EVALUATION OF BACILLUS AND TRICHODERMA SPECIES FOR BIOLOGICAL CONTROL OF BACTERIAL WILT CAUSED BY *Ralstonia Solanacearum* IN TOMATO

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DECLARATION

This is my original work and has not been presented for award of a degree or diploma in any other university.

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DEDICATION

This work is dedicated to dad Mr. Benjamin Kariuki and mum Mrs. Ellyjoy Muthoni Kariuki, my dearest daughter Beryl Nyakio, my niece Ivy, my loving sisters Viskey, Modester and her husband Michael for their support, encouragement and sacrifice throughout the research and thesis writing.

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ACRONYMS AND ABBREVIATION

AEZ	Agro-ecological zones
ANOVA	Analysis of Variance
AVRDC	Asian Vegetable Research and Development Centre OEPP
BCAs	Biological control agents
CFU	Colony forming unit
CPG	Casamino acid, bacto-peptone and glucose media
EU	European Union
FAO	Food and Agricultural Organization of the United Nation
HCDA	Horticultural Crops Development Authority
KARI	Kenya Agricultural Research Institute
KALRO	Kenya Agricultural Livestock and Research Organization
Kelman's TTC	Kelman's Triphenyl Tetrazolium Chloride
KES	Kenya Shillings
KEPHIS	Kenya Plant Health Inspectorates service
LSD	Least significant difference
NA	Nutrient agar
PDA	Potato dextrose agar
PGPA	Plant growth promoting agents
PH	Potential of Hydrogen
Pv	Pathovars
TSM	*Trichoderma* selective media
USAID	United State Agency for International Development
ZOI	Zones of inhibition

GENERAL ABSTRACT

Tomato (*Lycopersicum esculentum*) has been rated second most important vegetable crop in Kenya. Bacterial wilt caused by *Ralstonia solanacearum,* is a major biotic constraint to tomato productivity with yield losses of up to 64%. The available management strategies such as cultural practices and use of chemicals are limited in effectiveness. This study therefore focused on biological control agents (BCAs) in the management of *Ralstonia solanacearum*. *Bacillus* and *Trichoderma* isolates that are antagonistic to *Ralstonia solanacearum* and other important bacteria pathogens were screened and identified *in vitro*. The study also evaluated the effect of the *Bacillus* and *Trichoderma* isolates in management of bacterial wilt disease under field conditions.

Twenty-eight *Trichoderma* species isolated from local soils at Kabete and 19 *Bacillus* isolates retrieved from earlier screened isolates maintained at the Plant Science and Crop Protection Department, University of Nairobi were used as the antagonists against three pathogens. Paper disc method was used to test the antagonistic activity of the isolates against *Ralstonia solanacearum, Xanthomonas campestris* pv *campestris* and *Pseudomonas* spp *in vitro*. The experiment was conducted in a complete randomized design, with three replicates. Antagonistic activity was assessed by measuring the radius of zone of inhibition (ZOI) of the pathogen due to the antagonist. Field experiments were conducted in a randomized complete block design at Kabete and Mwea sites in Kenya. The treatments included; 3 *Trichoderma* isolates (T1, T2 and T4), 2 *Bacillus* isolates (CB64 and CA7), a mixture of T1, T2 and T4, chemical standard and distilled water as control. *Trichoderma* and *Bacillus* isolates were grown on sterilized sorghum grain and cow manure carriers, respectively. Antagonist's inoculation was carried out by dipping tomato plants for 30 minutes in each treatment suspension. Each treatment was applied at a rate of 150ml/plant hole and this was repeated after 35 days. Soils were sampled prior to transplanting, 60 days and 112 days after transplanting for quantification of *R. solanacearum* population and at 126 days for determining the total microbial count in the soil. Bacterial wilt incidence was assessed every week by counting the number of wilted plants in each plot. Yield parameters were assessed at physiological maturity.

In vitro studies showed that 10 *Bacillus* and 11 *Trichoderma* isolates had varied antagonistic activity against all the pathogens tested. *Trichoderma* isolate T1 was the most effective in inhibiting the growth of *Ralstonia solanacearum* with a mean ZOI measuring 13.5mm while *Bacillus* isolates CB64 was the best antagonist with a mean ZOI measuring 4.3mm.

Trichoderma isolate T28 and *Bacillus* isolate CA5 showed the highest ZOI of 15.2mm and 6.6mm, respectively against *Xanthomonas campestris.* The antagonists screened gave lower activity against *Pseudomonas* sp. compared to other pathogens. *Trichoderma* isolate T28 showed the highest ZOI of 9.3mm and *Bacillus* isolate CB14 and CB22 gave similar ZOI of 5.3mm. Isolates of *Trichoderma* showed better activity by more than 56.67% compared to isolates of *Bacillus*. All the treatments evaluated under field conditions significantly reduced bacterial wilt incidence and severity at $P \leq 0.05$ than the control at Kabete and Mwea sites. *Trichoderma* isolate T1 followed by *Bacillus* isolate CB64 were the best in reducing the disease incidence by more than 61.66% and 53%, respectively at both sites. Treatment CB64 and T1 had the highest reduction of *R. solanacearum* population in the soil by 93.17% and 92.07%, respectively. However, control had a pathogen increase of 20.40%. The total microbial count was highest in *Bacillus* treated plots in both sites. Isolate CB64 had the highest count of 1.32×10^5 CFU/ml at Kabete and 1.21×10^5 CFU/ml at Mwea site. CB64 and T1 performed significantly better compared to the standard, while the mixture of isolates T1, T2 and T4 performed poorest in all parameters. The treatments also increased the yield of tomato. Results from this study showed that *Trichoderma* and *Bacillus* isolates are effective biological control agents for use in management of bacterial wilt.

Keywords: Tomato, bacterial wilt, *Ralstonia solanacearum*, *Trichoderma*, *Bacillus*

CHAPTER ONE: INTRODUCTION

1.1 Background Information

Tomato (*Solanum lycopersicum*) has been documented as one of the most important vegetable crop belonging to the nightshade family of *Solanaceae* (Adams *et al.*, 2006). The crop is reported to have originated from South America (Jenkins, 1948). Taylor, (1986) confirmed tomato originated from South America by documenting geographical dispersal of the native wild relatives location at 0º to 20º S and 64º to 81º W where the species breed freely in the wild. Tomato was first domesticated in Mexico through pre-Hispanic culture (Jenkins, 1948). According to Champoiseau and Momol, (2008), during 1870, an American botanist Alexander W. Livingston devoted his entire life on this crop where he conducted selective breeding on improving and uplifting tomato into the customary form, we are aware of today. According to Athertonand, (1986), tomato was introduced in Africa by early Europeans during the pre-colonial period in the 16th century.

Food and Agriculture Organization (FAO) reported that in 2017, the area under tomato cultivation was 4.8 million hectares which produced 188 million tons with a market revenue of more than 190.4 billion USD globally (FAOSTAT, 2019). China is the leading tomato producers in the world accounting for 33% of the global production followed by India, United States of America, Turkey and Egypt (FAOSTAT, 2017 and 2019). In Africa, tomato is cultivated on 1.3 million ha which produces an average of 37.8 million tons annually (FAOSTAT, 2017). Egypt is the biggest tomato producer in Africa recording an average production of 7.3 million tons in 2017 followed by Nigeria, Tunisia and Morocco (FAOSTAT, 2019). Kenya is among the African's top ten leading producers of tomato, cultivated on 0.4 million hectares which produces about 280, 000 tons annually (FAOSTAT, 2017). Therefore, Kenya accounts for 0.2% of the world production. The crop in Kenya is mainly grown in open fields under irrigation and in greenhouses to meet the increasing demand for tomato (Monsanto, 2013). In Kenya, the key counties where tomato is grown includes; Kirinyaga, Laikipia, Kiambu and Kajiando (KARI, 2005).

Tomato has a wide range of uses and contribute to a healthy, well balanced diet. Tomato fruits are consumed fresh in salads or cooked in sauces, soup and meat or fish dishes. In addition, they can be processed into puree, jam, paste, powder, juices, tomato sauce and canned or dried into economically important processed products (Ochilo *et al.*, 2019). Tomato are excellent source of important micronutrients like iron, potassium, ascorbic acid and antioxidants like vitamins C, B, amino acids. These nutrients are what makes tomato highly recommended by

dieticians and nutritionists for controlling cholesterol and for weight reduction (Basco *et al.,* 2017).

Poor agricultural practices like continuous cropping of tomato crop without rotation, furrow irrigation, poor field hygiene and use of infected seedlings have led to increased disease incidences (Monsanto, 2013). Pests that include; leaf miner, American bollworm, nematodes, mites and diseases such as tomato blights, tomato wilts, blossom end rot not only cause reduction of yield and quality but also increase the cost of production (Monsanto, 2013). Among the serious diseases that attack tomato, bacterial wilt is one of the most devastating. Bacterial wilt caused by *Ralstonia* (formally *Pseudomonas*) *solanacearum* (Smith) causes massive death of plant resulting in yield and income reduction for farmers (Yabuuchi *et al.,* 1995).

Ralstonia solanacearum is a soil borne pathogen that once it infects the soil, it is easily spread within the adjacent fields (Virendra, 2017). This is mainly through contaminated irrigation/flowing water, transplants and this not only affect the crop but also renders the farm unusable to production of any *Solanaceae* crops (Champoiseau and Momol, 2008). The pathogen invades the roots and colonizes the xylem vessels causing wilt especially in tropical and sub-tropical regions (Champoiseau and Momol, 2008). The bacteria produces several phytotoxins such as extracellular polysaccharide and combination of plant cell wall degrading enzymes such as endoglucanase and polygalacturonase that enables it to invade plant's natural mechanism (Yendyo *et al.,* 2017). This pathogen infects a wide host range causing disease to approximately 450 crop species that include; tomato, potato, eggplant, tobacco, pepper, among others (Maji and Chakrabartty, 2014).

Management of bacterial wilt disease is challenging because the available methods are limited in their effectiveness. The long-term use of chemical products such as bactericides and fungicides induce resistance in the pathogen making it tolerant to chemical application (Maji and Chakrabartty, 2014). There are hardly available chemicals to manage bacterial diseases except the copper based and antibiotics that are used for human and animal medicine and are therefore highly restricted (Yendyo *et al.,* 2017). Use of cultural practices such as crop rotation has faced challenges due to unpredictable survival of the pathogen (Sequeira, 1993). *R. solanacearum* has ability to survive in the soil for long period of time even in the absence of vegetation (Champoiseau, and Momol, 2008). Use of resistant tomato variety is not effective since the pathogen has a number of distinct strains that makes a variety resistant to the disease in one region become susceptible in another (Onduso, 2014). It is also difficult to get good resistant tomato varieties at reasonable price with qualities preferred by consumers (Onduso, 2014). The limited

effectiveness of these management strategies calls for a focus on alternative methods to manage bacterial wilt disease. Biological methods that can be used to manage diseases have been an attractive area of study by many researchers.

Biological control agents (BCAs) are naturally occurring soil microorganisms that aggressively attack plant pathogen, suppress the diseases and help in control of pests and weeds (Singh *et al.,* 2014). The mechanism involved in biological control includes; parasitism, antibiosis, competition for nutrient and space, cell wall degradation by lytic enzymes and induced disease resistance (Singh, 2013). The idea of using microbes as a method of pests and disease management dates backs in the 19th Century. Since then, several studies have been done to control bacterial wilt of tomato with application of BCAs such as *Trichoderma* spp, *Bacillus* spp, *Pseudomonas* spp among others (Kumur and Ganesan, 2006; Singh, 2013; Maji and Chakrabartty, 2014; Yendyo *et al.,* 2017). It is therefore important to evaluate the antagonistic ability of BCAs against the pathogen and incorporate them into successful disease management strategy.

1.2 Problem statement

Tomato production in the country has been exposed to multiple risks that results in large losses both qualitative and quantitative hence decreasing tomato profitability. This decrease is caused by both pest and diseases. Insect pests that include leaf miner, flea beetles, fruit borers, aphids and whiteflies affect the crop from the time of emergence to harvest. Diseases that affect tomato include blights, mildews, cankers, and wilts with collective losses of up to 100%. Among the diseases affecting tomato production, bacterial wilt is one of the most destructive disease and is caused by *Ralstonia solanacearum*. Being a soil borne pathogen, it is difficult to manage *R. solanacearum* especially in the already infected fields and this has led to reduced income for small scale growers (Taylor *et al.,* 2011).

The farmers involved in horticultural export find it difficult to manage bacterial wilt disease because there are hardly known chemicals used to manage bacteria diseases. The available chemicals are normally copper based fungicides that are limited in effectiveness and antibiotics that are used for human and animal medicine hence highly restricted. Use of chemicals has led to more problems as they are non-biodegradable and they pollute the environment which has greatly contributed to the current climate change. In addition, chemical residues on the produce cause health problems to human beings. Furthermore, chemical pesticides are harmful to farmers applying them and continuous application has led to build up of resistance among

pathogens and pests (Maji and Chakrabartty, 2014). Target markets for tomato produce such as EU market have set strict quality requirement for food produce that includes: safe, clean produce free of chemical residues and safe for human consumption. This has greatly affected farmers who use chemicals intensively as their produce are rejected in the market.

Cultural practices like crop rotation used for management of *R. solanacearum* have challenges due to unpredictable nature of the pathogen. The pathogen has ability to survive in the soil for long period of time even in the absence of vegetation (Champoiseau and Momol, 2008). The pathogen is composed of a number of distinct strains hence varieties tolerant/resistant to the disease in one region may be susceptible in another (Onduso, 2014). All this factor has adverse effects on livelihood of everyone in the society due to aggravating issues of food insecurity which consequently leads to hunger. Most people especially the farmers whose livelihoods depend on agriculture end up in poverty and this may lead to decline in a country's economy.

1.3 Justification

Tomato is one of the most important vegetable crops in Kenya and the devastating effect of *R. solanacearum* on tomato production has increased loss of yields. In an effort to meet the demand of tomato, farmers result to use of chemicals. However, concerns on the toxicity of the chemical products used and their retention potential in the food chain has led to a shift from exclusive use of chemicals to use of biological control methods or the judicial use of chemicals in an integrated package.

Satisfying consumer needs, taking care of human health and the environmental safety can be achieved through sustainable, affordable and effective disease management strategies. The method used should guarantee continuous and increased production of quality/quantity tomato produce. This will ensure increased income for tomato growers and fair prices for the consumers (Taylor *et al.,* 2011).

BCAs are plant growth promoting bacteria and fungi that have antagonistic activity against *R. solanacearum.* Hence, they are able to reduce *R. solanacearum* inoculum intensity, disease incidence and severity with no residue effect on tomato produce. Moreover, they do not cause environmental pollution or other adverse effects that are associated with chemicals. In the long run they slow down or reduce the rate of climate change that has caused numerous problems. Use of BCAs is an effective method as some antagonists have broad spectrum of activity hence can manage different pathogens in one application and once, they establish, the strategy reduces the cost of production. Most microbial antagonists are found naturally in soils and their efficacy can be enhanced through addition of BCAs. The present study involves screening, identifying and evaluating *Bacillus* and *Trichoderma* spp that have antagonistic effect against *R. solanacearum in vitro* and under field conditions. This will assist researchers in coming up with effective BCAs to recommended for commercial use in management of bacterial wilt of tomato.

1.4 Objectives

The general objective is to contribute to sustainable horticultural production through the use of BCAs in the management of bacterial wilt of tomato.

Specific objectives:

1. To screen and identify *Bacillus* and *Trichoderma* species that are antagonistic to *Ralstonia solanacearum, Xanthomonas campestris* pv *campestris* and *Pseudomonas* spp *in vitro.*
2. To evaluate the potential of *Bacillus* and *Trichoderma* species in management of bacterial wilt disease of tomato under field conditions.

1.5 Hypothesis

1) *Bacillus* and *Trichoderma* isolates have no antagonistic activity against the three bacteria pathogens *in vitro*.
2) *Trichoderma* and *Bacillus* isolates have no effects on bacterial wilt incidence under field conditions.

CHAPTER TWO: LITERATURE REVIEW

2.1 Tomato Production in Kenya

Tomato is among the highly consumed vegetable ranked as number one in Kenya by KARLO (Smart farm, 2016). It is consumed as vegetable salad, tomato sauce and puree hence considered a must have in most kitchens. Tomato constitute 7% of total horticulture produce in Kenya and 14% of the entire vegetable produce (Ochilo *et al.,* 2019). Tomato is cultivated on 0.4 million hectares in Kenya, which produces about 280, 000 tons annually (FAOSTAT, 2017). In 2016, tomato production was at 410, 033 tons with a total value of 151 million USD (FAO, 2017). This production increased by 50% against the 271, 151 tons produced in 2001 (FAO, 2017). However, from 2016 to 2018 there has been about 30% decrease in tomato production against an increasing demand of 300,000 tons (Mwangi *et al.,* 2020). Studies have also shown that despite the growing demand of tomato attributed to growth in population, its productivity has also declined sharply from 25.5 t ha-1 in 2006 to 18.7 t ha-1 in 2016 (FAOSTAT, 2017). This decline has led to increase in tomato prices making them unaffordable and therefore, Kenya has resulted to importation of over 27, 000 tons from Ethiopia and Tanzania (Mwangi *et al.,* 2020).

Tomato production has a high-income potential for small scale farmers with land size between 0.5-2.5ha hence, a source of employment to rural and urban residents (Mbaka *et al.,* 2013). The main tomato growers are the small-scale farmers accounting for 80% of all the growers (Ochilo *et al.,* 2019), Kenyan tomato industry is set to improve particularly because of the governments' pursuit to realize the Big Four Agenda which aims at food security by improving productivity among smallholder farmers. The crop is produced both for domestic consumption and commercial purposes. Tomatoes have high nutritional value since they contain; ascorbic acid, bioflavonoids, enzymes, are rich in minerals (potassium, iron, and magnesium), vitamins C & B, amino acids (act as anti-oxidants) that promote good health (Shahid *et al.,* 2011). Tomato is important in diet against common cancer such as breast and prostate cancer (Marti *et al.,* 2016).

Tomatoes grow well in well drained clay or sandy loam soils that experiences temperature of between 20-27^{o}C with annual rainfall of over 600mm (Wiersinga *et al.,* 2008). Tomato crop is mainly grown in low rainfall regions particularly the semi-arid areas through irrigation. The crop is grown under open field and greenhouse condition with most educated youth preferring greenhouse production as it utilizes less land for high yields and quality produce (Mbaka *et al.,* 2013). The greenhouse varieties available include Anna F1, Nemoneta FI, Corazzon F1, Tylka

F1, Claudia F1, Chonto F1, Rambo F1 and Prostar F1 while Riogrande and Cal-J are the common varieties for open fields (Monsanto, 2013). Hybrid tomato varieties that include Kilele F1, Valoria F1, Shanty F1, Eden F1, Nuru F1, Tropicana F1 and Assila F1 that are disease tolerant and have increased yields are available in the market (Monsanto, 2013).

Tomato crop is grown all over the country and majority of production is done in Kirinyaga County (24%), Taita Taveta (7%), Muranga County, Kajiado, Narok, Nyandarua, Nyeri, Embu and Meru County among others with nearly 70% being produced by small-scale farmers (Otipa *et al.,* 2003: Monsanto, 2013). In Kirinyaga and Loitoktok counties, production is under irrigation scheme at Mwea and Namelock schemes, respectively (Lengai, 2016).

Tomato plants thrive well in areas free from frost since tomato is a warm season crop. It requires an average temperature ranging from 15 to 27^0C since high temperature above 30^oC inhibit fruit set, affects flavor and lycopene development (Waiganjo, 2006: Onduso, 2014). Tomatoes require well-drained soils with high organic matter content mainly the sandy loam soils. The most favorable soil pH for tomato growth ranges from 5.0 to 7.5 (Crop Nutrition, 2015). Tomatoes are grown in open fields during the long and short rain seasons according to the availability of rain and the anticipated pest/disease pressure but can also be grown in greenhouses under irrigation (Wiersinga *et al.,* 2008).

The total population growth in Kenya has risen to nearly 48 million in 2019 from 29 million in 1999 and 39 million in 2009 (KNBS, 2019). This population growth has increased tomato consumption per capita over the same period. Studies have shown that in 2018 only, the consumption per capita was 8.5kg which was a 41.7% increase from 2017 (KNBS, 2019). Therefore, the demand for tomato has also increased over time and this has created a wide market for the produce. The main market channels for the produce include direct orders to supermarkets, local hotels, middle men, and open air markets like Kangemi, Wakulima market and Githurai (Ochilo *et al.*, 2019). Well established market links exist that include sub-contract supply arrangement with farmers to sell produce to developed grocery channels in areas like Westlands and Parklands (Monsanto, 2013). Tomato farmers face serious market barriers that include quality issues, lack of consistent supply of produce to the market, pesticide residue on fruits, poor product hygiene, price fluctuation and perishability problems due to poor transport networks and storage facilities (Onduso, 2014).

2.2 Biotic constraints to tomato production in Kenya

Tomato production in the country is faced with major challenges that include pests, diseases, physiological disorders and marketing issues (Mwangi *et al.,* 2020) which greatly affect the yield and quality tomato produce hence reduction in market value. The major pests affecting tomato production include: African bollworm, aphids, whiteflies, Thrips, leaf miner and spider mites. Nematodes and weeds also affect tomato production. Leaf miner (*Tuta absoluta)* causes holes on fruits and the larvae burrows on leaves making them appear burnt (Real IPM, 2016). Whiteflies are most common during dry season and they suck the plant sap reducing growth rate and productivity, cause sooty molds on fruit and spread viral diseases such as tomato yellow leaf curl disease (Monsanto, 2013). Red spider mites (*Tetranychus* spp*)* are found when it is dusty during dry periods and they suck plant sap causing leaf curl, webbing and loss of leaves that leads to reduction of yields (Monsanto, 2013). Interference of weed during the early stage of production of tomato reduces the qualitative and quantitative attributes such as color, flavor and consistency. Weeds compete for space, light and nutrients with tomatoes and examples of weeds include: nightshade, black jack.

Nutrient deficiency, excess nutrient, water logging and drought are the main cause of physiological disorders and have a huge impact on production. Low level of calcium in the soil results to blossom end rot which causes black spot at the bottom of the fruit (Real IPM, 2016). Low potassium level in the soil causes uneven ripening and lack of red pigments on the fruits. Excessive nitrogen causes failure of the plant to produce fruits (Crop Nutrition, 2015).

Diseases are the major limiting factor in tomato production and they are more devastating in lowlands, highlands and in the tropics. There are more than 200 known diseases that attack different parts of tomato plant causing 15 to 95% economic losses of the crop (Muthoni *et al.,* 2012). These diseases are mainly caused by Bacteria, Fungi and Viruses. Early blight (*Alternaria solani*), late blight (*Phytophthora infestans*) Bacterial wilt (*Ralstonia solanacearum*), Bacterial canker (*Clavibacter michiganensis* pv. *michiganensis*), fusarium wilt and Root knot nematodes (*Meloidogyne* spp) are the main diseases affecting tomato production (Singh *et al.,* 2014). Other diseases include bacterial stem and fruit rot (*Erwinia carotovora* subsp. *carotovora*), bacterial speck (*Pseudomonas syringae* pv. tomato), powdery mildew (*Leveillula taurica*) and leaf mold (*Mycovellosiella fulva*) (Singh *et al.,* 2014). The most common virus diseases that constrains tomato production are yellow leaf curl virus and tomato spotted wilt virus (Yuging, 2018).

Among the diseases, bacterial wilt has been reported to be the most destructive in tomato production with 64% loss of crop grown under open field condition and up to 100% loss of crop in the greenhouse (Mbaka *et al.,* 2013). This is because the pathogen has a high destructive nature, wide host range, has ability to persist even in abandoned lands, has a wide geographical distribution that include tropics, sub tropics and warm temperate regions (Kelman, 1954: Mihovilovich *et al.,* 2017).

2.3 Bacterial wilt of tomato

Bacterial wilt is a major limiting factor in production of tomato among other Solanaceas plants in tropical and sub-tropical regions of the world (Sinha, 2016). It is caused by *Ralstonia solanacearum* Yabuuchi *et al.* (1995). This pathogen damages over 200 plant species in 50 different families (Buddenhagen and Kelman, 1963: Meng, 2013). *R. solanacearum* causes brown rot of potato with 0-90% yield loss, bacteria wilt or southern wilt of tomato, 10-30% yield loss of tobacco, causes Moko disease of banana with 80-100% loss, causes up to 20% yield loss in groundnuts, affects eggplant and some ornamentals (Elphinstone, 2005). *R. solanacearum* causes bacteria wilt of tomato has traditionally been grouped as race 1, biovar 3 (Virendra, 2017). In tomato production, bacteria wilt was identified and described in 1869 by E.F. Smith. Its origin and distribution remain unknown; however, the first documentation was done in the early 20th Century in South America, U.S.A and Australia where it was endemic.

The pathogen is soil borne (Virendra, 2017) and enters the plant through wounds on the roots from cultivation equipment, nematodes and insects or through cracks where secondary roots emerge (Mihovilovich *et al.,* 2017). Sources of inoculum in agricultural fields and method of spread include; use of contaminated irrigation water and surface water, use of contaminated planting materials (transplants) and contaminated farm tools and equipment (Mihovilovich *et al.,* (2017). Once the pathogen has established in the field, plant-to-plant spread may occur when bacteria move from roots of infected plants to roots of healthy plants (Sinha, 2016).

2.3.1 Causal organism

R. solanacearum is a Gram-negative, rod shaped, strictly aerobic bacterium that is inhibited in culture by low concentration (2%) of sodium chloride (Nacl) (Champoiseau and Momol, 2008: Sinha, 2016). This bacterium is cultured in liquid and solid agar medium at an optimum growth temperature range of 28 to 32^{o} C (Kelman, 1954). Bacterial colonies on solid agar are visible after 36 to 48 hours with morphological characteristic for the virulent type being entirely white or cream-colored, irregularly-round, fluidal and opaque (Kelman and person, 1961:

Champoiseau and Momol, 2008). Non- virulent *R. solanacearum* colonies usually appears uniformly round, smaller, and butyrous, or dry. Triphenyl Tetrazolium Chloride (TTC) medium (Kelman, 1954) is used to differentiate the two colony types and virulent colonies appear white with pink centers while non-virulent colonies are uniformly dark red.

