohydrates, photosynthesis and plant defense. Similarly, *Bacillus* spp. and diverse strains of *B. subtilis* increased tomato growth through solubilization of phosphorus and production of indole acetic acid (IAA), ammonia and siderophores under bacterial wilt pressure (Almoneafy *et al*., 2014; Singh *et al*., 2016).

### 4.5.3 Efficacy of plant extracts on management of bacterial wilt

Drenching of crude plant extracts to manage bacterial wilt exhibited varying level of effectiveness in reducing disease incidence, plant mortality and AUDPC. The findings concur with reports of Abo-Elyousr and Asran, (2009) and Kumar *et al*., (2017) who observed that crude extracts obtained from diverse plant sources exhibited varying levels of effectiveness in management of bacterial wilt. The findings are also in line with reports of Din *et al*., (2016), which demonstrated that incorporation of finely ground powder of *T. patula* into the soil effectively decreased bacterial wilt severity and increased tomato yield. The authors also observed that the results were comparable to that of standard antibiotics.

Moreover, Dutta, (2012) reported that application of a mixture of *C. longa* and *Ferula assa-foetida* powders reduced bacterial wilt incidence by 59.8% under field conditions in India. Similar findings were observed by Sharma and Kumar, (2009) who reported that *C. longa* and *Ferula assa-foetida* powders extracted in water reduced primary inoculum by 32.7% at 90$^{th}$ day post inoculation under field conditions.

Disease suppression by crude plant extracts can be mediated through direct antimicrobial action of active compounds, indirect activity through activation of competitive and antagonistic microbes and systemic resistance induction in host plants (Deberdt *et al*., 2012). These mechanisms are facilitated by a number of active compounds inherent of these medicinal plants.

Research on biocontrol potential of different species of Tagetes has revealed that this genus contains plants with bactericidal (Ramya *et al*., 2012), nematicidal, insecticidal and fungicidal properties (Obongoya et a., 2010). Physiochemical analysis of crude extracts of *Tagetes* sp. revealed the presence of flavonoids, saponins, quinones, sugars, tannins, terpenes, coumarins, alkaloids and terpernoids (Din *et al*., 2016). In *C. longa*, curcumin has been identified as the most vital compound with antimicrobial properties (Alavijeh *et al*., 2012; Gurjar *et al*., 2012) while in *R. officinalis*, phenolic groups of rosmarinic acid, carnosic acid (Ahmed *et al*, 2011; Nieto *et al.*, 2018) and caffeic acid (Nieto *et al.*, 2018) are the most important.

These compound act through coagulation of the bacterium cell protein and disruption of essential amino acids (Al-Obaidi, 2014), interfering with functions of the membrane sterol (Din *et al*., 2016), DNA intercalation and inhibition of DNA associated enzymes production (Wang *et al*.,

2000). Presence of these bioactive compound in crude extracts of the three plants could have caused the reduction in disease intensity compared to control. Furthermore, presence of bioactive secondary metabolites or unidentified compounds with bioactive activity has been associated with high levels of efficacy in managing bacterial wilt (Din *et al*, 2016).

### 4.5.4 Efficacy of plant extracts on growth and yield of tomato

Drenching of crude plant extracts resulted into varied increase in plant growth and fruit yield compared to untreated control. Similar observations have been made in other studies that reported variations in plant growth and yield increament in plants treated with crude plant expracts from diverse sources (Ji et al., 2005; Jang and Kuk, 2019). Ji *et al*., (2005) observed a significant yield increase in tomato crops treated with thymol to manage bacterial wilt.

Variation in growth promotion between extracts of different plant species and cultivars due to differences in their chemical composition has been reported (Jang and Kuk, 2019). These authors prepared 81 extracts from 31 different agricultural materials through different extraction techniques and found that leaf and stem extracts of *Glycine max* and *Allium tuberosum* exhibited the highest increase in fresh shoot weight of *Lactuca sativa* by up to 45%. Similarly, tomato plants sprayed with aqueous flower extracts of *T. minuta* showed an increase in shoot length, number of branches, number of bud and fruits by 75.9%, 27.4%, 42.7% and 66.2%, respectively (Nahak and Sahu, 2017). Analogous to the findings herein, Sharma and Kumar, (2009) demonstrated that tomato crops drenched with *C. longa* and *Ferula assa-foetida* powder extracted in water increased yield by up to 71.2% compared to untreated control while Dutta, (2012) reported that application of similar treatment combination resulted into an increased yield of 182.1 quintal /hac compared to untreated control which produced only 4.6 quintal /hac.

The contradictory results exhibited by *T. minuta* and *C. longa* in the two experiments could be due to concentration of active compounds, stage of growth when harvested for extraction, presence of an inhibitor (Nieto *et al.*, 2018), climatic conditions and growth conditions of the plant (Webster, 2008) which have been identified as factor affecting antimicrobial properties of plant extracts.

Although micro and macronutrients, amino acids and hormone like growth materials found in plants has been associated to bio stimulatory activities of plant extracts, the mechanism through which they promote plant growth has not been well elucidated due to presence of many bioactive

compounds (Jang and Kuk, 2019). Nahak and Sahu, (2017) suggested that growth promotion of tomato by *T. patula* extracts could be caused by flavonoids, quercetagenins, carotenoids, patuletins, quercetins and their derivatives in the extracts which are well known for their strong antioxidant and cyto-protectants properties. Correspondingly, Andresen and Cedergreen, (2010) demonstrated that application of tea seed powder on sterile *Lemna* caused a significant growth promotion effect through direct physiological plant response rather than indirectly through pest and disease control or by enhancing nutrient uptake.

These findings suggest that locally available crops possess strong ability to suppress soil borne phytopathogens while promoting plant growth and yield simultaneously. In simulating countries like Korea which have more than 45 plant extracts registered for managing pests and improving growth of crops (Jang and Kuk, 2019), these plants can be extracted into commercial products and used for management of phytopathogens of agriculturally important crops locally and abroad.

