SCREENING OF BENEFICIAL RHIZOSPHERIC BACTERIAL AND FUNGAL ISOLATES FOR CONTROL OF BACTERIAL WILT IN TOMATO (*Solanum lycopersicum L.*) IN KENYA

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Screening of Beneficial Rhizospheric Bacterial and Fungal Isolates for Control of Bacterial Wilt of Tomato (*Solanum lycopersicum L.*) in Kenya

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other University.

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DEDICTION

I dedicate this thesis to my beloved wife Emily Chepkemoi Kones; my children Elian Kiprop, Alexies Chepchumba, and Aldrine Kipkalia; My beloved mother Anna Ngenoh; brothers, sisters and many friends for their profound love, support, motivation and prayers. To the wonderful supervisors who inspired throughout the academic study period. Abundant blessings to you all.

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ABBREVIATIONS AND ACRONYMS

BCAs	Biological Control Agents
CFU	Colony Forming Units
CRD	Completely Randomized Design
DIA	Disease Incidence of Antagonists
DIC	Disease Incidence of Control
EPS	Extracellular Polysaccharides
GoK	Government of Kenya
HCD	Horticulture Crops Directorate
JKUAT	Jomo Kenyatta University of Agriculture and Technology
L.S.D	Least Significance Difference
NA	Nutrient Agar
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
ROS	Reactive Oxygen Species
TZC	Tetrazolium Chloride
USAID	United States Agency for International Development
VBNC	Viable but non cultural
VOCs	Volatile Organic Compounds

ABSTRACT

Ralstonia solanacearum is a soil-borne pathogen causing bacterial wilt disease in solanaceae crops, including tomatoes. Available control strategies have been limited because of pathogen's ability to develop resistance against pesticides, its wider host range, and ability to break host's innate resistance. Management strategies including chemical and cultural practices have all failed causing the pathogen to be a threat to production. Although this pathogen is reported to result in 100% yield losses in the fields, pockets of uninfected plants are always visible. Little is known about the rhizosphere microbiome that would contribute to successful survival of plants amidst the highly virulent pathogen. In the current study, purposive sampling of rhizospheres from healthy tomatoes from Bomet, Kiambu, Kajiado and Kirinyaga was done. Pour plate method was used to screen for potential beneficial bacterial using nutrient agar media. Sprinkling method was used to screen for potential beneficial fungal isolates using potato dextrose agar amended with chloramphenicol at rate of 25mg/l. Purification of bacteria and fungi was done 48 hours and 72 hours, respectively, post incubation at 28 ^{0}C. Population of isolates revealed 40 bacterial isolates distributed as Bomet (48%)>Kiambu (27%)>Kajiado (18%) >Kirinyaga (8%). Similarly, fungal isolates were distributed as Bomet (30%)>Kiambu (28%)> Kajiado (23)>Kirinyaga (19%). Biochemical, microscopic and morphological characterization for bacterial isolates revealed *Bacillus* sp. (27.5%), *Micrococcus* sp., (27.5%) and *Burkholderia* sp., (15%) as the most dominant bacterial organisms inhabiting the rhizosphere of healthy plants. Macroscopic and microscopic description of fungal isolates also identified *Aspergillus*, *Trichoderma,* and *Fusarium* species as the most dominant fungal isolates in the rhizospheres of healthy tomatoes. Hierarchical cluster analysis done using DARwin software V6 clustered fungal and bacterial isolates into four and three clusters respectively. Finally, greenhouse experiments studied the efficacy of four bacterial isolates prequalified in the lab bioassays, in controlling bacterial wilt in Rio-grande tomatoes. Data on disease incidence, severity and population of *R. solanacearum* in roots and rhizosphere soils from each treatment revealed that both *Bacillus* sp. KMB16 and *Pseudomonas* sp.KJ2 significantly ($p<0.05$) reduced bacterial wilt incidence and severity on tomatoes. Further, treatments with the two antagonists resulted in a significant reduction of *R. solanacearum* in the roots of the tests plants. *Bacillus* sp.KJ4, *Pseudomonas* sp.KJ2, and B*acillus* sp.BMT16 significantly ($P<0.05$) reduced population of *R. solanacearum* within the soil as compared to *Burkolderia* sp.KRN2. The result of this study contributes to knowledge of rhizosphere microbiome, diversity, and their potential. Further work needs to be done to formulate and utilize identified effective as an innovation that will shape the future of sustainable control of plant pathogens.

CHAPTER ONE

INTRODUCTION

1.1 Background Information

Ralstonia solanacearum is a gram-negative, nonspore-forming proteobacterium, causing bacterial wilt in over 200 plant species (Meng, 2013; Singh *et al*., 2014; Sakthivel *et al*., 2016). The pathogen is a soil borne bacterium (Phukan *et al*., 2019) widely distributed in tropics and subtropics (Kunwar *et al.,* 2019; Kumar *et al.,* 2022). The pathogen spread across cultivated fields through irrigation and surface water, contaminated soils, equipment and tools, hosts weeds, and plant materials with latent infection. Globally, *R. solanacearum* is considered an important plant pathogen because of its lethality, persistence in the soil, wide hosts range and its broad geographical distribution (Wei *et al*., 2018). In temperate countries, the pathogen has been implicated in cases of severe economic losses in potato, tomato and other important solanaceous crops (de Pedro-Jové *et al*., 2021). In some cases, crop losses going up to 100% are reported globally (Sikirou *et al*., 2009). Annually, tomatoes are grown by smallholder farmers in over 4.4 million hectares as source of income and important source of nutritional elements (Aloyce *et al*., 2017). These important elements include ascorbic acid, bioflavonoids, enzymes, and minerals like potassium, iron, and magnesium, vitamins C and B, and amino acids which act as anti-oxidants (Ali *et al.,* 2020). Further, tomato has been associated with lowering the risks of breast and prostate cancer (Marti *et al.,* 2016). Despite its nutritional and economic importance, tomato production have been severely affected by the pathogens (Aloyce *et al*., 2017), which include *R. solanacearum* causing bacterial wilt. The pathogen is known to spread quickly affecting crops and rendering farms unsuitable for the production of any solanaceous crops. The pathogen gains entry through natural openings or wounds, colonizes the cortex, invades the xylem vessels, before spreading to stem and aerial parts through the vascular bundles (Yadeta & Thomma, 2013). The bacterium multiplies rapidly within the host plant reaching high densities and blocking the waterway leading to wilting and plant death (Aloyce *et al.,* 2017).Conditions like high temperatures and humidity which are preferred by

tomatoes favors disease development and its devastation. Lack of effective and sustainable control methods in Kenya have subjected farmers to huge losses with majority failing to recover their high investments costs because of the pathogen. Utilization of biological controls could be the most effective control tool against *Ralstonia solanacearum* (Elsayed *et al*., 2020). Numerous studies have pointed out at the rhizosphere as an essential environment abundantly inhabited with microbes with inherent beneficial properties including disease suppression (Vishwakarma *et al*., 2020). Further, literature points at several biological controls agents as potential alternative strategies in the integrated management of diseases including bacterial wilt. Utilization of biocontrol agents from the rhizosphere presents a significant success because they have an added advantage of colonizing underground plants organs not reachable with pesticide.

Effective screening and evaluation of rhizosphere microbiome requires correct soil sampling, sample preparation (Romano *et al.,* 2020) and suitable tool for analyzing the role of these microbes. The emergence of techniques like computational biology, sequencing technology and omics analysis have led to the discovery of the useful role that a community of beneficial microbes play (Li *et al*., 2021) in enhancing plant health and growth. Different methodological approaches suitable for identification techniques are important for harnessing these naturally occurring community of microbes driving their interaction with the plants. Numerous laboratory-based methods including culture-dependent approach have been advanced to assist in unravelling the diversity and evaluating the properties that antagonistic microbes inhabiting rhizosphere environments possess against pathogens causing plant diseases. The culture-dependent method follows a culturing steps where after screening and isolation, plating on the petri dishes is done to obtain pure cultures required for other important analysis (Anguita-Maeso *et al.,* 2020). The method can be used with other approaches like next generation sequencing and metagenomics which are commonly used to profile microbial assemblages (Li *et al*., 2021) in the rhizosphere, and their efficacy in controlling bacterial wilt diseases.

Biocontrol agents like plant growth rhizobacteria (PGPRs) used against bacterial wilt are isolated from healthy plants but are known to be scarce within the rhizosphere of

disease plants (Huang *et al*., 2013). It is believed that plant disease tolerance is because of these rhizosphere dwellers. A number of organisms isolated from rhizosphere soils have demonstrated their efficacy towards control of plant associated pathogens. For instance, under *in vitro* experiment, *Pseudomonas* and *Bacillus sp*. isolated from healthy tomato rhizophere significantly inhibited virulent *R. solanacearum* isolated from wilted tomato plant (Gashaw *et al*., 2022). Though there has been increasing attempts to screen rhizospheric microorganisms against plant pathogens *in vitro,* there is insufficient information that has been generated so far to specifically support isolation and use of *in situ* microbes in heavily infected rhizosphere. For this reason, it was crucial to screen and evaluate the efficacy of the rhizosphere dwellers to antagonize bacterial wilt for it to be incorporated as one of the sustainable disease management strategy sustainable to smallholder farmers.

1.2 Statement of the Problem

Across the globe, bacterial wilt disease caused by *R. solanacearum* is the leading devastating pathogen currently known because of its ability to cause rapid and fatal wilting symptoms in many host plants (Yuliar *et al.,* 2015).The pathogen is a threat to most important agricultural crops, including tomatoes, an important commercial vegetable crop playing a significant role in enhancing food security and creating employment (Vu *et al*., 2017). Significant losses caused by the pathogen occur in the fields in many regions of the world but vary based on crop's cultivar (Vanitha *et al.,* 2009), local climates, and soil types, cropping management practices and tolerance or susceptibility of the crop preferred by the farmer. Farmers have abandoned the majority of susceptible crops due to heavy losses incurred, which has been reported to exceed $ 950 million annually in over 80 countries (Guchi, 2015) worldwide. If effective control measure is not put in place, important source of livelihood and nutrients like vitamins and dietary fiber to increasing world population will be lost.

In the US and Canada, *R. solanacearum* is considered a quarantine organism (Swanson, 2005) and this has derailed the developing countries from exporting high value horticulture crops known to hosts *R. solanacearum.* In Kenya, farmers have cited bacterial wilt as the most problematic tomato disease to manage, hindering

production of any solanaceous crops in major production regions in the country (Kaguong'o *et al*., 2010, Sharma *et al.*, 2022). For instance, 77% of previously surveyed farms in 10 former districts reported the prevalence of the disease (Kaguong'o *et al*., 2010). As a result, majority of farmers have been forced to abandon their formerly productive greenhouses and fields immediately the pathogen is established (Aloyce *et al*., 2017). Farmers' lack of adherence to seed regulations in Kenya has contributed to the rampant spread of the pathogen (Muthoni *et al*., 2014). Cultural control methods, for instance, crop rotation is not practical among smallholder farmers due to land scarcity. Though some tomatoes may tolerate the pathogen, most of the infected tomato seedlings die, but those surviving the disease are stunted and do not flower (Jogaiah *et al*., 2013) and this results in the loss of yields. Additionally, such cultivars have been disregarded by farmers due to poor yield and unpleasant taste perceived to be associated with those cultivars.