2.3.2 Taxonomy

Since it was first described as *R. solanacearum*, it has been grouped into multiple genera. It was classified into genus *Bacillus* as *Bacillus solanacearum* in 1896 by E.F smith. Later on, it advanced to *Bacterium* genus, *Pseudomonas* genus and called *Pseudomonas solanacearum* (Smith *et al.,* 1995). Afterwards in 1992 it was grouped under the genus *Burkholderia* and in 1995 under the genus *Ralstonia* hence called *Burkholderia solanacearum* (Yabuuchi *et al.,* 1995). From then on it was named *Ralstonia solanacearum*, Yabuuchi *et al* (1995) and associated with the family *Ralstoniciae.*

2.3.1 Symptoms of bacterial wilt on tomato

Bacterial wilt pathogen penetrates the plant through the roots and it clogs xylem tissues in the stem preventing water and nutrients from reaching the branches and leaves (Sinha, 2016). The first symptoms are wilting of younger leaves during the heat of the day (Champoiseau and Momol, 2014). Wilt usually may occur in only one stem. The wilting may recover during the evening when the temperatures are low and then becomes progressive (Muthoni *et al.*, 2012). Wilting eventually becomes permanent leading to stunting, yellowing and death of the plant (Kelman, 1954: Meng, 2013)

When the plant is infected at an early stage of growth, the pathogen damages the vascular system and the stem may show long, narrow, dark brown streaks externally from soil level upward (Kelman, 1954). This may lead to the collapse of the stem. Under favorable conditions the disease manifests itself within the plant without showing symptoms a condition called latency and spread to other plants in the field (Mihovilovich *et al.,* 2017).

The stem also reveals brown hollow center as a result of rot from the inside out which is the brown discoloration of the water conducting elements (vascular system) (Muthoni *et al.*, 2012). When cut, stems exude grey-brown bacterial ooze from the vascular tissue (Virendra, 2017). Bacterial streaming of infected tomato plant is used as a quick diagnostic test to distinguish *R. solanacearum* from other vascular wilts such as fusarium and veticillium wilt that are caused by fungal pathogens (Mihovilovich *et al.*, 2017). A stem section of 3 inches is cut from plant

showing vascular discoloration using a sharp blade and the cut section is suspended in a transparent glass filled with clear water. Bacteria cells and extracellular polysaccharide are seen streaming out of the xylem cut end in the clear water as milky white strands (Kelman, 1954: Sinha, 2016: Virendra, 2017).

3.3.2 Identification of *Ralstonia solanacearum*

Recognizing and scouting of symptoms is vital for advance diagnosis of bacterial wilt on tomato. Multiple molecular and microbiological techniques have proved precise in identification of *R. solanacearum* from soil samples, water, symptomatic and asymptomatic plants (Fajinmi and Fajinmi, 2010). Molecular techniques have assisted in identification of *R. solanacearum* as it is contemplated to be "species complex" as a result of diversity of gene among the species (Álvarez *et al.,* 2010). A series of procedures are undertaken for identification of the pathogen in the laboratory. They include; pathogen isolation which involve the removal of the pathogen form the infected plant. Stem sections (2cm long) of diseased plant are cut and sterilized using 2% sodium hypochlorite for two minute then rinsed three times in sterile distilled water. Using a mortar and pestle they are mashed and macerated in 50ul of distilled water and allowed to stand for 2 minutes. Streaks are made on nutrient agar media and incubated at 28^{o}C for 48 hours. Creamy-white irregular flat and fluidal colonies are formed with a whorl in the center (Elphinstone, 2005).

Use of screening test helps in scouting and pinpointing bacteria in contaminated water, infected plants and soil contaminated by *R. solanacearum* (EU, (2006). However, the test is not applicable for identification to the biovar or race of the pathogen. Examples of these tests are: use of susceptible host for pathogenicity assessment, use of *R. solanacearum* specific primers, and planting on a half selective medium, serological agglutination test and poly B-hydroxybutyrate granule detection (Lelliott and Stead, 1987: Sinha, 2016).

Biochemical growth test is also utilized for biovar identification of *R. solanacearum* (Buddenhagen and Kelman, 1963). This test is utilized based on the ability of different pathogen strains to produce acid from multiple carbohydrates (Buddenhagen and Kelman, (1963): Yabuuchi *et al.,* 1995). DNA analysis test which revolves around utilization of Polymerase Chain Reaction amplification and DNA probe hybridization methods which assesses multiple nucleic-acid of strains of *R. solanacearum* has greatly assisted in identifying different strains of this pathogen (Khasabuli *et al.,* 2017).

3.3.3 Life cycle of *Ralstonia solanacearum*

Ralstonia solanacearum is a soil borne pathogen that penetrates the host plant through the wounds on the roots and colonizes the xylem vessels (Muthoni *et al.,* 2012). Colonization of the xylem bundle takes place due to the massive multiplication of *R. solanacearum* in the vessels hence secreting enzymes which degrades the cell wall (Elphinstone, 2005). Under favorable conditions, within the host plant there is high expression of virulence genes and exopolysaccharides production which are the main determinant of pathogenicity (Meng, 2013). High volume of the pathogen within the plant increases gene pathogenicity compared to non-host environment where they are repressed by low densities of bacteria (Muthoni *et al.,* 2012: Meng, 2013).

In the ecosystem, *R. solanacearum* is motile and moves toward plant by the sense of stimuli through swimming using flagella (Sinha, 2016). The stimuli are tantalized to plant host root exudates, multiple organic acids and amino acids. During the host pathogen interaction, three stages are encountered: namely colonization of the roots, infection of cortical and penetration of xylem vessels (Nguyen and Ranamukhaarachchi, 2010). *R. solanacearum* locates the host through the sense of stimuli, the pathogen penetrates the roots through wounds and natural wound (areas where roots emerge) and attach to axil of flourish roots or root extension zone (they have epidermal weaker barrier) (Denny, 2006). Due to the damaging enzymes produced by the pathogen the cell wall of the host is destroyed and the pathogen occupies the intercellular spaces where it obtains nourishments from the lamella by the help of pectinolytic enzymes. The bacteria move to the cortex of the pre-eminent roots and enormous intercellular pockets are formed and retrogression of the cells is seen (Álvarez *et al.,* 2010).

Bacteria crosses the endodermis and invades the vascular bundle through the axils of secondary roots, roots extremities and regions where there is no fully differentiated endodermis (Sinha, 2016). The pathogen then occupies the intercellular spaces of the vascular system and destroy the parenchyma cells (Sinha, 2016). The pathogen then proceeds to the upper part of the plant through the stem and multiplies sporadically. The population of the pathogen increases up to 10^{10} per cell and this inhibits the flow of xylem fluids. Prolonged multiplication of the pathogen in the xylem vessels leads to clogging and wilting of the whole plant (Nguyen and Ranamukhaarachchi, 2010). The pathogen adapts itself to a saprophytic life after the plant crumble and dies until it contacts another host (Nguyen and Ranamukhaarachchi, 2010).

R. solanacearum can remain the soil for a long time in the absence of desired host and its population can be influenced by prevailing biotic and abiotic factors (Nyangeri, 1982). Presence of alternative host such as weeds and reservoir plants aids in the existence of the pathogen in water and soil in absence of the host (Sinha, 2016). *R. solanacearum* can survive in the soil for a long period of up to 10years even in absence of any vegetation (Nyangeri, 1982: Champoiseau and Momol, 2008). This is greatly influenced by the soil moisture content, soil type/structure, organic matter content, presence of antagonists and temperature.

3.3.4 Ecological conditions favoring distribution of the pathogen

Pathogenicity relates to both the virulence (infection ability) and aggressiveness of the pathogen on the plant that is usually determined by the plant variety, condition of the plant and the environment. *R. solanacearum* is favored by high temperature and moist acidic soils with a pH < 7.0 (Sinha, 2016). Under high temperatures between 24 and 35^0C and moisture ranging from 155.6 mm to 495.2 mm, the virulence of the pathogen in the soil is high as a result of high multiplication rate (Fajinmi and Fajinmi, 2010). The pathogen causes high infection within an altitude ranging from 1520m to 2120m above the sea level (Johnson, 1982). In Kenya, these conditions are commonly encountered in Central, Rift Valley and Eastern region and the disease occurrence usually declines outside these zones.

Weak plants are highly damaged by the pathogen as compared to healthy plants that have high resistance mechanism towards the pathogen (AVRDC, 2005). Plants with wounds provide source of entry for the pathogen as compared to the unwounded one. It has been reported that there is a synergistic interaction between root-knot nematode (*Meloidogyne* spp*)* and bacterial wilt of tomato. The nematode infestation accelerates disease development (AVRDC, 2005). Presence of weeds in the field also influences the pathogen virulence because they act as alternative hosts for *R. solanacearum* (Salamanca, 2015). During adverse conditions, the pathogen can survive in the weeds until the condition are favorable for disease development in the tomato plant.

2.4 Management of bacterial wilt of tomato

Ralstonia solanacearum being a soil borne pathogen is not easily managed once it infects the soil and the plants (AVRDC, 2005). Integrated disease management strategies have been adopted to control bacterial wilt in tomatoes. They include cultural method, chemical method and use of resistance varieties but they are limited in success (Mbaka *et al.,* 2013). Disease diagnosis is vital for the identification of the right pathogen when designing any disease management program (Maloy, 2018).

2.4.1 Cultural practices and field sanitation

Phytosanitary measures undertaken within the country include use of disease-free seeds and assessing pest risk to prevent introduction, spread and establishment of the pathogen (KEPHIS, 2012). Cultural measures include intercropping, crop rotation, use of resistance varieties and soil amendment. This creates unfavorable environment for the pathogen to develop, thrive and this reduces the amount of inoculum in the field (Muthoni *et al.,* 2012). Use of disease-free tomato seeds from certified seed producing company to prevent the introduction of *R. solanacearum* inoculum in the field has been used to manage the disease (Kareem, 2015).

Crop rotation involves planting crops of different families on the same piece of land the next season after tomato crop (Smith *et al.,* 1995). This has been witnessed to improve tomato production and yields as compared to monocropping (Monsanto, 2013). Use of resistance crops and crop rotation for 5-7 years can reduce infections by *R. solanacearum* (Smith *et al.,* 1995: Onduso, 2014). However, this is limited by the fact that the pathogen is able to survive in the soil for a long time up to 10 years even without vegetation (Nyangeri, 1982: Champoiseau and Momol, 2008). The pathogen has a wide host range and existence of weeds and volunteer crops especially the *Solanaceous* family limit the success of crop rotation (Onduso, 2014). Small scale farmer owns small size land that limit crop rotation which greatly impact on the management of bacterial wilt within the country (Muthoni *et al.,* 2012).

Sanitation are measures taken to reduce the dissemination of bacterial wilt pathogen by reducing the level of inoculum in the fields and greenhouses. This involves the removal of plant residues, uprooting infected tomato plants, removing disease asymptomatic plants (Salamanca, 2015). Use of sterilized tools and equipment and washing hand are vital in management of bacterial wilt (Kareem, 2015). There are various disinfectants used for sanitation that include Hydrogen peroxide, Chloride dioxide Ethanol and Chlorine bleach (Salamanca, 2015).

2.4.2 Chemical control

Chemical control has been practiced since 1960 in reducing losses of tomato production due to pathogens (Yuliar *et al.,* 2015). However chemical control of bacterial wilt has been reported to be difficult (Hartman *et al.,* 1994). Some bactericides, bleaching powder and streptomycin have shown to be effective in reducing bacterial wilt (Yuliar *et al.,* 2015). Combination of thymol and Acibenzolar-S-methyl has increased tomato yield as well as suppressing the disease incidence in the field. When tomato seeds are soaked in low sodium chloride solution, they tend to increase seed growth and resistance towards *R. solanacearum* (Yuliar *et al.,* 2015). Di-Bromo Di-nitro propane 1, 3-diol is a chemical product from Osho Chemical Industries Limited, Kenya that has been reported effective in managing bacterial wilt (PCPB Manual, 2020). The available chemicals usually have adverse effect on health and cause environmental degradation (Fajinmi and Fajinmi, 2010). Furthermore, there are hardly available chemicals to manage bacterial diseases except the copper based and antibiotics that are used for human medicine and are therefore highly restricted.

2.4.3 Biological methods

Biological control measures in disease management involving the use of modified living organisms or their constituents to manage diseases have been used in the recent past (Pal, 2018).

2.4.3.1 Suppressive soil and amendment of soil

Suppressive soils are soils which contain microbial organism which are hostile towards the pathogen (Kareem, 2015). *R. solanacearum* is a soil borne pathogen that once present in suppressive soils, the microorganisms in the soil produces lytic enzymes and antibiotics as they compete for food with the bacteria wilt pathogen (Kareem, 2015). This hostile condition reduces the pathogen population directly to a level below the economic threshold.

Soil amendment is the manipulation of the top soil through addition of farmyard manure, compost manure and green manure (Yadessa *et al.,* 2010). In amended soil there is low level of bacterial wilt pathogen as compared to the soil not amended (Yadessa *et al.,* 2010). This is because use of soil amendment increases the nutrients level in the soil which increases the plant growth and vigor hence facilitating crop resistance against bacterial wilt disease. The nutrients also increase the microbial population and activity in the soil which has negative effect on *R. solanacearum*. Use of silicon fertilizer and sugarcane bagasse as a soil amendment has been reported to significantly reduce bacterial wilt incidences (Ayana *et al.,* 2011).

2.4.3.2 Use of antagonistic microorganisms

This is the use of microorganisms to suppress bacterial wilt. Multiple mixture of beneficial microorganisms is introduced in the soil to shield the plant against bacterial wilt pathogen and also to improve the plant performance (Kareem, 2015). The effects attributed by antagonistic microorganisms towards *R. solanacearum* pathogen include: Food competition, excretion of toxic substances which affect the pathogen, parasitism towards the pathogen and release of metabolic byproducts which are volatile toward the pathogen (Mukherjee *et al.*, 2013). A mixture of bacteria has been proven to suppress bacterial wilt disease and improve tomato performance in the field. This includes *Azotobacter, Lactobacillus* and *Streptomyces* species which boost the plant photosynthesis, accelerate the breakdown of lining material and high nitrogen fixation in the soil (Lwin, 2006). Some fungal microorganisms present in the soil are hostile towards bacterial wilt in the soil for example *Trichoderma harzianum* which parasitize *R. solanacearum* (Kumar, 2017).

2.4.3.3 Use of avirulent mutants of *Pseudomonas solanacearum*

Avirulent strain of *Pseudomonas solanacearum* isolated from *Sterilzia reginae* has proven to suppress the growth and development of bacterial wilt in the greenhouse (Arwiyanto *et al.*, 1994). This is incited by bacteriocin-sensitive or bacteriocin-resistant strains. Various studies have reported significant protection of tomato plants from *R. solanacearum*, a pathogen that causes bacterial wilt disease by making simultaneous or sequential root inoculations of avirulent mutants' strain of *Pseudomonas solanacearum* (Yendyo *et al.*, 2017: Maji and Chakrabartty, 2014: Liza and Bora, 2009: Kumur and Ganesan, 2006). A study by Trigalet and Trigalet-Demery, (1990) reported that Tn5-induced avirulent mutants that were derived from virulent strain GMI1229 of *Pseudomonas solanacearum* significantly reduced bacterial wilt disease incidence compared to the control. The study also reported that the possibility of antagonistic activity of avirulent mutant strain of *Pseudomonas solanacearum* against the virulent strain was observed when the relative inoculum ratio of the virulent *Pseudomonas solanacearum* strain to avirulent cells was equal to or lower than 0·1 (Trigalet and Trigalet-Demery, 1990).

2.4.3.4 Use of *Trichoderma* in control of *Ralstonia solanacearum*

Trichoderma genus are soil borne ascomycetes that are distributed around the world (Schuster and Schmoll, 2010). *Trichoderma* can be located where there is availability of plant residue and decaying material and they are able outcompete their competitors in the ecosystem by instigating antagonistic activity and systemic resistance against pathogens (Kumar and Ganesan,

2006: Howell, 2018). Once they have established themselves in the field, they have an expeditious growth by high production of bright green conidia and highly branched conidiophore (Yendyo *et al.,* 2017). In addition, they unleash their degradation mechanism such as lytic enzymes for breaking down of heterogeneous substrate (Schuster and Schmoll, 2010: Boukaew *et al.,* 2011). *Trichoderma* species poses a high defense mechanism, high dissemination and phylogeny under good environmental conditions (Howell, 2018). They have damaging association with the host, sexual development, enzymes secretion and production which result to having high competition towards other pathogens. Biological control strains of *Trichoderma* genus are favorable biological fungicides as they are high colonizers in their habitat through effective utilization of substrates and high secretion and production of enzymes and antibiotic metabolites (Schuster and Schmoll, 2010).

Trichoderma has ability to parasitize and colonize other fungi. They parasitize *Rhizoctonia solani* hyphae by penetrating the pathogen and coiling around the hyphae and continuous termination of the host cytoplasm (Howell, 2018). Mycoparasitism and antibiosis are the main principal and vital mechanism in control of *R. solanacearum* by *Trichoderma* as they compete for nutrients and space within the ecosystem (Howell, 2018). *Trichoderma* species have a rapid growth together with the developing root system of tomato plant. This results in excellent colonization of the roots against *R. solanacearum* in the soil (Howell, 2018). *In vitro* studies by Ramesh *et al.* (2009), Narasimha and Srinivas, (2012) and Yendyo *et al.* (2017) have reported that *Trichoderma* strains have potential antibacterial activity against *R. solanacearum.*

Trichoderma species produces glucanases and chitinases enzymes which suppress *R. solanacearum* pathogen by destroying cell wall unification (Howell, 2018). They degrade chitin, polysaccharides and beta glucan which are vital in the rigidity of cell wall. *Trichoderma* species induces resistance in tomato plant when they are inoculated in the seedlings roots. This is by penetrating the cortex and the epidermis of the tomato roots which accelerates the production of toxic compounds (peroxidase activity) and also stimulates high chitinases activity (Howell, 2018). They also influence high root and shoot growth of tomato plant, tolerance to changes in nutrition and other stress within the ecosystem, enhance high amount of mineral absorption (Copper, Zinc, Iron, Manganese and Sodium) (Sharma *et al.,* 2012).

2.4.3.5 Use of *Bacillus* in control of *Ralstonia solanacearum*

Multiple species of bacteria have been utilized to regulate bacterial wilt in tomato. This includes *Bacillus* spp, *Actinomyces* and *Pseudomonas fluorescens* (Yendyo *et al.,* 2017). *Bacillus* represents a genus of widely distributed gram positive, aerobic and facultative aerobic bacteria (Stein, 2005). The bacteria in this genus are catalase positive and are able to produce resistant endospores which are metabolically active even under harsh environmental conditions (Logan *et al.,* 2007). Several *Bacillus* species have a high survival mechanism and tolerance toward desiccation and contributes to high plant growth (Singh and Yadav, 2016). *Bacillus* species secrets catalytic enzymes that include proteases, chitinases, glucanases and peptide antibiotics such as bacteriocin, bacilli, bacitracin among others which are known as antibacterial and antifungal substances (Gross and Vidaver, 1990: Stein, 2005). Examples of *Bacillus* species includes: *Bacillus pumilus, Bacillus vallismortis, Bacillus cereus, Bacillus lincheniformis, Bacillus amyloliquefaciens* and *Bacillus megaterium* (Nguyen and Ranamukhaarachchi, 2010). These strains have shown high potential for bacterial wilt suppression, increase in fruit weight, biomass and plant height (Nguyen and Ranamukhaarachchi, 2010). The most studied *Bacillus* species used for biological control is *Bacillus subtilis* which is considered to be harmless. Antibiotics such as polymyxin, circulin and colistin produced by majority of *Bacillus* strains are active against gram positive and gram-negative bacteria (Maksimov *et al.,* 2011). These secondary metabolites have a broad spectrum of activity against various pathogens and also promote tomato plant growth by improving the height of plants, increase the yields and quality of fruits produced (Singh and Jagtop, 2017: Narasimhamurthy *et al.,* 2018).

Endophytic *Bacillus* spp have ability to inhibit the growth of *R. solanacearum in vitro* by inducing the zones of inhibition (Singh *et al.,* 2012: Sultana, 2015: Revathi *et al.,* 2017). A study by Yendyo et al., 2017 has reported significant inhibition of the growth of *R. solanacearum in vitro* by *Trichoderma species, Pseudomonas fluorescens* and *Bacillus subtilis*. Kumar and Ganesan (2006) evaluated biological control agents, *Glomus mosseae* UHR, *Bacillus subtilis* HHR-1, *Pseudomonas fluorescens* IIHR+3, *Trichoderma harzianum* HHR P1 and *T. viride* IIHR P22 against tomato bacterial wilt caused by *R. solanacearum* in pathogen infested plots. Per cent survivability of tomato increased with the use of all biological control agents tested.

2.4.3.6 Limitation of application of Biological control agents (BCAs) in the soil

Several authors have reported the importance of BCAs in the management of plant diseases especially the fact that they are environmental friendly, absence of harmful residues and low production cost (Ramesh *et al.,* 2009: Bonaterra *et al.,* 2012: Revathi *et al.,* 2017). However, at present, there are scientific evidences indicating that the use of BCAs has some disadvantages. The main disadvantage is that the BCAs are highly specific against the target disease and pathogen. This implies that the BCAs with antagonistic activity against one pathogen may not have activity against another (Bonaterra *et al.,* 2012). Therefore, management of a certain diseases may require multiple BCAs to be used. Another disadvantage is the variable efficacy of the BCAs due to the influences of various biotic and abiotic factors since they are living organisms (Bonaterra *et al.,* 2012). The complex microbial community of soils imposes a buffering effect on population increase by individual species which operates as much against introduced BCAs as against pathogen (Askary, 2015). Therefore, there is need for methods to analyze the population of autochthonous micro-biota to estimate the qualitative and quantitative alteration in the microbial community structure caused by the application of the BCAs (Bonaterra *et al.,* 2012: Askary, 2015).

The role of BCAs have been well proven by research work (Seleim *et al.,* 2011: Yendyo *et al.,* 2017), however, BCAs remain active for a relatively short-time and therefore unable to provide long lasting control (Askary, 2015). This is why continuous application of BCAs to boost the microbial population is necessary. In addition, the BCAs applied in the soil are inconsistent and slower in action due to the dynamic nature of the soil and this calls for more research to improve the performance of the BCAs. Moreover, it is generally uneconomical to add any BCA directly to the soil in amount sufficient to control pathogen. This is why application of BCAs is done using carriers such as manure and sorghum to provide nutrient and facilitate their multiplication (Sharma and Kumur, 2009). Production and formulation of selected BCA strains requires suitable industrial production to increase their shelf-life and retain Biocontrol activity similar to that of fresh cells (Powell and Jutsum, 1993: Bonaterra *et al.,* 2012). Therefore, proper methods of industrial scale-up and fermentation have to be developed.

CHAPTER THREE

In vitro screening of *Bacillus* and *Trichoderma* antagonists against *Ralstonia solanacearum, Xanthomonas campestris* pv *campestris* and *Pseudomonas* sp.

ABSTRACT

Bacterial pathogens cause devastating diseases on economically important crops like tomato, cabbage and bulbous ornamentals such as Ornithogalum causing severe losses. There are limited strategies for managing these diseases. This study was aimed at screening isolates of *Bacillus* and *Trichoderma* spp for antagonistic activity against important bacterial plant pathogens *in vitro*. Bacterial plant pathogens tested were *Ralstonia solanacearum, Xanthomonas campestris* pv *campestris* and *Pseudomonas* sp. Twenty eight *Trichoderma* isolates from local soils collected at Kabete Field Station of the University of Nairobi and 19 *Bacillus* isolates retrieved from earlier screened isolates maintained at the Plant Science and Crop Protection Department, University of Nairobi were used as antagonists. Molten nutrient agar was seeded with 2×10^8 CFU/ml of each pathogen and paper disc method was used to test the efficacy of the antagonists. Filter paper discs (5mm diameter) soaked for 10 minutes in *Bacillus* broth prepared on shaker flasks for 7 days and 5mm discs of actively growing Trichoderma on potato dextrose agar were placed on the seeded media. Antagonistic activity was assessed by measuring radius of zone of inhibition (ZOI) of the pathogen by the antagonist. Ten *Bacillus* and 11 *Trichoderma* isolates had varied antagonistic activity against the pathogens tested. *Trichoderma* isolate T1 was the most potent in inhibiting the growth of *Ralstonia solanacearum* with a mean ZOI measuring 13.5mm while *Bacillus* isolate CB64 was the best antagonist with a mean ZOI measuring 4.3mm. *Trichoderma* isolate T28 and *Bacillus* isolate CA5 showed the highest ZOI of 15.2mm and 6.6mm, respectively against *Xanthomonas campestris*. The antagonists screened gave lower activity against *Pseudomonas* sp. compared to other pathogens. *Trichoderma* isolate T28 showed the highest ZOI of 9.3mm and *Bacillus* isolate CB14 and CB22 gave similar ZOI of 5.3mm. Isolates of *Trichoderma* showed better activity by more than 56.67% compared to isolates of *Bacillus*. The results showed that twelve *Trichoderma* isolates (with ZOI of ≥ 10mm) and nine *Bacillus* isolates (with ZOI of ≥ 4.3mm) have high potential in managing various bacterial diseases.

Keywords: Tomato, *Ralstonia solanacearum, Trichoderma, Bacillus*.

3.1 INTRODUCTION

Management of bacterial plant diseases to increase productivity of various important crops like tomato has been a great challenge. Management of bacterial diseases has been achieved through the use of cultural practices, resistant plant varieties and chemicals (Narasimhamurthy *et al.*, 2018). Most bacterial pathogens such as *R. solanacearum*, *X. campestris* pv *campestris* and *Pseudomonas* sp. cause devastating diseases of important crops (Sundin *et al.,* 2016). *R. solanacearum*, the causal agent of bacterial wilt, is the most devastating disease in tomato production (Sinha, 2016). In Kenya, bacterial wilt disease causes 64 to 100% yield loss (Mbaka *et al.,* 2013). *X. campestris* pv *campestris* causes black rot, a major disease of vegetables in brassica family (Massomo *et al.,* 2003). *Pseudomonas* sp. that causes bulb rot disease of Ornithogalum has led to 40% losses in the cut-flower production (Mwangi, 1993).

Available strategies for control of bacterial pathogens are limited in effectiveness hence management of the diseases is difficult (Mbaka *et al.,* 2013). New strains of the pathogens keep on arising and are capable of infecting resistant crop varieties (Onduso, 2014). Furthermore, there are hardly known chemicals to manage bacterial diseases except antibiotics that are used for human and animal diseases which are highly regulated to prevent development of resistance of human pathogens (Yendyo *et al.*, 2017).