# CHAPTER FIVE : GENERAL DISCUSSION, CONCLUSION AND RECOMMENDATION

## 5.1 General Discussion

The findings herein have shown that the most antagonistic bacteria were dominated by gram positive bacteria. This corroborate previous findings which also reported dominance of gram positive bacteria from various ecological niches including subsurface aquifer soils (Bone and Balkwill, 1988) and soils collected from tea estates (Wafula *et al*.,2015). The abundance of gram positive bacteria can be attributed to their ability to tolerate harsh environmental stress because of the thick peptidoglycan wall and endospores (Schimel *et al*., 2007).

This study also showed that different AEZs greatly varied in number of antagonistic microorganisms. Related findings were reported by Berg *et al*., (2006) which revealed that soil and site location had an effect on relative abundance and diversity of bacteria with antagonistic potential against *Verticillium dahlia*. The authors isolated more antagonistic bacteria in soils collected from Berlin, Germany, compared to two other experimental sites located in Rostock and Braunschweig, Germany respectively. In contrast, an earlier study conducted by Berg *et al*., (2005) on bioprospecting for fungal antagonists against *Verticillium dahlia* indicated that soil from Rostock harbored the highest diversity antagonistic fungi.

Consequently, majority of the bacterial antagonists isolated belonged to *Bacillus* spp., followed by *Paenibacillus* spp. even though the largest zone and clearest zone of inhibition against *R. solanacearum* was conferred by gram negative *Serratia* spp. and *Acinetobacter* spp., respectively. Studies investigating occurrence and biocontrol efficacy of soil bacteria have also reported that members of genus *Bacillus* are the most frequently isolated bacteria with antagonistic potential against diverse genera of phytopathogenic fungi (Imran *et al*., 2012) and different strains of *R. solanacearum* (Tahir *et al*., 2016). Endospore formation by gram positive bacteria facilitate their survival under harsh environmental conditions (Haas and Defago, 2005) resulting in elevated population of gram positive bacteria in dryer soils. Tahir *et al*., (2016) also observed that *Serratia* spp., with antagonistic potential against *R. solanacearum* were the least popular among the isolated antagonists.

In the case of fungi, majority of the most active isolates were diverse strains and species of *Trichoderma.* The genus *Trichoderma* is known to host complex species with biocontrol potential

against *R. solanacearum* (Abbas *et al*., 2017) and many other fungal phytopathogens (Cherkupally *et al*., 2017; Meena *et al*., 2017; Redda *et al*., 2018). They were found to be the most predominant antagonists against major soil and foliar pathogens of French beans (Fulano *et al*., 2016) and were also the most effective in inhibiting growth of pathogenic and toxic *Fusarium* spp. (Popiel *et al*., 2008).

These findings also demonstrated superior activity of plant extracts from spices and herbs in inhibiting growth of both fungal and bacterial phytopathogens *in vitro*. Previous studies have also demonstrated strong antifungal and antibacterial activity of commonly used spices against phytopathogenic microorganisms (Gurja *et al*., 2012; Muthomi *et al*., 2017). Insightful enquiries revealed that medicinal plants produce secondary metabolite with antimicrobial activity (Verma *et al*., 2012; Gurjar *et al.*, 2012) for protecting themselves against a variety of pathogens (Gurjar *et al.*, 2012; Sen and Batra, 2012). Using the appropriate techniques, these compounds can be tapped and formulated into products for management of plants pests and diseases (Muthomi *et al*., 2017). Moreover, pesticidal plants are more accessible and cheaper compared to synthetic products and can even be produced by local farmers commercially (Anjarwalla *et al*., 2016).

Generally, microbial antagonists exhibited better disease suppression under field conditions compared to plant extracts. Among the fungal antagonists, *T. hamatum* which produced the largest zone of inhibition *in vitro* also conferred the highest disease suppression under field conditions and was followed by *T. atroviride* while *T. harzianum* gave the least suppression. A similar trend in biocontrol potential was initially observed under *in vitro* experiments and therefore corroborating reports that some antagonists exhibit positive correlation between *in vitro* activity and efficacy in plants (Yang *et al*., 2012). Yang *et al*., (2012) observed that *B. subtilis* exhibited superior activity against *R. solanacearum* both *in vitro* and under greenhouse conditions, outperforming *B. amyloliquefaciens*, *Myroides odaratiminu*s and *Strenotrophomonas maltophilia* in the process.

However, for bacterial antagonists and plant extracts, the most active isolates or extracts *in vitro* were not always the most active in the field. For instance, *Serratia* spp., was the most active *in vitro* but always exhibited lower efficacy than *B. subtilis* in the field. Similarly, *T. minuta* which was superior in activity among the plant extracts *in vitro* only performed well in experiment 1 but gave the poor results in experiment 2. This lack of consistency in *in vitro* activity and disease

management *in vivo* has also been reported. Subedi *et al*., (2019) observed that antagonistic isolates against bacterial wilt did not present any correlation between *in vitro* activity and in planta control of bacterial wilt under greenhouse conditions. This observation suggest that positive correlation does not always exist between *in vitro* inhibition in the laboratory and management of the disease under greenhouse or field conditions.

Bacterial antagonists exhibited relatively better and stable performance in suppressing bacterial wilt in tomato compared to fungal antagonists even though *T. hamatum* showed superior activity in the first experiment. Specifically, the activity of fungal antagonists, which were all different species of *Trichoderma*, exhibited a reduction in efficacy in experiment 2. In contrast, bacterial antagonists' performance improved in the experiment 2. These variations in efficacy can be attributed to abiotic and biotic influence of the environment and soil properties. Among the abiotic factors, solar radiation, soil temperature, soil moisture, type of soils, soil pH and nutrition have been identified as the major factors affecting efficacy of biocontrol agents (Stewart *et al*., 2010; Moosavi and Zare, 2015) while soil microorganisms and crop species are the main biotic factors influencing performance of biocontrol agents (Stewart *et al*., 2010).