There is hardly single management measure reported to be successful in suppressing *R. solanacearum* (Wubshet, 2018) and the current effects presents a challenge in uplifting food production for the increasing population (Pathak *al.,* 2017). The destructive nature, broad host range, and persistence of *R. solanacearum* compounded with wide genome plasticity (Elnaggar *et al.,* 2018) requires biological control interventions. The unique survival and pathogenicity strategies possessed by *R. solanacearum* have rendered its identification and control difficult since the host plant can highly harbor infection in a latent state without showing any symptoms (Subedi, 2015).

The pathogen's unique way of infecting and multiplying in the xylem, which has been regarded as the 'black hole' with unfavorable conditions for growth (Meng *et al.,* 2015) is a challenge even to innate host resistance mechanisms. Efforts to harness recent resistance for breeding tactics have failed. However, biological agents have demonstrated considerable potential against such vascular diseases. Being available in all the plants parts, biological control agents play a significant role in suppressing plant diseases while enhancing growth promotion (Ahmed *et al.,* 2022). Identification of the pathogen using symptoms such as wilting and yellowing of leaves is not reliable because they can be confused with other biological and non-

biological factors that can exhibit similar symptoms. In some instances, the pathogen can latently exist in plants with no visual symptoms. This scenario can ease the spread and survival of *R. solanacearum* since control strategies by farmers would not be timely. Sophisticated methods of identification like Polymerase Chain Reaction (PCR), use of specific antibodies, semi-selective media are lab-based and out-of-reach to many farmers. Though these methods could be effective in evaluating the presence of the pathogen in the soil, the techniques are however only effective with a bacterial population exceeding 10^4 CFU/g soil (Pontes *et al.,* 2017). Management approaches including cultural methods such as crop rotation have shown no significant success as the pathogen is reported to survive under oligotrophic environments like pure water. Physical barriers are also not reliable since the pathogen can spread through water and uncertified planting materials commonly used by farmers (Elnaggar *et al.*, 2018). Use of varieties which had been regarded as the most effective, economical and environmentally friendly has however recorded limited success (Yuliar *et al.*, 2015) due to unstable resistant genes which can easily be broken down by the complex virulent machinery possessed by pathogen (Elnaggar *et al*., 2018). Breeders have a hard task of finding resistance durability that can withstand the pathogen (Gopalan-Nair *et al.,* 2020). Moreover, varieties that possess partial resistance can get latently infected and thus easing the spread of the pathogen among the farmers (Muthoni *et al*., 2012). Because of these challenges, it has become necessary to research into and bring out alternative solutions to protect crops against devastating pathogens and biological controls through the use of microorganisms which have gained popularity.

Microbes that have been attributed to possess beneficial properties like plant growth promotion, disease suppression, and assimilating nutrients to plants are known to be abundant within the plant rhizosphere (Vishwakarma *et al.,* 2020). Recent studies on crops microbiome during attack from the pathogen have pointed out at the selective nature of plants towards microbiome. These studies have also demonstrated that beneficial microbes intended to suppress pathogens are recruited within the rhizosphere when wheat infected with *R. solani AG8* are recycled (Yin *et al.,* 2021). Further, plants can accurately differentiate pathogenic and beneficial microbes thereby maintaining the dynamic balance between their growth and defenses (Zhang

et al., 2021). In a study conducted using never-ripe mutant (*Nr*) and wild type (WT) tomatoes, results revealed that *Nr* mutant significantly differed with WT in not only the way it functions, but also in the composition of the community of rhizosphere bacteria which resulted from the changes in the metabolites exuded in the roots of both WT and *Nr* tomatoes (Fu *et al.*, 2021). Apart from inducing resistance against pathogen, the rhizosphere microbes can confer broad spectrum benefits including fixing nitrogen, plant growth hormones and antimicrobial compounds (Vishwakarma *et al.,* 2020). Efforts towards finding sustainable control strategy for *R. solanacearum* should, therefore, focus on the rhizosphere of selected plants due to abundance of microbes that qualify to be utilized as biocontrol agents. In that regard, the study was aimed at screening the rhizosphere of tolerant tomato plants for beneficial microbes and evaluating their efficacy in the management of bacterial wilt diseases in tomatoes.

1.3 Justification

Majority of smallholder farmers attempting to manage pests are aware of the losses caused by abiotic stressors (Ochilo *et al*., 2019). These losses can be considerably reduced if the alternative control strategies are made available. Biocontrol methods have remained to be the most promising because they do not impact negatively on the environment (Nyarieko *et al.,* 2018). Diversification of management strategies, can likely improve the efficacy of control and reduce the cost associated with management of both abiotic and biotic stressors (Ochilo *et al*., 2019) and thus enhance profitability in tomato farming in Kenya. Despite BCAs being crucial component in integrated pests and disease management, availability and costs have been the limiting factor among the smallholder farmers (Ochilo *et al*., 2019). This leaves farmers with limited options who resort to heavy use of synthetic pesticides. A wider array of affordable and available BCAs are required in the country to address this problem (Ochilo *et al*., 2019). Rising environmental awareness among the consumers, depletion of natural resources and human health nutritional concerns have witnessed a paradigm shift among the farmers to use ecofriendly biological agents for production as opposed to agrochemicals (Herrero *et al.,* 2017; Alori & Babalola, 2018).

BCAs minimize health hazards that are common with local farmers who do not have an understanding of how to adopt safety precautions on labels of most agrochemicals (Alori & Babalola, 2018). BCAs can increase yield, suppress diseases and at the same time avoids environmental pollution (Alori & Babalola, 2018). Satisfactory suppression of bacterial wilt pathogen using commercially available mix of effective microorganisms have been reported (Lwin & Ranamukhaarachchi, 2006). Utilization of BCAs in management of plant diseases enhance food safety among consumers since they are biodegradable and leave no residues (Gupta *et al.,* 2014). This will address the challenges of interceptions of tomato products witnessed in the export markets.

Isolation, screening and utilization of potential microbes within the rhizosphere of bacterial wilt tolerant tomatoe*s* is considered as potential alternative to overcome adaptive nature of *R. solanacearum*. Novel bio-control agents naturally found within the soil have excellent antagonistic and mycoparasitic effects against plant pathogens including bacterial wilt disease (Yendyo *et al*., 2018) Microorganisms like *B. cereus, B. subtilis, Paenibacillus spp.*, and *Providencia vermicola* found to be dominant in bacterial wilt resistant potato rhizosphere (Chamedjeu *et al*., 2019) present a significant potential in complementing other management tools that are commonly used. Further, strains of *Streptomyces* have attracted considerable attention because of its ability to compete and inhabit similar ecological niche with pathogenic microbes (Diallo *et al*., 2011). *Streptomyces sp.* CB-75 is a good example of bio-control agent that has been demonstrated to suppress *Colletotrichum musae* disease in banana seedlings while improving its growth parameters like the leaf area, root length and root diameter (Chen *et al*., 2018).This beneficial microbe limit activities of plant pathogens by outcompeting them for space, secreting antibiotics and parasitizing the pathogenic microbes (Shishido *et al.,* 2007). Others enhance plant growth, for instance, *Pseudomonas, Azospirillum, Klebsiella, Enterobacter, Arthrobacter, Burkholderia, Bacillus, Rhizobium, and Serratia* (Zaefarian & Rezvani, 2016) thus enabling plant to fight off infections. Other organisms like *Bacillus cereus* AR156 potentially elicit immune reactions in plants and suppress numerous plant diseases including wilts and fungal infections (Wang *et al*., 2019).

Although the beneficial role played by plants microbiome for plant growth is widely known, there is scarce knowledge in the role of *In situ* rhizosphere microbiome associated with bacterial wilt tolerant plants. Yet, soil environment harbors many species of antagonistic microorganisms that live in the rhizosphere of higher plants and provides immunity to plants against certain microbes that can cause diseases. Additionally, these microorganisms significantly lower the severity of the diseases that are induced by pathogenic fungi and bacteria. The understanding and utilization of these natural phenomena under this study, provide a possible environmentally friendly approach to control devastating diseases like bacterial wilt of tomatoes.

1.4 General Objective

To contribute to sustainable tomato production in Kenya through effective management of bacterial wilt caused by *R. solanacearum* using effective rhizosphere bacterial and fungal isolates.

1.4.1 Specific Objectives

i. To isolate and characterize beneficial bacterial and fungal isolates from the rhizosphere of bacterial wilt tolerant tomato plants.
ii. To determine the efficacy of identified bacterial and fungal isolates against bacterial wilt in tomato plants.

1.5 Null Hypotheses

$H_0$1: The rhizosphere of bacterial wilt tolerant tomato plants do not harbor beneficial bacteria and fungi

$H_0$2: The rhizospheric beneficial bacterial and fungal isolates are not effective against bacterial wilt in tomato plants.

CHAPTER TWO

LITERATURE REVIEW

2.1 Scope

This part gives details on tomato production in Kenya, constrains, pathogenicity determinants of *R. solanacearum*, hosts range, disease symptomatology, geographical distribution, and microbes' diversity in the rhizosphere. It ends with the importance of beneficial microbes in bacterial wilt management.

2.2 Tomato Production in Kenya

Kenya is ranked 6th among Africa's leading tomato producers, with a total production of approximately 397007 tons (Karuku *et al.,* 2017). The crop is approximated to account for 7% of the total horticultural products and approximately 14% of the total vegetable produced in the country (GoK, 2010). Greenhouses production of tomato in Kenya accounts for 5% of the total yield whereas the amount produced in the open field is approximated to be 95% (Mwangi *et al*., 2020). It is one of the most popular fruit vegetable cultivated in various counties (Karuku *et al*., 2016) particularly by smallholder farmers holding farms ranging between 0.2 to 3 hectares and accounting for 70% of total agricultural output (Ndirangu *et al.,* 2018). Tomatoes produced in Kenya are consumed locally and some are exported to East African market (Geoffrey *et al.,* 2014) with little (cherry and dried tomatoes) getting access to markets outside East Africa. Kenya exported a total of 3380 metric tons of processed tomatoes to Tanzania, Uganda and Sudan between 2006 and 2010, earning a total of 209.7 million shillings ($2.9M) (USAID, 2012). The area under production and yield volumes among the major producing counties varied with the period of 2017-2018 as witnessed in Table 2.1.