Alternative strategies for the management of bacteria diseases are necessary (Kago *et al.,* 2019). Fungi and bacteria that are antagonistic against the pathogens and promote plant growth have been used to manage the diseases (Brotman *et al.,* 2008: Singh *et al.,* 2012: Nikolic *et al.,* 2013). Biological control of bacterial wilt disease of solanaceous crops through the use of antagonistic agents has been reported (Narasimha and Srinivas, 2012: Kumar, 2017). Plant growth promoting rhizobacteria have been found to have antagonistic activity against *Xanthomonas campestris* pv *campestris* (Compant *et al.,* 2005: Liu *et al.,* 2016). The action of certain plant growth promoting agents (PGPA) that include *Bacillus* strains and *Trichoderma* spp has been reported effective in management of *Pseudomonas* spp (Kloepper *et al.,* 2004: Sundin *et al.,* 2016). Studies have reported that microbial antagonists such as *Rhizobium*, *Bradyrhizobium*, and *Trichoderma* spp have shown greater residue effects after application in the soil in one season (Yaqub and Shahzad, 2011). The ability of these Biocontrol agents to persist in the soil can provide protection in the next crop. Application of the antagonist after every season increases their population in the soil which in turn increases their competitiveness and effectiveness (Yaqub and Shahzad, 2011). The aim of this study was to screen and identify biological control agents that are antagonistic to these important bacterial pathogens *in vitro*.

3.2 MATERIALS AND METHODS

3.2.1 Experimental site and experimental design

The experiment was conducted in Plant Pathology laboratory at the University of Nairobi, Kenya. The experiment was conducted using complete randomized design with three replicates.

3.2.2 Isolation of bacterial pathogens

Stem samples of bacterial wilt diseased tomato plants were collected from heavily infected fields in Mwea region, Kirinyaga County for isolation of *R. solanacearum*. The stems were cut into small pieces (2cm long) which were surface sterilized with 2% sodium hypochlorite solution for 2 minutes followed by 3 rinses in sterile distilled water. The plant pieces were macerated using sterile glass rod in sterile distilled water contained in sterile universal bottle. A sterile wire loop was dipped in the upper layer of the suspension and was streaked on petri plate containing 2, 3; 5 Triphenyl Tetrazolium Chloride (Kelman's TZC agar) medium for isolation of *R. solanacearum* (Kelman, 1954). *Pseudomonas* sp. was isolated on sucrose nutrient agar (SNA) medium (EPPO, 2014) from bulb rot diseased Ornithogalum samples collected from infected field at Kabete Field Station. *X. campestris* pv *campestris* was isolated on yeast peptone sucrose agar (YPSA) medium (Schaad, 1988) from black rot diseased cabbage leaves collected from heavily infected fields at Kabete Field Station using the same procedure outlined above.

The petri plates were incubated upside down at 28^{o}C for 48 hours. Isolated pathogens were purified on nutrient agar (NA) and then transferred to NA on agar slants and incubated for 48 hours. Bacterial suspension was prepared by adding 2ml sterile distilled water in each slant aseptically and shaken to dislodge the bacteria cells. Bacterial suspension for each pathogen was transferred into empty sterile universal bottles to which 5ml distilled water was added for dilution and preservation. Bacteria suspensions were then stored at room temperature (23 ±2^{o}C) as stock solution for later experimental use (Kelman and Person, 1961: Khasabuli *et al.*, 2017). Confirmation of identity of the pathogens was done through the cultural and morphological characteristics of each pathogen.

3.2.3 Pathogenicity test

Pathogenicity test was only carried out for isolated *R. solanacearum* because it was main pathogen of interest in this study. The test was carried out on Riogrande tomato cultivar which

is a susceptible tomato variety to bacterial wilt. Healthy seedlings that were 25 days old were selected from the nursery and used for pathogenicity assay. Bacterial inoculum was prepared by taking 1ml of *R. solanacearum* stock suspension and was added to Casamino acid, peptone glucose (CPG) broth and incubated at 28^{o}C for 48 hours on a rotary shaker at 150rpm (Kelman, 1954). The culture broth was centrifuged at 12, 000rpm for 10 minutes at 10^{o}C and the resultant bacterial pellet was suspended in sterile distilled water. The concentration of the suspension was adjusted to 1×10^{8}CFU/ml by serial dilution technique (Ran *et al.,* 2005: Narasimhamurthy *et al.,* 2018).

Inoculation of *R. solanacearum* was done using root dip and soil drench methods. For root dip method, the roots were cut at 1cm from the tip with a sterile pair of scissors. The seedlings were dipped in the bacterial suspension for 30 minutes (Mwangi *et al.,* 2008). The inoculated seedlings were then transplanted into 3kg pots containing sand and forest soil with no history of *R. solanacearum* at the ratio of 1:1 and placed in the greenhouse. For soil drench method, the root system of each seedling previously transplanted into the 3kg pots containing sand and soil was wounded with a sterile needle. Five milliliters of the inoculum suspension was poured around the wounded roots of each plant (Mwangi *et al.,* 2008). Non inoculated tomato seedlings transplanted as above were used as control. Three plants were planted per pot and four pots served as the replicate for each treatment.

3.2.4 Retrieval of *Bacillus* strains and isolation of *Trichoderma*

A total of 19 coded *Bacillus* spp. preserved in sterile modified loam soil were obtained from the Culture Collection Center of the Department of Plant Science and Crop Protection, University of Nairobi. *Bacillus* strains were retrieved by sprinkling particles of the modified loam soil on nutrient agar as described by Wagacha *et al.* (2007). The petri plates were incubated for 48 hours at room temperature (23±2^{o}C). Pure cultures of the isolates were obtained by sub-culturing each isolate on fresh NA and incubating for 24h at 23±2^{o}C.

Soil samples for *Trichoderma* isolation were acquired randomly from cultivated fields, coffee fields, pasture land, kitchen waste disposal site, tomato field and non-cultivated area at Kabete Field Station. The samples from the six areas where composited into one sample and transported in paper bags and stored at 4^{o}C in the laboratory. *Trichoderma* strains from the soil were isolated using serial dilution technique. One milliliter aliquots of the suspension from the fourth dilution were plated on *Trichoderma* selective medium (TSM) as described by Maina *et al.* (2015). The petri plates were incubated for 10 days in the dark at 20^{o}C to allow fungal growth (Mclean *et al.*, 2005). Pure cultures of *Trichoderma* isolates were maintained on potato dextrose agar (PDA) at 28^{o}C (Watts *et al.*, 1988). Identification of the isolates was carried out using morphological and microscopic characteristics as described by Samuel *et al.* (2004). The isolates obtained were coded T1 up to T28.

3.2.5 Preparation of antagonistic broth of *Bacillus* isolates

One hundred milliliters of TSCHEN's medium consisting of 15g glucose, 15ml glycerol, 15g soybean meal, 5g $(NH_4)SO_4$, 1g yeast extract, 5g NaCl and 5g $CaCO_3$ in 1000ml distilled water with a pH of 7.5 was prepared in 500ml conical flask for each *Bacillus* isolate (Tschen and Kou, 1984). The medium was sterilized at 121^{o}C for 15 minutes at 1 bar pressure and then cooled to about 40^{o}C. *Bacillus* suspension for each isolate was prepared by flooding a 10 days old pure cultures with sterile distilled water and scrapping the culture gently using sterile glass slide. Ten milliliters of each suspension was transferred to the conical flask with TSCHEN's medium. The conical flasks were corked using cotton wool and properly sealed with aluminium foil to avoid contamination. The inoculated liquid media were incubated at room temperature (23±2^{o}C) for 7 days on a rotary shaker at 120rpm (Wagacha *et al.*, 2003).

3.2.6 *In vitro* screening of the antagonists against the bacterial pathogens

3.2.6.1 Bacterial antagonists (*Bacillus* isolates)

Paper disc method was used to screen the antagonists against *Ralstonia solanacearum*, *Xanthomonas campestris* pv *campestris* and *Pseudomonas* sp. Virulent *R. solanacearum* isolate was multiplied on nutrient broth and cultured for 48 hours at 28^{o}C. Ten milliliters of 48 hour old bacterial suspension containing 2×10^{8} CFU/ml was mixed with 1litre of 50^{o}C molten sterilized nutrient agar so as to get a thick lawn of bacteria on the surface of the petri plate (Singh and Jagtap, 2017). The seeded NA medium was poured into sterile petri-plates and allowed to solidify. Previously sterilized filter paper discs measuring 5mm in diameter were soaked in different antagonist broths of *Bacillus* isolates for 10 minutes. Three to five paper discs were placed at equidistant points, one centimeter from the edge of each plate. The plates were replicated three times and incubated at 28±2^{o}C for 48 hours. Observations on formation of zones of inhibition (ZOI) between the antagonists against the pathogen around the filter paper discs were made and their radius in mm recorded at 24, 48 and 72 hours of incubation. Filter paper discs dipped in sterile water served as control (Singh and Jagtap, 2017).

3.2.6.2 Fungal antagonists (*Trichoderma* isolates)

Ten ml of 48-hour old *R. solanacearum* suspension containing 2×10^{8} CFU/ml previously grown on nutrient broth was mixed with 1litre of 50^{o}C molten sterilized PDA so as to get a thick lawn of bacteria on the surface of the petri plate (Singh and Jagtap, 2017). The seeded PDA medium was poured into sterile petri-plates and allowed to solidify. Fungal discs of *Trichoderma* isolates measuring 5mm in diameter from the margin of actively growing 4 days old cultures were used. The fungal discs were removed with a cork borer and placed at the center of the plates containing pathogen seeded PDA. The plates were replicated 3 times and incubated at 28±2^{o}C for 4 days. Observations and measurements of the zones of inhibition around the mycelial discs against *R. solanacearum* were recorded after 2, 4 and 6 days. Discs of solidified sterile PDA medium that had not been cultured with anything served as control.

The same procedure was used for evaluating the antagonistic activity of *Bacillus* and *Trichoderma* isolates against *Xanthomonas campestris* pv *campestris* and *Pseudomonas* sp. The medium was seeded with each of the respective pathogen in place of *Ralstonia solanacearum.*

3.3 Data analysis

Analysis of variance for data recorded on measurements of zones of inhibition was carried out using Genstat 15th edition to evaluate the antagonistic ability of *Bacillus* and *Trichoderma* isolates. Mean comparisons were conducted using Fisher's Protected Least Significant Difference (LSD) test ($P \leq 0.05$).

3.4 RESULTS

3.4.1 Isolation of *Ralstonia solanacearum*

3.4.1.1Symptoms of *R. solanacearum* on tomato plants collected for isolation

The observed symptoms of bacterial wilt on infected tomato plants used for isolation of *R. solanacearum* were; lower leaves turning pale yellow, loss of leaf turgidity followed by dropping of leaves and sudden wilt of plants (Figure 3.1). The vascular bundle of the infected plant showed a brown discoloration (Figure 3.2). Infected tomato plants showed a milky white bacterial exudate from the stem hence a positive ooze test after bacterial streaming was done (Figure 3.3).

Figure 3.1: Tomato plant showing bacterial wilt symptoms (drooping leaves, yellow

Figure 3.2: Brown discoloration of the vascular bundle.

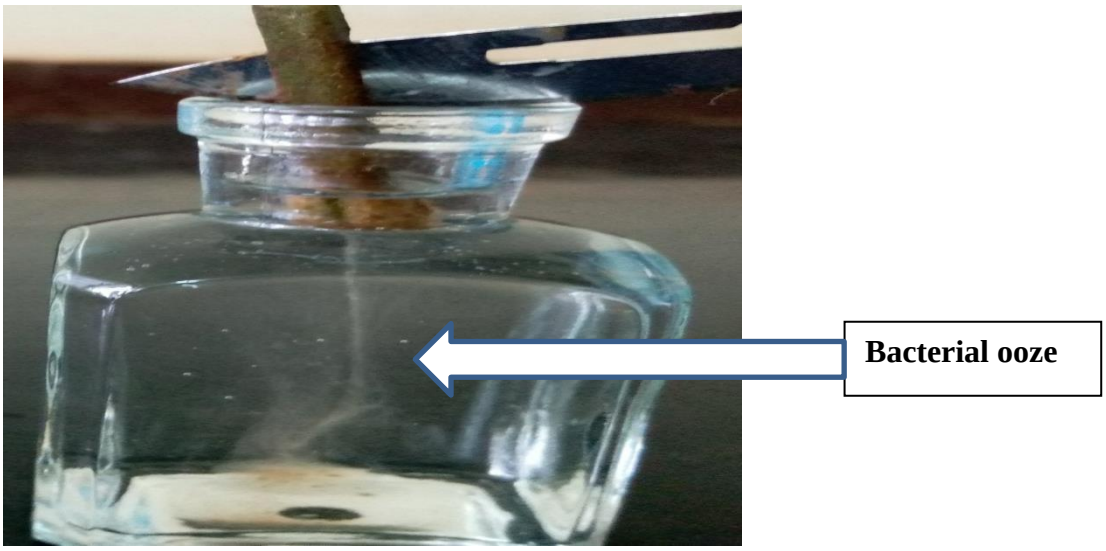

Figure 3. 3: Bacteria oozing from tomato plant showing bacterial wilt leaves,

3.4.1.2 Colony characteristics of *Ralstonia solanacearum*

White, fluidal, pinkish red centered colonies of *R. solanacearum* with round irregular margins of 8.0mm were observed on TZC medium (Figure 3.4). In addition, some colonies appeared round, deep red with narrow bluish border.

Colonies of *Xanthomonas campestris* pv *campestris* appeared mucoid, circular, convex shiny yellow when grown on YPDA media. *Pseudomonas* sp. colonies on SNA media were smooth, elevated, round with entire margins, pearly whitish-yellow in color.

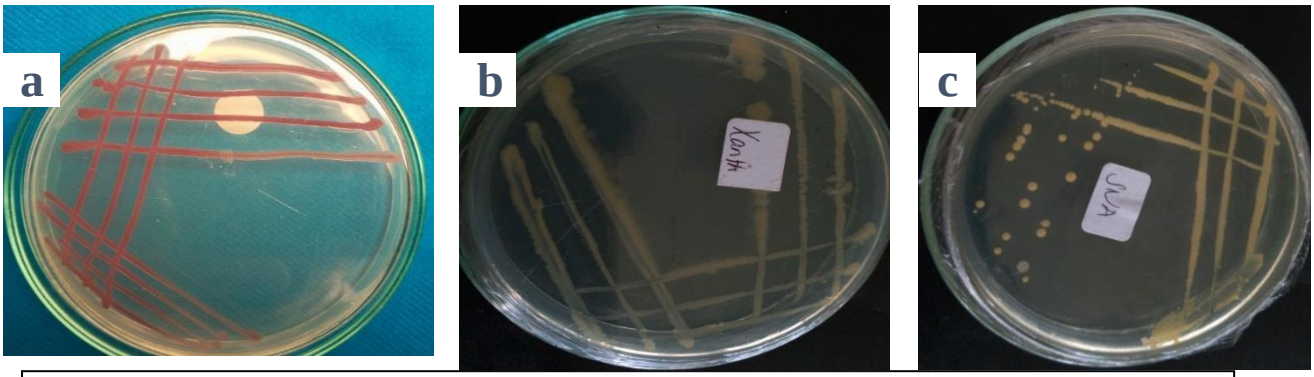

Figure 3. 4*:* a; large elevated fluidal, white colonies of *Ralstonia solanacearum* with pink center on Kelman's TZC medium. b; Circular, mucoid, convex shaped, shiny yellow colonies of *Xanthomonas campestris* pv *campestris* on YPDA medium. c; Smooth, elevated, round with entire margins, pearly whitish-yellow colonies of *Pseudomonas* sp on SNA

3.4.2 Pathogenicity test

Bacterial wilt symptoms were observed one week after transplanting. *R. solanacearum* was confirmed as the causal agent of the bacterial wilt symptoms observed on the inoculated tomato plants. The causal agent was confirmed by the presence of bacteria ooze as referred to in the materials and methods. Colonies of the pathogen isolated from wilted plants and cultured on TZC medium were white with pink center. In soil drench method, 10 out of 12 transplanted plants showed typical bacterial wilt symptoms. In root dip method, 7 plants showed bacterial wilt symptoms and no plant in control showed any symptoms. Disease incidence progressed rapidly inducing 70 to 80% wilt of plants in both methods after 25 days of transplanting.

3.4.3 Isolation and identification of *Trichoderma*

A total of 28 different *Trichoderma* isolates were identified from the samples collected. Pure cultures of each *Trichoderma* isolate grown on PDA medium showed different growth patterns and colony characteristics. Colony color varied from light green to dark green (Figure 3.5).

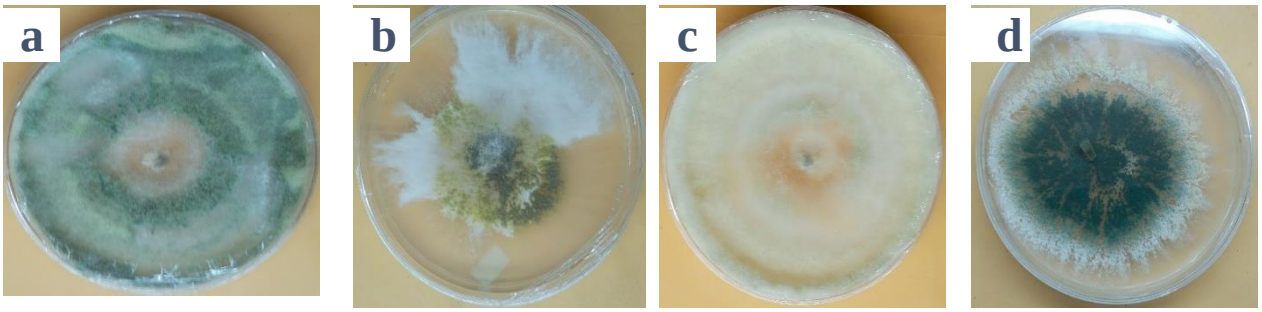

Figure 3.5*:* *Trichoderma* isolates T2 (a), T1 (b), T3 (c) and T7 (d), respectively used against the pathogens with colony colors varying from light to dark green.

3.4.4 *In vitro* screening of antagonists against *Ralstonia solanacearum*, *Xanthomonas campestris* pv *campestris* and *Pseudomonas* sp

3.4.4.1Bacterial antagonists (*Bacillus* isolates)

There were clear zones of inhibition (ZOI) around the filter paper discs by some of the *Bacillus* isolates against the test (Figure 3.6). Control plates had no visible ZOI. From the 19 *Bacillus* isolates screened, the isolates that had no zones of inhibition were discarded. Eight *Bacillus* isolates had activity in more than one pathogen.

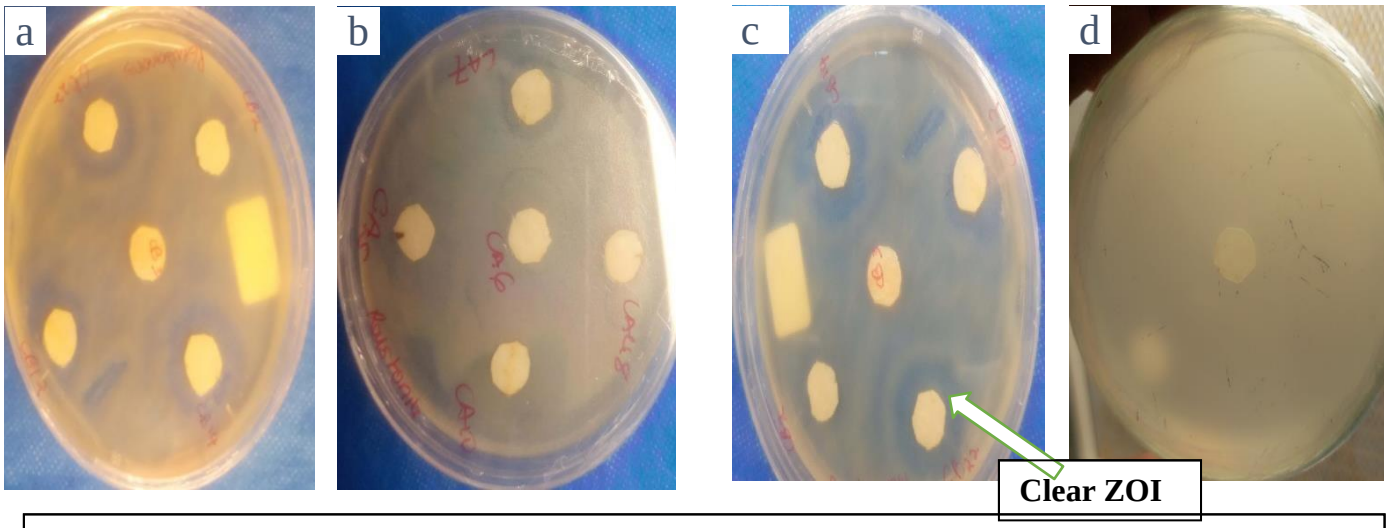

Figure 3.6: ZOI of *Bacillus* isolates against (a) *Pseudomonas* sp, (b) *Ralstonia solanacearum*, (c) *Xanthomonas campestris* pv *campestris* and (d) control (filter paper disc dipped in sterile water).

3.4.4.1.1 *Bacillus* isolates against *Ralstonia solanacearum*

Four isolates of *Bacillus* (CB64, CA7, CA5 and CA10) showed activity against the pathogen. The zones of inhibition (ZOI) of *Bacillus* isolates against *R. solanacearum* were significantly different at $P \leq 0.05$. Isolate CB64 gave the highest ZOI mean of 4.3mm at 24 hours of incubation and remained constant up to 48 hours of incubation. This was followed by CA7 with a ZOI of 3.4mm; while ZOI by other isolates was $\leq$ 2.2mm. However, the ZOI of CB64 decreased to 3.0mm at 72 hours. Similarly, the other three *Bacillus* isolates recorded reduced ZOI after 48 hours of incubation as shown in Table 3.1. No ZOI was observed for control.

Table 3. 1: Radius of the zone of inhibition (mm) due to *Bacillus* against *Ralstonia solanacearum* after 24, 48 and 72 hours of incubation

	Measurements of ZOI radius in mm		
***Bacillus* isolates**	**24 hours**	**48 hours**	**72 hours**
CB64	4.3a	4.3a	3.0a
CA7	3.4b	3.4b	2.4b
CA5	2,2c	2,2c	1.8c
CA10	1.5c	1.5c	0.0d
Control	0.0d	0.0d	0.0d
Mean	2.3	2.3	1.4
CV (%)	19	19	16
LSD (P≤0.05)	0.79	0.79	0.42

Means that have similar letter along a column are not significantly different at P≤0.05. CV: Coefficients of Variation. LSD: Least significance differences of means. ZOI: Zones of inhibition.

3.4.4.1.2 *Bacillus* isolates against *Xanthomonas campestris* pv *campestris*

For *Xanthomonas* spp, 10 *Bacillus* isolates showed activity against the pathogen. The isolates had high significant difference in the radius of ZOI at P≤0.05 compared to control (Table 3.2). Isolate CA5 had the largest ZOI of 6.6mm, followed by CA51 and CB8 at 5.7 and 4.1mm, respectively. The other seven isolates had significantly lower ZOI of ≤2.5mm compared to the first three isolates. Control had no clear ZOI. There was no significant difference among the antagonist at 24, 48 and 72 hours of incubation.

Table 3.2: Radius of zone of inhibition in mm due to *Bacillus* against *Xanthomonas campestris* pv *campestris* after 24, 48 and 72 hours of incubation

	Measurements of radius of ZOI in mm		
***Bacillus* isolates**	**24 hr.**	**48 hr.**	**72 hr.**
CA5	6.6a	6.6a	6.6a
CA51	5.7ab	5.7ab	5.7ab
CB8	4.1c	4.1c	4.1c
CA48	2.5d	2.5d	2.5d
CB4	2.2de	2.2de	2.2de
CB24	2.2de	2.2de	2.2de
CB12	2.0de	2.0de	2.0de
CA9	1.8de	1.8de	1.8de
CA10	1.8de	1.8de	1.8de
CA7	1.5e	1.5e	1.5e
Control	0.0f	0.0f	0.0f
Mean	2.8	2.8	2.8
CV (%)	19.50	19.50	19.50
LSD (P≤0.05)	0.91	0.91	0.91

Means that have similar letter(s) along a column are not significantly different at P≤0.05. CV: Coefficients of Variation. LSD: Least significance differences of means. ZOI: Zones of inhibition.

3.4.4.1.3 *Bacillus* isolates against *Pseudomonas* sp.

Ten isolates of *Bacillus* had activity against the pathogen; however seven of these isolates showed activity with a ZOI of more than 3.7mm. *Bacillus* isolates with the highest ZOI against *Pseudomonas* sp. were CB14 and CB22 which had similar measurements of 5.3mm while control had no clear ZOI as indicated in Table 3.3. There was a significant difference at P≤0.05 in ZOI between the isolates. However, for each isolate, the ZOI remained constant at 24, 48 and 72 hours of incubation.

Table 3.3*:* Radius of zone of inhibition (mm) due to *Bacillus* against *Pseudomonas* sp. at 24, 48 and 72 hours of incubation

	Measurements of radius of ZOI in mm		
***Bacillus* isolates**	**24 hr.**	**48 hr.**	**72 hr.**
CB14	5.3a	5.3a	5.3a
CB22	5.3a	5.3a	5.3a
CA48	4.4b	4.4b	4.4b
CB12	4.2bc	4.2bc	4.2bc
CA10	4.0bc	4.0bc	4.0bc
CB8	3.8cd	3.8cd	3.8cd
CA5	3.7cd	3.7cd	3.7cd
CB24	3.3de	3.3de	3.3de
CA51	3.0e	3.0e	3.0e
CA7	1.6f	1.6f	1.6f
Control	0.0g	0.0g	0.0g
Mean	3.5	3.5	3.5
CV (%)	11.20	11.20	11.20
LSD (P≤0.05)	0.67	0.67	0.67

Means followed by the same letter(s) along a column are not significantly different at P≤0.05. CV: Coefficients of Variation. LSD: Least significance differences of means. ZOI: Zones of inhibition.

Ralstonia solanacearum recorded the least ZOI compared to other pathogen with the highest ZOI mean of 4.3mm. This was followed by *Pseudomonas* sp. at 5.3mm and then *Xanthomonas* spp which showed the highest ZOI of 6.3mm. However, isolate CB64 that gave the highest ZOI of 4.3mm against *R. solanacearum* had no activity against *Xanthomonas* and *Pseudomonas* sp. Isolate CA7 that was second best against *R. solanacearum* with a ZOI mean of 3.4mm performed poorly against *Xanthomonas* sp. at 1.5mm and *Pseudomonas* sp. at 1.6mm. It was also observed that ZOI started reducing after 48 hours of incubation in *R. solanacearum* but stayed constant in other pathogens. The ZOI were reduced by the growing *R. solanacearum* pathogen but in other pathogens it remained constant.