The prevailing weather conditions at the time of research was acquired from https://power.larc.nasa.gov/ (Appendix 1) and soil physiochemical properties results ( Appendix 2) from soil sample analyzed at KALRO-NARL were examined. From the data, it was evident that weather conditions were harsher in experiment 2 compared to experiment 1. For instance, the average amount of precipitation, solar insulation and maximum atmospheric temperatures for experiment 1 were 52.6mm,18.0 MJ/m^2/day and 26.0°C while in experiment 2, they were 29.1mm, 22.8 MJ/m^2/day and 29.2°C (Appendix 1). This indicated that in experiment 2, the amount of rainfall was lower while temperatures and solar insulation were at their peak. These weather trends corroborate the reports by Cooper and Law (1977) and Mace (2012) which also found that the months from December to April were the hottest and driest in most regions in Kenya.

Among the abiotic factors, soil temperature is one of the most important factors directly affecting establishment, persistence and survival of biocontrol products in the soil (Stewart *et al*., 2010). Even though data on soil temperatures was not recorded in the current experiment previous research on soil temperatures from different lowlands and highland located in different environments in Kenya showed that atmospheric temperatures differed from soil surface

temperatures by up to 24.3°C in the hottest lowlands and a minimum of 10°C in the highlands (Mace, 2012). Based on the above studies, in addition to the fact that the soil in the experimental site was dark cotton soils, which are known to absorb more radiant heat than lighter soils (Onwuka *et al*, 2018), then it can be estimated that the soil temperatures were higher than the ambient temperature. At high temperatures, the efficacy of fungal antagonists is highly suppressed due to a reduction or total inhibition in the infection process and establishment (Stewart *et al*., 2010).

A study by Pietikainen *et al*., (2005) on bacterial and fungal activity in soils at different temperatures ranging from 0°C-45°C indicated that both bacterial and fungal growth and activity was optimum at temperatures between 25°C and 30°C. However, a decline for both groups was noted beyond 30°C and was highly drastic for fungi than bacteria. These authors reported that the maximum growth temperature for fungi was 40°C though some activity was observed in the case of bacteria at 45°C, and suggested that soil bacteria had a higher maximum growth temperature than that tested in their work. Similarly, Wei *et al*., (2017) demonstrated that seasonal variations in temperature affected the efficacy of antagonistic *Ralstonia picketti* against bacterial wilt under field conditions. They recorded maximum disease suppression when the temperature was around 20°C which drastically reduced with increasing mean seasonal temperatures leading to the highest disease levels during the warmest seasons. *In vitro* evaluations revealed that the pathogen multiplied faster at higher temperatures while antagonists grew faster at lower temperatures.

Soil pH and nutrition have also been cited as major influencers affecting efficiency of soil applied microbial antagonists. Surprisingly, the site soils had a near neutral pH with relatively moderate to high amounts of major mineral elements except for total soil nitrogen (Appendix 2). A lot of research has reported that neutral pH favor abundant growth and establishment of bacteria compared to fungi (Rousk *et al*., 2009; Fernandez-Calvino and Baath 2010; Wang *et al*., 2017). Soil pH has been found as a major constrain in effectiveness of biocontrol potential of *Trichoderma* spp. Specifically, efficient suppression of soil borne diseases by *Trichoderma* spp. has been shown to be favored in low soil pH (<7) and constrained in alkaline soils (Stewart *et al*., 2010). Similarly, Abeyratne and Deshappriya, (2018), demonstrated that the highest survival rate and biocontrol efficacy of *Trichoderma* spp. were achieved at soil pH ranging from 4.6 to 5. In contrast, Ownley *et al*., (2003) reported that biocontrol potential of *P. fluoresces* against take-all of wheat increased with increasing pH.

Other soil edaphic factors including the type of soil and moisture content have also been known to affect biocontrol potential of antagonists. However, studies investigating the effects of soil types on natural incidence, persistence and biocontrol efficacy has yielded contradicting results (Stewart *et al*., 2010). While maximum populations of bacteria were isolated from soils with highest proportion of clay, fungi population did not show any significant variation to soil texture (Hamarashid *et al*., 2010; Mohammad, 2015). Effectiveness and persistence of biocontrol products on diverse soil types is rarely identical (Moosavi and Zare, 2015) and the ability of biocontrol agents to perform in different soil types differ significantly even to closely related organisms (Stewart *et al*., 2010).

Even though soil moisture can have a major impact on establishment and survival of antagonists, it can be expected that such adverse effects did not occur in the current study since the plants were watered regularly to compensate for the low rainfall levels during the research period. Nevertheless, it has been found that fungi have low levels of osmotolerance and hence low moisture levels can lead to restrained spore survival, germination of conidia and growth of germ tube and mycelia (Moosavi and Zare, 2015; Stewart *et al*., 2010). In contrast, excess water drastically reduces survival of fungal biocontrol agents and affects efficacy of both bacterial and fungal antagonists through leaching of conidia and endospores (Moosavi and Zare, 2015).

When considering biotic factors, then interaction with resident microbes and crop species, in this case tomato, might have also contributed significantly to the performance of introduced antagonists. Resident soil microorganisms are constantly competing for limited space and resources in the rhizosphere (Mazzola and Freilich, 2017) and therefore respond with inhibitory action towards introduced biocontrol agents, constraining their ability to establish (Steward *et al*., 2010). In 2005, Bae and Kundsen reported that high levels of microbial biomass present in the soil together with fungistatic effects of resident *Pseudomonas* spp. resulted into reduced biocontrol efficiency *of T. harzianum*. Accordingly, pre-trial studies in the experimental field revealed that the site supported very high populations of bacteria, that is, up to $4.5 \times 10^5$ CFUs/g of soil. Such high populations are known to reduce effective establishment of *Trichoderma* spp.

In this study, isolation of antagonists was done from the rhizosphere of tomato and potato since these two crops are the most grown vegetables affected by bacterial wilt in Kenya. Correspondingly, only two isolates tested for efficacy under field conditions, that is, *Acinetobacter*

spp., (Tul 15R) and *T. atroviride* (Abo 14A) were isolated from potato rhizosphere. Nevertheless, their performance was relatively comparable and even better, in the case of *T. atroviride*, than other isolates obtained from tomato rhizosphere. Since high rhizocompetence of biocontrol products is a major prerequisite for successful biocontrol efficacy (Schreiter *et al*., 2018), then these isolates seem to have adapted well to the rhizosphere of tomato. Previous research by Berg *et al*., (2005) and Berg and Smalla, (2009) had shown that each plant species was capable of selecting its own specific antagonist independent of soil type (Berg *et al*., 2005). They suggested that individual crop species produced root exudates with different composition of amino acids, sugars and organic acids which are important nutritional sources for the microorganisms. In their findings, Berg *et al*., (2005) also observed that *Trichoderma* spp. was ubiquitously isolated from the bulk soil and rhizosphere of oilseed rape and strawberry.