Table 2.1: Production of Tomato in Kenyan counties from 2017- 2018

COUNTY	2017			2018			% of Total
	Area (Ha)	Volume (MT)	Value (KES)	Area (Ha)	Volume (MT)	Value (KES)	
Kajiado	2,452	54,827	1,914,835,250	3,024	71,250	2,379,680,250	12.0
Kirinyaga	3,219	60,490	2,247,500,000	2,460	60,587	2,037,800,000	10.2
Narok	2,277	54,220	1,700,200,000	2,420	54,082	1,886,227,500	9.5
Machakos	2,453	39,255	1,029,775,000	4,075	56,225	1,328,475,000	6.7
Kiambu	544	7,099	270,033,750	769	24,499	1,249,126,000	6.3
Taita Taveta	726	22,990	904,500,000	783	28,610	1,238,650,000	6.2
Makueni	575	22,250	893,600,000	931	27,675	941,600,000	4.7
Homabay	1,143	8,490	482,811,240	1,541	12,104	743,706,000	3.7
Lamu	275	10,700	242,508,000	491	16,242	693,153,000	3.5
Kisumu	663	16,341	542,320,000	536	19,030	592,650,000	3
Trans Nzoia	672	19,804	613,560,000	441	14,633	518,266,000	2.6
Kitui	311	6,743	245,790,000	735	13,588	459,685,000	2.3
Murang'a	1,258	8,888	417,409,550	1,315	9,250	448,946,300	2.3
Bungoma	538	10,041	456,710,000	564	11,129	442,570,000	2.2
Siaya	741	10,674	442,675,000	628	9,523	431,532,500	2.2
Laikipia	578	19,670	674,420,300	321	10,999	376,500,000	1.9
Bomet	545	7,535	236,650,000	550	9,849	320,578,000	1.6
Kwale	448	6,989	320,023,000	420	6,966	319,660,000	1.6
Meru	549	12,386	485,356,018	498	9,702	316,985,000	1.6
Nyeri	273	6,670	239,619,940	356	11,348	299,950,768	1.5
Others	6,812	101,214	3,018,982,134	5,405	97,167	2,877,810,938	14.5
Total	27,053	507,275	17,379,279,182	28,263	574,458	19,903,552,256	100

Source: AFA-Horticultural Crops Directorate, 2019

2.3 Tomato Production Constrains.

Tomato production is faced with numerous challenges despite being an important crop. Overtime, yield have remained low due to abiotic factors like poor soils, high temperatures, and erratic rainfall (wright *et al*., 2016). Arthropods pests, fungal, bacterial and viral diseases have also been reported as one of the major constrain bedeviling tomato production in the country (Toroitich *et al*., 2014). Additionally, failure of farmers to adopt technologies like improved seeds, post-harvest handling, physiological disorders (Geoffrey *et al.,* 2014) and overdependence and improper use of synthetic chemicals have witnessed increased losses.

2.4 Bacterial Wilt Disease

Among the diseases, bacterial wilt caused by *R. solanacearum* is a major production constrain responsible for up to 90% losses in both open field and greenhouse conditions (Chengo *et al.,* 2022). Its rampant spread of bacterial wilt has derailed production of not only tomato, but other solanacea crops as well. The extensive presence of pathogen across major tomato producing counties is attributed to high dependence of solanaceous crops (Kago *et al*., 2016), as an important source of vitamins and dietary fiber as well as income. Lack of elaborate strategies for disease management among smallholder farmers have witnessed disease persistence in the fields. Further, unregulated seed systems have contributed immensely to the increased widespread, high prevalence and increased cases of incidences of the diseases (Kago *et al.,* 2016). Despite efforts to regulate the seed industry to curb spread of diseases through contaminated planting materials, sharing of seeds in the informal seed industry has derailed the efforts. This implies that production areas that are free from bacterial wilt are not safe from infection.

Cultural strategies like crop rotation and fallowing is not practical in Kenya where productive land is scarce. Some farmers resort to improved seed, cultural practices and homemade botanical and non-botanical strategies as a way to control plant pests. Other farmers, however, do not utilize any management tool to control the pathogen (Ochilo *et al*., 2019). Though some of the strategies employed may work on other pests and diseases, their efficacy will not overcome the destructive nature of *R.*

solanacearum. High costs of chemicals, customer preference for chemical free products, developed pathogen's resistance to chemicals and environmental toxicity has made chemical control unpopular. As a result, farmers will adopt inexpensive, effective and environmentally friendly measures including formulated beneficial rhizosphere bacteria and fungi associated with bacterial wilt tolerant tomatoes.

2.4.1 Pathogenicity and Disease Symptomatology

Ralstonia solanacearum has the ability to survive saprophytically in the soil until it comes into contact with a new host (Álvarez *et al*., 2010). In the presence of susceptible host (Yao & Allen, 2007), the pathogen mobilizes pathogenic resources to initiate the process of infection (Tans-kersten *et al.,* 2001). They utilize flagellar and pili to move towards plant roots upon sensing chemical compounds from the host's roots (Tans-kersten *et al.,* 2001; Yao & Allen, 2007). During the interaction with the plants, the pathogen involves sophisticated invasion strategies to allow their survival and ability to overcome plant defense systems (Li *et al*., 2021). The pathogen utilize lipopolysaccharides to attach to the root surface before colonizing sites of root elongation. As a response, plants develop a powerful network of defense systems, including layers of physical barriers, preformed defenses, and inherent immune systems to keep off the pathogens (Zhang *et al*., 2020a). Unfortunately, these barriers, in the presence of favorable conditions and highly virulent pathogen are overcome and successful infection takes place.

Expression analysis of the pathogen's gene has indicated many virulence determinants and its regulatory network (de Pedro-Jové *et al*., 2021). The pathogen can enter into the plant through wounds induced mechanically or natural wounds formed at root axils (Wubshet, 2018) during lateral root elongation. The invasion of the host plants starts with the pathogen entering the roots then colonizing its intercellular spaces before eventually entering the xylem vessels where they vigorously replicate to reach high population density (Khokhani *et al*., 2017). Even though resistant cultivars reduce the multiplication of bacteria by limiting its movement across the xylem tissues (Kim, *et al.*, 2016a), the bacterium overcomes this resistance with time. The pathogen turns on its own metabolisms and alter sap

biochemistry in the high flow xylem vessels of the host's plant as a survival strategy to survive under harsh condition within the xylem (Lowe-Power *et al.,* 2018). It systematically spreads inside the vascular system and reaches the aerial parts of the plant (Ombiro *et al*., 2018). The rapid multiplication increases the level of EPS which eventually block the flow of water to plant shoots, leading to wilting (Hikichi *et al*., 2017). Symptoms of the disease depend on the internal and external factors.

Common symptoms include wilting of young leaves, stunting, yellowing of the foliage (Lowe-power *et al*., 2018) and eventual death of the plant (Agrios, 2005). Other symptoms include downward bending of leaves and growth of adventitious roots on the stems, followed by the formation of narrow dark stripes that correspond to infection of vascular bundles found beneath the epidermis (Aslam & Mukhtar, 2023). The aggressiveness of *R. solanacearum* strain and the susceptibility of the host determine the expression of the symptoms and the rate at which the disease develops. Vascular tissues, mostly the xylem get discolored at early stages of infection (Kim *et al*., 2016b). As the disease progress, discoloration of portions of the pith and cortex takes place, which can later progress to necrosis. The appearance of slimy viscous ooze usually at the points that correspond to vascular tissues will be seen if the stem is transversely cut (Álvarez *et al*., 2010). As a result of the destruction of surrounding tissues and xylem degradation, collapse and death of the plant will occur (Kim *et al*., 2016b; Wubshet, 2018). After host's destruction, the pathogen can continue surviving by getting back into soil, water, and even other wild hosts plants acting as reservoirs (Prakasha *et al*., 2016). Under the harsh environmental conditions, *R. solanacearum* can change into viable but not culturable (VBNC) state, allowing it to survive and later build up inoculum to a level capable of causing a disease, which ultimately results in substantial economic losses (Elsas *et al*., 2001; van Overbeek *et al*., 2004).This step by step infection and life cycle of the pathogen is depicted in Figure 2.1 below.

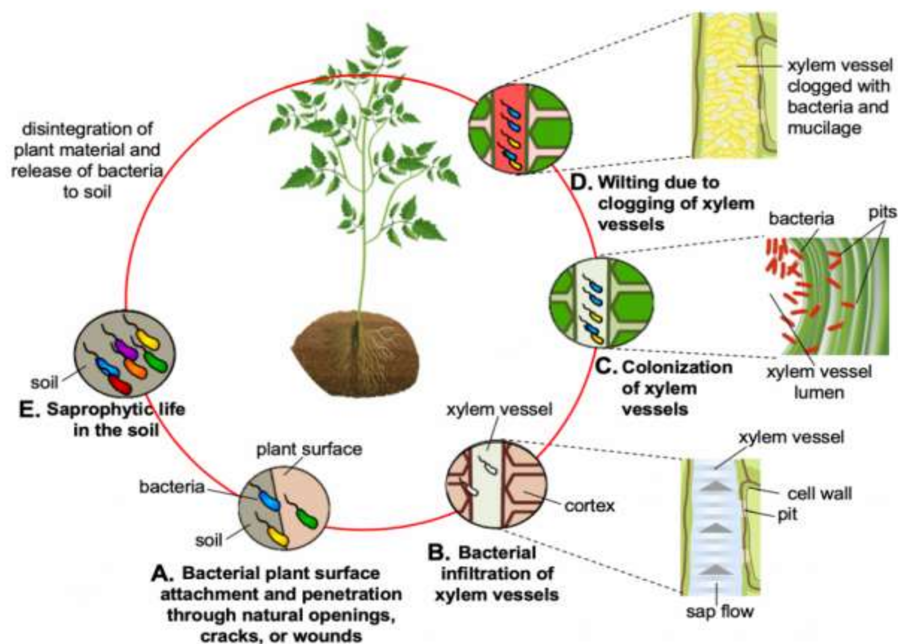


Figure 2.1: Different phases of *R. solanacearum* life cycle (Alam & Rustgi, 2020).

The detection of the pathogens using various means are further complicated by its ability to convert into VBNC state (Ascarrunz *et al*., 2011) which does not manifest signs of the disease. For instance, *R. solanacearum* biovar 2 strain KZR-5 survives conditions of starvation as viable colony forming units and responds to stress by enhancing its tolerance to stressors, allowing it to easily adapt unfavorable environmental conditions (Álvarez *et al.,* 2008). This makes detection and control quite difficult.

2.4.2 Pathogenicity Determinants of *R. Solanacearum*

Ralstonia solanacearum requires specialized gene network to aid in getting into the stem of the host plants from the soil environment. These complex gene products are useful not only in avoiding plant innate defense systems, but also in the acquisition of nutrients, penetration of xylem tissues and movement across the plant cellular networks. Important extracytoplasmic virulence and transcriptional factors encoding for virulence in the pathogen are regulated by an intricate network of unique pathways of signal transductions that interacts with one another (Álvarez *et al*., 2010). Five gene *Phc* systems are the core in this sensory network regulating

exopolysaccharides, exoenzymes that degrade the cell wall (Allen *et al*., 2007) and other factors that control pathogens growth and its response to the environment. Additionally, *Prh* genes activates the secretion of type III enzymes following a complex transduced signaling pathway derived from cells of the plant using a six-gene complex (Schell, 2000). Additionally, *R. solanacearum* possess genes that contain several enzymes that scavenge reactive oxygen species (ROS), allowing it to tolerate ROS during pathogenesis. They express a diverse response to ROS stress or simply tolerate that harsh oxidative environment (Flores-Cruz & Allen, 2009). R. *solanacearum* also secretes numerous cell wall degrading enzymes, for instance, polygalacturonases and endoglucanase (Breed *et al*., 1988), that contribute significantly to pathogen's ability to induce bacterial wilt disease (Meng, 2013).