Isolate CA5 performed best against *Xanthomonas campestris* at 6.6mm followed by its activity

against *Pseudomonas* sp. at 3.7mm and the least performance was against *R. solanacearum* at 2.2mm. The effects of isolate CA5 were significant in all the pathogens. Isolate CA51 that was second best against *Xanthomonas campestris* with a ZOI mean of 5.7mm, was also active against *Pseudomonas* sp. with a mean of 3.0mm but this was significantly lower than in *Xanthomonas* sp. The isolate had no activity against *R. solanacearum*. Isolate CB8 that was the third best against *Xanthomonas campestris* with a ZOI mean of 4.1mm, had comparable activity against *Pseudomonas* sp. at 3.8mm but no activity against *R. solanacearum*.

Among the seven best *Bacillus* isolates against *Pseudomonas* sp, five isolates (CB14, CB22, CA48, CB12 and CA10) had ZOI $\geq$ 4.0mm. Out of these five isolates, none of them had similar activity in *Xanthomonas* sp or *R. solanacearum*. Isolate CB8 that was the sixth best against *Pseudomonas* sp. with a ZOI mean of 3.8mm was the third best for *Xanthomonas campestris* at 4.1mm. Isolate CA5 that was the seventh best against *Pseudomonas* sp. with a ZOI mean of 3.7mm had the highest activity against *Xanthomonas campestris* at 6.6mm. Isolate CA51 which was among the nine best antagonists against *Pseudomonas* sp. at 3.0mm was the second best for *Xanthomonas campestris* at 5.7mm. It was noted that isolate CA7, the tenth antagonist against *Pseudomonas* sp. that performed poorly at 1.6mm, also had poor performance against *Xanthomonas campestris* at 1.5mm but was second best for *R. solanacearum* at 3.4mm.

There was a significant difference between the means of the ZOI of the three pathogens at $P \leq 0.05$. *Pseudomonas* sp. had the highest average mean of 3.5mm followed by *Xanthomonas campestris* at 2.8 and then *R. solanacearum* at 2.3mm (Figure 3.7).

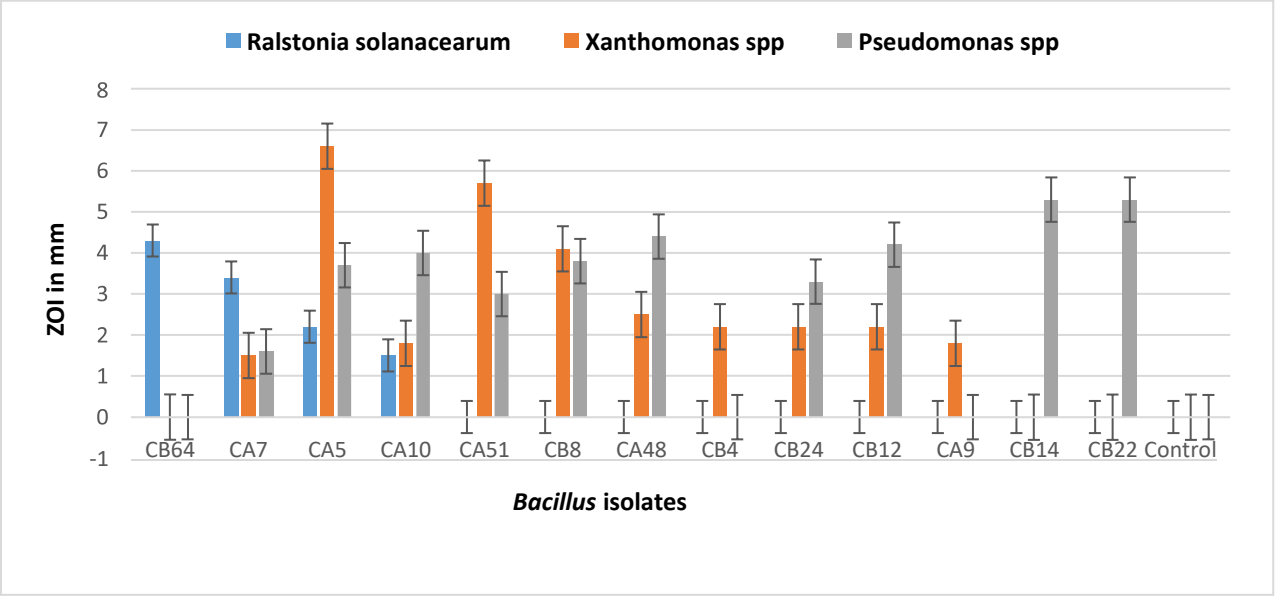

Figure 3.7: Mean radius of zone of inhibition (ZOI) in mm (Millimeters) induced by *Bacillus* isolates against three plant pathogens after 48 hours of incubation

3.4.4.2 Fungal antagonists (*Trichoderma* isolates)

Out of the 28 *Trichoderma* isolates screened, 21 isolates showed activity against the three pathogens. This was indicated by formation of clear ZOI around the mycelia discs of *Trichoderma* isolates that had activity against the three test pathogens (Figure 3.8). The isolates that had no zones of inhibition had no activity against the pathogens, hence were not recorded. The maximum ZOI of *Trichoderma* isolates against the pathogens were formed at 4 days of incubation and generally remained stable and constant even at every 6 days.

3.4.4.2.1 *Trichoderma* isolates against *Ralstonia solanacearum*

At 4 days of incubation, nine *Trichoderma* isolates had the highest activity against *R. solanacearum* with ZOI of ≥ 9.2mm. There was a significant difference between the ZOI of *Trichoderma* isolates against *Ralstonia solanacearum* at P≤0.05 as shown in Table 3.4. These isolates were grouped into four clusters. Cluster one was the best Isolates T1, T4 and T2 with ZOI of 13.5mm, 13.1mm and 12.5, respectively. Cluster two had T8, T3 and T7 with ZOI of 11.6mm, 11.2, and 11.0mm, respectively. Cluster three had two isolates, T6 and T14 that a ZOI mean of 10.8 and 10.2mm, respectively. Isolate T16 formed the last cluster with ZOI mean of 9.2mm. Control had no clear ZOI.

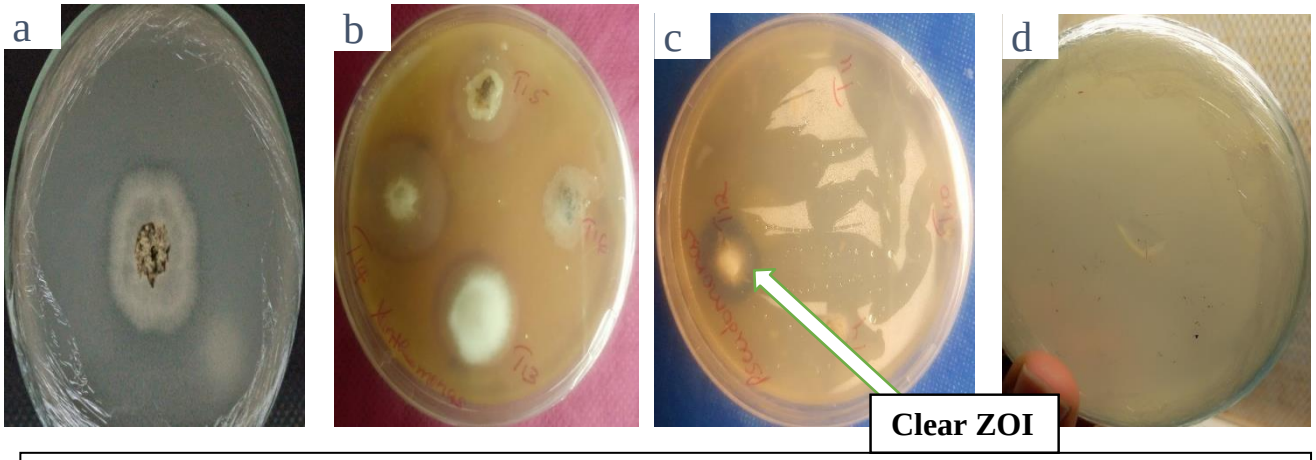

Figure 3.8: ZOI of *Trichoderma* isolates against *R. solanacearum* (a), *Xanthomonas campestris* (b) and *Pseudomonas* sp. (c). d; Control (5mm disc of PDA)

Table 3. 4: Radius of zone of inhibition (mm) due to *Trichoderma* isolates against *Ralstonia solanacearum* at 2, 4 and 6 days of incubation

	Measurements of radius of ZOI in mm		
***Trichoderma* isolates**	**2 days**	**4 days**	**6 days**
T1	7.7bc	13.5a	13.5a
T4	7.6bc	13.1ab	13.1ab
T2	9.1bc	12.5b	12.5b
T8	10.9a	11.6c	11.6c
T3	9.2b	11.2cd	11.2cd
T7	7.4c	11.0cd	11.0cd
T6	7.7bc	10.8de	10.8de
T14	8.2bc	10.2e	10.2e
T16	8.0bc	9.2f	9.2f
Control	0.0d	0.0g	0.0g
Mean	7.6	10.3	10.3
CV (%)	12.90	3.70	3.70
LSD (P≤0.05)	1.67	0.64	0.64

Means that have similar letter(s) along a column are not significantly different at P≤0.05. CV: Coefficients of Variation. LSD: Least significance differences of means. ZOI: Zones of inhibition.

3.4.4.2.2 *Trichoderma* isolates against *Xanthomonas campestris* pv *campestris*

There was a significant difference between the ZOI of *Trichoderma* isolates against *Xanthomonas campestris* pv *campestris* at P≤0.05 as shown in Table 3.5. From the screened *Trichoderma* isolates, 11 isolates showed activity against the pathogen with ZOI of 7.7mm and above. The isolates were grouped in five clusters. First cluster had isolate T28 that performed best with a ZOI of 15.2mm followed by isolate T12 in the second cluster with a ZOI mean of 11.4mm. Third cluster had isolates T14, T18, T1, and T5 with ZOI mean of 10.7, 10.6, 10.3 and 10.0mm, respectively. Isolates T6, and T19 formed the fourth cluster with a ZOI of 9.4mm. Last cluster had T13, T10 and T17 with ZOI of 8.5, 8.4 and 7.7mm, respectively. No ZOI were formed in control.

Table 3. 5: Radius of zone of inhibition (mm) induced by *Trichoderma* against *Xanthomonas campestris* pv *campestris* at 2, 4 and 6 days of incubation

	Measurements of radius of ZOI in mm		
***Trichoderma* isolates**	**2 days**	**4 days**	**6 days**
T28	9.6a	15.2a	15.2a
T12	8.7b	11.4b	11.4b
T14	5.2cd	10.7bc	10.7bc
T18	5.4c	10.6bc	10.6bc
T1	5.1cd	10.3cd	10.3cd
T5	4.5de	10.0cd	10.0cd
T6	3.8ef	9.4de	9.4de
T19	3.5f	9.4de	9.4de
T13	3.2fg	8.5ef	8.5ef
T10	5.4c	8.4ef	8.4ef
T17	2.4g	7.7f	7.7f
Control	0.0h	0.0g	0.0g
Mean	4.7	9.3	9.3
CV (%)	10.00	6.70	6.70
LSD (P≤0.05)	0.8	1.04	1.04

Means that have the same letter(s) along a column are not significantly different at P≤0.05. CV: Coefficients of Variation. LSD: Least significance differences of means. ZOI: Zones of

inhibition.

3.4.4.2.3 *Trichoderma* isolates against *Pseudomonas* sp.

There was a significant difference in the ZOI of *Trichoderma* isolates against *Pseudomonas* sp. at P≤0.05 (Table 3.6). From the isolates screened, 11 *Trichoderma* isolates showed activity against the pathogen. The isolate with the largest ZOI was T28 with a mean ZOI of 9.3mm followed by T12 at 7.7mm. The other nine isolates had ZOI of ≤3.4mm which was significantly lower compared to the ZOI recorded for T28 and T12. Control had no ZOI.

Table 3. 6: Radius of zone of inhibition (mm) induced by *Trichoderma* isolates against *Pseudomonas* sp. at 2, 4 and 6 days of incubation

	Measurements of radius of ZOI in mm		
***Trichoderma* isolates**	**2 days**	**4 days**	**6 days**
T28	9.3a	9.3a	9.3a
T12	7.7b	7.7b	7.7b
T1	3.4c	3.4c	3.4c
T16	3.3c	3.3c	3.3c
T3	3.3c	3.3c	3.3c
T14	3.2cd	3.2cd	3.2cd
T11	2.8de	2.8de	2.8de
T26	2.4e	2.4e	2.4e
T25	2.2ef	2.2ef	2.2ef
T4	2.2ef	2.2ef	2.2ef
T9	2.2ef	2.2ef	2.2ef
Control	0.0g	0.0g	0.0g
Mean	3.5	3.5	3.5
CV (%)	9.70	9.70	9.70
LSD (P≤0.05)	0.57	0.57	0.57

Means that have the same letter(s) along a column are not significantly different at P≤0.05. CV: Coefficients of Variation. LSD: Least significance differences of means. ZOI: Zones of inhibition.

Isolate T1 which had the highest activity against *R. solanacearum* with a ZOI of 13.5mm also showed high activity against *Xanthomonas campestris* at 10.3mm but recorded significantly lower activity against *Pseudomonas* sp. with a ZOI of 3.4mm. Isolate T8 which was best in cluster two against *R. solanacearum*, had no activity against the other pathogens. Isolate T3, the second best of cluster two performed well against *R. solanacearum* with a ZOI of 11.2mm, but performed poorly against *Pseudomonas* sp. at 3.3mm and was not active against *Xanthomonas campestris*. Isolate T7 was only active against *R. solanacearum* where it recorded ZOI of 11.0mm. Isolate T6 which had 10.8mm with *R. solanacearum* performed fairly well with *Xanthomonas campestris* at 9.4mm but had no activity against *Pseudomonas* sp.

Isolate T14 performed better against *R. solanacearum* and *Xanthomonas campestris* at 10.2 and 10.7 mm, respectively compared to its performance against *Pseudomonas* sp. at 3.2mm. The last cluster T16 that was the lowest performer against *R. solanacearum* with a ZOI of 9.2mm was significantly poor against *Pseudomonas* sp. at 3.3mm but showed no activity against *Xanthomonas campestris* pv *campestris*.

Isolates T28 and T12 that had the highest ZOI of 15.2 and 11.4mm, respectively against *Xanthomonas campestris* pv *campestris* also had the highest activity against *Pseudomonas* sp. at 9.3 and 7.7mm, respectively. However, the activity of these isolates (T28 and T12) against *Xanthomonas campestris* pv *campestris* was significantly higher compared to *Pseudomonas* sp. Isolate T18 that was third best against *Xanthomonas* sp. at 10.6mm had no activity against the other pathogens. *Trichoderma* isolates T5, T19, T13 and T17 that had activity against *Xanthomonas campestris* with a ZOI of more than 7.7mm, had no activity against *Ralstonia solanacearum* and *Pseudomonas* sp. Among the three test pathogens, the means of ZOI showed significant difference at $P \leq 0.05$. *R. solanacearum* had the highest average mean of 10.3 mm followed by *Xanthomonas campestris* pv *campestris* at 9.3mm and then *Pseudomonas* sp. at 3.5mm (Table 3.7).

Table 3. 7: Radius of the zone of inhibition (mm) induced by *Trichoderma* against three bacteria pathogens after 4 days of incubation

Measurements of radius of ZOI in mm			
***Trichoderma* isolates**	***Ralstonia solanacearum***	***Xanthomonas campestris***	***Pseudomonas* sp.**
T1	13.5a	10.3cd	3.4c
T4	13.1ab	NA	2.2ef
T2	12.5b	NA	NA
T8	11.6c	NA	NA
T3	11.2cd	NA	3.3c
T7	11.0cd	NA	NA
T6	10.8de	9.4de	NA
T14	10.2e	10.7bc	3.2cd
T16	9.2f	NA	3.3c
T28	NA	15.2a	9.3a
T12	NA	11.4b	7.7b
T18	NA	10.6bc	NA
T5	NA	10.0cd	NA
T19	NA	9.4de	NA
T13	NA	8.5ef	NA
T10	NA	8.4ef	NA
T17	NA	7.7f	NA
T11	NA	NA	2.8de
T26	NA	NA	2.4e
T25	NA	NA	2.2ef
T9	NA	NA	2.2ef
Control	0.0g	0.0g	0.0g
Mean	10.3	9.3	3.5
CV (%)	3.70	6.70	9.70
LSD (P≤0.05)	0.64	1.04	0.57

Means followed by similar letter(s) along a column are not significantly different at P≤0.05. CV: Coefficients of Variation. LSD: Least significance differences of means. ZOI: Zones of inhibition. NA: No Activity

3.5 DISCUSSION

Isolation and identification of *Ralstonia solanacearum*

Ralstonia solanacearum colonies that were white fluidal with pinkish center on TZC medium were Virulent *R. solanacearum* colonies while those that appeared round, deep red with narrow bluish border were avirulent. These results are comparable to those reported by Chaudhry and Rashid, 2011 and Khasabuli *et al.* (2017). Virulent colonies appear as large, elevated, fluidal and white with a pale red to pink center while avirulent mutant colonies appear as butyrous, deep red with bluish border on TZC media as described by Champoiseau and Momol, (2008) and Chamedjeu *et al.* (2018). Virulence of *R. solanacearum* can be determined on the basis of colony color on TZC media (Seleim *et al.,* 2014). Present results agreed with those reported by Popoola *et al.* (2015) and El- Habbaa *et al.* (2016) that culture traits on different media are important tools for identification of *R. solanacearum*. According to Seleim *et al.* (2011) and Garcia *et al.* (2019), Triphenyl Tetrazolium Chloride medium is used to distinguish *R. solanacearum* from other bacteria during isolation. This media also shows the difference between virulent and avirulent colonies (Fajinmi and Fajinmi, 2010: Rahman *et al.,* 2013). Hence in the current study the colonies appeared fluidal whitish with a pink center which showed that the isolated *R. solanacearum* was virulent. Pathogenicity test carried out confirmed the virulence of *R. solanacearum*. This was indicated by 80% disease incidence observed on Riogrande plants. The soil drench method was a better method for inoculating the pathogen as compared to the root dip method. These findings agree to those reported by Narasimha and Srinivas, (2012).

Colonies of *Xanthomonas campestris* pv *campestris* appeared mucoid, circular, convex shiny yellow when grown on YPDA media. EPPO, (2014) reported that *X. campestris* pv *campestris* appears as mucoid, circular, convex, shiny yellow colonies when grown on yeast peptone sucrose agar medium which supports the findings of the current study. *Pseudomonas* sp. colonies on SNA media were smooth, elevated, round with entire margins, pearly whitish-yellow in color. These observations are similar to those reported by Schaad, (1988) and EPPO, (2014).

Isolation and identification of *Trichoderma*

Identification of *Trichoderma* isolates using morphological characteristic is a conventional method that remains a practical method to identify *Trichoderma* up to genus level (Samuel *et al.,* 2004). Isolates in the present study had different colony morphology and color that varied

from light green to dark green similar to characteristics reported by Kannangara *et al.* (2017). All the 28 isolates isolated were confirmed to belong to genus *Trichoderma*, hence they were all screened against the bacterial pathogens. Similar colony morphology may indicate same species of *Trichoderma* hence sporulating structures such as conidiophore branching, phialides and spores were used to identify the successful isolate after screening against the bacterial pathogen to species level (Rifai, 1969).

Screening of *Bacillus* and *Trichoderma* isolates against *Ralstonia solanacearum*, *Xanthomonas campestris* pv *campestris* and *Pseudomonas* sp

In vitro assessment of the ability of various antagonistic agents that acts against different plant pathogens is the first step in the selection of potential biological control agents (Ramesh *et al.*, 2009). These antagonists use different mechanisms that include: competition, lysis, antibiosis, siderophore production and hyper parasitism (Revathi *et al.*, 2017). In this study, strains of *Bacillus* and *Trichoderma* effectively suppressed the growth of *Ralstonia solanacearum*, *Xanthomonas campestris* pv *campestris* and *Pseudomonas* sp. *in vitro*. These findings agree with those reported earlier by (Lwin and Ranamurkhaarachchi, 2006): Liza and Bora, 2009). Biological control agents in the current study had activity against *R. solanacearum in vitro.* This is possibly because according to Cook and Sequeira, (1991), *R. solanacearum* is a poor competitor outside the host plant and this allows for biological control of the pathogen. The present results showed that different isolates of *Trichoderma* and *Bacillus* clearly hinder the growth of *R. solanacearum*. These findings are comparable to earlier reports by Guo, (2004), Revathi *et al.* (2017) and Virendra, (2017). The radius of zones of inhibition caused by *Bacillus* isolates against the *R. solanacearum* ranged between 1.5mm and 10mm which was comparable to the findings reported by Ramesh *et al.* (2009) and Singh *et al.* (2012). The antagonistic ability of *Bacillus* isolates against *R. solanacearum* is in line with results observed by Chandrasekaren and Chun, (2016). *Bacillus* isolate CB64 had the highest activity (ZOI= 4.3mm) against *R. solanacearum.* These results showed that *Bacillus* secrete antibiotics and secondary metabolites that suppress the pathogen through antibiosis. The antagonistic ability of *Bacillus* isolates against *R. solanacearum* observed in the present study was similar to the results observed by Seleim *et al.*, (2011). Seleim *et al.*, 2011 reported that *Pseudomonas fluorescens, Pseudomonas putida* and *Bacillus subtilis* had broader inhibition zones against the pathogen *Ralstonia solanacearum*.

Zones of inhibition of *Trichoderma* isolates against *R. solanacearum* ranged from 9.2 mm to 13.5 mm, similar to the findings of Yendyo *et al.* (2017). *Trichoderma* isolate T1 was the most potent in inhibiting the growth of *R. solanacearum* with a mean ZOI of 13.5mm. *Trichoderma* secretes different compounds that act against *R. solanacearum* and produce secondary metabolites that promotes plant growth and yield in the field (Sharma *et al.,* 2012: Singh and Jagtap, 2017). The results herein showed that most *Trichoderma* isolates performed better than *Bacillus* isolates against all the pathogens. This was because *Trichoderma* spp are fungi that produce volatile compounds such as β-1, 3-glucanase, chitinases, protease, gliotoxis and dermadine among others with higher inhibitory properties compared to *Bacillus* isolates. Similar findings have been reported by Hernandez-Castillo *et al.* (2020) of higher inhibition of fusarium spp when *Trichoderma* species are used (62.4 to 54.8%), in contrast to when *Bacillus* species (44.5 to 36.9%) are used *in vitro*. Similarly, these findings of the current study are in line with the earlier findings of the *in vitro* studies by Narasimha and Srinivas, (2012) that showed *Pseudomonas fluorescence*, *Trichoderma* spp and plant extracts have antibacterial activity against *R. solanacearum* and most strains of *Trichoderma* performed better than *Pseudomonas* spp. Diffusible metabolites secreted by *Bacillus* and *Trichoderma* isolates inhibited the growth of *R. solanacearum* indicating the role of secondary metabolites in suppressing the pathogen *in vitro* (Ramesh *et al.,* 2009: Virendra, 2017). According to Yendyo *et al.* (2017), the activity of native *Trichoderma* spp against *R. solanacearum* as observed by the formation of inhibition zones was significantly higher compared to *Bacillus* spp. These observations are comparable to the findings of the present study.

Bacillus isolates tested against *Xanthomonas campestris* pv *campestris* and *Pseudomonas* sp. in the present study were found to be more effective compared to their activity against *R. solanacearum.* It has been reported that certain endophytic bacteria can reduce the *in vitro* growth of *Xanthomonas campestris* through production of siderophore and antibiotic compounds (Compant *et al.,* 2005). This report is in agreement with results herein that, *Bacillus* isolates in the present study were able to reduce the growth of *Xanthomonas campestris* as indicated by formation of zones of inhibition. The zones of inhibition of *Bacillus* isolates against *Xanthomonas campestris* pv *campestris* ranged between 1.5mm to 6.6mm in radius and isolate CA5 showed the highest ZOI (6.6mm). These findings agree with those reported by Liu *et al.* (2016) of the antagonism of *Xanthomonas campestris* by plant growth promoting rhizobacteria. Similarly, Monteiro *et al*. (2005) reported control of black rot of *Brassicas* caused by *Xanthomonas campestris* pv *campestris* using strains of *Bacillus subtilis*. Zones of

inhibition of *Trichoderma* isolates against *Xanthomonas* sp. ranged between 7.7 mm to 15.2mm in radius, which was comparable to earlier studies (Nikolic *et al.,* 2013: Sharma, 2018). *Trichoderma* isolate T28 showed the highest ZOI (15.2mm) against *Xanthomonas campestris* pv *campestris*. The activity of isolate against the test pathogen was due to production of metabolites and secondary compounds that are able to suppress the growth of the pathogen (Nikolic *et al.,* 2013).

Bacillus isolates had higher antagonistic activity against *Pseudomonas* sp. compared to the other test pathogens and control. Zones of inhibition ranged from 3.0mm to 5.3mm in radius and isolate CB14 and CB22 gave similar ZOI (5.3mm). These results are comparable to earlier reports by Bais *et al.* (2004) that *Bacillus subtilis* has been used against infections of *Pseudomonas* spp. The present study showed that *Trichoderma* isolates had significantly lower activity against *Pseudomonas* sp. compared to other test pathogen. This is because *Pseudomonas* species produce their own metabolites that have antagonistic properties against some pathogen and this could also lower the activity of *Trichoderma*. *Trichoderma* isolate T28 had the highest activity (9.3mm) against the pathogen. These findings are in line with earlier studies by Brotman *et al.* (2008) and Hill *et al.* (2015) who reported that there was minimal activity of *Trichoderma* spp against *Pseudomonas* spp *in vitro*.

Bacillus isolate CA10, CA7, CA5 and *Trichoderma* isolate T1 and T14 had antagonistic activity against all the pathogens. This was possibly because the secondary metabolites synthesized by the antagonists and chemical factors like antibiotics have a broad spectrum of activity (Gross and Vidaver, 1990). A larger ZOI was formed after 48 hours of incubation compared to that of 24 hours maybe due to the fact that antagonists secretes more metabolites with increased time of incubation (Singh *et al.*, 2012: Chen *et al.*, 2013). The study revealed that ZOI decreased or remained constant over time, after 48 hours and 4 days of incubation for *Bacillus* and *Trichoderma* isolates, respectively. This may be attributed to reduced ability of the antagonist to produce more metabolites and the pathogen being able to withstand the metabolites due to reduced effectiveness (Sultana, 2015). *Bacillus* strains secretes different antibiotics such as polymyxin, circulin and colistin that are active against gram positive and gram negative as well as many pathogenic fungi (Tapwal *et al.,* 2011: Maksimov *et al.,* 2011: Mukherjee *et al.*, 2013). According to Montesinos *et al.* (1996) and (Lwin and Ranamurkhaarachchi, 2006), the method used for screening the activity of biological control agents against the pathogen may affect the effectiveness of the antagonists. The antagonistic ability of the isolates against the pathogens is lower in filter paper disc method as compared to cross-streaking method (Nguyen and

Ranamurkhaarachchi, 2010). In filter paper disc method, the antagonists are introduced at the same time as the pathogen with little time for metabolites production Mandal *et al.* (2017). Hence use of different methods may give higher measurements of the ZOI.