Generally, treatments which had higher efficacy in reducing disease incidence and crop mortality also produced higher yield output. Consequently, antagonistic microorganisms have been reported to boost yield output either indirectly through prevention and reduction of harmful effects of plant pathogens or directly through secretion of growth regulators such as cytokinins, auxins, gibberellins or facilitation of nutrient uptake through phosphate solubilization and nitrogen fixation (Seleim *et al*., 2011; Stewart and Hill, 2014). In this study, reduction of disease incidence resulted in higher number of healthy and productive plants hence more plants and fruits per unit area. However, the role of direct influence of individual antagonist such as secretion of growth regulators and facilitation of nutrient uptake, and the synergism between the indirect and direct influence cannot be overruled.

None of the antagonists and plant extracts was capable of totally protecting the crops, though the level of disease incidence and crop mortality was significantly reduced by some isolates compared to untreated control. Similar observations have been reported in lots of previous studies investigating efficacy of biocontrol agents against plant disease. Moreover, biocontrol agents can only suppress the population of the pathogen but cannot completely eradicate it (Kohl *et al*., 2019). Equally, the continuous exposure of the pathogen population to extreme environmental stress has led to selection and adaptation (Kohl *et al*., 2019) of the most fastidious strains of soil borne pathogens which might outcompete an effective biocontrol agents based on ecological constrains. The superior performance of bacteria can be associated by their relatively higher tolerance and

adaptation to wide variations in soil properties compared to fungi (Papiernik *et al.*, 2007; Srivastava *et al*., 2014).

### 5.2 Conclusion

*Trichoderma* spp. exhibited superior *in vitro* activity compared to other fungal genera in inhibition of phytopathogens. Specifically, *T. hamatum* and *T. harzianum* showed superior inhibition of *R. solanacearum* and *F. oxysporum*, respectively. In the case of bacterial antagonists, *Serratia* spp. and *Bacillus* spp. exhibited higher growth inhibition on *R. solanacearum* and *F. oxysporum*, respectively. Similarly, efficacy of crude plant extracts against the test pathogens varied from one plant species to the other. Consequently, *T. minuta* exhibited superior *R. solanacearum* inhibition while *C. longa* showed strong inhibition of both bacterial and fungal test pathogens.

Application of most active microbial antagonists and crude plant extracts to manage bacterial wilt under field conditions resulted in varying level of disease suppression. The performance of antagonists and plant extracts significantly ($P \leq 0.05$) differed between the first and repeat experiments. *T. hamatum* and *B. subtilis* were superior in activity compared to commercial standard checks and other treatments. In the case of plant extracts, *T. minuta* and *C. longa* performance varied in experiments 1 and 2, respectively. *Tagetes minuta* exhibited the best disease suppression in the experiment 1 while *C. longa* showed superior activity in experiment 2. These finding therefore indicate that the local environment is a rich harbor of potential biocontrol agents that can be sourced and exploited for management of bacterial wilt.

### 5.2 Recommendation

I. The most active microbial isolates, that is, *B. subtilis* and *T. hamatum* should be formulated into commercial products for management of bacterial wilt.
II. The most active microbial isolates and crude plant extracts should be tested for activity against other diseases both *in vitro* and under field conditions.
III. The effects of antagonistic isolates and plant extracts on non-target organisms and potential toxicity to humans, wildlife and marine life should be researched on.
IV. The modes of action of the biological control agents and active ingredient in plant extracts should be studied.
V. Additional studies should be conducted to verify the performance of plant extracts given that their performance was not steady in the current work.

VI. Factors influencing microbial community structure, abundance, diversity and distribution of soil microbial antagonists should be investigated.

VII. Further exploration on best and economically viable methods for mass production and commercialization of the microbial antagonists and plant extracts is needed.

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## APPENDICES

Appendix 1: Monthly weather conditions of the experimentation field site.

| | Weather Conditions | | | | | |
|---|---|---|---|---|---|---|
| Month | Precp. (mm) | Temp. Range (°C) | Insln. | Temp. Max (°C) | Temp. Min (°C) | RH |
| 2018 Calender Year | | | | | | |
| July | 33.65 | 10.19 | 14.13 | 22.51 | 12.31 | 79.28 |
| August | 32.47 | 11.36 | 16.45 | 23.95 | 12.59 | 73.27 |
| September | 30.00 | 13.04 | 22.09 | 26.52 | 13.48 | 65.88 |
| October | 59.39 | 12.44 | 20.56 | 27.00 | 14.55 | 67.20 |
| November | 88.53 | 11.15 | 12.86 | 26.62 | 15.47 | 72.39 |
| December | 143.93 | 10.33 | 20.24 | 25.40 | 15.10 | 76.83 |
| 2019 Calender Year | | | | | | |
| January | 29.12 | 12.63 | 19.45 | 27.03 | 14.4 | 66.12 |
| February | 8.07 | 13.48 | 24.05 | 28.8 | 15.33 | 58.91 |
| March | 21.20 | 15.00 | 25.36 | 30.86 | 15.85 | 50.70 |
| April | 58.10 | 13.03 | 22.33 | 29.92 | 16.89 | 61.28 |

Source: https://power.larc.nasa.gov/

Key: - Precp: Precipitation in millimeters

Temp. Range: Temperature Range at 2 Meters (°C)

Temp. Max: Maximum Temperature at 2 Meters (°C)

Temp. Min: Minimum Temperature at 2 Meters (°C)

RH: Relative Humidity at 2 Meters (%)

Insln. All Sky Insolation Incident on a Horizontal Surface (MJ/m^2/day)