2.4.3 Management of Bacterial Wilt

Integrated pests management that involves judicious use of chemical, physical and utilization of cultural practices are currently the most option by most farmers. Cultural control methods commonly used by smallholder farmers encompass techniques that increase quality and quantity of yield while reducing impacts of plant pathogens (Ajilogba & Babalola, 2013). These methods include, for instance, sanitation, use of resistant cultivars, and crop rotation. Crop rotation reduces pathogen population in the soil, but one must entirely eliminate plant residues containing the pathogen if the pathogen must be controlled effectively. Sanitation, on the other hand, involves completely destroying infected plants, using sanitized footwear and clothing and cleaning farm tools before reusing them during field operations (Acero *et al*., 2008). This ensures that contaminated soils containing *R. solanacearum* inoculum are not spread within the farm. Survival of the pathogen deep in the soil limits the efficacy of physical strategies, for instance, soil solarization (Barchenger *et al.,* 2022), bio-fumigation and steaming. These habitats makes it hard for the strategies to come in contact with the pathogen.

Utilization of resistant cultivars developed through genetic insertion, has over a long time been considered as the most effective control strategy (Yuliar *et al*., 2015) for bacterial wilt. Such cultivars have indeed demonstrated reduced susceptibility of

vascular tissues to the disease (Kurabachew & Ayana, 2016), but public acceptance is always required before their commercialization (Mamphogoro *et al*., 2020). Physical and cultural methods, on the other hand, are environmentally and cost friendly, but their efficacy is derailed by the existence of diverse pathogen's strains that allows their survival in the soil, various plant's organs and broad host range (Imada *et al.,* 2015; Aloyce *et al*., 2017; Ahmed *et al*., 2022). Chemical control, which involves utilization of different chemicals to suppress bacterial wilt, have been relied over the years but most are rendered not highly effective when used alone (Yuliar *et al.* 2015). The inefficiency of the chemicals is attributed to bacterium's ability to invade the plant and get sheltered in the xylem vessels of infected plants where chemicals would not reach (Aloyce *et al.,* 2017). Additionally, farmers have reluctance in using them, due to costs and ineffectiveness that result from pathogen's developed resistance (Xue *et al.,* 2009)**.** Reported cases of chemical residues found in food products confirms the negative effects of pesticides on environment and human health (Romano *et al*., 2020).

One of the method that has been identified to have greatest potential to overcome bacterial wilt menace is the use of beneficial rhizospheric microbes. This strategy utilizes living organisms adapted to specific environment commonly preferred by bacterial pathogens, to kill such non-beneficial organisms. It is considered as an important element in integrated pest management aimed at reducing use of synthetic fungicide (Mamphogoro *et al.,* 2020). According to Zhang *et al*. (2007), antagonistic bacteria inhabiting the rhizosphere have shown positive impact on the growth of the plant and management of the plant pathogens. Such potential rhizosphere microbes can potentially sustain itself and spread after successful initial establishment, and thereby effectively offering a longer suppressive impact to plant diseases (Quimby *et al.,* 2002; Whips *et al*., 2007). As a result, there has been an upward trend in the preference for the use of beneficial bacteria and fungi to control the spread of *R. solanacearum*.

2.5 Isolation, Characterization and Utilization of Rhizosphere Microbes as Biocontrol Agents

Lack of effective, affordable, and environmentally friendly methods of controlling persistent plant pathogens including bacterial wilt have greatly driven research towards looking at use of beneficial microbes as an alternative (Tang *et al.,* 2023). Harnessing of these beneficial microbes for agricultural application depends on suitable source and isolation techniques, proper identification and test of their viabilities in suppressing plant pathogens. This informs the approaches needed towards harnessing their natural plant induced tolerance. Healthy rhizosphere is considered as one of the excellent sources for the isolation of the beneficial microbes (Yang *et al.,* 2012) as compared to the surrounding bulk soils (Praeg *et al.,* 2019). The rhizosphere microorganisms associated with tolerant plants could act as commensal and symbiotic organisms through diverse mode of action including inhibition or displacement of pathogens within the niche, thus supporting plant immune system and acting as plant growth promoters (Anguita-Maeso *et al.,* 2020). The diversity and composition of microbes within the rhizosphere is majorly considered a function of soil properties and that diverse species of plants and the soil properties (Bulgarelli *et al*., 2013; Leach *et al*., 2017; Jiang *et al.,* 2017). Further, studies have suggested that plants play significant role in recruiting beneficial microbes during pathogen attack (Hacquard *et al*., 2017; Yin *et al*., 2021). Sophisticated communication between plants roots and microbes via chemical signaling within the rhizosphere results in bio-filming of the beneficial microorganisms, which in turn induce systemic resistance in hosts plants (Mhlongo *et al*., 2018). A number of lab based techniques have been fronted assist in unravelling the diversity and evaluating the antagonistic properties of rhizospheral microbes against plant pathogens.

Culture-dependent method can be used with other approaches like next generation sequencing and meta 'omics' to profile microbial assemblages (Li *et al.,* 2021) in the rhizosphere. Through the use of various isolation approaches, many PGPRs have been positively isolated from the of healthy plants' rhizosphere soils (Huang *et al*., 2013) and their efficacy under greenhouse and *in vitro* trials have been tested (Wei *et*

al., 2011). Evidences of isolated microbes effectively working against pathogen have been extensively documented. Plant growth promoting rhizobacteria (PGPRs) which are abundant in the rhizosphere of healthy plants have been relied upon to manage bacterial wilt caused by *R. solanacearum* (Huang *et al*., 2013*).* Majority of these microbes inhabiting the rhizosphere facilitate acquisition of nutrients by the plants and, on the other hand, suppresses pathogenic invasions (Leach *et al*., 2017; Bulgarelli *et al.,* 2013). These include, for instance, *Bacillus* spp. (Miljaković *et al.,* 2020; Hashem *et al*., 2019; Cao *et al*., 2011), *Pseudomonas* spp. (Qessaoui *et al*., 2019), and *Streptomyces* spp. (Le *et al*., 2021; Le *et al*., 2022; Viaene *et al*., 2016). Moreover, bacteria utilize diverse mechanisms of antagonism that may include the release of low weight molecular compounds like antibiotics, lytic enzymes, siderophores, and even toxins (Le *et al.,* 2021; Pliego *et al*., 2008). This strategy gives the microbes an added advantage to survive a competitive rhizosphere environment where there is a stiff competition for nutrients. Apart from enhancing nutrients availability through nitrogen fixation, and solubilization of soil phosphorus, *Bacillus* species can as well produce siderophores that can promote plant growth while suppressing the pathogens (Hashem *et al.,* 2019).

Microorganisms present in the soil produce metabolites that are useful in the gene signaling within the host plants. One of the metabolites is extracellular polysaccharides (EPS) which have been reported to significantly reduce bacterial wilt of eggplant by 51% under *in vitro* and greenhouse conditions (Prakasha *et al*., 2017). Extracellular polysaccharides for instance, have been demonstrated to induce systemic resistance thus reducing the accumulation of hydrogen peroxide in plant tissues (Valepyn *et al*., 2014). *Flavobacterium* isolated from the rhizosphere of resistant plant is another examples of effective rhizosphere bacteria that suppressed *R. solanacearum* of tomatoes in a pot experiment (Kwak *et al.,* 2018), through production of metabolites. Characterization and tests of viabilities of isolated rhizosphere microbe is essential in harnessing their prospective ability to suppress pathogens that can potentially cause plant diseases.

2.6 Application of Beneficial Microbes in Disease Management

Rhizosphere soil is regarded as a system in which its interaction with the soil microbes creates complex soil microbial activities that may promote plant growth. Diversity of microbes in the soil is important in influencing the quality and health status of the soils (Zhao *et al*., 2018). The composition of rhizosphere microbes is collectively determined by plant genotype, biochemical properties of the rhizosphere soils and the available bacterial sources (Cheng *et al.,* 2020). Apart from utilization of structural modification, exudation of secondary metabolites and coordinating mechanisms for defense strategies, plants also detect pathogenic microbes through the surveillance of soil niches around the root (Pascale *et al.,* 2020). The release of chemicals signals into the environment by the plants during interaction with its microbiome can lead to either a negative or positive impact on other plants or other members of the rhizosphere microbiome (Jones *et al.,* 2019). Numerous studies focus on the use of beneficial microbes that can inhibit growth and multiplication of pathogens through complex metabolic activities resulting from interactions between plant-beneficial microbes and pathogen-plant-microbes interaction (Mhlongo *et al*., 2018). Possible biocontrol mechanisms include production of antimicrobial compounds, niche exclusion, nutrients acquisition, direct antagonists and resistance induction (Ahmed *et al.,* 2022). These mechanisms not only protects the plants from diseases (Aarab *et al.,* 2015) but also improve their growth.

The presence of complex and diverse rhizosphere microbes is quite essential for the maintenance of homeostatic balance within the soil ecosystem (Liu *et al.*, 2016). Rhizosphere soils which are influenced by root growth and microbial activities have gained serious agricultural research attention because of its importance in promoting the production of crops and enhancement of soil health (Singh *et al*., 2014). Microorganisms like the gram-positive *Bacillus* sp. have been found attractive since they form heat and desiccation resistant endospores which allow them to be easily formulated as a stable powder for proper storage and better shelf life (Wulff *et al.,* 2002). The ability of beneficial microbes to induce growth in plants species is unique in terms of hosts specificity and interactions. This unique relationship between plant growth promoting regulators could involve specific interaction as well as the

recognition process. This can be through bio-fertilization, stimulation of root growth, and suppression of soil-borne pathogens using different mechanisms (Dinesh *et al.*, 2015).

Isolated microbes can be used to create products like bio-fertilizer which contains living organisms that can promote plant growth, health and increased yield (Fuentes-Ramirez & Caballero-Mellado, 2005). For instance, *Lysinibacillus sp., Bacillus subtilis* and *Pseudomonas cepacea* that solubilize phosphates, were able to suppress *R. solanacearum* in chili by 66.7-80 % when combined (Istifadah *et al.,* 2017) as one treatment during application. According to Dawwam *et al.* (2013), different mechanisms are involved in plant promotion by the rhizosphere microbes. Bacteria produce plant growth regulators that play a role as chemical messengers capable of inducing plant growth and development. *Burkholderia cepacia* MPC-7 bacteria, for example, solubilize insoluble phosphate through various processes that include the production of gluconic acid (Pathak *et al.*, 2017) acidification, and chelation and exchange reaction. These processes make the phosphate readily available to plants, thereby enhancing root growth and optimal uptake of other required nutrients within the soil (Chabot *et al.,* 1996).