3.6 CONCLUSION

Ten *Bacillus* and 11 *Trichoderma* isolates had varied antagonistic activity against the pathogens tested. *Trichoderma* isolates T1, T28 and T28 were the most potent in inhibiting the growth of *R. solanacearum*, *X. campestris* and *Pseudomonas* sp, respectively. *Bacillus* isolates CB64 and CA5 had the highest activity against *R. solanacearum* and *X. campestris*, respectively while CB14 and CB22 had the highest activity against *Pseudomonas* sp. Isolates of *Trichoderma* showed better activity by more than 56.67% compared to isolates of *Bacillus.* The high difference in means of ZOI implied that *Bacillus* isolates had the lowest activity against *R. solanacearum* followed by *X. campestris* but showed high activity against *Pseudomonas* sp. The results showed that twelve *Trichoderma* isolates (with ZOI of ≥ 10mm) and nine *Bacillus* isolates (with ZOI of ≥ 4.3mm) have high potential in managing various bacterial diseases.

However, since the activity of *Bacillus* and *Trichoderma* isolates under field conditions may be different from the *in vitro* activity, there is need for greenhouse and field trails to confirm these findings.

CHAPTER FOUR

Effect of *Bacillus* and *Trichoderma* species in management of bacterial wilt disease caused by *Ralstonia solanacearum* on tomato

ABSTRACT

Bacterial wilt caused by *R. solanacearum* is one of the most devastating diseases in tomato cultivation. This study aimed to evaluate the effect of *Bacillus* and *Trichoderma* isolates to manage the bacterial wilt disease under field conditions. Field experiments were conducted in a randomized complete block design at Kabete and Mwea sites in Kenya. The treatments included; 3 *Trichoderma* isolates (T1, T2 and T4), 2 *Bacillus* isolates (CB64 and CA7), a mixture of T1, T2 and T4, chemical standard and distilled water as control. *Trichoderma* and *Bacillus* isolates were grown on sterilized sorghum grain and cow manure carriers, respectively. Antagonist's inoculation was carried out by dipping tomato plants for 30 minutes in each treatment suspension. Each treatment was applied at a rate of 150ml/plant hole and this was repeated after 35 days. Soils were sampled prior to transplanting, 60 days and 112 days after transplanting for quantification of *R. solanacearum* population and at 126 days for determining the total microbial count in the soil. Bacterial wilt incidence was assessed every week by counting the number of wilted plants in each plot. Yield parameters were assessed at physiological maturity.

All the treatments evaluated under field conditions significantly reduced bacterial wilt incidence and severity at $P \leq 0.05$ than the control at Kabete and Mwea sites. *Trichoderma* isolate T1 followed by *Bacillus* isolate CB64 were the best in reducing the disease incidence by more than 61.66% and 53%, respectively at both sites. Treatment CB64 and T1 had the highest reduction of *R. solanacearum* population in the soil by 93.17% and 92.07%, respectively. However, control had a pathogen increase of 20.40%. The total microbial count was highest in *Bacillus* treated plots in both sites. Isolate CB64 had the highest count of 1.32×10^5 CFU/ml at Kabete and 1.21×10^5 CFU/ml at Mwea site. CB64 and T1 performed significantly better compared to the standard, while the mixture of isolates T1, T2 and T4 performed poorest in all parameters. The treatments also increased the yield of tomato. Results from this study showed that *Trichoderma* and *Bacillus* isolates are effective biological control agents for use in management of bacterial wilt.

Keywords: Tomato, bacterial wilt, *Ralstonia solanacearum*, *Trichoderma*, *Bacillus*

4.1 INTRODUCTION

Tomato (*Lycopersicum esculentum*) is one of the most consumed vegetables in Kenya (Smart Farm, 2016). Cultivation of tomato crop suffers high losses due to several viral, fungal and bacterial diseases that affect the crop (Yuging, 2018). Among the diseases, bacterial wilt caused by *Ralstonia solanacearum* has been reported to be the most rampant disease in tomato production (Kago *et al.,* 2019). In Kenya, bacterial wilt causes 64% losses on crops grown under open field conditions and up to 100% loss on the crops in the greenhouse (Mbaka *et al.,* 2013).

Management of bacterial wilt is difficult since *R. solanacearum* pathogen has a wide host range and damages over 200 plant species in 50 different families that include: tomato, potato, eggplant, pepper, tobacco (Meng, 2013). The pathogen has a high destructive nature, ability to persist even in abandoned lands and a wide geographical distribution (tropics, sub-tropics, warm temperate regions) (Mihovilovich *et al.,* 2017). There are hardly known chemicals to manage bacterial diseases, except antibiotics that are used for animal and human diseases (Yendyo *et al.,* 2017). These antibiotics are highly regulated to avoid development of resistance if carelessly used.

The effectiveness of available conventional management strategies for bacterial wilt is very limited (Aguk *et al.,* 2018). Hence, biological control measures that use antagonistic fungal and bacterial agents are an attractive option (Mandal *et al.,* 2017). Biological control of bacterial wilt disease of solanaceas crops caused by *R. solanacearum*, using antagonistic agents, has been reported earlier (Singh, 2013 and Kumur, 2017). Plant growth promoting bacteria and fungi like *Bacillus* and *Trichoderma* species have been reported to be promising bio-control agents for management of *R. solanacearum*. It has been found that species of *Bacillus* and *Trichoderma* are able to reduce bacterial wilt incidence on tomato plants and increase the yields (Thongwai and Kunopakarn, 2007 and Narasimhamurthy *et al.,* 2018). Hence, the aim of this present study was to evaluate the efficacy of *Bacillus* and *Trichoderma* strains in managing the bacterial wilt of tomato under field conditions.

4.2 MATERIALS AND METHODS

4.2.1 Description of experimental site

Field experiments were conducted during the period (March-August, 2019). Tomato fields heavily infected with bacterial wilt disease were identified at Kabete and Mwea sites. Kabete Field Station is located in Kiambu County, Kenya at an agro-ecological zone (AEZ) III, at 01°

15’S; 036°44’ E and an altitude of 1820m above sea level. It has a bimodal rainfall of 1059 mm annually and a temperature ranges between 12.3 and 22.5°C. The soils are deep, dark brown to brown humic nitosols with kaolinite clay minerals and a good drainage ideal for tomato production (Lengai, 2016). Mwea site, Kirinyaga County is located in agro-ecological zones II at 0.5420° S, 37.2735° E and at an altitude of 1570m above sea level. The region experiences a bimodal rainfall of 1470 mm annually and temperature ranges between 15.6 and 28.6°C. The soils are deep humic nitisols that are moderately fertile with a pH of about 5 (Waiganjo *et al.,* 2006).

4.2.2 Growth and survival of *Bacillus* isolates in manure carrier

Two *Bacillus* isolates CB64 and CA7 that had the highest activity against *R. solanacearum in vitro* were multiplied in cow manure used as a carrier for field experiments. Manure was expected to provide food and support for *Bacillus* isolates and make them easy to handle and apply. Cow manure which had decomposed for a period of 3 months was sun dried, crushed and sieved through 2mm sieve. The C: N ratio of the manure was determined before sterilization at 121^{o}C at 1.5 bars for 15 minutes. This process of sterilizing manure was repeated once for 3 days. Two hundred grams of the sterile manure was poured in sterile sandwich boxes used as incubating chambers. Isolates CB64 and CA7 previously grown on nutrient agar at 27^{o}C for 48 hours were harvested by flooding the plates of each isolate with 5ml sterile distilled water. Bacterial colonies were then scraped off, using sterile glass slide and each *Bacillus* isolate suspension was emptied in different sterile conical flasks. The concentration of the bacterial suspension was adjusted to 1×10^{9}CFU/ml by serial dilution technique. Using a sterile pipette, 0.5 ml of sterile distilled water was added to one gram of dry sterile cow manure to make it moisturized and to facilitate bacteria multiplication. Using a sterile syringe, the sterile manure was inoculated by injecting 0.3ml of each *Bacillus* suspension into one gram of manure following a modified protocol by Macharia, (2002). *Bacillus* isolates were allowed to multiply in the carrier at 28^{o}C for 48 hours. The concentration of *Bacillus* isolates in the manure was determined through serial dilution technique.

4.2.3 Growth and survival of *Trichoderma* isolates in sorghum carrier

Three *Trichoderma* isolates; T1, T2 and T4 that had the highest activity against *R. solanacearum in vitro* were maintained by regular sub-culturing at intervals of 10 days on PDA. The isolates were multiplied in white sorghum grains following a modified protocol by Kumur, (2017). The C: N ratio of the sorghum grains was determined before sterilization. Two hundred and fifty grams of sorghum grain were placed in 1litre conical flask and supplemented with 5% anhydrous dextrose in 250ml distilled water. The sorghum was then parboiled and autoclaved at 121^{o}C at 1.5 bars for one hour for two consecutive days. Five mm discs of 7 day old culture of each *Trichoderma* isolates was placed in each of the flasks with sterilized sorghum grains. The flasks were corked with cotton wool and aluminium foil then incubated at 28±1^{o}C for 18 days, with regular shaking after every 3 days. Colonized sorghum was air dried and ground into powder using a grinding machine.

4.2.3.1 Evaluation of *Trichoderma* isolates population in sorghum carrier

Spore count per gram of the colonized carrier was determined on the 7th, 11th, 14th and 18th day after inoculation. Serial dilution technique was used. Ten grams of *Trichoderma* colonized carrier was suspended in 90ml sterile distilled water and was shaken for 30 minutes on a rotary shaker at 500rpm. One milliliter was drawn as the suspension was being shaken and was added to 9ml of sterile water blank in a conical flask. This procedure was repeated up to 10^{-6} folds and the amount of spores in 1ml of the suspension was determined using haemocytometer. The average spore count for each dilution was multiplied by the reciprocal of the dilution factor to get the initial concentration following a protocol by Muriungi *et al.* (2013).

4.2.4 Experimental design

The experimental design was randomized complete block design (RCBD) with 8 treatments and 3 replicates. Each treatment plot was 3.6m by 2m, separated from other plot by 1m of weed free bare ground. The blocks were separated by 2m paths. Riogrande tomato variety that is moderately resistant to bacterial wilt disease was used. There were 20 plants in each plot with a spacing of 60cm by 60cm. The 8 treatments tested were; *Trichoderma* isolates T1, T2 and T4 each multiplied in sorghum carrier, *Bacillus* isolates CB64 and CA7 each multiplied in cow manure carrier and a chemical standard used by farmers (Enrich® contained; Di- Bromo Di nitro Propane 1, 3- diol). A mixture of T1, T2 and T4 in the ratio of 1:1:1 was included and distilled water was used as control treatment.

4.2.4.1 Preparation and application of the treatments in the field

One gram of well colonized manure that contained 5.86×10^{15} CFU/g for *Bacillus* isolate CB64 and 6.73×10^{15} CFU/g for isolate CA7 were mixed each in 10ml distilled water. For each *Trichoderma* isolate, 1g of well colonized ground sorghum that contained an average of 2.6×10^{9} spores/g was mixed in 40ml distilled water. The mixture of T1, T2 and T4 was prepared in a ratio of 1:1:1 and the concentration applied was 2.6×10^{9} spores/g of sorghum carrier. One gram of the standard chemical was mixed in 3litres distilled water, following the manufactures recommended application rates. Four weeks old tomato plants were uprooted from the nursery. The roots of the tomato plants were first dipped for 30 minutes in the treatments before being transplanted into the plots for the respective treatments. This was to ensure that isolates applied were given enough time for interaction with the plants before being transplanted into the field. Immediately after transplanting, a soil drench of 150ml of each treatment was applied around the plant roots following a modified protocol of Rosyidah *et al.* (2013). The soil drench with freshly prepared treatments was applied again after 35 days of transplanting.

4.2.5 Determination of bacterial wilt incidence

Bacterial wilt incidence was assessed every week after treatment application up to the end of the experiment by counting the number of wilted plants in each plot. Disease incidence was assessed as percentage of wilted plants within each treatment.

i.e. $I = \frac{NPSWS}{NPPT} \times 100$

Where: I- wilt incidence, NPSWS- number of plants showing wilt symptoms and NPPT- number of plants per treatment (Ayana *et al.,* 2011).

4.2.6 Assessment of the disease severity based on stem browning and bacterial ooze

Assessment of bacterial wilt disease severity was done once during the crop growth and at the end of the experiment at 126 days. The severity during the crop growth was done through destructive sampling where six plants were randomly uprooted per treatment. The uprooted stems were cut longitudinary at 2cm above the soil level and the lower part was split open using a sharp blade. Any browning observed on the split stem was recorded using a score of 0-3 where, 0- no browning, 1- light brown color restricted to 2cm from base of stem, 2- light brown color spread more than 2cm from the base of the stem and 3- dark brown color on the vascular tissue (Elphinstone *et al.,* 1998). About 10cm of the upper part of the uprooted plants was checked for bacterial ooze using bacterial streaming method. Bacterial streaming involved suspending the cut stem in clear water in a beaker to allow bacterial cells out of plant stem into

the water. Bacterial ooze was scored using scale 0- 3 where 0- no ooze, 1- thin strands of bacteria ooze that stops in 3 minutes, 2- continuous thin flow that is unrestricted and 3- heavy ooze turning the water turbid in 2 minutes (Elphinstone *et al.*, 1998). The same procedure was repeated at 126 days to evaluate stem browning and bacterial oozing. Percentage severity index (PSI) was calculated using the method described by Cook, (2006)

i.e. $PSI = \sum score \times \frac{100}{number\ of\ plants\ rated \times maximum\ scale\ of\ the\ score}$

4.2.7 Evaluating the effect of *Bacillus* and *Trichoderma* isolates on *Ralstonia solanacearum* population in the soil

The pathogen population was determined 3 times during the experiment. The first sampling was carried out before tomato plants were transplanted to determine the population of *R. solanacearum* in the experimental area. The second and third samplings were undertaken at 60 and 112 days after treatment application. Six plants were randomly sampled from each treatment to determine the population of *R. solanacearum* in the soil rhizosphere around the plant root zone. Soil scoops of about 50g were randomly picked from a depth of 10cm and were composited in polythene bags for laboratory analysis. Ten grams of the sampled soil was placed into 250ml flasks containing 90ml sterile water. After vigorous shaking for 30 min in a rotary shaker at 200rpm, the suspension was serially diluted up to 7 fold as described by Xu *et al.* (2012). One ml from each of the 6th and 7th dilutions was plated on Kelman's TZC media. After 48 hours of incubation at 28ºC, colonies typical to *R. solanacearum* were counted. Colony forming units were calculated per gram of soil. Reduction of *R. solanacearum* population by the treatment expressed as a percentage was calculated following procedure by Xu *et al.* (2012) with minor modifications. I.e. the difference between the amount of initial inoculum in the soil before treatment and the population of *R. solanacearum* at 60 or 112 days after treatment, divided by the initial inoculum (before treatment) expressed as a percentage.

4.2.8 Assessing the effect of *Bacillus* and *Trichoderma* isolates on total microbial population and diversity in the soil

Soil microbial population and communities were evaluated 126 days after treatments application by serial dilution technique following a modified protocol of Larkin, (2008). Fifty grams soil samples collected randomly from tomato plant roots of each treatment were divided into two 10g sub samples. Each 10g was added to 90ml sterile distilled water in 250ml conical flasks and was shaken for 5 minutes. The soil solution was serial diluted up to 10^{-5} folds. General population of bacteria and fungi that can be cultured were determined by plating 1ml of the 3rd, 4th and 5th dilutions on nutrient agar and PDA. Nutrient agar plates were incubated at 28°C for 48 hours to allow bacteria growth and PDA plates were incubated at 23°C for 7 days to allow fungi growth. Counting of the viable colonies was done after incubation.

For microbial diversity, different types of bacterial and fungal colonies were determined. The determination was based on the morphological characteristics of the colonies observed on the obverse side of the plate that included colony color, size and appearance.

4.2.8.1 Survival of *Bacillus* and *Trichoderma* isolates in the soil

Following the serial dilution technique and plating 1m of the 3rd, 4th and 5th dilutions on nutrient agar and PDA as described in 4.2.8 above, some *Bacillus* and *Trichoderma* isolates were retrieved after 126 days of treatment application in the soil. *Bacillus* isolates retrieved were identified on nutrient agar plates using the morphological characteristics of the colonies. These characteristics included colonies that are circular, rough, opaque and fuzzy white with jagged edges as described by Ming, (2008). Some *Trichoderma* isolates were retrieved on fungal plates by their distinctive greenish yellow to white colony characteristics and were counted separately.

4.2.9 Evaluating the effect of *Bacillus* and *Trichoderma* isolates on tomato yields and fruit size

Tomato yields were assessed weekly from the first harvest of mature fruits at 85 days after transplanting by measuring the weight of the total fruit from each plot using a weighing balance. The yields per plot were converted to tons per hectare (Diogo and Wydra, 2007). The quality of tomato fruits was determined by measuring the size in diameter of each fruit per plot using Vernier calliper.

4.3 Data analysis

Data on *Ralstonia solanacearum* population was transformed using log10. All the data collected on field experiments was subjected to analysis of variance (ANOVA) using Genstat 15th edition. ANOVA test was conducted in a general treatment structure in randomized block design to evaluate the significant differences due to treatments (BCAs), between the test location (Kabete and Mwea site) and among the days after treatment application. Means were separated using Fischer's protected LSD at 5% significance level.

4.4 Results

4.4.1 Determination of the population density of *Trichoderma* isolates in sorghum carrier

There was growth and survival of all the *Trichoderma* isolates in the sorghum carrier. The color of the parboiled sorghum carrier changed, assuming the characteristic greenish yellow and whitish color of the *Trichoderma* species by 14 days of incubation. Growth as determined by spore count per gram of sorghum was found to increase with time after inoculation for the first 10 days. Sorghum grains proved to be good medium for the growth and survival of *Trichoderma* isolates. Isolate T4 had the highest conidia count of 5.5×10^9 conidia/g followed by T1 at 2.6×10^9 conidia/g and isolate T2 had the least count of 9.8×10^8 conidia/g after 18 days of incubation (Table 4.1).

Table 4.1: Mean number of *Trichoderma* conidia per gram of carrier at 7, 11, 14 and 18 days of incubation

Incubation period (days)				
Treatment	**7 (**$\times10^6$)	**11 (**$\times10^8$)	**14 (**$\times10^9$)	**18 (**$\times10^9$)
Trichoderma isolate T1	8.10b	9.70a	2.70ab	2.60a
Trichoderma isolate T2	60.00a	3.60ab	1.10b	0.98b
Trichoderma isolate T4	0.01c	0.69b	5.50a	5.50a
Mean	22.70	4.70	3.10	3.03
CV (%)	13.9	67.6	60.2	62.1
LSD (P≤0.05)	6.30	6.30	3.73	3.75

Means that have the same letter(s) along a column are not significantly different at P≤0.05. The LSD of the interaction between treatments and days was 2.25 with Fpr. < .001. CV: Coefficients of Variation. LSD: Least significance differences of means.

4.4.2 Determination of bacterial wilt incidence

At 61 days after planting (DAP) when data collection started to 126 days, all the treatments (T1, T2, T4, CB64, CA7, chemical standard, and the mixture of T1, T2 and T4) showed consistent significant difference at $P \leq 0.05$ in the disease incidence compared to the control that recorded 88.33% incidence at Kabete site (Table 4.3). However, there were variations among the treatments on the disease incidences. At 61 days, there were no significant differences among the treatments at Kabete site. By the end of the experiment at 126 days, T1 and CB64 were the best treatments with the lowest percentage disease incidence of 26.67% and 30.00%, respectively. The second-best treatments T2, T4 and chemical standard recorded significantly higher disease incidence of 50%, 50% and 51.67%, respectively which was more than 20% higher compared to T1 and CB64. Treatment CA7 that recorded a disease incidence of 76.67% was significantly lower than the first and second-best treatments. The mixture of T1, T2 and T4 performed poorest among the treatments with a disease incidence of 83.33%. The same trend of performance was observed at Mwea site. All the treatments had significant differences in disease incidence from 61 DAP up to the end of the experiment at 126 DAP compared to control at $P \leq 0.05$. Treatment T1 followed by CB64 were the best performers with the lowest disease incidence of 26.67% and 40.00%, respectively. The next three treatments T2, T4 and chemical standard recorded a disease incidence of 50%, 51.57% and 51.67%, respectively which was comparable in performance to the results recorded at Kabete site. Treatments T2, T4 and chemical standard were significantly lower in performance compared to T1 and CB64 by ≥20% as observed at Kabete site. Treatment CA7 recorded 73.33% disease incidence and this was close in performance to 76.67% recorded at Kabete site. The mixture of T1, T2 and T4 recorded disease incidence of 88.33% which was close to 83.33% disease incidence recorded at Kabete. From the results, it was noted that the best two treatments T1 and CB64 outperformed the chemical standard at both sites. Treatments T1, T2 and T4 when applied separately, recorded significantly lower disease incidence compared to the mixture of the same at both sites. Treatment CA7, a *Bacillus* isolate, though recorded significantly lower disease incidence than control, was among the poorly performing treatment and recorded significantly higher disease incidence compared to CB64 at both sites. Control was the poorest performer followed by the mixture of T1, T2 and T4 at both sites. Control and the mixture of T1, T2 and T4 had higher disease incidence at Mwea than Kabete site by 5%. The mean difference between the two sites showed that Mwea site had higher disease incidence in all the treatments compared to Kabete site. The disease progressed significantly faster at Mwea than Kabete site with average mean

difference of 21.88% and 11.2% respectively at 61 DAP. During the experiment, the disease incidence at Mwea remained consistently higher than at Kabete (Figure 4.1 and 4.2). Mwea site was located in AEZ II which had higher temperatures compared to Kabete site located at AEZ III (Table 4.2). Higher maximum temperatures that ranged from 26 to 31.3^{o}C were recorded at Mwea site and those of Kabete site ranged from 20.41 to 26^{o}C.

Table 4.2: Temperature readings during the experiments in ^{O}C for year 2019

	Kabete site		Mwea site	
Month	**Maximum**	**Minimum**	**Maximum**	**Minimum**
April	22.41	16.50	31.30	18.00
May	26.19	15.74	29.00	17.70
June	20.41	14.00	26.20	17.30
July	21.90	12.70	27.40	16.90
August	22.16	12.60	28.00	16.90

The values in the table are average of the daily temperature data in each month

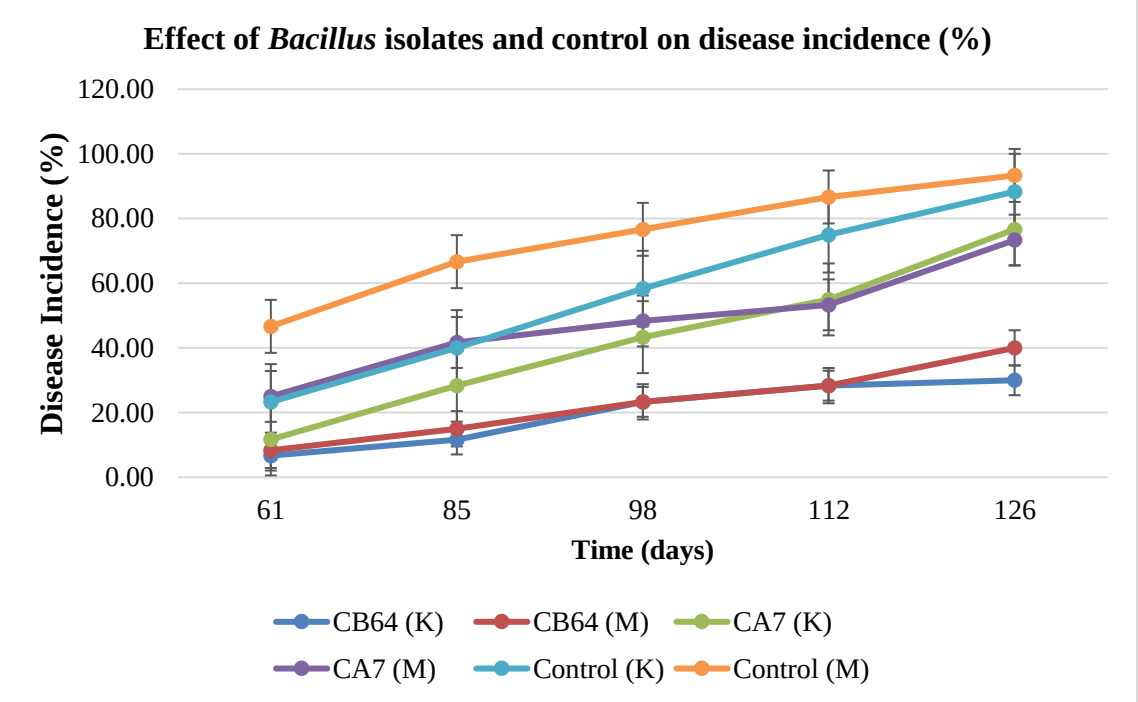

Figure 4.1: Disease progress curve for percentage disease incidence due to *Bacillus* isolates and control at Kabete site (K) and Mwea site (M)