Appendix 2: Chemical and physical properties of soils collected from field site for efficacy experiment.

| Fertility results | value | class |
|---|---|---|
| Soil pH | 7.30 | slight acid |
| Total Nitrogen % | 0.15 | low |
| Total Org. Carbon % | 1.54 | moderate |
| Phosphorus (Olsen) ppm | 22.00 | adequate |
| Potassium me% | 0.40 | adequate |
| Calcium me% | 43.20 | high |
| Magnesium me% | 4.80 | high |
| Manganese me% | 0.40 | adequate |
| Copper ppm | 2.00 | adequate |
| Iron ppm | 20.20 | adequate |
| Zinc ppm | 7.50 | adequate |
| Sodium me% | 1.06 | adequate |
| % Clay | 58 | |
| % Sand | 30 | |
| % Silt | 12 | |
| Textural class | Clay | |

Appendix 3: Population (CFU/g of soil $\times 10^4$) of different bacterial isolates from soil samples collected in 10 agro ecological zones of the five counties from February to May, 2017

| | | Embu | Kirinyaga | | Meru | | Muranga | | Nyandarua | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Morphological descriptors | LM 3 | LM 3 | LM 4 | LH1-UM1 | LH3-LH4 | UM 3 | UM4 | UH1/UH2 | UH2 | UH2/UH3 |
| A | Cream,concave,large,glistening,circular | 19.4 c | 96.0d | 80.7d | 70.0c | 22.4d | 40.0 c | 49.4c | 34.2cde | 23.3c | 40.0cd |
| B | Cream,concave,large,glistening,irregular | 10.0 c | 18.0d | 2.7d | 6.7d | 5.7de | 0.6c | 1.1c | 5.8cde | 2.5c | 0.0e |
| C | cream,concave,medium,glistening,entire | - | - | - | 6.2d | 0.0e | - | - | - | - | - |
| D | Cream,concave,medium,glistening,smooth,irregular | 11.7c | 54.7d | 6.0d | 15.2d | 42.9c | 56.7c | 32.2c | 11.7cde | 9.2c | 13.3de |
| E | Cream,drop-like,small,circular,glistening | 165.6b | 566.7b | 447.3b | 267.6b | 133.3b | 337.8b | 289.4b | 103.3b | 148.3b | 129.2b |
| F | cream,drop-like,small,glistening,produce clear zone | 2.8c | - | - | - | - | - | - | - | - | - |
| G | cream,glistening,concentric,crateriform | | | | 0.0d | 1.9e | | | | | |
| H | cream,large,flat,glistening,circular,wrinkled | 2.2c | - | - | - | - | - | - | - | - | - |
| I | Cream,large,glistening,rhizoid | 2.2c | 8.7d | 11.3d | 3.3d | 0.5e | 5.0c | 6.7c | 7.5cde | 5.0c | 7.5de |
| J | cream,large,glistening,translucent centres,circular | 11.1c | - | - | - | - | 0.0c | 2.8c | 2.5de | 7.5c | 1.7e |
| K | Cream,large,lobate | 1.7c | 0.7d | 1.3d | 1.0d | 0.0 e | 0.0c | 0.6c | 3.3de | 0.0c | 0.8e |
| L | cream,punctiform | - | 41.3d | 195.3c | - | - | - | - | 0.0e | 0.0c | 13.3de |
| M | Cream,raised,large,dull,irregular | - | 7.3d | 2.0d | 1.0d | 0.0e | 23.3c | 11.7c | 3.3de | 0.0c | 2.5e |
| N | Cream,raised,very large,glistening,circular | - | 6.0d | 3.3d | 0.5d | 2.9e | 2.2c | 3.3c | 9.2cde | 0.8c | 0.0e |
| O | Cream,raised,wrinkled,medium,circular,glistening | 0.6c | - | - | - | - | 0.0c | 2.2c | - | - | - |
| P | Cream,rough,dull,undulate | 3.9c | 8.0d | 6.7d | 3.8d | 0.0e | 0.0c | 5.0c | 1.7de | 2.5c | 2.5e |
| Q | Cream,umbonate,circular,entire | 7.8c | 0.0d | 5.3d | 12.9d | 4.3de | 3.3c | 0.6c | 36.7cd | 26.7c | 3.3e |
| R | cream,very large,irregular,lobate | 0.6c | - | - | 0.0d | 1.9e | - | - | - | - | - |
| S | Cream,white centre,drop-like,small,entire | - | - | - | 1.4d | 0.5e | 0.0c | 1.1c | - | - | - |
| T | cream-white,concave,medium,producing clear zones | 16.7c | - | - | 0.0d | 1.0e | - | - | - | - | - |
| U | Cream-yellow,small,dull,irregular,scalloped surface | 2.2c | - | - | 25.7d | 0.0e | - | - | - | - | - |
| V | pink,circular,rough | 0.6c | - | - | 2.4d | 0.5e | 1.1c | 1.7c | - | - | - |
| W | red,small,dull | - | - | - | 0.0d | 0.5e | - | - | - | - | - |