Microbes out-compete plant pathogens by colonizing the rhizosphere and through the production of diverse antibiotic compounds like lipopeptides (Alvarez *et al.,* 2012). *Pseudomonas* species, including *P. aeruginosa*, *P. syringae* and *P. fluorescens* suppress *R. solanacearum* growth through production of enzymatic materials which included lipase, protease and a-amylase enzymes, capable of disintegrating the genetic make-up of the pathogen (Mohammed *et al.,* 2020). The antagonistic activities of the microbial toxins degrade polymers by breaking down compounds of the pathogen (Wilhite *et al.,* 2001), as a way of accessing carbon as a source of energy (Heydari & Pessarakli, 2010).Volatile organic compounds (VOCs) made up of alkanes, alkenes and ketones; sulfur compounds (Mhlongo *et al*., 2018) produced by bacteria like *Bacillus amyloliquefaciens* FZB42 and *Bacillus artrophaeus* LSSC22 are low molecular compounds synthesized using different metabolic pathways. These volatiles have been demonstrated to reduce wilt index and induced systemic resistance on tobacco inoculated with *R. solanacearum*, enhancing the

concept that VOCs can suppress plant pathogens (Tahir *et al.,* 2017). *Bacillus cereus* AR156 is an example of efficient bacterial strain with the ability to control a broad spectrum of plant diseases while eliciting an immune response in plants. The interaction between the plants and *B. cereus* AR156 can result in changes in the composition of root exudates from plant and this could directly affect the interaction of plant pathogens and *B. cereus* AR156, therefore, affecting the development of both microorganisms (Wang *et al.*, 2019).

Antimicrobial VOCs induce systemic resistance in plants and destroy the integrity of pathogenic deoxyribonucleic acids (DNA), thus hampering its ability to cause infection (Tahir *et al*., 2017). This event of interfering and distorting the integrity of pathogenic genetic machinery induces a change in the level of enzymes that are related to ontogenesis expression. This in turn can alter the growth of the targeted organisms. Inoculation of plants with isolated and formulated rhizosphere microbes can induce protection against destructive plant diseases that cause significant yield losses. These microbes induce plants to activate responsive mechanisms that can allow aversion or tolerance of infection, resulting in considerably increased yield and plant quality at lesser ecological destabilization (Ramirez & Mellado, 2005; Vishwakarma *et al*., 2020). Additionally, the microbial consortia currently utilized in the agricultural management systems can return the soil whose chemical, physical and biological balance has been altered by the use of chemical inputs (Woo & Pepe, 2018). This achievements can point out at a possibility of achieving sustainability in agricultural production through adoption of BCAs as part of important agricultural inputs.

CHAPTER THREE

MATERIAL AND METHODS

3.1 Scope

This chapter is composed of sub-sections with detailed information on the sampling areas, rhizosphere sampling, isolation, morphological and biochemical characterization of rhizosphere microbes. It also includes pathogenicity tests of isolated *R. solanacearum*, laboratory and greenhouse tests of potential antagonists, experimental design, data collection, processing and analysis.

3.2 Sampling Areas

To identify pockets of bacterial wilt tolerant plants, areas corresponding to tomato production were selected from two specific points per county across the four counties namely Bomet (Kapletundo), Kiambu (Gatundu north), Kajiado (Kitengela), Kirinyaga (Kianguku). Coordinates corresponding to the sampling sites were taken and recorded (Table 4.1). These areas are considered to be suitable for production of solanaceaous crops including tomato and potatoes over continuous period of time. This makes it possible for *R. solanacearum* to be persistence because of continuous availability of hosts.

3.3 Climate and Soil Conditions of the Sampling Sites

The climatic conditions affect the plant physiology which in turn impacts rhizosphere microorganisms. The potentiality of plant growth promoting microorganisms to enhance plant health, growth, diversity and their survival is influenced by non-biological factors that include soil nutrients, pH, and temperature (Hernández-Montiel *et al*., 2017). Soil pH have a significant effect on nutrients availability to plants which in turn affects selection of microbes in the rhizosphere soils. With this in mind, and with an aim to clearly understand the relationship of the climatic conditions and the soil microbiota that would determine its richness, data on below ground conditions, rainfall and temperature were collected from meteorological stations from the counties in question. Additionally, the composite soil obtained from

the four counties were analyzed for level of nitrogen, phosphorous, potassium, pH and electrical conductivity (EC) using different validated procedures. Determination of the soil pH and EC was done using a pH meter (AD8000 from ADWA) buffered using two buffer solutions (buffer 7 and buffer 4). Washing of the dipped electrode was done using distilled water before dipping it into suspension and recording the pH of the sample. The electrical conductivity, on the other hand, was determined using an EC meter. Salt content in the soil solution was displayed digitally after the electrodes were dipped into solution of the soil samples. Further, analysis of essential nutrients that included nitrogen, potassium, and phosphorous in the collected samples was also done. The amount of phosphorous in the soil was evaluated using a modified Bray No2 method, where calibration curve of absorbance was plotted against the amount of phosphate in the standards (FAO, 2021a). The amount of phosphate in the filtrate was then read from the absorbance and the calibration curve.

The available phosphate (P_2O_5) mg/kg= (C*20/V*0.001*1000*f), where C=the amount of phosphate in the v ml of the filtrate (ug-P_2O_5), V is the sample volume placed into the volumetric flask (ml), and f= is the correction factor in the sample. Soil were analyzed for nitrogen through the use of Kjeldahl procedure (FAO, 2021b).The percentage of total nitrogen available in soils was calculated as; %N in the soil sample = (a-b) *0.01*V*100/*W*al. Where a=the volume of the 0.01N HCL standard consumed by the sample, b= the volume of the standard 0.01N HCL consumed by the blank, V= the final volume of the digestion (100ml), W= the sample weight taken (0.3g) and al. = aliquots taken for the analysis (10ml). Determination of potassium content in the soil sample was done by calculating the concentration of potassium as follows; K=C*V*F*100/1000*W where; C= (a-b), and a=the concentration of potassium in the sample extract, b =concentration of the element in the blank extract, V=the volume of extract solution, W=weight of the sample and F= was the dilution factor.

3.4 Sampling of Rhizosphere Soils and Plants

Rhizosphere soils and the plants samples of both infected and healthy tomato plants were purposively collected (Gashaw *et al.,* 2022) in the fields in four counties

namely; Bomet, Kirinyaga, Kiambu, and Kajiado (Kenya). This is where tomato production is intensive and bacterial wilt disease is known to be severe. Typical symptoms associated with bacterial wilt viz., leaves epinasty, yellowing, necrosis and the emergence of the adventitious roots on stems and vascular browning (Narasimha & Srinivas, 2012; Seleim *et al*., 2014) were used to identify infected plants for the isolation of *R. solanacearum.* Two samples of both infected and healthy plants (rhizosphere soils and whole plant) were collected from sampled fields for a total of two fields per county. The collected samples were placed in khaki bags (Yanti *et al*., 2016) and tagged according to the location and date of collection and taken to JKUAT lab for storage in a refrigerator at 4° C to preserve the microbes until they were isolated a day after storage.

3.5 Isolation of Rhizosphere Microbes

Isolation of *R. solanacearum* from freshly diseased plants from each sample was done by plating 0.1ml aliquot of suspension containing bacterial ooze in TZC media before incubating the plates at 28^{0} C for 48 hours. Pink centered and mucoid colonies were aseptically picked and placed in viol tubes containing sterile distilled water (Seleim *et al.,* 2014) at room temperature for subsequent experiments. Isolation of potentially beneficial bacteria and fungi from the rhizospheric soils collected from healthy plants was done using serial dilution method. Ten grams of each soil sample were placed in a 250ml Erlenmeyer flasks with 90ml of sterile distilled water and agitated for 10 minutes with a magnetic shaker before picking suspensions for the preparation of an eight-fold serial dilution. Isolation of potential beneficial bacteria was done by plating an aliquot of 0.1 ml serial dilution from each soil sample in nutrient agar (from HiMedia laboratories) and plates were incubated for 48 hours at 28 ^{0}C. On the other hand, fungi were isolated by sprinkling soil from healthy rhizospheres on potato dextrose agar (from HiMedia laboratories) plates prepared and left to cool overnight. The media for isolation of fungi was amended with 25 mg/l of chloramphenicol to hinder the growth of bacteria. The plates were then placed in an incubator at 28° C for 72 hours (Mwashasha *et al*., 2016) for fungi to grow. After incubation period, observations were made and individual isolated colonies were purified by sub-culturing individual colonies on plates containing their

respective freshly prepared media. After incubation period, pure isolates of bacteria were kept under 20% glycerol stock at -80^0 C. Fungi were stored in potato dextrose agar slants flooded with 20% sterile glycerol before keeping them under -80^0 C.

3.6 Characterization of Fungal and Bacterial Isolates

Fungal isolates were characterized morphologically whereas bacterial isolates were characterized both morphologically and chemically. Fungal isolates were morphologically characterized based on the surfaces, reverse, and peripheral color. Additionally, their elevation, and the shapes of the margins were also used. Illustrations found in Barnet and Hunter (1987) were then used to compare and identify the genus level that each isolate belonged based on microscopic observations. The identification of pure fungal colonies was done to *Genus* level based on morphological characters, which included the elevation, margin, color of the surface, reverse, and periphery. Other information included the nature of their hyphae, whether they were septate or non-septate, was also used as shown in Table 4.3 and as presented on Plate 4.1.

3.6.1 Morphological Characterization of the Fungal Isolates

Fungal isolates were morphologically characterized according to Barnette and Hunter (1987) through microscopic and macroscopic observations. Small pieces of fungal growth were cut using a sterile inoculating needle then mounted on glass slides with two drops of lactophenol blue. To spread the fungal growth, teasing using teasing needle was done to sparsely spread hyphae before covering it with a cover slip. The slides were then mounted on a light microscope for observation. Heavy sporing isolates like *Aspergillus sp.* were washed with 70% ethanol to remove the clumps. Compound microscope was used to observe the type and shapes of spores, surface, peripheral and reverse color, hyphae septation, elevation, and margins of the purified fungal isolates. The *Genus* name of each fungal isolate was concluded after comparing spores against those found in Barnett and Hunter (1987) key.

3.6.2 Morphological Characterization of Bacterial Isolates

Characterization of the bacterial isolates involved examination of colony morphology and culture features that included color, pigmentation, margin, elevation, shape, size and growth form. By using the slide procedure, isolates were stained with safranin and observed using dissecting and compound microscope. The identification of different bacterial isolates was done, according to Robert *et al*., (1957) where the unknown group numbers were first determined. Once the group number was known, more information were obtained by going to the pages of the groups identified within the manual. Other descriptive features of the bacterial isolates were observed by looking at their morphological characteristics which included shapes, elevation, color, their margins *(*Swarupa *et al*., 2014), Gram status, and their motile status.