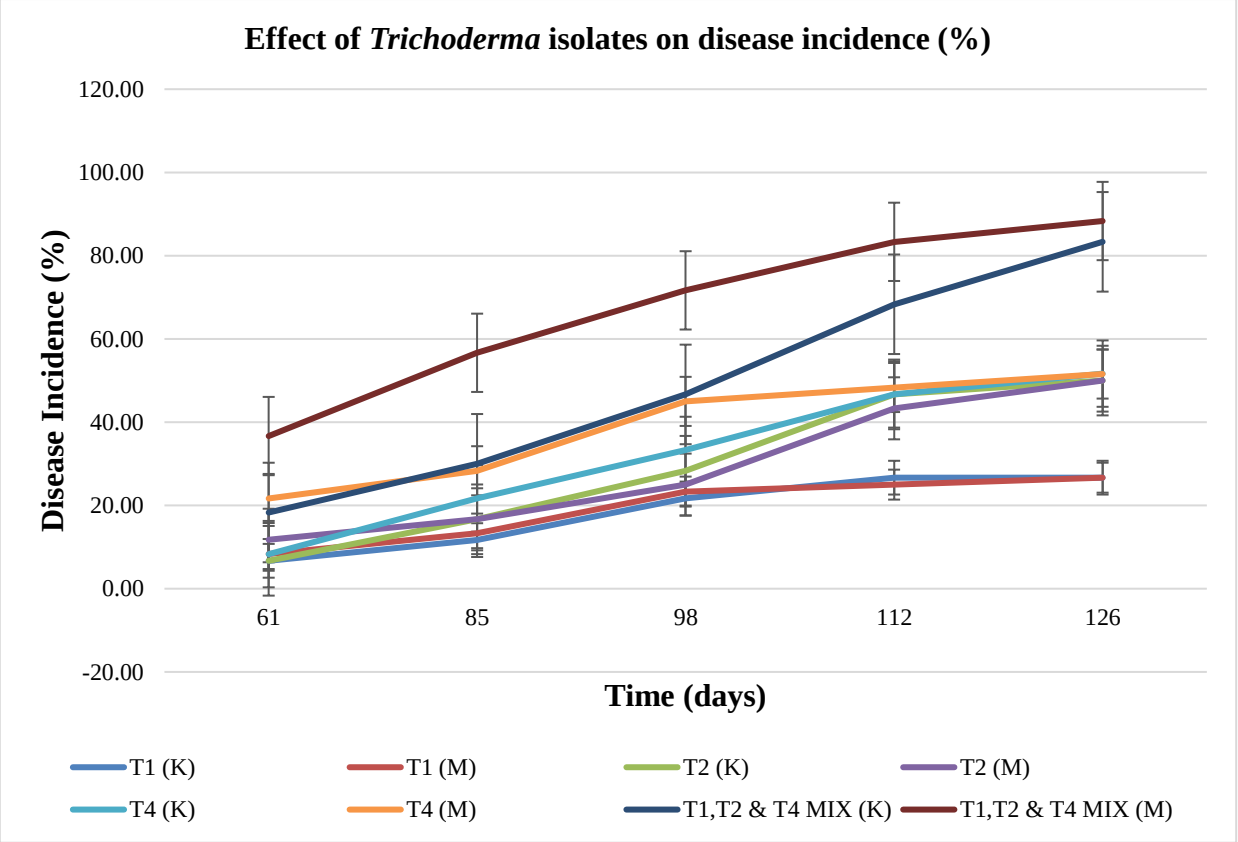

Figure 4. 2: Disease progress curve for percentage disease incidence due to *Trichoderma* isolates at Kabete site (K) and Mwea site (M)

Table 4. 3: Disease incidence assessed as a percentage (%) of wilted plants within each treatment, days after transplanting

	Kabete					Mwea				
Treatment	**61 days**	**85 days**	**98 days**	**112 days**	**126 days**	**61 days**	**85 days**	**98 days**	**112 days**	**126 days**
T1	6.70a	11.67a	21.67a	26.67a	26.67a	8.33a	13.33a	23.33a	25.00a	26.67a
CB64	6.70a	11.67a	23.33a	28.33a	30.00a	8.33a	15.00a	23.33a	28.33a	40.00b
T2	6.70a	16.67abc	28.33a	46.67b	50.00b	11.76ab	16.67a	25.00a	43.33b	50.00c
STANDARD	8.30a	15.00ab	30.00a	46.67b	50.00b	16.67bc	30.00b	43.33b	46.67b	51.67c
T4	8.30a	21.67abc	33.33ab	46.67b	51.67b	21.67cd	28.33b	45.00bc	48.33bc	51.57c
CA7	11.70a	28.33bcd	43.33bc	55.00c	76.67c	25.00d	41.67c	48.33c	53.33c	73.33d
T1,T2 & T4 MIX	18.30ab	30.00cd	46.67cd	68.33d	83.33d	36.67e	56.67d	71.67d	83.33d	88.33e
CONTROL	23.30b	40.00d	58.33d	75.00e	88.33e	46.67f	66.67e	76.67e	86.67de	93.33f
Mean	11.2	21.9	35.6	49.17	57.08	21.88	33.54	44.58	51.88	59.38
CV (%)	60.1	35.1	19	6.3	4.1	21.7	8.4	5.5	6.3	4.4
LSD (P≤0.05)	11.84	13.46	11.86	5.45	4.05	8.33	4.92	4.33	5.7	4.53
F pr	0.061	0.004	< .001	< .001	< .001	< .001	< .001	< .001	< .001	< .001

Means that have similar letter(s) along a column are not significantly different at P≤0.05. CV: Coefficients of Variation. LSD: Least significance differences of means.

4.4.3 Assessment of the disease severity based on stem browning and bacterial ooze

All the treatments had significant effects on bacterial wilt disease severity at $P \leq 0.05$ at Kabete and Mwea sites compared to control (Table 4.4). Treatment T1 had the lowest percentage severity index of 50.42% for bacterial ooze and 46.44% for stem color at Kabete site. Treatment CB64 was the second best with severity index of 50.61% and 53.89%, respectively. These observations were similar at Mwea site. Treatment T1 had the lowest severity index of 63.33% for bacterial ooze and 58.89% for stem color and CB64 followed at 71.11% and 62.22%, respectively. There was no significant difference between treatments CA7, the mixture of T1, T2 and T4, and control on the severity index of bacterial wilt disease at both sites. Control had more than 98% disease severity index which was higher than the best treatment by more than 47%. Treatments T1 and CB64 recorded significantly lower severity index of bacterial wilt compared to standard in both sites. Treatment T1 was lower by 24.02% for bacterial ooze and 28.00% for stem color at Kabete site. At Mwea site T1 was lower than standard by 24.45% and 31.49%, respectively. Treatment CB64 was lower than standard by 23.83% and 20.55% for bacterial ooze and stem color, respectively at Kabete site and 16.67% and 27.67% at Mwea site. Treatments T1, T2 and T4 each applied as a single treatment recorded lower severity index compared to the mixture of the three treatments at both sites. The mean difference of all the treatments showed that the percentage severity index was higher at Mwea site than Kabete site by 8.83% for bacterial ooze and 7.72% for stem color.

Table 4.4: Severity index (%) of bacterial wilt disease, 126 days after treatment application

	Kabete		Mwea	
Treatment	**Bacterial ooze**	**Stem color**	**Bacteria ooze**	**Stem color**
T1	50.42a	46.44a	63.33a	58.89a
CB64	50.61a	53.89a	71.11ab	62.22a
T2	71.67b	75.78b	79.78bc	80.89b
STANDARD	74.44b	74.44b	87.78cd	89.89bcd
T4	76.44b	81.56bc	87.11cd	88.11bc
CA7	87.89bc	89.78bc	89.89de	91.22cd
T1, T2, T4 MIX	93.44c	89.78bc	96.00de	97.22cd
CONTROL	98.11c	93.67c	98.44e	98.67d
Mean	75.35	75.67	84.18	83.39
CV (%)	12.4	12.2	6.2	6.5
LSD (P≤0.05)	16.4	16.18	9.09	9.47
F pr.	< .001	< .001	< .001	< .001

Means that have the same letter(s) along a column are not significantly different at P≤0.05.
CV: Coefficients of Variation. LSD: Least significance differences of means.

4.4.4 Evaluating the effect of *Bacillus* and *Trichoderma* isolates on *Ralstonia solanacearum* population in the soil

Before application of the soil treatments, there was no significant difference in the population of *R. solanacearum* among the plots in Kabete and Mwea sites at P≤ 0.05 (Table 4.5). At 60 days after treatment application, all the treatments except the mixture of T1, T2 and T4 showed significant reduction in the population of *R. solanacearum* at P≤ 0.05 compared to control at both sites (Figure 4.3). At Kabete site, *R. solanacearum* population in the control increased from 7.35×10^6 CFU/ml before treatment application to 8.61×10^6 CFU/ml at 60 days. This was 17.11% increase in the pathogen population. Similarly, at 112 days the pathogen population in control plots increased by 20.40%. After 112 days of treatment application, CB64 and T1 had the lowest *R. solanacearum* population of 0.65 and 0.77×10^6 CFU/ml, respectively. This was a reduction in pathogen population of 93.17% for CB64 and 92.07% for T1. The second-best treatment T2 and standard recorded *R. solanacearum*

population of 2.86 and 3.01×10^6 CFU/ml, respectively. These was a 67.34% and 65.61% reduction in pathogen population, respectively. However, this reduction was lower by 24% compared to the best treatment CB64 and T1. This demonstrated that the best treatment CB64 and T1 were significantly better than the standard. The third best treatment was T4 which had *R. solanacearum* population of 4.18×10^6 CFU/ml and pathogen reduction of 56.94%. The last treatment CA7 had a population of 8.20×10^6 CFU/ml and recorded pathogen reduction of 8.96%. Treatment CA7 although significantly better than control was significantly lower than other effective treatments. The treatment recorded lower *R. solanacearum* reduction percentage by 84% compared to the best treatment CB64. The mixture of T1, T2 and T4 performed poorest among the treatments and *R. solanacearum* population was 9.18×10^6 CFU/ml. The inoculum increased by 1.82% after 112 days of treatment application.

The second site Mwea, had similar trends in results as Kabete site. However, the population of *R. solanacearum* in the soil was significantly higher at Mwea than Kabete site in all the treatments (Figure 4.4). At 60 days after treatment application, the pathogen population in the control increased from 8.76×10^7 CFU/ml before treatment application to 9.40×10^7 CFU/ml. This was 7.31% increase in pathogen population. Similarly, at 112 days, *R. solanacearum* population increased by 20.89% in control plots. After 112 days of treatment application, T1 and CB64 had the lowest *R. solanacearum* population of 7.94×10^6 and 1.17×10^7 CFU/ml, respectively. This was percentage pathogen reduction of 92.75% for T1 and 88.78% for treatment CB64. The second best treatment T2 recorded *R. solanacearum* population of 3.28×10^7 CFU/ml and this was 68.47% pathogen reduction. The third best treatment T4 and standard which recorded *R. solanacearum* population of 3.98 and 4.70×10^7 CFU/ml, respectively gave a 61.37% and 56.92% pathogen reduction, respectively. Treatment CB64, T1, T2 and T4 were significantly better than the standard. The mixture of T1, T2 and T4 performed poorest among the treatments and *R. solanacearum* population was 1.08×10^8 CFU/ml. The pathogen population increased by 3.15% after 112 days of treatment application. This mixture of T1, T2 and T4 had a significantly lower percentage reduction of pathogen compared to the same treatments applied separately. The mean difference of the treatments showed that Mwea site had higher initial inoculum level at 1.05×10^8 CFU/ml compared to Kabete site at 9.04×10^6 CFU/ml.

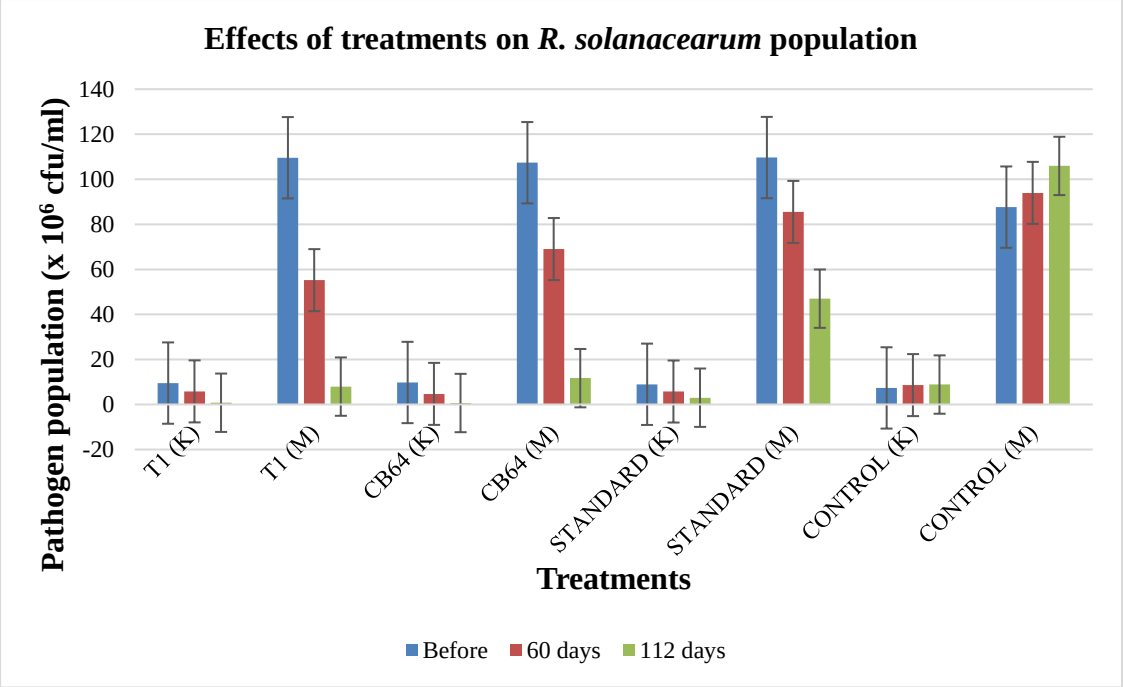

Figure 4.3: Effects of *Trichoderma* (T1), *Bacillus* (CB64) and chemical standard *on R. solanacearum* population in the soil (x 10^6 cfu/ml) after 60 and 112 days of application at Kabete (K) and Mwea (M) sites.

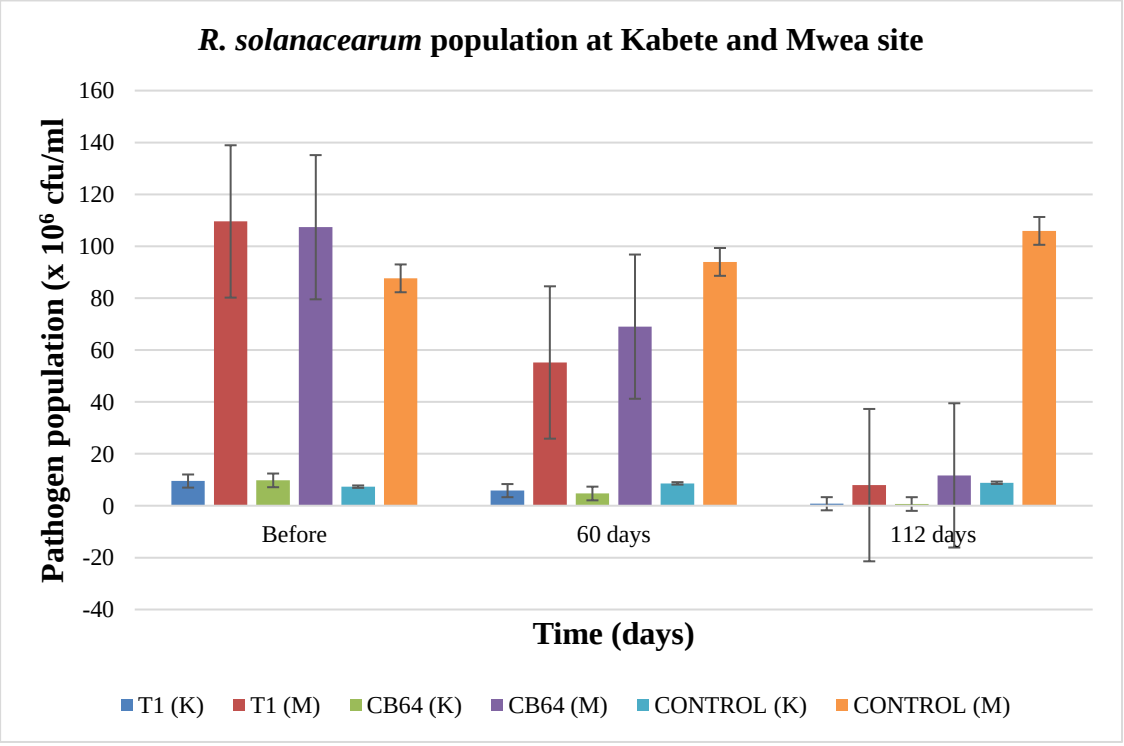

Figure 4. 4: *R. solanacearum* population in the soil (x 10^6 cfu/ml) at Kabete (K) and Mwea (M) sites after 60 and 112 days of treatment application.

Table 4. 5: Quantification of *Ralstonia solanacearum* population in the soil after application of treatments (×10^6) in CFU/ml

	Kabete					Mwea				
	***R. solanacearum* population**			**Pathogen reduction (%)**		***R. solanacearum* population**			**Pathogen reduction (%)**	
Treatment	**Before**	**60 days**	**112 days**	**60 days**	**112 days**	**Before**	**60 days**	**112 days**	**60 days**	**112 days**
T1	9.51a	5.82ab	0.77a	40.14b	92.07a	109.57b	55.21a	7.94a	49.57a	92.75a
CB64	9.77a	4.71a	0.65a	50.44a	93.17a	107.35b	69.02ab	11.69b	35.51b	88.78a
T2	8.91ab	6.42bc	2.86b	27.89c	67.34b	106.05b	78.34bc	32.81c	26.12c	68.47b
STANDARD	8.97a	5.76ab	3.01b	35.51bc	65.61b	109.65b	85.51bcd	46.99d	21.69cd	56.92c
T4	9.73a	8.32cd	4.18b	14.31d	56.94c	104.11b	78.34bc	39.81cd	24.75c	61.37bc
CA7	9.04a	8.45cd	8.20c	6.25de	8.96d	108.34b	91.20cd	75.34e	15.83d	29.82d
T1, T2, T4 MIX	9.02a	9.18d	9.18c	-1.85e	-1.82e	105.20b	106.91d	108.39f	-1.92e	-3.15e
CONTROL	7.35b	8.61cd	8.85c	-17.11f	-20.40f	87.64a	93.97cd	105.93ef	-7.31f	-20.89f
Mean	9.04	7.16	4.71	19.45	45.23	104.74	82.31	53.61	20.53	46.76
CV (%)	0.7	1.1	1.5	25.8	10.4	0.5	0.7	1.1	24.6	12.9
LSD (P≤0.05)	1.84	1.85	1.466	8.79	8.25	18.65	17.03	11.74	8.83	10.58
F pr.	0.209	0.003	< .001	< .001	< .001	0.196	0.001	< .001	< .001	< .001

Means that have similar letter(s) along a column are not significantly different at P≤0.05. CV: Coefficients of Variation. LSD: Least significance differences of means. Analysis was done on data transformed to log10.

4.4.5 Assessing the effect of *Bacillus* and *Trichoderma* isolates on total microbial population and diversity in the soil

All the treatments had significantly higher soil microbial population compared to control at both sites at P≤ 0.05 (Table 4.6). Microbial population recorded in control plot was 2.63×10^4 CFU/ml at Kabete site and 2.13×10^4 CFU/ml at Mwea site. Treatment CB64 had the highest total microbial count of 1.32×10^5 CFU/ml at Kabete site and 1.21×10^5 CFU/ml at Mwea site. Treatment CA7 was the second best with total microbial count of 1.09×10^5 CFU/ml at Kabete site and 1.03×10^5 CFU/ml at Mwea site. Treatments T4, T2 and T1 were third best in performance and had no significant difference at P≤ 0.05 in both sites. The fourth treatment was the mixture of T1, T2 and T4 which had microbial population of 4.60×10^4 CFU/ml at Kabete site and 3.80×10^4 CFU/ml at Mwea site. The fourth treatment in both site was significantly higher in microbial population than the standard which had 3.83×10^4 CFU/ml and 3.23×10^4 CFU/ml at Kabete and Mwea sites, respectively. The standard had significantly lower microbial count compared to the best treatment CB64 which had 1.32×10^5 CFU/ml at Kabete site and 1.21×10^5 CFU/ml at Mwea site. The total microbial count consisted of various types of bacteria and fungi counts. In all the treatments, the bacterial counts were much higher than the fungal counts in both sites. At Kabete site, the bacterial population ranged from 3.53×10^4 CFU/ml to 1.24×10^5 CFU/ml and at Mwea site, the population ranged from 2.53×10^4 CFU/ml to 1.00×10^5 CFU/ml. The fungal population ranged from 0.17×10^4 CFU/ml to 1.23×10^4 CFU/ml at Kabete and from 0.20×10^4 CFU/ml to 2.27×10^4 CFU/ml at Mwea site. The first two treatments that had the highest microbial count were *Bacillus* isolates CB64 and CA7 at both sites. These isolates were multiplied in cow manure carrier. The fungal treatments T1, T2 and T4 were multiplied in sorghum carrier. From the observations, the proliferation of soil microbes was higher where manure was used as a carrier compared to where sorghum was used as a carrier. Kabete site had higher total microbial count as shown by the mean difference of 7.55×10^4 CFU/ml compared to Mwea site that 6.71×10^4CFU/ml.

Table 4. 6: Total microbial counts ($\times 10^4$) in CFU/ml after 126 days of soil treatment

	Kabete			Mwea		
Treatments	**Microbial**	**Bacteria**	**Fungi**	**Microbial**	**Bacteria**	**Fungi**
T1	8.73c	7.80c	0.93ab	7.43c	5.17d	2.27a
CB64	13.17a	12.43a	0.73bc	12.13a	10.03a	2.10a
T2	7.70c	7.27c	0.43cd	6.93c	5.97cd	0.97bc
STANDARD	3.83de	3.53de	0.30d	3.23de	2.53ef	0.70c
T4	8.83c	7.60c	1.23a	7.70c	6.70c	1.00bc
CA7	10.87b	9.77b	1.10a	10.33b	8.63b	1.70ab
T1, T2, T4 MIX	4.60d	4.43d	0.17d	3.80d	3.60e	0.20c
CONTROL	2.63e	2.40e	0.23d	2.13e	1.67f	0.47c
Mean	7.55	6.9	0.64	6.71	5.54	1.18
CV (%)	10.9	12.7	32.2	10.8	11	41
LSD ($P \leq 0.05$)	1.44	1.54	0.36	1.27	1.07	0.84
F pr.	< .001	< .001	< .001	< .001	< .001	< .001

Means that have the same letter(s) along a column are not significantly different at $P \leq 0.05$. CV: Coefficients of Variation. LSD: Least significance differences of means.

Further observation on the microbial composition in the treatments showed different types of bacteria and fungi based on the morphological characteristics. Morphological characteristics included colony color, size and appearances. Categorization based on the color of bacterial colonies ranged from pink, yellow, white and cream white. For fungi, the rate of mycelia growth, mycelia color and appearance in terms of mycelia growth pattern were characteristics used for general grouping of fungal colonies. Based on these characteristics, the bacteria and fungi colonies were placed in different groups as shown by (Table 4.7). At Kabete site, CB64 had the highest diversity of bacterial colonies at 5 groups. Treatments CA7, T4, T2 and T1 performed second best in bacterial colony diversity. These treatments had similar number of groups (four groups) of bacteria colonies. The mixture of T1, T2 and T4, standard and control had the lowest bacteria colony diversity with no significant difference. There was no significant difference at $P \leq 0.05$ in the diversity of fungal colonies in treatments CB64, T4, T1 and CA7. These treatments had more than three groups of fungal colonies and they were the highest compared to other treatments. *Trichoderma* isolate T2 was the second best treatment and had two groups of fungal colonies followed by standard. The mixture of T1, T2 and T4 and control had the least number of fungal colony group.

At Mwea site, treatment CB64 had the highest bacterial diversity of 5 colony groups followed by T4 at 4 groups. Treatments T1 and CA7 were third best and had similar population diversity of bacteria colonies of 3 groups. The standard treatment, control and the mixture of T1, T2 and T4 had the least bacteria colony diversity. Treatments T4 and CB64 had the highest fungal colony diversity of 4 groups followed by T1 with 3 groups. Kabete site had slightly higher bacterial diversity and fungal diversity compared to Mwea site as indicated by the mean differences.

Table 4. 7: Bacteria and fungi diversity after 126 days of soil treatment based on colony groups (with similar characteristics)

	Kabete		Mwea	
Treatments	**Bacteria**	**Fungi**	**Bacteria**	**Fungi**
T1	3.67b	4.67a	4.33b	4.00a
CB64	6.67a	3.67b	5.67a	3.00ab
T2	2.33c	3.33b	2.33cd	2.33cd
STANDARD	1.67c	1.33c	2.00de	1.00e
T4	4.00b	4.67a	3.67b	4.33a
CA7	5.67a	3.33b	5.33a	2.67bc
T1, T2, T4 MIX	1.67c	1.67c	1.67e	1.33de
CONTROL	1.34c	0.67c	1.33e	1.00e
Mean	3.38	2.92	3.29	2.46
CV (%)	22.3	32.5	18.5	21
LSD ($P \leq 0.05$)	1.22	1.50	0.94	1.00
F pr.	< .001	< .001	< .001	< .001

Means that have similar letter(s) along a column are not significantly different at $P \leq 0.05$. CV: Coefficients of Variation. LSD: Least significance differences of means.

4.4.6 Survival of *Bacillus* and *Trichoderma* isolates in the soil

Bacillus isolates CB64 and CA7 were easily re-isolated from the soil at the end of the experiments than *Trichoderma* isolates except T4 at both Kabete and Mwea site (Table 4.8). Isolate CB64 had more bacterial colonies retrieved at 3.30 and 4.7×10^4 CFU/ml for Kabete and Mwea site, respectively. This was followed by treatment CA7 at 5.0 and 2.7×10^3 CFU/ml for Kabete site and Mwea site, respectively. *Trichoderma* isolate T4 was re-isolated at both sites. The concentration of T*richoderma* isolate T4 retrieved was 2.7×10^3 CFU/ml and 2.0×10^3 CFU/ml for Kabete and Mwea site, respectively. *Trichoderma* isolate T2 had 1.3×10^3 CFU/ml and was only retrieved at Kabete site. Kabete site had more antagonists retrieved than Mwea site. Only the isolates with morphological and visual characteristics similar to the isolates applied during treatment application were retrieved.

Table 4.8: *Bacillus* and *Trichoderma* isolates retrieved (CFU/ml) after 126 days of treatment application

		Kabete	Mwea
Treatment	**Concentration applied (×10^9)**	**Antagonist re-isolated (×10^4)**	**Antagonist re-isolated (×10^4)**
CB64	5900000a	3.30a	4.70a
CA7	6700000a	0.50b	0.27b
T1	2.60c	0c	0b
T2	0.98d	0.13bc	0b
T4	5.50b	0.27bc	0.20b
CONTROL	0e	0c	0b
Mean	2.10×10^6	0.70	0.86
CV (%)	32	38.7	53.3
LSD (P≤0.05)	1.20×10^6	0.48	0.82

Values are means of three replicates. Means that have the same letter(s) along a column are not significantly different at P≤0.05. CV: Coefficients of Variation. LSD: Least significance differences of means.