| X | White center,yellow,margins,medium,rough,round | - | - | - | - | - | 5.6c | 0.0c | 15.0cde | 0.8c | 0.0e |
| Y | white punctiform | - | 360.0c | 543.3b | - | - | - | - | 41.7c | 0.0c | 57.5c |
| Z | white, circular with radiating,wooly margins | 21.7c | - | - | 67.6c | 56.7c | - | - | - | - | - |
| Z1 | white, flat, large,rhizoid,with cream wrinkled centre | - | - | - | 1.0d | 0.0e | - | - | - | - | - |
| Z2 | white, small, concave,concetric,glistening,circular | 0.6c | - | - | 0.0d | 0.5e | - | - | - | - | - |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Z3 | White,concave,circular with radiating margins | | - | - | - | - | 0.0c | 3.3c | 6.7cde | 1.7c | 0.0e |
| Z4 | White,filiform green centre,medium, clear zones | - | - | - | - | - | 0.0c | 3.3c | - | - | - |
| Z5 | White,filiform,irregular | - | 0.0d | 0.7d | - | - | - | - | - | - | - |
| Z6 | White,filiform,rhizoid | - | 0.0d | 2.0d | - | - | - | - | - | - | - |
| Z7 | White,large, concave,circular,glistening | 0.6c | 0.0d | 8.7d | 6.2d | 5.7de | - | - | 5.8cde | 0.0c | 0.0e |
| Z8 | White,large,filamentous | 2.8c | 2.0d | 2.7d | 1.9d | 1.9e | 0.6c | 0.0c | 0.8de | 9.2c | 8.3de |
| Z9 | White,large,flat,irregular,rough,dull | - | 2.7d | 0.0d | 1.0d | 0.0e | 0.0c | 0.6c | 90.0b | 0.0c | 0.8e |
| Z10 | White,large,flat,irregular,rough,glistening | - | - | - | - | - | 0.0c | 0.6c | 1.7de | 0.0c | 0.0e |
| Z11 | White,large,flat,rhizoid | - | - | - | 8.6d | 0.0e | - | - | - | - | - |
| Z12 | White,large,raised,rough,scalloped | 1.1c | 0.7d | 0.0d | 1.0d | 0.0e | 3.9c | 0.6c | 0.0e | 0.0c | 1.7e |
| Z13 | white,medium,flat,circular,concentric | 10.6C | 14.0d | 12.7d | 6.7d | 1.4e | 1.7c | 3.3c | 1.7de | 0.8c | 1.7e |
| Z14 | White,medium,flat,circular,rough | - | 2.7d | 5.3d | 0.0 d | 1.0e | 7.2c | 3.3c | 0.0e | 2.5c | 4.2e |
| Z15 | white,medium,flat,dull,irregular,scalloped,crateriform | - | - | - | 0.0 d | 0.5e | - | - | - | - | - |
| Z16 | White,medium,raised,rough,circular | 2.2c | 4.0d | 3.3d | 6.7d | 2.9e | 0.0c | 1.1c | 3.3de | 7.5c | 5.8e |
| Z17 | white,small,circular,smooth,glistening | - | - | - | - | - | 11.7c | 2.2c | - | - | - |
| Z18 | White,small,drop-like,dull | 0.6c | 0.0d | 0.7d | 4.3d | 0.0e | 2.2c | 6.1c | 0.0e | 37.5c | 20.8de |
| Z19 | White,small,drop-like,filamenous | - | - | - | - | - | 0.0c | 9.4c | 5.0de | 3.3c | 0.0e |
| Z20 | White,small,flat,dull,circular | - | 0.0d | 4.7d | 1.0d | 0.5e | 4.4c | 1.7c | 7.5cde | 0.0c | 3.3e |
| Z21 | White,small,flat,dull,irregular | 10.0c | - | - | 3.3d | 0.0e | 1.1c | 0.0c | 0.0e | 0.0c | 0.0e |
| Z22 | white,small,flat,scalloped | 1.1c | - | - | 1.0d | 0.0e | - | - | - | - | - |
| Z23 | white,very large,irregular,flat,rough | - | - | - | 21.0d | 0.0e | 0.0c | 2.2c | - | - | - |
| Z24 | White,very large,raised,rough | - | - | - | 3.8d | 1.0e | 1.1c | 0.0c | - | - | - |
| Z25 | White-cream,punctiform,glistening | 473.3a | 1087.3a | 810.0 a | 512.4a | 244.3a | 821.7a | 422.8a | 510.8a | 338.3a | 277.5a |
| Z26 | Yellow,large,circular | - | - | - | 2.9d | 1.0e | 0.0c | 2.8c | 0.0e | 0.0c | 3.3e |
| Z27 | yellow,punctifom | - | - | - | 1.0d | 0.0e | - | - | - | - | - |
| Z28 | Yellow,small,circular,glistening | - | 2.7d | 14.0 d | 4.8d | 1.0e | 7.2c | 2.2c | 3.3de | 3.3c | 0.0e |
| Grand Mean | | 14.0bc | 40.8a | 39.8a | 19.1bc | 9.6c | 23.9b | 15.6bc | 16.3bc | 11.3c | 10.7c |
| LSD (Isolates) (P≤0.05) | | 38.0 | 111.2 | 105.9 | 36.9 | 19.2 | 83.6 | 66.5 | 36.6 | 46.4 | 32.7 |
| CV % | | 415.5 | 380.6 | 371.7 | 317.6 | 330.6 | 525.2 | 639.9 | 280.4 | 514.0 | 381.6 |