Gram staining of pure isolates of bacteria was done by spreading a thin layer of bacterial isolates on a glass slide before mixing it gently and letting it to dry. Fixation was done by briefly passing the smear through a flame. The bacterial cells were then mixed gently then flooded with crystal violet and leaving it for one minute. While holding the slide in the slanting position, sterile water was used to wash crystal violet stains before using Gram's iodine to flood the cells and allowing it to stand for one minute before re-washing. To decolorize the stains cells, 95% ethyl alcohol were added drop by drop to the slides held gently in slanting position, until the drops of ethyl alcohol turned clear. Rinsing using sterile distilled water was again done before safranin was used to counterstain for 45 seconds. The slides were then blot dried before using a light microscope to observe the cells under oil immersion (Cappuccino and Sherman, 2014). Results obtained during gram staining were confirmed by adding 3% KOH drop by drop (Halebian *et al*., 1981) before introducing loop full bacterial cells and mixing it thoroughly for about 1 minute then lifting the wire loop gently. Observation was done and those identified as gram positive formed watery suspension that could not follow the wire loop. On the other hand, bacteria that formed a thread of slime following the wire loop for about 0.5 to 2 cm were considered as positive for KOH and, therefore, were concluded as gram negative (Dida *et al*., 2018) bacterial isolates.

All bacterial isolates were also subjected to motility tests using sulfide indole motility (SIM) medium from HiMedia laboratories. This was done by inoculating tubes containing SIM agar prepared and left to col overnight with 24 hour old bacterial isolates. With the help of sterile needle, bacterial cells were inoculated when a single stab was made at the epicenter of the tube to almost a half depth of the medium. The tubes were placed in the incubator at 37 ^{0}C for 24 h. When the inoculated medium changed from golden clear to turbid, bacteria was recorded as positive for motility whereas those that remained restricted to the line of inoculation were recorded as negative for motility.

3.6.3 Biochemical Characterization of Isolated Bacteria

Biochemical tests often reveal vital information useful in the accurate identification of the genera of various bacterial isolates from the rhizosphere soils. These tests included motility, MR-VP, catalase activity, starch hydrolysis, triple iron sugar and citrate utilization tests (Dinesh *et al*., 2015). Both microscopic descriptions and biochemical characteristics that were demonstrated by the bacterial microbes were used to identify bacterial isolates to the *Genus* level according to Krieg et al. (2010).

3.6.3.1 Methyl Red-Voges- Proskauer (MR-VP) Test

This test was conducted by inoculating bacterial isolates obtained into MR-VP broth in tests tubes in duplicates before shaking and incubating them at 37 ^{0}C for 72 h. 5 drops of methyl red indicator was added to an aliquot of each bacterial culture and observation for color change was made. Those bacterial cultures that turned red upon adding drops of methyl red indicator were recorded as positive for methyl reaction whereas those that turned to pale yellow were recorded as negative for methyl reaction. On the other hand, VP tests were done by adding Barrit's reagent (4% KOH and 5% alpha naphthol in 95% ethanol) before observation was made. Isolates recorded as positive for VP reaction produced deep coloration whereas those that produced deep red coloration were recorded as negative for VP tests (Cappuccino & Sherman, 2002).

3.6.3.2 Catalase Activity

Catalase tests helps in detecting the activity of catalase enzyme available in anaerobic bacteria that contain cytochrome complex. Microbes containing catalase enzymes are able to decompose hydrogen peroxide to water and oxygen, thus safeguarding the microbes from the harmful effects of reactive oxygen species. Bacterial colonies incubated for 24 hours were aseptically picked from the plates and then placed on glass slides. The colonies placed on the slides were flooded with a 3% hydrogen peroxide solution (H_2O_2) before observations were made. Those that formed effervescence, as a result of breaking down hydrogen peroxide to water and oxygen were recorded as catalase positive. On the contrary, those isolates that were recorded as catalase negative did not produce effervescence due to lack of catalase enzyme that would break hydrogen peroxide to water and hydrogen. Catalase enzyme is critical in defending the bacteria against oxidative stress catalyzing the decomposition of H_2O_2. Additionally, catalases are crucial in diverse cellular processes like metabolite production, cell development and differentiation.

3.6.3.3 Starch Hydrolysis Test

Gram's solution were used to flood bacterial isolates that were streaked on plates containing starch agar and incubated for 48h at 37^0 C before observations were made. Bacterial isolates whose growth margins showed clear zones were recorded as positive for starch hydrolysis, whereas those whose margins were not cleared were recorded as negative for starch hydrolysis. Those that hydrolyzed starch can easily hydrolyze complex sugars into sub-units transportable into cells for metabolism. This therefore enhance bacterial survival in its environment.

3.6.3.4 Triple Sugar Iron Agar Tests

To determine whether bacterial isolates could produce hydrogen sulphide (H_2S) and gas, triple sugar iron tests were performed on all the bacterial isolates. Bacterial isolates were streaked on the agar slants of triple sugar iron that had been prepared and left to cool overnight. After 24 h at 37 ^{0}C in an incubator, the fermentative activities of individual bacteria were determined by observing the color of slants and

butts of the tubes. The presence of yellow color on both butts and slants implied that both the slants and butts acidic. Contrary, presence of red color indicated alkaline slants, and red or orange-red color implied that the butt was alkaline. Production of hydrogen sulphide within the medium is always indicated by blackening of color. However, all the purified bacterial isolates obtained were not able to produce any blackening in the slants and, therefore, it was concluded that none was positive in the production of hydrogen sulphide gas. Further, the presence of the gas as a result of bacterial isolate was indicated by the formation of a transparent space between the slants and the butts. In this case, only one bacterial isolate was able to form transparent space between the butt and the slant.

3.6.3.5 Citrate Utilization

Citrate utilization test was done to determine whether isolated bacteria could utilize citrate as the only carbon source. Sterile wire loop was used to tab bacteria isolated before using the same wire loop to tap on Korsa citrate medium that had been prepared and left overnight to cool in the test tubes. Observation for color change, for instance, from clear to turbid was observed after incubating the tabbed test tubes for 24 h at 37^0 C. Those that change color of the medium to blue were recorded as positive for citrate utilization, whereas those that did not record any change in color were negative for citrate utilization.

3.7 Determination of Efficacy of Fungal and Bacterial Antagonists against *R. Solanacearum*

3.7.1 Pathogenicity Test for Isolated *R. Solanacearum*

Pathogenicity test of isolated *R. solanacearum* was first done before assessing the efficacy of isolated potential beneficial fungal and microbial isolates obtained from the four endemic regions in Kenya. Pre-cultures of *R. solanacearum* were plated on 2,3,5-triphenyltetrazolium chloride (TZC) medium, and incubated for 24 hours before observing the presence of pink centered, irregular shape, mucoid colony (Kelman 1954) typical to virulent *R. salanacearum.* Sterile pipette teats were used to pick pink centered and fluidal colonies and placed into fresh prepared CPG medium

(0.1% Casamino Acids, 0.5% glucose and 1% peptone) (Hendrick & Sequeira, 1984) before placing them in a rotary shaker at 28 ^{0}C with agitation of 200 rpm for 72 hours. The medium were then centrifuged at 5000 rpm for 10 minutes at room temperature before they were suspended again in sterile distilled water then adjusted to pathogen density of 10^8 cfu/ml by adding more bacterial cells if the suspensions were found to be low or adding more sterile distilled water where the suspension was too concentrated (Chamedjeu *et al.,* 2019). Haemacytometer was used to determine the cell concentration in the suspension intended for *in vitro* bioassays.

Three weeks old Rio-grande tomato seedlings at 4-true leaves stage raised using sterile media and nutrients supplied in form of Hoagland's solutions were uprooted then washed with distilled water before cutting the roots tips using sterile scalpel. The seedlings were placed on sterile plastic half-filled with bacterial suspensions and firmly fitted with sterile Styrofoam to hold the seedlings firmly upright. The experiment consisted of five treatments, with the fifth set up containing sterile distilled water, instead of bacterial suspensions, to act as control experiment. The set up were placed in growth chamber at 25^0 C and 80% relative humidity. Tomato plants were monitored regularly for development of typical wilt symptoms, and the severity of bacterial wilt disease while following the severity scale; percentage of shoot wilted, using a scale of 0-5 where 0=No symptoms, 1=one leaf wilted (1%-25%), 2=2 or 3 leaves wilted (26%-49%), 3=half plant wilted (50%-74%), 4=all leaves wilted (75%-100%), 5=Plant dead) (Tans-Kersten *et al*., 2001). After observation of bacterial wilt symptoms, infected tomatoes were uprooted and washed with tap water before dipping into 70% ethanol for 3 minutes. They were then rinsed with distilled water and dried using paper towel before longitudinally cutting them to observe brown discolorations on the vascular bundles. Streaming of *R. solanacearum* from each infected set up was done by cutting two stems above the crown from each treatment and leaving them in the clean bench before they were dipped into glass beakers containing sterile distilled water. Ooze were plated in NA medium amended with TZC and placed in an incubator for 48 hours at a temperature of 28 ^{0}C before morphological observation were made (Baitani, 2017). The presence of brown discoloration on the vascular bundles in the dissected stem and presence of irregular,

pink centered and mucoid colonies confirmed that wilting of tomato plants were as a result of virulent *R. solanacearum*.

3.7.2 Multiplication of Potentially Beneficial Bacterial and Fungal Isolates

Pure isolates of potential fungal and bacterial isolates were multiplied on freshly prepared media. Fungal isolates were plated on freshly prepared PDA amended with chloramphenicol at the rate of 0.25g/l (Carnot *et al*., 2017) to suppress bacterial growth. The plates were placed in an incubator at temperature of 28^0 C for 72 hours until they were fully grown. On the other hand, potential bacterial antagonists were multiplied using nutrient broth (10g peptone, 10g beef extract, 5g sodium chloride, final pH 7.3 ± 0.1 in one liter of sterile distilled water) 48 hours prior to maturity of fungal isolates. This was done by picking a colony of a 2-day old culture of isolated bacterial antagonists using sterile wire loop and aseptically inoculating into the freshly prepared broth contained in 250ml conical flasks.

The flasks were incubated in a rotary shaker for 72 hours at 150 rpm at 28± 2˚C then amended using sterile distilled water (Yanti *et al*., 2016) to a desired concentration of 1.0 x 10^9 CFU/ml (Lwin & Ranamukhaarachchi, 2006) after spectrophotometrically observing its otical density using a spectrophotometer (PD-3000UV from APEL). Spores from potential beneficial fungi were harvested from petri dishes grown on PDA by flooding the petri dishes using 50ml of 0.85% normal saline (Santiago et al., 2015). Sterile glass spreader was used and the solutions were passed through four sterile absorbent cotton wool plugs stacked together, to remove any fragments of hyphae that were present. The number of spores were counted using haemocytometer and amended to 10^9 CFU/ml by using sterile distilled water. The amended suspense was poured into sterile universal tubes and placed in a rotary shaker for 24 hours for agitation of spores and extraction of secondary metabolites.