4.4.7 Evaluating the effect of *Bacillus* and *Trichoderma* isolates on tomato yields and fruit size

All the treatments had significantly higher total tomato yields at P≤ 0.05 compared to control at both sites (Table 4.9). At Kabete site, treatment CB64 had the highest yield of 39.51 tons/ha followed by T1 , T2 and chemical standard which recorded a total yield of 37.13, 31.99 and 30.37 tons/ha, respectively. This treatments recorded significantly higher yields by more than 155.64% compared to control. The mixture of T1, T2 and T4 recorded significantly lower yields at 17.49 tons/ha compared to the same treatments applies individually that recorded 37.13, 31.99 and 29.20 tons/ha for T1, T2 and T4, respectively. Treatment CA7 recorded significantly higher yield than control at 24.21 ton/ha but this was lower than the best treatment CB64 and T1. Similar trend of results was observed at Mwea site with T1 and CB64 recording the highest yields at 20.72 and 20.58 tons/ha, respectively. Treatment T2, chemical standard and T4 were second best with 16.84, 16.73 and 14.50 tons/ha, respectively. Similar to the results observed at Kabete site, the mixture of T1, T2 and T4 recorded significantly lower yields at 8.29 tons/ha compared to the same treatments applies individually at Mwea. All the treatments recorded significantly higher yield by 79.44% compared to control P≤ 0.05. The mean difference showed that the yield at Kabete were significantly higher than those recorded at Mwea site in all the treatments.

The size of tomato fruits (mm) was significantly different at P≤ 0.05 in all the treatments compared to control at both sites (Table 4.9). Control had the lowest measurements of fruit size of 36.61mm at Kabete and 36.90mm at Mwea site. Treatment T1, CB64, T2 and chemical standard had no significant difference in fruit size and recorded 49.56, 49.76, 47.02 and 48.30mm, respectively at Kabete. Similarly, treatments T1, CB64 and T2 had no significant difference at Mwea site with fruit size of 48.45, 48.42 and 46.13mm, respectively. The mixture of T1, T2 and T4 recorded a fruit size of 39.93 at Kabete and 41.00mm at Mwea which was significantly lower than the fruit size of the same isolates applied individually. Treatment CA7 recorded the least fruit size of 43.79 and 44.15mm at Kabete and Mwea site, respectively after the mixture of T1, T2 and T4. The difference in means showed that there was no significant difference in the fruit size measurements between Kabete and Mwea sites at P≤ 0.05.

Table 4. 9: Total yield in each treatment converted to tons per hectare and fruit size (mm) from the first to seventh week of harvest, at Kabete and Mwea site

	Kabete		Mwea	
Treatment	**Total Yields (tons^{-ha})**	**Fruit size (mm)**	**Total Yields (tons^{-ha})**	**Fruit size (mm)**
T1	37.13ab	49.56a	20.72a	48.45a
CB64	39.51a	49.76a	20.58a	48.42a
T2	31.99abc	47.02a	16.84b	46.13ab
STANDARD	30.37bc	48.30a	16.73b	44.86b
T4	29.20bc	42.51bc	14.50bc	45.04b
CA7	24.21cd	43.79b	12.60c	44.15b
T1, T2, T4 MIX	17.49de	39.93c	8.29d	41.00c
CONTROL	11.88e	36.61d	4.62e	36.90d
Mean	27.72	44.68	14.36	44.37
CV (%)	6	3.9	11.2	3
LSD (P≤0.05)	8.257	3.07	2.816	2.37
F pr.	< .001	< .001	0.001	< .001

Means that have the same letter(s) along a column are not significantly different at P≤0.05. CV: Coefficients of Variation. LSD: Least significance differences of means.

4.5 Discussion

Determination of population density of *Trichoderma* isolates in sorghum carrier

Trichoderma isolates were able to grow, survive and multiply in sorghum grain carrier. Sporulation of each *Trichoderma* isolate in sorghum carrier started between 5 to 21 days after incubation. Colonization of the parboiled sorghum was characterized by the sorghum grains assuming whitish, yellow and greenish color of the *Trichoderma* spp. Strains of *Trichoderma* are known to colonize substrates containing lignin and cellulose such as sorghum, rice, maize grains, and farmyard manure among others. They produce enzymes that degrade polysaccharides in sorghum and other substrates (Harman *et al.*, 2004: Kumar, 2017).

Trichoderma isolate that gave the best growth as demonstrated by sporulation was isolate T4 followed by isolate T1 and then isolate T2. Sporulation of all the *Trichoderma* isolates increased from 10^6 to 10^9 conidia per gram of sorghum carrier between 7 and 14 days of incubation (Table 4.1). These findings are in agreement with those reported by Singh *et al.* (2014) that at 7 days after inoculation, sorghum grain substrate had the highest population of

Trichoderma harzianum of 2.25×10^8 CFU/g. The results herein are also comparable to those reported by Rajput *et al.* (2014) that the highest population of 10.03×10^9 CFU/g of *Trichoderma harzianum* was recorded in sorghum grain carrier compared to other substrates.

The C: N ratio of the white sorghum was 13.4. This ratio showed that there was high nitrogen content in the sorghum carrier. The availability of N in sorghum carrier increased sporulation and hypha growth essential for substrate colonization which was in agreement with reports by Okoth *et al.* (2009). Similar findings by Srivastava *et al.* (2010) also reported that out of 6 substrates used for mass multiplication of *Trichoderma harzianum*, sorghum grains, with high N content was the best carrier and gave significantly higher numbers of *Trichoderma* conidia compared to other substrates. The results in the current study shows that sorghum grain is a good substrate for multiplication and for use as carrier for *Trichoderma* isolates in large application of the isolates in the field. The results herein confirm those reported by Pandey, (2009) and Kumar, (2017). However, from 14 days to 18 days of incubation, the concentration of *Trichoderma* isolates T4 and T1 remained constant at 10^9 conidia/g of sorghum while for isolate T2, the conidia population reduced slightly to 10^8. These results are comparable to the findings reported by Muriungi *et al.* (2013) who found that there was an increase in *Trichoderma* isolates conidia per gram of sorghum carrier but the increase was not indefinite. These result indicates that two weeks are enough to give maximum spore population density of the *Trichoderma* isolates in sorghum carrier. This is because with time when the available nutrients in sorghum substrate are exhausted, the population of *Trichoderma* remains constant or falls rapidly similar to findings reported by Singh *et al.* (2014). Also moisture content in the substrate reduce with time as most of the parboiled sorghum gets colonized. Moisture content in substrate greatly affects the population of *Trichoderma* spp. High moisture contents may be helpful to increase the population of *Trichoderma* in any substrate (Rahman *et al.,* 2011).

Determination of bacterial wilt incidence and severity in Kabete and Mwea field

All the treatments in the present study showed significantly lower disease incidence and severity compared to control at $P\leq 0.05$ as shown in tables 4.3 and 4.4. A minimum disease incidence of 26.67% was observed after 126 days of incubation in plots treated with *Trichoderma* isolate T1 at both Kabete and Mwea sites. This performance was significantly better than control plots which gave 88.33% and 93% disease incidence at Kabete and Mwea site, respectively. These results are in agreement with Kumar, (2017) findings who reported a tomato bacterial wilt disease incidence range of 30-40% when plots were treated with *Trichoderma harzianum*. The best treatment, *Trichoderma* isolate T1 which had more than

61.66% reduction of bacterial wilt disease incidence also gave the lowest disease severity of 63.33% compared to control that had more than 98% severity at both sites. The percentage severity index observed in the best treatment T1 was lower than control plots by more than 47%. These findings are in line with those reported by Narasimhamurthy *et al.* (2018) that *Trichoderma asperellum* treated plots showed reduction of bacterial wilt disease by 51.06%. Other reports by Kumar and Ganesan, (2006): Rosyidah *et al.* (2013): Tinatin and Saykal, (2016): Yendyo *et al.* (2017) also found that the use of *Trichoderma* strains reduced bacterial wilt disease incidence compared to the control treatment. The reduction of bacterial wilt incidence and severity may be due to *Trichoderma* strains producing antagonistic compounds against *R. solanacearum* as observed in the *in vitro* results of the current study. These findings are in line with Tapwal *et al.* (2011) who reported that different strains of *Trichoderma* produce various metabolites and secondary compounds that have antagonistic activity against *R. solanacearum*.

Bacillus isolate CB64 was the second best treatment that showed bacterial wilt disease incidence of 30% at Kabete and 40% at Mwea after 126 days of transplanting. These findings are comparable to those reported by Singh *et al.* (2012) that a minimum disease incidence of 12.67% at 50 days after incubation was observed in *Bacillus* BS-5 treated soil. In the present study, the disease incidence at 60 days of incubation was 6.70% and 11.70% for plots treated with *Bacillus* isolate CB64 and CA7 respectively.

Treatment CB64 had an average of 50% reduction in bacterial wilt disease incidence compared to control. This was similar to earlier reports by Lemessa and Zeller, (2007) that *Bacillus subtilis* strain B_2G reduced bacterial wilt incidence by 60%. Similarly, Akintokun *et al.* (2019) reported 67% disease reduction in plants inoculated with *Bacillus thuringiensis*. Ramesh *et al.* (2009) also found *Bacillus* strains EC4 and EB66 reduced bacterial wilt incidence by 70% when compared to control. *Bacillus* strains are able to produce antibiotics such as 2, 4-diacetylphloroglucinol (DAPG) among other compounds that are able to induce resistance in tomato plants. This may explain the results observed herein. The *in vitro* experiment in the current study showed that *Bacillus* isolates induced ZOI against *R. solanacearum*. The ZOI were due to the ability of *Bacillus* isolates to produce metabolites such as antibiotics that had antagonistic activity against the pathogen and isolate CB64 had the highest ZOI. Comparable findings have been reported by Ji *et al.* (2008): (Wei *et al.*, 2011): Huang *et al.* (2014) of the ability of *Bacillus* strains to produce various metabolites and compounds that are able to reduce bacterial wilt incidence and severity in tomato plants. These are among the important

mechanisms used by BCAs to reduce bacterial wilt disease in the field (Ramesh *et al.,* 2009). Plots treated with CB64 had the lowest disease severity at an average of 59.46% compared to control. Antagonism of *Bacillus* strains towards *R. solanacearum* and reduction of bacterial wilt incidence and severity in tomato has been demonstrated by many researchers (Guo *et al.,* 2004: Aliye *et al.,* 2008: Nguyen and Ranamukhaarachchi, 2010: Wei *et al.,* 2011: Singh *et al.,* 2012: Chen *et al.,* 2013: Maji and Chakrabartty, 2014).

Plots treated with *Bacillus* isolate CA7 performed poorly in reducing the disease incidence and severity at both sites. The isolate reduced the disease incidence by 11.66% and 20% at Kabete and Mwea, respectively which was much lower than *Bacillus* isolate CB64. The disease severity was reduced by an average of 23.56% compared to control in both sites. This performance was also much lower than 38.54% reduction observed in isolate CB64. Treatment CB64 performed way better than CA7 in reducing disease incidence and severity though they are both *Bacillus* isolates. This is because strains of *Bacillus* produce various secondary metabolites that vary in their effectiveness. This difference in the behavior of *Bacillus* strains in relation to antagonistic effects against the pathogen was also noted by other researchers who reported that *Bacillus* strains produce different kinds of lipopeptides and secondary metabolites that vary in their effectiveness (Ongena *et al.,* 2007).

The concentration of *Bacillus* isolate CB64 applied in the soil for managing bacterial wilt disease of tomato in the present study was 5.9×10^{15}CFU/ml. This concentration exhibited a 30% and 40% disease incidence at Kabete and Mwea site, respectively. Seleim *et al.* (2011) found that when a concentration of 10^8 CFU/ml of *Bacillus subtilis* was applied in the soil, it exhibited a 45% bacterial wilt disease incidence. Therefore, it was noted that the concentration of *Bacillus* isolates to be applied in the soil may be a key factor to consider when planning to reduce bacterial wilt incidence in the field.

Plots treated with *Trichoderma* isolate T1 and *Bacillus* isolate CB64 performed better than the ones treated with the standard chemical by more than 20% in reducing bacterial wilt disease incidence and severity. These findings were similar to those reported by Yendyo *et al.* (2017) that the best treatment outperformed the chemical control by more than 3% in reducing the disease. The standard treatment was a chemical composed of Di-Bromo Di nitro propane 1, 3-diol. Multitude of factors in the soil may have contributed to the performance of the chemical standard applied as a soil drench as opposed to *Trichoderma* and *Bacillus* isolates. These findings are comparable to those reported by Yendyo *et al.* (2017). There are different microorganisms in the soil that are responsible for breaking down the active ingredient in the

chemical standard which may have led to its degradation. Furthermore, chemical standard may have directly interacted with soil through adsorption and covalent bond formation which affected its availability to crops thus limiting the effectiveness (Tseng, 2019).

Plots treated with *Trichoderma* isolates T1, T2 and T4 had significantly lower disease incidence and severity compared to the plots treated with the mixture of the same treatments. Plots treated with the mixture of *Trichoderma* isolates had bacterial wilt disease incidence of 83.33% at Kabete and 88.33% at Mwea which was only 5% better than the control. Plots treated with *Trichoderma* mixture also recorded disease severity of more than 89.78%. These results are in agreement with those reported by Akrami *et al.* (2011) that the mixture of *Trichoderma asperellum, virens* and *harzianum* performed significantly lower in reducing *Fusarium oxysporum* disease severity compared to the same isolates applied individually. A study by Robinson-Boyer *et al.* (2009) reported comparable findings that the mixtures of two or three *Trichoderma* isolates resulted in lower efficacy against *Botrytis cinerea* infections on strawberry flowers compared to when the isolates were applied alone. Freeman *et al.* (2004) also reported that combination of *Trichoderma* isolates T-39 with T-166 did not significantly reduce the grey mold disease incidence on strawberry compared to the same isolates applied singly. According to Xu *et al.* (2010), application of *Trichoderma* isolates as a mixture gives poor results in reducing disease incidence and severity of *Botrytis cinerea* compared to the same isolates applied individually. This is because each *Trichoderma* isolate produces antimicrobial compounds which might inhibit any BCA that they are applied in combination with. Hence, may cause interference among the BCAs leading to the observed results. These finding supports the results observed in the current study. However, further studies needs to be carried out to test possible synergistic and antagonistic effects of the mixture of *Trichoderma* isolates.

The results observed in the present studies under field conditions were in harmony with those recorded during the *in vitro* studies. *Trichoderma* isolate T1 emerged as the best treatment among *Trichoderma* isolates by inducing the largest ZOI of 13.5mm against *R. solanacearum in vitro* and also gave the highest percentage in reducing the disease incidence and severity (more than 61.66% and 34.67%, respectively) in the field. Among *Bacillus* isolates, CB64 was the best in both *in vitro* and *in vivo* studies. The isolate induced the highest ZOI of 4.3mm *in vitro* and gave more than 50% and 38.54% reduction in disease incidence and severity, respectively. *Trichoderma* isolate T1 provided better results in controlling bacterial wilt disease incidence in tomato plants compared to *Bacillus* isolate CB64. These results are comparable to

those reported by Yendyo *et al.* (2017) that a 92% disease prevention was recorded in *Trichoderma* treated plots and 84% in *Bacillus* treated plots. *Trichoderma* isolates performed better than *Bacillus* isolates in both studies. However, retrieval of the isolates at the end of the experiment showed significantly higher population of *Bacillus* isolate CB64 compared to the *Trichoderma* isolates. This demonstrated that *Bacillus* isolate CB64 had a higher survival rate in the soil. It is known that *Bacillus* species produce endospores that resist harsh conditions in the soil hence gives better antagonism and survival. These studies indicate that rhizospheric bacteria and fungi have antagonistic ability to suppress the bacterial pathogen both in the field and *in vitro*.

Bacterial wilt disease incidence and severity progressed significantly faster at Mwea than Kabete site during the experiments. The mean difference between the two sites showed that Mwea site had higher disease incidence by more than 7.26% compared to Kabete site. The disease severity was also higher at Mwea site by more than 8.3% compared to Kabete site. Mwea site is located in AEZ II which had higher temperatures compared to Kabete site located at AEZ III (Table 4.2). Higher maximum temperatures that ranged from 26 to 31.3^{o}C were recorded at Mwea site and those of Kabete site ranged from 20.41 to 26^{o}C. High temperatures and moisture favor the growth of *R. solanacearum*. This could have been the reason for the significant difference in the results observed in the two sites. These findings are in agreement to those reported by Alvarez *et al.* (2010): Yendyo *et al.* (2017).

Evaluating the effect of *Bacillus* and *Trichoderma* isolates on *R. solanacearum* population in the soil

At the onset of the experiments, the amount of *R. solanacearum* inoculum in the soil was an average of 9.04×10^{6} CFU/ml at Kabete and 1.05×10^{8} CFU/ml at Mwea sites. This level of inoculum was able to cause 23.30% and 46.67% disease incidence on control plots at Kabete and Mwea sites, respectively 60 days after transplanting. These findings confirm those reported by Pradhanang *et al.* (2003) who found that *R. solanacearum* initial inoculum containing 4.5×10^{6} CFU/ml was able to cause 100% bacterial wilt incidence in control plots at the end of the experiment. However, at 60days after treatment application, all plots treated with *Bacillus*, *Trichoderma* and standard chemical showed significant reduction in *R. solanacearum* population in the soil at $P\leq 0.05$ compared to control at Kabete and Mwea site. Plots treated with the mixture of *Trichoderma* isolates T1, T2 and T4 and control showed significant increase in the pathogen population. Control plots showed an increase in *R. solanacearum* population of 17.11% at Kabete and 7.31% at Mwea site. This increase of the pathogen

population in the soil was higher at Kabete site than Mwea site though the disease incidence was higher at Mwea site as observed earlier in this study. This is because it is known that bacteria disease expression is higher in warmer temperatures even when the inoculum level in the soil is low as compared to cooler temperatures. These findings are in agreement to those reported by Wei *et al.* (2011) that higher temperatures influenced *R. solanacearum* development and this led to high disease incidence even in areas with low inoculum levels.

Just like in bacterial wilt incidence and severity reduction, plots treated with *Trichoderma* isolate T1 and *Bacillus* isolate CB64 had the highest percentage reduction of the pathogen population in the soil. *Bacillus* isolate CB64 was the best treatment at Kabete and second best at Mwea site and reduced the pathogen population by 50.44% and 35.51%, respectively. Plots treated with *Trichoderma* isolate T1 reduced the pathogen population in the soil by 40% in both sites. Towards the end of the experiment, after 112days of treatment application, all the treated plots gave more than 50% reduction of *R. solanacearum* population in the soil. *Trichoderma* T1 gave the highest percentage reduction of the pathogen of 92% at both sites. Plots treated with *Bacillus* isolate CB64 gave 93.17% and 88.78% reduction of *R. solanacearum* population in the soil at Kabete and Mwea site, respectively. These results showed that *Bacillus* CB64 performed better at Kabete where the temperatures were cooler than at Mwea site where the temperatures were warmer while *Trichoderma* isolate T1 performed consistently well in both sites. Control plots gave 20% increase of the pathogen population at both sites. The results herein are in line with those reported by Sharma and Kumar, (2009) that plots treated with *Trichoderma viride* reduced *R. solanacearum* population in the soil by 29% at 90 days of treatment application. Similar studies by Guo *et al.* (2004) and Rosyidah *et al.* (2013) have reported that soil application of *Trichoderma* strains significantly reduces the population of *R. solanacearum* in the soil. The pathogen reduction in the soil may be because *Trichoderma* strains have the ability to adapt in extreme soil conditions. This adaptation allows *Trichoderma* spp to colonize the soils, outcompete and suppress the pathogen. *Trichoderma* produces antibiotics that are able to suppress the development of *R. solanacearum* as observed in the *in vitro* results of this study. Comparable studies by Sharma and Kumar, (2009) have reported that strains of *Trichoderma* produce antibiotics like dermadine and gliotoxins that are able to suppress the development of bacterial pathogens such as *R. solanacearum*. Several studies have found a wide number of plant pathogens particularly the soil borne fungi such as *Fusarium* spp, *Pythium, Rhizoctonia* spp among other to be controlled by *Trichoderma* spp. The studies have reported *Trichoderma* as a microorganism that has broad spectrum of activity

as observed in the present study (Barari, 2016: Basco *et al.,* 2017).

The antagonism between bacterial inoculum of *Bacillus* strains and *R. solanacearum* that occurs in the rhizosphere of tomato plant explains why there was reduction of the pathogen population in plots treated with *Bacillus*. Results from the *in vitro* work of the current study showed that the antagonistic effect of *Bacillus* against *R. solanacearum* was by antibiosis. This was indicated by the formation of zones of inhibition induced by *Bacillus* isolates against *R. solanacearum* on nutrient agar plate. These findings herein showed that the mechanism by which *Bacillus* was using to reduce the pathogen population in the soil was antibiosis. Comparable field findings have reported a more than 64% reduction of the pathogen population when the soils where treated with *Bacillus* fortified organic fertilizer (Wei *et al.,* 2011: Ding *et al.,* 2013). Studies by Tan *et al.* (2013) and Huang *et al.* (2014) have also reported findings that are in agreement with the present study that application of *Bacillus amyloliquefaciens* strongly reduced *R. solanacearum* population in the soil rhizosphere.

Plots treated with chemical standard which had propane as the active ingredient gave 65.61% reduction of *R. solanacearum* population in the soil at Kabete and 56.92% at Mwea site after 112days of treatment application. However, this reduction was lower by 26.46% and 35.83% than plots treated with *Trichoderma* isolate T1 at Kabete and Mwea site, respectively. *Bacillus* CB64 that was also the best treatment, reduced the pathogen population in the soil by more than 27.56% and 31.86% at Kabete and Mwea site, respectively compared to standard chemical. These results are in line to Sharma and Kumar, (2009) findings that soil drench with chemical treatment reduced bacteria pathogen population in the soil by only 36.2% at 90days. Plots treated with the mixture of T1, T2 and T4 gave an average increase of 2% in *R. solanacearum* population at Kabete and Mwea sites. However, the increase was lower compared to the 20% increase in control plots. This mixture of *Trichoderma* isolates performed poorly compared to the same isolates applied as individual treatment. The observed results may be because *Trichoderma* produces different compounds that vary from each other. When the three *Trichoderma* isolates are combined, these compounds are not able to reduce the pathogen population compared to the same isolates applied individually hence the effectiveness of the mixture treatment was not additive. These results are comparable to Guo *et al.,* (2004) findings who reported that mixing strains of *Trichoderma* provided significantly lower suppression of bacterial wilt disease in the soil compared to same isolates applied individually. It was noted that before application of BCA mixtures in the field, it is important to test whether they combine effectively and have synergistic effective against the pathogen.

The mean difference of the treatments showed that Mwea site had higher initial inoculum level of 1.05×10^8 CFU/ml compared to Kabete site that had 9.08×10^6 CFU/ml. This could have explained why despite application of the same treatments in both sites, the percentage reduction of *R. solanacearum* population in the soil was lower at Mwea compared to Kabete. The higher temperatures observed at Mwea compared to Kabete site may have favored the multiplication of the pathogen in the soil and this explains the high populations recorded at Mwea than Kabete site. The population of the pathogen in the soil was significantly correlated with the temperatures. According to Wei *et al.* (2011), temperatures and not relative humidity are the factors influencing the survival of *R. solanacearum* in the soil and disease development and this finding support the results observed herein.

The trend of the results observed in the performance of all the treatments in reducing the pathogen population in the soil was in harmony with the findings recorded for the disease incidence and severity reduction. This was especially in plots treated with *Trichoderma* isolate T1 and *Bacillus* isolate CB64 that performed best in all the parameters. Plots that had a high % reduction of *R. solanacearum* population in the soil also had low bacterial wilt incidence and severity. This is because there is a positive and significant correlations between the disease incidence and the pathogen population in the soil. These findings are in agreement to those reported earlier (Wei *et al.*, 2011) that plots treated with *Bacillus* fortified organic fertilizer had significantly reduced *R. solanacearum* populations in the soil and hence the plots had decreased disease incidence compared to the control.

Cow manure and sorghum grains were used as carriers for *Bacillus* and *Trichoderma* isolates respectively. These carriers are known to provide beneficial strains in the soil with abundant nutrients that aid in their survival, production of antibiotics and in suppressing soil pathogens such as *R. solanacearum*. In this study, it was observed that total microbial counts increased significantly in plots treated with BCAs that were in cow manure and sorghum carrier compared to chemical standard and control that did not have any carrier. This could explain why isolates of *Bacillus* and *Trichoderma* significantly reduced the pathogen population in the soil compared to control. These findings are comparable to those reported by Tan *et al.* (2013) that use of organic substrates like manure, rice husks, and sorghum or rice grains as carriers for BCAs can provide nutrients for the desired antagonists. This therefore, increases their opportunity for establishment in the soil which then increases their effectiveness. As observed in the *in vitro* work of this study, part of mechanism used by the BCAs in reducing disease incidence, severity and pathogen population was through antibiosis that was shown by formation of ZOI induced

by the BCAs against the pathogen. During the *in vitro* work of the current study, competition for space and nutrients was observed as another mechanism used by *Trichoderma* and was indicated by the pathogen being overgrown and overrun by *Trichoderma* isolates. Comparable studies by Kloepper *et al.* (2004) and Seleim *et al.* (2011) have also reported that reduction of bacterial wilt incidence, severity and pathogen population in the soil was through production of antimicrobial substances by the BCAs and through competition.

Assessing the effect of *Bacillus* and *Trichoderma* isolates on the total microbial population and diversity in the soil

At 126 days of treatment application, all the treated plots gave significantly higher total microbial population compared to control in both Kabete and Mwea sites at $P \leq 0.05$ (Table 4.6). *Bacillus* treated plots had the highest total microbial count in both sites. Plots treated with *Bacillus* isolate CB64 gave the highest microbial count of 1.32×10^5CFU/ml at Kabete and 1.21×10^5CFU/ml at Mwea site. Total microbial population in control plots was 2.63×10^4CFU/ml at Kabete and 2.13×10^4CFU/ml at Mwea site. These findings are in agreement with those reported by Larkin, (2008) who reported that application of *Bacillus subtilis* in the soil significantly increased the microbial population and the population ranged from 1.2 to 2.5×10^8 CFU/g of soil. *Bacillus* isolates in this study were multiplied in cow manure carrier that had C: N ratio of 15.2. Manure is an organic matter applied in the soil that is known to support most microorganisms. This is because manure acts as a substrate that provides nutrients for microorganisms and this encourages microbial development and multiplication in the soil. Similar findings by Lupwayi *et al.* (2005) have reported that cow manure increased the microbial population in bulk soil by 26% after one year of application compared to inorganic fertilizer and control which reduced the microbial count. The moderate C: N ratio observed showed that manure was a good substrate for microbial breakdown and this reason could explain the high microbial populations observed. These findings are in confirmation with those reported by Jindo *et al.* (2012) that moderate C/N ratio in composts manure leads to availability of N during decomposition of organic compounds, resulting in high initial decomposition rates of hemicellulose and cellulose and hence high microbial populations. Larkin, (2008) also reported comparable findings that support the results herein that application of most soil amendments such as microbial inoculants, commercial bio control agents and aerobic compost increases microbial population and alters their activity. Bernard *et al.* (2012) reported findings that are in agreement with the results herein that compost-amended plots gave between 20% and 60% significantly higher bacterial and fungal cfu's than the un-amended plots.