Means accompanied by different letters within a column are significantly different according to Fishers Protected LSD test (P ≤0.05). Data fields marked with – are for isolates that were not detected.

Appendix 4: Population (CFU/g of soil $\times 10^3$) of different fungal isolates from soil samples collected in ten agro ecological zones of five counties from February to May, 2017

| Codes | Morphological descriptors | Embu | Kirinyaga | | Meru | | Muranga | | Nyandarua | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | LM 3 | LM 3 | LM 4 | LH1-UM1 | LH3-LH4 | UM3 | UM4 | UH1-UH2 | UH2 | UH2/UH3 |
| A | Beige,raised,cottony,large,entire | - | - | - | - | - | - | - | 2.0b | 0.0b | 4.0bcd |
| B | Black with gray brown center,concave,small | - | - | - | 0.0c | 11.3ab | - | - | - | - | - |
| C | Black with white margin,concave, circular | - | - | - | 0.0c | 10.7ab | - | - | - | - | - |
| D | Black with yellow- brown centre, medium, smooth | - | 8.0cdef | 4.7bc | 0.0c | 0.7b | - | - | - | - | - |
| E | Black, concave, verrucose, circular | - | 6.7cdef | 16.0a | 8.7abc | 0.7b | 10.0b | 0.7cd | - | - | - |
| F | Black, sparse mycelia, spreading, raised | 0.7cd | - | - | - | - | - | - | 7.3b | 6.7b | 0.7d |
| G | Black,concave,small, rugose, undulate | - | - | - | 3.3bc | 5.3b | 0.0c | 2.7bcd | - | - | - |
| H | Black,raised,small,circular, smooth | - | - | - | - | - | - | - | 0.0b | 4.0b | 0.0d |
| I | Black,raised,wooly,irregular | - | 16.7abc | 0.0c | - | - | - | - | - | - | - |
| J | Brown,verrucose,powdery,curled, raised | - | 0.0f | 4.0bc | - | - | - | - | - | - | - |
| K | Buff, concave ,smooth, small | - | 16.0abcd | 0.0c | - | - | - | - | 0.0b | 0.0b | 0.0d |
| L | Buff, glistering,granular,flat | - | 4.7cdef | 0.0c | 3.3bc | 2.7b | - | - | - | - | - |
| M | Cream gray,cottony, raised,irregular | - | - | - | 0.0c | 3.3b | - | - | 1.3b | 0.0b | 0.0d |
| N | Cream gray,veruccose,circular,undulate | - | - | - | 4.0abc | 2.7b | 0.0c | 8.7abc | 2.0b | 2.0b | 2.0d |
| O | Cream with white center, umbonate, circular | - | - | - | - | - | - | - | 3.3b | 0.0b | 0.0d |
| P | Cream yellow,small,concave,circular,smooth | 3.3cd | 4.0def | 0.0c | 0.0c | 0.7b | - | - | 7.3b | 3.3b | 3.3cd |
| Q | Cream, circular, velvety, smooth, circular | - | - | - | 0.0c | 1.3b | - | - | 5.3b | 0.0b | 0.0d |
| R | Cream, crateriform, cottony, large, entire | 2.0cd | 6.7cdef | 0.0c | 4.7abc | 2.7b | 10.0b | 6.7abcd | - | - | - |
| S | Cream, with dull white concentric ring, flat | - | 4.0def | 2.0bc | 4.0abc | 0.0b | - | - | - | - | - |
| T | Cream, wooly,raised,irregular, spreading | - | - | - | - | - | 0.0c | 0.7cd | 0.0b | 1.3b | 0.0d |
| U | Cream,circular, flat,dull,large | - | - | - | - | - | 4.0bc | 0.0d | - | - | - |
| V | Cream,very large,umbonate,raised | - | 14.0bcde | 0.0c | - | - | 0.7c | 0.0d | - | - | - |
| W | Dark gray to black with white margins, raised,smooth | - | - | - | 2.7bc | 0.0b | - | - | - | - | - |
| X | Dark gray, circular, cottony, concave | - | 4.7cdef | 10.7abc | 12.7a | 4.0b | 6.0bc | 14a | 4.0b | 0.0b | 0.0d |
| Y | Dark gray, crateriform, wooly, entire | 1.33cd | 0.0f | 3.3bc | 2.0bc | 0.0b | - | - | 4.7b | 4.7b | 4.7abcd |
| Z | Dark gray,flat,granular,medium | - | 0.0f | 2.7bc | 2.7bc | 5.3b | 2.7bc | 0.0d | - | - | - |
| Z1 | Dark gray,raised, irregular, cottony,undulate | - | 0.0f | 3.3bc | 0.0c | 0.7b | 1.3c | 0.0d | - | - | - |
| Z2 | Dark green to gray, raised, irregular, medium | - | - | - | - | - | - | - | 0.0b | 5.3b | 0.0d |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Z3 | Dark green, wooly, raised,circular,smooth | - | - | - | 4.0abc | 0.0b | - | - | - | - | - |
| Z4 | Dark green,rugose,concave, circular, undulate | - | 2.7ef | 0.0c | 0.7c | 2.0b | - | - | - | - | - |
| Z5 | Gray brown,raised,concave, small,circular | - | 0.0f | 4.0bc | 2.7bc | 0.7b | 2.0bc | 0.7cd | - | - | - |
| Z6 | Gray cream,rugose, powdery,umbonate | - | 6.0cdef | 12.7ab | 0.7c | 2.7b | - | - | - | - | - |
| Z7 | Green with cream margins,small,concave,entire | - | 7.3cdef | 0.0c | - | - | - | - | 0.0b | 2.0b | 0.0d |
| Z8 | Green with white margins, concave, small,rugose | - | 5.3cdef | 5.3abc | 2.7bc | 0.0b | - | - | - | - | - |
| Z9 | Khaki brown,granulated concentric rings,flat, circular, entire | - | 9.3bcdef | 2.7bc | 4.7abc | 19.3a | - | - | - | - | - |
| Z10 | Light gray,circular,verrucose,undulate,small | - | - | - | 4.7abc | 0.0b | 2.0bc | 0.0d | - | - | - |
| Z11 | Light gray,woolly,circular,entire,large | - | - | - | 2.7bc | 0.0b | 0.7c | 0.0d | - | - | - |
| Z12 | Light gray,circular,rugose,medium,undulate | - | - | - | - | - | 0.0c | 4.7bcd | 4.7b | 0.0b | 0.0d |
| Z13 | Pale gray,flat,circular, | 2.0cd | - | - | 0.0c | 0.7b | 4.0bc | 0.0d | 4.7b | 4.7b | 4.7abcd |
| Z14 | Pearl white,raised,umbonate,cottony,large, entire margin | - | - | - | - | - | 1.3c | 2.7bcd | 0.0b | 1.3b | 0.0d |