3.7.3 Laboratory Screening of Antagonists

Fresh prepared petri dishes containing NA were seeded with an aliquot of 100µl of $1x10^8$ CFU/ml virulent *R. solanacearum* suspensions and 3 sterilized paper discs measuring 6mm were placed at equidistant. The discs were impregnated with 10 µl

of 24 hour old potential beneficial bacterial isolates that were amended to a concentration of $1x10^9$ CFU/ml (Chen *et al*., 2018). For potential beneficial fungi, the discs were dipped in the normal saline suspension left in the rotary shaker overnight, and then placed on petri dishes at equidistant. The petri dishes were placed in an incubator for 48 hours at a temperature of 28 ^{0}C before observation were made. After 48 hours, plates were removed and observation were made, where clear halo around the disc represented the level of inhibition from bacterial and fungal isolates. The diameter of clear halos were measured and recorded (in mm) as inhibition distance (Khan *et al*., 2018). Both fungal and bacterial isolates that showed an inhibition diameter of less than 11 were discarded because they are not viable in field environments.

3.7.4 Determination of Efficacy of Microbial Antagonists Against R. Solanacearum Under Greenhouse Conditions

3.7.4.1 Experimental Design

The experiment had five treatments replicated thrice and pot arrangements were done in a completely randomized design (CRD) set up. The treatments were as follows; T1- *Bacillus sp*. BMT16+ *R. solanacearum*, T_2- *Burkholderia* sp. KRN2+*R.solanacearum,* T_3-*Bacillus* sp.KJ4+*R. solanacearum*, T_4-*Pseudomonas* sp.KJ2+*R.solanacearum* and T_5- Sterile distilled water+ *R. solanacearum*.

3.7.4.2 Greenhouse Assays

Greenhouse experiments were carried out in a glass house in Kiambu, JKUAT, Juja campus. Seeds of Rio-grande, a tomato variety highly susceptible to bacterial wilt were bought from an agrovet in Juja and raised in starter trays containing sterile coco-peat in the greenhouse. Watering was done in the morning and evening daily until germination. After germination, fertigation using polyfeed was done until the seedlings were ready to be transplanted. 2 days prior to setting up the experiment, media (mixture of forest soil, and sand at a ratio 2:1) was autoclaved at 121^0 C for 2 hours to kill any microorganisms, including *R. solanacearum* that might have been present in the media (Aliye *et al*., 2008). After cooling, the media were emptied into

a 5-kg pots and placed in the green house ready for experimental set up. Bacterial antagonists used in the treatment were selected after *in vitro* bioassays were multiplied and quantified using the procedure followed in 3.7 above.

Twenty one-days old seedlings were uprooted and their roots systems were gently washed with tap water before rinsing with sterile distilled water. The washed seedlings were placed in sterile aluminum foil and a sterile scalpel was used to cut off the root tips to create entry for bacterial antagonists. The seedlings were first dipped for 30 minutes in each treatment made up of 50 mls mixture of bacterial antagonists and *R. solanacearum* (25mls each) to allow interaction of plants and antagonists before transplanting into the pots (Kariuki *et al.,* 2020). Using drench method, the pots were inoculated with the mixture at the same time (Shashitu, 2021) and plants were maintained and observations on the manifestation of bacterial wilt symptoms were made.

Data on incidences of bacterial wilt on the tomatoes was then calculated according to Wang *et al*., (2019) as follows; Disease incidence (%) = (Disease Index X No. of disease plants in this index) ⁄ (total No. of plants investigated X highest disease index) X 100. The treatments that indicated lower percentage of disease incidence indicated greater efficacy of the antagonists in controlling bacterial wilt disease. On the other hand, data on disease severity was collected daily based on a scale of 0-5 where, 0 = healthy, 1 = partial wilting of one lower leaf, 2 = wilting of two to three lower leaves, 3 = wilting of all leaves except top two to three leaves,4 = wilting of all leaves and 5 = plant dies (Horita &Tsuchiya, 2007). The disease severity was determined according to (Hyakumachi *et al*., 2013) as follows; Disease severity = {(5A+4B+3C+2D+E)}/5N, where A were number of plants on the scale 5; B were number of plants on a scale of 4; C were number of plants on a scale of 3; D were number of plants on a scale of 2; E were number of plants on a scale of 1 and N were total number of plants used for that particular treatment. Finally, the efficacy (B.E) of bacterial antagonists used in the greenhouse experiment was calculated according to Wang *et al*., (2019) using the formula; Bio-control Efficacy (%) = {(D.I.C-D.I.A) ⁄ D.I.C}; where; DIC is disease incidence of control treatment, and DIA is the disease incidence of antagonists treated group.

3.7.4.3 Quantification of *R. Solanacearum* Population in Roots and Soils

Quantification of roots and soil colonizing bacteria was done according to (Saad *et al*., 2018) but with slight modification. After the completion of data collection, the tomato plants were uprooted and about 50g of rhizosphere soils adhering to the roots systems were gently shaken and placed in sterile double ziplock bags while the roots systems were washed with running tap water before rinsing with sterile distilled water and finally blot drying them using sterile paper towel. The roots were later cut, weighed and placed in 50ml ependorf tube then grounded in sterile distilled water. A serial dilution up to 7-folds was done before spotting 10µl (in triplicate) from each dilution on CPG agar medium incubated at 28^0 C until the colonies were clearly visible. For the rhizosphere soils, 10g of each sample was placed in a 250ml flasks and 90ml of sterile distilled water were added before placing them on a rotary shaker for 30 minutes at 200 rpm at $28^0 \pm 2^{\circ}$C. Serial dilution was also done up to 7 folds according to before spot-plating 10µl in triplicate as done for the fresh roots. For *R. solanacearum*, similar procedure was followed but the media was amended with TZC to enhance observation and counting of colonies typical to *R. solanacearum*. The colonies for *R. solanacearum* were counted and quantities obtained were later normalized to the root fresh weight of the plant (Shashitu, 2021), whereas that from rhizosphere samples were calculated as colony forming units per unit weight of soils.

3.8 Data Analysis

Data collected on the population of *R. solanacearum* in the roots and rhizosphere soils, disease severity and disease incidence of greenhouse biossays were subjected to analysis of variance (ANOVA), using Genstat 14.0 (VSN International, US) software. Where there was a significant difference, the means were separated using Least Significance Difference (L.S.D) at 5% probability level (Steel & Torrie, 1982). A graph was generated using a Origin version 9.1 (Origin LAB USA). Hierarchical cluster analysis of bacteria and fungi based on microscopic, chemical and macroscopic characteristics was done using DARwin software V6, where hierarchical clustering was done using weighted pair group method with averaging (WPGMA). Where there was a dissimilarity among the isolates, a dendrogram was drawn and the distance between characters were determined using Euclidean metric.

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 Scope

The results and discussion of chemical composition of the sampled soils, isolated microbial and fungal microbes and agro meteorological conditions of the regions where sampling was done are presented in this chapter. Morphological and biochemical characterization of the isolated fungal and microbial isolates obtained, with their level of efficacy in controlling *R. solanacearum* under *in vitro* assays and in the greenhouse is also presented. Further, effects of these microbes on the population of *R. solanacearum* both in soil and in fresh roots of test plants is also presented in this chapter.

4.2 Climatic and Soil Conditions of Sampling Sites

Across the selected counties, data obtained during sampling from the meteorological stations of the respective sampling sites revealed the average annual temperature and rainfall (Table 4.1). All the regions had varying annual average rainfall and temperature with Kirinyaga recording the highest range followed by Bomet, Kiambu and finally Kajiado. The pattern was similar with average annual temperature. However, among the highest temperature range, Kajiado recorded the highest, followed by Kirinyaga, Kiambu. Bomet had the lowest highest temperature among the four counties.

Table 4.1: Sampling Points across the Four Counties with Corresponding Agrometeorological Conditions.

Areas	**Fields**	**Coordinates**	**Average annual Rainfall (mm)**	**Average annual temperature (^{0}C)**
Bomet	1	0°38'37"S, 35°12'06"E		16-24 (County government
(Kapletundo)	2	0°38'25"S, 35°13'08"E	1000-1400	of Bomet,2018)
Kajiado		1°30'05"S, 36°56'21"E		10-34 (County government
(Olchonyori)	2	1°29'57"S, 36°56'15"E	300-1250	of Kajiado, 2018)
Kiambu	1	1°01'31"S,37°04'06"E		11.8-26 (County government
(Mangu)	2	1°03'52"S,37°02'39"E	600-1300	of Kiambu, 2018)
Kirinyaga	1	0°45'21"S, 37°17'03"E		17-27 (County government
(Makutano)	2	0°45'40"S, 37°17'00"E	1212-2146	of Kirinyaga, 2018)

The composition of soil from the sampling sites were different from one another in terms of mineral composition, pH and electrical conductivity. The results of minerals composition, as compared to that of normal range recommended for the tomato production, were as presented on a tabular format shown in Table 4.2 below.

Table 4.2: Composition of Minerals Found in the Composite Soil Sampled from Four Bacterial Wilt Endemic Regions.

Location	**Fields**	**EC (Ms/cm)**	**pH**	**P (mg/kg)**	**K (me/100g)**	**N (%)**
Kiambu	1	0.17	6.20	123.50	8.87	0.25
	2	0.15	6.30	123.00	8.45	0.23
(Mangu)						
Bomet	1	0.12	6.50	166.40	8.79	0.68
	2	0.10	6.40	166.00	8.25	0.65
(Kapletundo)						
Kajiado	1	0.16	5.70	115.00	5.61	0.34
(Olchonyori)	2	0.16	5.80	115.70	5.30	0.32
Kirinyaga	1	0.09	6.79	164.90	8.38	0.55
(Makutano)	2	0.10	6.81	164.50	8.65	0.52
Normal range		**<0.80**	**5.50-6.50**	**30.00-80.00**	**0.20-0.60**	**>0.25**

The dissimilarity of soil mineral composition can be associated to difference in the level of precipitation and temperature which in turn affect spatial distribution and diversity of beneficial microbes across selected areas. Fields in Kirinyaga recorded the highest pH range of (6.79, 6.81) whereas those in Bomet and Kiambu recorded pH of 6.4, 6.5 and 6.2, 6.3 respectively. The two fields sampled in Kajiado had the least pH range of 5.7 and 5.8. In terms of nitrogen, fields in Bomet had the highest level of nitrogen (0.65 and 0.68%) followed by Kirinyaga (0.52 and 0.55%) and Kajiado (0.32 and 0.34%). Fields sampled in Kiambu recorded the least amount of nitrogen (0.23 and 0.25%) as compared to the other three counties. The highest electrical conductivity range was recorded in the soil sampled from Kiambu (0.15 and 0.17Ms/cm), followed by Kajiado (0.16 Ms/cm) in both fields and then Bomet (0.1 and 0.12 Ms/cm). Kirinyaga recorded the least electrical conductivity of (0.09 and 0.1 Ms/cm). The level range of phosphorous also varied from soil to soil, with the highest level recorded from soil samples from Bomet (166 and 166.4 mg/kg), and followed by Kirinyaga (164.5 and 164.9mg/kg), Kiambu (123 and 123.5 mg/kg) and Kajiado (115 and 115.7 mg/kg). Similarly, potassium also varied with Kiambu recording the highest (8.45 and 8.87 me/100g), followed by Bomet (8.25 and 8.79 me/100g), Kirinyaga (8.38 and 8.65 me/100g) and sampled fields from Kajiado had the least potassium (5.3 and 5.61 me/g).