Plots treated with *Trichoderma* isolates T1, T4 and T2 were second best after *Bacillus* isolates. The isolates had no significant difference among themselves in total microbial count which was an average of 8.42×10^4CFU/ml at Kabete and 7.35×10^4CFU/ml at Mwea site. This was significantly higher than the total microbial count in the control plots. These findings are comparable to those reported by Cai *et al.* (2015) that colonization of *Trichoderma harzianum* strain SQR-T037 on tomato roots significantly increased the microbial population compared to control. *Trichoderma* isolates were grown and multiplied in sorghum grain carrier that had low C: N ratio of 13.4. Sorghum provides high nutrients level for microorganisms especially during the initial stages of application. The low C: N ratio in sorghum is known to contribute to enhanced proliferation of microbial population in the soil. Kumar, (2017) also reported that low sorghum C: N ratio encouraged high growth and multiplication of microbial population in the soil. However, the total microbial counts in cow manure carrier were significantly higher than those recorded in plots with sorghum carrier. These findings are in agreement with those reported by Singh *et al.* (2014) that the proliferation of soil microbes is known to be high in manure even after a long time since the nutrients are released slowly and do not get depleted easily. The results herein are in line to Bonanomi *et al.* (2010) findings who reported that application of organic amendments in the soil significantly influenced the microbial population in a positive way.

Plots treated with the mixture of *Trichoderma* isolates gave total microbial count of 4.60 and 3.80×10^4 CFU/ml at Kabete and Mwea site, respectively. However, this microbial population was significantly lower compared to the population recorded by plots treated with the same *Trichoderma* isolates applied individually in both sites. These findings showed that the effect of the mixture of *Trichoderma* isolates were not additive as compared those of the individual isolates. The interaction in the mixture did not encourage proliferation of the microbes in the soil.

Chemical standard treated plots had significantly lower total microbial count compared to other treated plots in both sites. The microbial population was lower by more than 8.9×10^4CFU/ml compared to CB64 which was the best treatment in both sites. It was noted that the chemical standard did not stimulate the microbial population in the soil as compared to *Bacillus* and *Trichoderma* isolates. These findings are also comparable to those reported by Wang *et al.* (2015) that higher microbial populations were observed in soils treated with bioorganic fertilizer that contained *Bacillus amyloliquefaciens* compared to chemical fertilizer treatment.

Total microbial population consisted of various types of bacteria and fungi count. In all the

treated plots, the bacteria counts were significantly higher than the fungi counts in both sites (Table 4.6). The bacterial counts were more than the fungal count by an average of 1.12×10^5 CFU/ml. Plots treated with *Bacillus* isolate CB64 had the highest bacterial count of 1.24×10^5CFU/ml at Kabete and 1.00×10^5CFU/ml at Mwea site whereas bacterial count in plots treated with *Trichoderma* isolate T1 that had the highest fungal count was 7.80 and 5.17×10^4CFU/ml at Kabete and Mwea site, respectively. This was lower by more than 4.63×10^4CFU/ml than the bacterial count in plots treated with *Bacillus* CB64. However, this was expected because the carrier for CB64 was cow manure that was well decomposed hence encouraging fast growth and multiplication of bacteria. These findings are in agreement with those reported by Larkin, (2008) that *Bacillus* strain GBO3 and *Trichoderma viride* GI- 21 significantly increased the population of bacteria that could be cultured at $P\leq 0.01$ compared to control. Liu *et al.* (2018) reported comparable findings that treatment of soil with bioorganic fertilizer and organic fertilizer significantly increased rhizospheric and bulk soil bacteria abundance.

For fungal counts, *Trichoderma* isolates T4 and T1 had the highest fungal count of 1.23×10^4CFU/ml at Kabete and 2.27×10^4CFU/ml at Mwea, respectively whereas the fungal count in CB64 was lower by 5.0×10^3CFU/ml. Fungi are known as primary decomposers because they produce enzymes that are able to breakdown cellulose and lignin (Harman *et al.,* 2004). This explains why the fungal counts were higher in *Trichoderma* treated plots which had sorghum carrier. These findings are in line with Kumar *et al.* (2014) findings that application of *Trichoderma* spp. in the soil significantly increased the fungal population dynamics in the soil. Bernard *et al.* (2012) also reported comparable finding that bacterial and fungal cfu's were significantly higher in plots amended with *Trichoderma virens* than control plots. These findings support the results herein. The mean difference showed that Kabete site had higher total microbial count of 7.55×10^4CFU/ml compared to Mwea site that had 6.71×10^4CFU/ml.

Microbial diversity in the soil was determined based on morphological characteristics of different types of bacterial and fungal colonies (Table 4.7). *Bacillus* and *Trichoderma* isolates that were in cow manure and sorghum carriers, respectively, significantly stimulated diverse microbial communities in the soil compared to the control at $P\leq 0.05$. Comparable findings were reported by Shen *et al.* (2013) that application of organic treatments gave different rhizospheric soil bacteria and fungi communities and hence a wide microbial diversity. Lupwayi *et al.* (2005) also reported findings that are in line with the results herein that

application of organic manure significantly increased the microbial diversity because the soil microorganisms mineralize nutrients from organic soil amendments.

Plots treated with *Bacillus* stimulated eleven different types of microbial population at Kabete and nine types at Mwea site. *Trichoderma* treated plots stimulated nine and eight different types of microbial population at Kabete and Mwea sites, respectively while control stimulated only two types of microbial population in both sites. These findings are in agreement with those reported by Larkin, (2008) that application of *Bacillus subtilis* in the soil resulted in the highest microbial diversity compared to other treatments and control. At Kabete site, plots treated with *Bacillus* isolate CB64 that was in cow manure carrier had the highest diversity of bacterial colonies given by seven different groups as compared to *Trichoderma* that had four groups of different types of bacterial colonies. At Mwea CB64 was also the best with six different groups of bacterial colonies compared to *Trichoderma* that had four groups. Control had only one group of bacterial colonies in both sites. These findings correspond to Liu *et al.* (2018) reports that treatment of soil with bioorganic fertilizer that contained *Bacillus amyloliquefaciens* and organic fertilizer significantly increased rhizospheric and bulk soil bacteria abundance compared to Fungal population. In all the treatments, bacteria colony diversity was higher compared to fungal colony diversity in both sites. This may be because bacteria is known to develop and multiply faster compared to fungi. Larkin, (2008) also reported that bacterial populations were observed in plots treated with *Trichoderma* isolates and this indicated that there was some stimulation of bacteria activity by the treatment.

For fungi diversity, *Trichoderma* isolates T1 and T4 which were in sorghum carrier had five and four different fungal groups at Kabete and Mwea sites, respectively. This was significantly higher compared to *Bacillus* isolate CB64 that had four and three different fungal groups at Kabete and Mwea sites, respectively and control that had one group in both sites. The fungal diversity was not significantly different among the *Trichoderma* treatments. Similar findings were reports by Naseby *et al.* (2000) that fungal populations and diversity in the soil in absence of *Pythium* spp. were significantly higher than control with inoculation of *Trichoderma* strains.

The mixture of *Trichoderma* isolates had the least number of bacterial and fungal diversity similar to the trend of the results observed where the mixture had the least total microbial count. Plots treated with chemical standard also had the least number of bacterial and fungal diversity similar to the trend of the results observed where the chemical standard had the least microbial count. Similar findings have been reported by Scheepmaker and Kassteele, (2011) that microbial Biocontrol agents induced higher microbial populations and diversity in the soil compared to

chemical control agents.

The above study has demonstrated that microbial population, microbial diversity and mechanisms of action of the BCAs namely antibiosis and competition are the major contributors in controlling *R. solanacearum* pathogen. From the above results, it was observed that the best performing antagonists namely *Bacillus* isolate CB64 and *Trichoderma* isolate T1 were able to combine several factors that contributed to the control of *R. solanacearum*. These factors included the ability to increase microbial population, microbial diversity and the ability for antibiosis and competition. Therefore, *Bacillus* isolate CB64 that had the highest bacteria colony diversity and microbial population in both sites and *Trichoderma* isolate T1 that had the highest fungal colony diversity and microbial population. In addition, the isolates recorded the highest percentage reduction of *R. solanacearum* population, disease incidence and severity at both sites. It is known that when there is a high microbial population in the soil, then there are more microorganisms that produce antibiotics against bacterial wilt pathogen and this could explain the results herein. Furthermore, more types of bacteria and fungi, means there is a high diversity of microbial population in the soil as observed in plots treated with CB64 and T1. This diversity influences different strains of antagonistic microorganism that have various antagonistic activity that leads to suppression of pathogenic bacteria and fungi such as *R. solanacearum*.

Survival of *Bacillus* and *Trichoderma* isolates in the soil

Bacillus isolates were easily re-isolated from the soil at 126 day after treatment application at the end of the experiments compared to *Trichoderma* isolates in both sites (Table 4.8). Plots treated with *Bacillus* isolate CB64 had the highest number of bacteria colonies re-isolated with more than 3.30×10^4 CFU/ml of the isolate in both sites. Retrieval of *Trichoderma* isolate T4 from the soil was less than 2.7×10^3CFU/ml at both sites. This was comparable to the findings reported by Beagle- Ristaino and Papavizas, (1985) that *Trichoderma viride* strain T- 1- R9 was recovered at 10^3CFU/g of soil after 130 days of application. Retrieval of *Trichoderma* isolate T4 was 3.03×10^4 CFU/ml lower than the population of *Bacillus* isolates retrieved in both sites. *Trichoderma* isolate T2 was also re-isolated only at Kabete site and the population was 1.3×10^3CFU/ml. Isolate T1 was not retrievable. These findings correspond to those reported by Larkin, (2008) who demonstrated that *Bacillus subtilis* and some *Trichoderma* spp. can persist in the soil since the isolates were retrieved from the soil at the end of the experiments.

It had been noted that during the *in vitro* work of the present studies, cultures of T1 left at room temperatures (23± 2^{o}C) for more than two months died out and could not be retrieved by sub-culturing in PDA. This was a clear indication that survival of *Trichoderma* in the soil is low and retrieval is very poor hence the results observed herein. Comparable studies by Beagle-Ristaino and Papavizas, (1985) have also reported that population densities of *Trichoderma* spp did not significantly increase in numbers when added to soil as conidia and that *Trichoderma harzianum* were not retrieved from the soil rhizosphere. These findings corroborate the results observed herein. The results indicated that *Trichoderma* isolates needs to be boosted for subsequent crop seasons. Papavizas, (1981) also reported that at 130 days of *Trichoderma harzianum* incubation, only 3% of the original number of conidia were recovered due to reduced survival and lack of sporulation of the fungi in the soil. However, *Bacillus* which was recorded with the highest survival rate showed that there are mechanisms that helps them survive in the soil. It is known that *Bacillus* strains produce endospores that are highly resistant to both chemical and physical stress and has ability to inhabit and survive in the soil (Huang *et al.,* 2014). This reason explains why *Bacillus* is well suited for bio control application.

Evaluating the effect of *Bacillus* and *Trichoderma* isolates on tomato yields and fruit size

In the present study, it was observed that all the treatments had significantly higher yields and fruit size compared to control at P≤ 0.05 (Table 4.9). Plots treated with *Bacillus* isolate CB64 had the highest average yields of 39.51 and 20.58 tons per hectare at Kabete site and at Mwea site, respectively. Control had an average yield of 11.88 t/ha at Kabete and 4.62 t/ha at Mwea site. The average yields observed in CB64 were 232.58% and 345.45% more than those recorded in control at Kabete and Mwea site, respectively. Similar findings were reported by Seleim *et al.* (2011) that *Bacillus subtilis* and *Pseudomonas fluorescence* achieved 91% and 348% increase of tomato yields per plant respectively compared to control. These yields show that *Bacillus* isolate CB64 had growth promoting activity indicated by the higher yields compared to control. Comparable findings of *Bacillus* strains ability to promote growth and yields of tomato plants have been reported (Lemessa and Zeller, 2007: Aliye *et al.,* 2008: Yendyo *et al.,* 2017).

Similarly, *Trichoderma* T1 treated plots recorded significantly higher yield of 37.13 and 20.72 t/ha at Kabete and Mwea site, respectively than control. The yields observed were 212.54% and 348.48% more than those recorded in control at Kabete and Mwea site, respectively. These results correspond to Narasimhamurthy *et al.* (2018) findings, who reported that plots treated

with *Trichoderma asperellum* (T4 and T8) enhanced tomato yields at 5.45 t/ha and 5.50 t/ha compared to untreated plots. Comparably, Sharma and Kumar, (2009) also reported a 142.1% increase of tomato yields compared to control after application of *Trichoderma viride* in the soil. Kumar, (2017) also reported findings that are in agreement with the results described herein that plots treated with *Trichoderma harzianum* had significant increase in all the growth parameters and 50% increase in tomato yields.

Not only were the fruit yields significantly higher, the fruit sizes were also observed to be more than 13.57mm larger compared to the control. Plots treated with *Trichoderma* isolate T1 gave the highest average measurement in fruit size of 49.56 and 48.45mm at Kabete and Mwea site, respectively. *Bacillus* isolate CB64 gave average measurement in fruit size of 49.76mm and 48.42mm at Kabete and Mwea sites, respectively. These fruit size measurements were significantly higher by more than 36% compared to the control that had an average fruit size measurement of 36mm in both sites. Strains of *Bacillus* and *Trichoderma* are known to produce plant growth hormones that are responsible for increased yields and fruit size (Singh *et al.*, 2012). According to Seleim *et al.* (2011) and Singh *et al.* (2012), the mechanism involved in plant growth promotion by the BCAs occurs directly through secretion of plant growth hormones such as auxins, cytokinins and gibberellins. Also, the BCAs promote plant growth by facilitating the nitrogen uptake through nitrogen fixation and solubilisation of phosphorous (Singh *et al.*, 2012).

In this study *Bacillus* and *Trichoderma* isolates produced metabolites against *R. solanacearum* as observed in the *in vitro* studies and also secreted secondary compounds that promoted tomato plant growth and increase in yield. Comparable findings by Ongena *et al.* (2007) and Yendyo *et al.* (2017) have reported production of metabolites and secondary compounds by strains of *Bacillus* and *Trichoderma* that have antagonistic activity against *R. solanacearum* and are able to promote plant growth and yield increase. It is known that bacterial wilt is one of most devastating disease of tomato that causes more than 64% losses in yields and leads to formation of small sized and shriveled tomato fruits (Mbaka *et al.*, 2013). Therefore, management of the disease will also reduce the yield loss and will also increase the fruit quality. This study demonstrated that, the BCAs used were able to reduce the pathogen population in the soil, disease incidence and severity and therefore, contributed greatly to the increase in tomato yields and fruit size. These results are supported by Seleim *et al.* (2011) and Singh *et al.* (2012) findings, who reported that plant growth promotion by the BCAs may occur indirectly when they reduce or prevent the injurious effect of the pathogen on the plant.

From this study, isolate CB64 and T1 recorded the highest yields and fruit sizes and outperformed the standard chemical treatment while the mixture of *Trichoderma* isolates was among the treatments that recorded the least yields and fruit size when compared to the same isolates applied individually. These results were not surprising considering the mixture of *Trichoderma* isolates also performed poorly in relation to reducing the disease incidence, severity and pathogen population in the soil. The mean difference of the treatments showed that Kabete site had higher yields compared to Mwea. This was because the disease incidence and severity were higher at Mwea than Kabete site as observed earlier in the present study.

CHAPTER FIVE: GENERAL DISCUSSION CONCLUSION AND RECOMMENDATION

5.2 GENERAL DISCUSSION

Bacillus and *Trichoderma* isolates effectively suppressed the growth of *R. solanacearum* as observed in the *in vitro* work of the present study. These findings were in agreement with those reported by Guo, (2004): Lwin and Ranamurkhaarachchi, (2006): Revathi *et al.* (2017). The antagonistic activity of *Bacillus* and *Trichoderma* against the pathogen was through antibiosis. This was observed by formation of zones of inhibition induced by the metabolites and different compounds secreted by the antagonists against the pathogen. These findings are comparable to those reported by Singh and Jagtap, (2017) that, *Bacillus* and *Trichoderma* are able to secrete different compounds and produce secondary metabolites that induce the formation of zones of inhibition against *R. solanacearum*. *Trichoderma* isolates were observed to have antagonistic activity against the pathogen through competition which was demonstrated by isolates outgrowing and overrunning *R. solanacearum* in plates containing PDA.

Among the *Bacillus* isolates, strain CB64 had the highest zone of inhibition measuring 4.3mm against *R. solanacearum*. For *Trichoderma*, isolate T1 induced the highest ZOI of 13.5mm against the pathogen. These results indicated that *Trichoderma* had significantly higher ZOI compared to *Bacillus* at $P \leq 0.05$. Comparable results have been reported by Yendyo *et al.* (2017) that the activity of native *Trichoderma* spp. against *R. solanacearum* observed by formation of inhibition zones was significantly higher compared to *Bacillus* spp.

However, the *in vitro* findings of the higher performance of *Trichoderma* isolates against the pathogen as compared to *Bacillus* isolates were not consistent under field conditions. This was because, it was observed that *Bacillus* isolate CB64 which recorded the highest performance in all the parameters tested under field conditions was not significantly different from *Trichoderma* isolate T1 at $P \leq 0.05$. Plots treated with CB64 had a reduction of more than 50% in bacterial wilt incidence compared to control at Kabete and Mwea sites. These results are comparable to those reported by Lemessa and Zeller, (2007) that *Bacillus subtilis* strain B2G reduced bacterial wilt incidence by 60%. Similarly, plots treated with *Trichoderma* isolate T1 reduced the disease incidence by 61% and this reduction was not significantly different from the one recorded in *Bacillus* CB64 treated plots. These findings are in line with those reported by Narasimhamurthy *et al.* (2018) that *Trichoderma asperellum* treated plots showed reduction of bacterial wilt disease incidence of 51.06%.

All plots treated with *Bacillus* and *Trichoderma* isolates recorded significantly higher percentage reduction of *R. solanacearum* population in the soil compared to control at $P \leq 0.05$ after 112 days of treatment application. Plots treated with CB64 recorded 93.17% and 88.78% reduction of *R. solanacearum* population at Kabete and Mwea site, respectively. Isolate T1 recorded 92% reduction at both sites while control plots recorded 20% increase of the pathogen population at both sites. These findings are in agreement with those reported by Wei *et al.* (2011) that more than 64% reduction of *R. solanacearum* population was recorded when soils were treated with *Bacillus* fortified organic fertilizer. Comparable results to these herein have been reported by Sharma and Kumur, (2009) that plots treated with *Trichoderma viride* reduced *R. solanacearum* population in the soil by 29% at 90 days.

The reduction of bacterial wilt incidence and *R. solanacearum* population in the soil was because *Bacillus* and *Trichoderma* are able to produce antagonistic compounds and metabolites against the pathogen as observed during the *in vitro* work of the present study. Comparable findings have been reported by Tapwal *et al.* (2011): Wei *et al.* (2011): Huang *et al.* (2014) that, *Trichoderma* and *Bacillus* strains have ability to produce various metabolites and compounds that are able to reduce bacterial wilt incidence in tomato plants.

The *in vitro* results of the present study showed that, *Bacillus* isolate CA7 induced a ZOI of 3.4mm against *R. solanacearum*. This was significantly lower compared to the ZOI induced by *Bacillus* isolate CB64 that had 4.3mm. Similar trend was also recorded under field conditions where plots treated with *Bacillus* isolate CA7 performed poorly in reducing disease incidence and pathogen population in the soil compared to CB64. The isolate reduced the disease incidence by 11.66% and 20% at Kabete and Mwea site, respectively which was significantly lower than 88% recorded in CB64 treated plots.

Treatments were applied as soil drench in each tomato plant in the field. *Bacillus* strains are known to produce endospores that are highly resistant to both chemical and physical stress and has ability to inhabit and survive in the soil (Huang *et al.,* 2014) as compared to *Trichoderma*. This would explain why *Bacillus* isolate CB64 performed significantly better under field condition despite the significantly lower ZOI recorded in the *in vitro* study as compared to *Trichoderma*. Moreover, *Bacillus* isolates were multiplied in cow manure carrier before application in the field. Cow manure is known to provide beneficial strains in the soil with abundant nutrients that aid in their survival, production of antibiotics and in suppressing soil pathogens such as *R. solanacearum*. In this study, it was observed that total microbial

population in the soil increased significantly in plots treated with *Bacillus* isolates multiplied in cow manure. The findings were comparable to those reported by Tan *et al.* (2013) that use of organic substrates like manure as carriers for BCAs can provide nutrients for the desired antagonists and therefore, increasing their opportunity for establishment in the soil and their effectiveness.

5.2 CONCLUSION

The present study clearly demonstrated that the evaluated isolates of *Trichoderma* and *Bacillus* have potential for use as biological control agents to manage bacterial wilt disease of tomato. *Trichoderma* and *Bacillus* isolates used as biological control agents were able to reduce the percentage disease incidence. Results in the current study showed that at the end of the experiment, the best treatment (*Bacillus* CB64 and *Trichoderma* T1) recorded disease incidence below 40% compared to control which had more than 90% disease incidence at Kabete and Mwea site.

These BCAs were also able to reduce the population of *Ralstonia solanacearum* in the soil by more than 90% as opposed to control that had an increase in the pathogen population. The disease severity in tomato plants was greatly reduced by *Bacillus* and *Trichoderma* isolates by more than 50%.

All the *Bacillus* and *Trichoderma* isolates evaluated under field conditions in the present study greatly increased the yields of tomato and fruit size compared to control. Plots treated with *Bacillus* isolate CB64 and *Trichoderma* isolate T1 had the highest performance in the all parameters.

Under field conditions, it was observed that *Bacillus* isolate CB64 and *Trichoderma* isolate T1 outperformed the standard chemical in the management of bacterial wilt disease, in reducing *R. solanacearum* population in the soil and in the total microbial population.

The result also demonstrated that *Trichoderma* isolates T1, T2 and T4 when applied individually performed way better than the mixture of the same treatments that performed poorly in all the parameters.

The study also showed that *Bacillus* and *Trichoderma* isolates had very high antagonistic activity against *Ralstonia solanacearum, Xanthomonas campestris* pv *campestris* and *Pseudomonas* sp. *in vitro*.

The *in vitro* work of this study showed that *Trichoderma* isolates had higher activity against

the bacteria pathogens compared to *Bacillus* isolates. *Bacillus* isolates induced the highest antagonistic activity against *Pseudomonas* sp. compared to the other pathogens. *Trichoderma* isolates showed the highest activity against *Ralstonia solanacearum* compared to the other pathogens and the least activity was recorded in *Pseudomonas* sp.

Growth of *Trichoderma* isolates in sorghum carrier and *Bacillus* isolates in cow manure increased their proliferation *in vitro* and also increased the microbial populations and diversity in the soil under field conditions.

During the *in vitro* work of the present study, *Bacillus* isolate CB64 had significantly lower ZOI against *R. solanacearum* compared to the *Trichoderma* isolates. However, under field conditions, CB64 was the best treatment only comparable with *Trichoderma* isolate T1.

Bacillus isolate CB64 was more dominating in the soil compared to *Trichoderma* isolates as observed by the highest population of the isolate recorded during re-isolation of the BCAs from the soil. The isolate was also multiplied in cow manure carrier that favored the microbial dynamics in the soil and hence influencing the good performance of *Bacillus* isolate CB64.

5.3 RECOMMENDATIONS

1. For effective utilization of the *Bacillus* and *Trichoderma* isolates, *in vitro*, testing for the efficacy of BCAs should be done before testing under greenhouse and field conditions. This helps to narrow down on the effective BCAs following the mechanisms of antagonism *in vitro*.
2. *Bacillus* isolate CB64 and *Trichoderma* isolate T1 recorded the highest percentage reduction of bacterial wilt incidence and severity in the field. Therefore, CB64 and T1 are recommended for use as biological control agents in the management of bacterial wilt disease caused by *R. solanacearum* on tomato in the field.
3. Plots treated with *Bacillus* isolate CB64 and *Trichoderma* isolate T1 had the highest percentage reduction of *R. solanacearum* population in the soil. Hence, *Bacillus* CB64 and *Trichoderma* T1 are recommended for use in reducing *Ralstonia solanacearum* inoculum level in the soil.
4. Cow manure and sorghum grains were used in the present study as carriers for *Bacillus* and *Trichoderma* isolates, respectively and were found to work effectively. Hence, for cultivation of any antagonist on a relatively large scale to be used for field experiments, cow manure and sorghum grains are recommended for use as carriers for *Bacillus* and *Trichoderma,* respectively.

5. There is need to partner with different stockholders that include financial institutions and research Centre to enhance technology adoption for the use of *Bacillus* and *Trichoderma* isolates to make formulations for commercial use.

5.4 Further work

The current efficacy study was the initial step in the process of developing *Bacillus* and *Trichoderma* isolates as biological control agents for productivity and sustainability. However accurate identification of the isolates to species level using molecular characters that can distinguish the isolates through their DNA characters is recommended.

Bacillus and *Trichoderma* in the current study have been found to have antagonistic activity against *Ralstonia solanacearum* through antibiosis and competition. However, further studies are recommended on additional mechanisms used by *Bacillus* and *Trichoderma* in reducing bacterial wilt incidence, severity and reducing pathogen population in the soil and their impact on host plant.

The mixture of *Trichoderma* isolates T1, T2 and T4 in the current study was found to have poor performance in reducing bacterial wilt incidence, severity, the pathogen population in the soil and in increasing the microbial populations in the soil as compared to the same isolates applied individually. Therefore, further work on the impact of mixing *Trichoderma* isolates in the management of bacterial wilt is recommended.

Bacillus isolates in present study were easily retrieved as compared to *Trichoderma* isolates. However, further work on the residual effects of *Bacillus* and *Trichoderma* treatments in managing *Ralstonia solanacearum* after the season is recommended.

For effective utilization of the *Bacillus* and *Trichoderma* isolates that were screened against *Xanthomonas campestris* and *Pseudomonas* sp. *in vitro,* further studies and evaluation under field conditions are recommended.

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