| Z15 | Pink,cottony,irregular,raised,undulate | - | 2.0ef | 4.7bc | 6.7abc | 4.7b | - | - | - | - | - |
| Z16 | Pink,creamy,circular,rugose,undulate | - | - | - | - | - | 0.0c | 3.3bcd | - | - | - |
| Z17 | Pink,wooly,raised,regular,spreading | - | - | - | - | - | 3.3bc | 2.0bcd | - | - | - |
| Z18 | Pinkish gray,flat,concetrics,circular,entire | - | - | - | 2.0bc | 0.0b | - | - | - | - | - |
| Z19 | Pinkish gray,rugose,flat,sparse mycelia,circular | - | - | - | 1.7c | 0.0b | - | - | - | - | - |
| Z20 | Pinkish white,cottony,raised,large,irregular,spreading, | - | - | - | - | - | - | - | 0.0b | 0.0b | 10.0ab |
| Z21 | Purple gray,concave,large, circular,entire margin | - | - | - | - | - | 0.7c | 0.0d | - | - | - |
| Z22 | Purple whitish,raised,cottony,large,spreading | - | 0.0f | 1.3c | 3.3bc | 1.33b | - | - | - | - | - |
| Z23 | Purple,sparse mycelia, granular,flat | - | 4.0def | 0.0c | - | - | - | - | - | - | - |
| Z24 | Purplish white,circular,cottony,concave,entire | - | 20.7ab | 0.0c | 2.6bc | 0.0b | - | - | - | - | - |
| Z25 | Purplish white,large,wooly,irregular,undulate | - | - | - | 2.2bc | 1.3b | 0.7c | 2.7bcd | 0.0b | 0.0b | 4.7abcd |
| Z26 | White circular,fluccose,producing inhibition zone | - | - | - | - | - | - | - | 0.0b | 0.0b | 0.7d |
| Z27 | White with a black concentric ring,rugose,circular | - | - | - | 0.0c | 0.7b | - | - | - | - | - |
| Z28 | White with cream center,circular,cottony,large,irregular | - | 0.0f | 4.0bc | - | - | 1.3c | 7.3abcd | - | - | - |
| Z29 | White with dark green center,flat,rugose,small | - | - | - | 0.0c | 1.3b | - | 2.7bcd | - | - | - |
| Z30 | White with dark green granules, flat, spreading | - | 0.0f | 4.0bc | - | - | - | - | - | - | - |
| Z31 | White with gray center,raised,cottony,small,circular | - | - | - | - | - | 4.7bc | 0.0d | - | - | - |
| Z32 | White with green concentric, small,concave,circular | - | 0.0f | 6.0abc | - | - | 1.3c | 0.0d | - | - | - |
| Z33 | White with rusty brown granules,rugose,medium,flat | - | - | - | - | - | 1.3c | 0.0d | - | - | - |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Z34 | White,cottony,dark brown spots in the center, concave, circular | - | 27.3a | 0.0c | 3.3bc | 0.7b | - | - | - | - | - |
| Z35 | White,circular,concentrics,cottony,large,entire | - | - | - | - | - | - | - | 0.0b | 4.7b | 0.0d |
| Z36 | White,concave,verrucose,rugose,growing into media | - | 6.0cdef | 1.3c | 4.2abc | 0.0b | 7.3bc | 0.0d | 0.0b | 2.7b | 0.0d |
| Z37 | White, cottony, steep raised plateau shaped,large,circular | - | - | - | - | - | - | - | 0.0b | 1.3b | 0.0d |
| Z38 | White,cottony,flat, irregular | 7.3bc | - | - | 0.6c | 5.3b | - | - | 3.3b | 4.7b | 10.7a |
| Z39 | White,cottony,small,concave,circular | 18.0a | 10.0bcdef | 8.7abc | 10.2ab | 10.7ab | 24.0a | 8.7abc | 24.0a | 12.0a | 2.7cd |
| Z40 | White,crateriform,circular,sperse mycelia | - | - | - | - | - | 0.7c | 0.0d | - | - | - |
| Z41 | White,crateriform,fluccose,circular,medium | - | 4.0def | 4.7bc | 4.1abc | 2.7b | - | - | - | - | - |
| Z42 | White,flat, spreading,sparse mycelia | - | 2.0ef | 0.0c | - | - | 0.0c | 7.3abcd | - | - | - |
| Z43 | White,flat,granulated with pale green dots,spreading | 5.3bcd | 1.3f | 2.7bc | 4.7abc | 0.7b | 2.7bc | 0.0d | 2.7b | 2.0b | 2.0d |
| Z44 | White,fluccose,spread all over,irregular | 2.7cd | 4.0def | 0.0c | 0.5c | 0.7b | - | - | 2.7b | 3.3b | 4.7abcd |
| Z45 | White,granular,small,circular,flat,entire | - | - | - | 3.3bc | 0.0b | - | - | - | - | - |
| Z46 | White,large,irregular, cottony,raised with flat surface | - | - | - | - | - | - | - | 0.0b | 0.0b | 0.7d |
| Z47 | White,raised to petri dish top,cottony | - | - | - | - | - | 0.0c | 2.7bcd | - | - | - |
| Z48 | White,raised,cottony,irregular,small,undulate | - | 0.0f | 10.0abc | 0.7c | 5.3b | 0.0c | 3.3bcd | 6.0b | 0.0b | 0.0d |
| Z49 | White,raised,medium,irregular,undulate | - | 10.7bcdef | 4.7bc | 8.7abc | 5.3b | 2.0bc | 9.3ab | - | - | - |
| Z50 | White,small,concave,powdery,glistering | 7.3bc | - | - | 0.0c | 3.3b | - | - | 3.3b | 0.0b | 0.0d |
| Z51 | White,sparse mycelium,large,circular,concave | - | - | - | 2.4bc | 0.0b | 0.0c | 1.3bcd | - | - | - |
| Z52 | White,velvety,rugose | - | 4.7cdef | 0.0c | - | - | - | - | - | - | - |
| Z53 | White,wriggled, irregular,umbonate,flat | 12.0ab | 0.0f | 4.7bc | - | - | 7.3bc | 0.0d | 0.0b | 0.0b | 8.7abc |
| Z54 | Yellow brown,granular,small,umbonate,flat,circular | 5.3bcd | 0.0f | 6.0abc | 4.7abc | 5.3b | 4.0bc | 4.0bcd | - | - | - |
| | Means | 0.8cd | 2.6a | 1.7b | 1.7b | 1.6bc | 1.3bcd | 1.2bcd | 1.1bcd | 0.8d | 0.8d |
| | LSD isolates (P≤0.05) | 3.1 | 5.6 | 5.2 | 4.1 | 5.8 | 3.7 | 3.8 | 3.8 | 3 | 2.9 |
| | CV % | 522.4 | 297.5 | 440.6 | 340.7 | 518.8 | 397.8 | 447.4 | 488.3 | 543.1 | 511.3 |

Means accompanied by different letters within a column are significantly different according to Fishers Protected LSD test (P ≤0.05). Data fields marked with – are for isolates that were not detected.