The variation in the soil mineral composition significantly influences rhizobacterial diversity and population in diverse ways. Soil properties, for instance, structure, pH and nutrient status affects microbial biodiversity because it selectively creates conducive environment favoring certain types of microorganisms and regulating available root exudates, thus affecting the selection of microbes by plants (Igiehon & Babalola, 2018). High nitrogen, phosphorous and optimum pH level found within the range preferred by most microbes (5.5- 6.5), in Bomet is attributed to the highest number of both fungi (13) and bacterial isolates (19) obtained as compared to other counties. Ambient temperatures have also been pointed to have different impacts not only on both soil carbon and nitrogen cycle, but also on the growth and development of plant growth. Lack of optimum temperature limits growth and respiration of soil micr.oorganisms and enzyme dynamics (Liu *et al.,* 2022). Low temperature negatively impact soil bacterial community structure through inhibition of nitrogen

and carbon accumulation (Zhang *et al.,* 2022). Further, diurnal variation or low season temperatures significantly impact the turnover efficiency of both soil-carbon and nitrogen (Kurihara et al., 2018).

Precipitation level is another environmental parameter that affects mineral composition in the soil, which in turn impact the selectivity of rhizosphere microbes. According to Naylor *et al*. (2017), significant shift in the composition of microbes associated within the rhizosphere, for instance, *Actinobacteria*, have been associated to limited precipitation. Because of diversity in the response of plants to such changes in the environment, population of these microbes also changes. Under this study, Bomet had the highest level of precipitation compared to other counties, and consequently the highest number of fungal and microbial isolates. This concurs with Frindte *et al*. (2019), who found a correlation between soil moisture levels and the development of distinct microbial communities. Their study pointed out that some communities like *Geobacteraceae*, *Comamonadaceae*, or *Myxococcales* prefer flooded soils. Root exudates and organic acids within the rhizosphere accelerate solubilization of phosphorous and potassium whereas alkaline salts restricts availability of phosphorous as a result of formation of less soluble calcium phosphate compounds (Randall *et al.,* 2001). These effects can have a significant effect on the growth of plant, and thus impacting on the exudates and metabolites released to the rhizosphere. The results further concurs with findings of Marschner *et al*., (2011) that the composition of rhizosphere microbes vary depending on the various factors including the root zone, species of the hosts plants, phenological phase of plants, stress, and disease events.

4.3 Isolated Rhizosphere Microbes

Results on enumeration of microorganisms revealed that the highest number of microbes isolated from the healthy rhizosphere soils sampled were from Bomet, which accounted for 30% and 48% of total fungi and bacteria isolated respectively (Figure 4.1). Kiambu accounted for 28% of total isolated fungi and 27% of the total isolated bacteria, while Kajiado, which was ranked third had 23% and 18% of total

fungal and bacterial isolates respectively. Lastly, Kirinyaga accounted only 19% and 8% of the total isolated fungi and bacteria respectively.

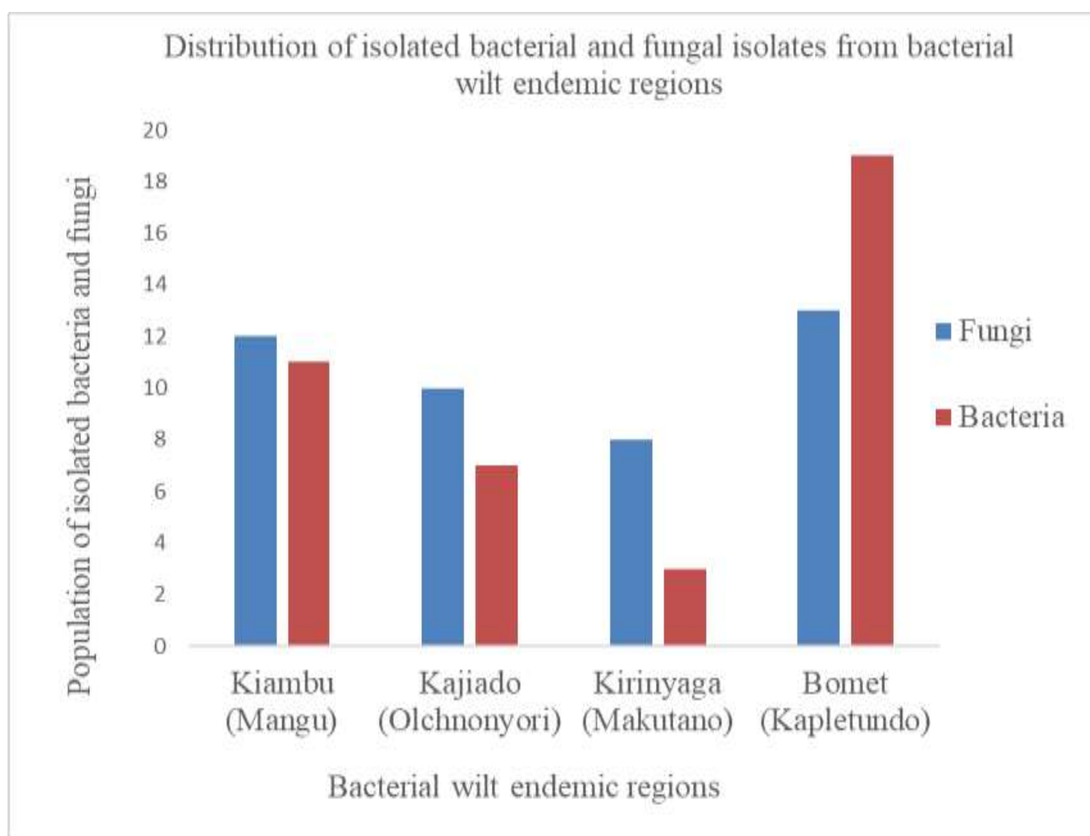


Figure 4.1: Distribution of Bacteria And Fungi Isolated From Bacterial Wilt Endemic Regions

The result on the distribution of bacteria and fungi from four endemic regions (Figure 4.1) concurs with those found by Durán *et al.* (2019) which revealed that occurrence of fungi is determined by the soil chemical properties. As a results of diverse metabolites and exudates that plants release as a response to nutrient imbalance in the soil, the diversity and population of rhizosphere microbes is also affected (Hartmann *et al*., 2007; Marschner 2008). The results obtained concurs with the findings by Larkin (2003) that changes in the soil microbiology is driven by soil characteristics, and environmental conditions in combination with management strategies. However, the results contradicts some of their other findings that associated low fungal diversity to high nitrogen level in the soils. In this study, soils

samples from Bomet recorded the highest diversity of fungal isolates as compared to other counties despite recording highest level of nitrogen. Apart from pH, Msenya *et al.* (2021) also found out that the distribution and growth of fungi is influenced by other environmental factors which include levels of moisture, degree of aeration, amount and types of nutrients, and human activities. Understanding the impacts of different environmental parameters is critical in obtaining beneficial microbes that can be harnessed for their natural resistance as biological control agents. According to Frey-Klett *et al.* (2011), fungal and bacterial organisms play a critical role in the enhancement of the survival of the interacting partners. However, the results contradicts that of Brulé *et al*., (2001) who discovered that not at all times can bacterial and fungal microbes have beneficial impacts on the hosts plants associated with them. A good examples is *P. fluorescens BBc6R8* which promotes *Laccaria bicolor's* viability, a kind of mycorrhizal fungus found in adverse soil conditions. However, Deveau *et al.* (2010) holds that the same fungus, on the other hand, can as well promote the bacterium's survival

4.4 Characterization of Isolated Microorganisms

Morphological, biochemical and microscopic description of 43 fungal and 40 bacterial isolates revealed the genera of each isolate.

4.4.1 Fungal Isolates

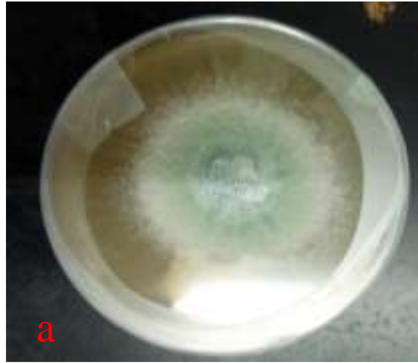
Macroscopic description of fungal isolates indicated a wide variation in terms of surface, reverse and peripheral color (Table 4.3). The colors ranged from green to cream with the majority (13 out of 43) featuring black surface (*Aspergillus sp.).* Fungal isolates with white surface color (*Phytophthora sp*., *Chladosporium sp.,* and *Sporidesmium sp.)* were the second majority followed by green (*Trichoderma and Penicillium sp*.) and pink (*Fusarium sp.*), then cream color (*Oedecephalum sp.).* *Aspergillus, Phytophthora, Sporidesmium,* and *Chladosporium sp*. had cream reverse color while *Trichoderma, Penicillium and Odecephalum* sp. were yellow. *Fusarium sp*. was the only fungal isolate that showed red reverse color. All fungal isolates showed white peripheral color except *Trichoderma* and *Fusarium sp*. that showed

cream and pink color respectively. As indicated in table 4.3, the margins of the fungal isolates were smooth except *Oedecephalum sp.*, which were serrated.

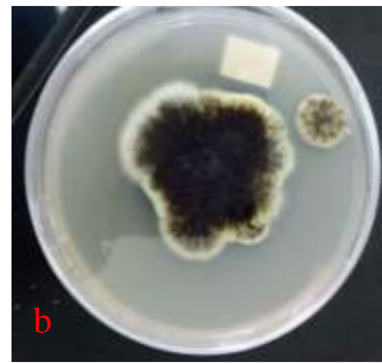
Table 4.3: Colony Characteristics of Fungal Isolates Obtained from the Rhizospheres

Total No.	Surface	Reverse	Periphery	Margin	Hyphae	Elevation	Genus
13	Black	Cream	White	Smooth	Septate	Raised	*Aspergillus sp.*
8	Green	Yellow	Cream	Smooth	Septate	Raised	*Trichoderma sp.*
8	Pink	Red	Pink	Smooth	Non-Septate	Raised	*Fusarium sp.*
5	White	Cream	White	Smooth	Non-septate	Raised	*Phytophthora sp.*
3	Cream	Yellow	White	Serrated	Septate	Raised	*Odecephalum sp.*
2	Green	Yellow	White	Smooth	Septate	Raised	*Penicillium sp.*
2	White	Cream	White	Smooth	Septate	Raised	*Chladosporium sp.*
2	White	Cream	White	Smooth	Septate	Flat	*Sporidesmium sp.*

When pure fungal isolates were cultured in fresh media and incubated, each isolate demonstrated different morphological characteristics as witnessed in Plate 4.1 (a and b) and their corresponding spores when observed under a light microscope are shown in a similar Plate 4.1 (c and d). *Fusarium sp*. showed non-septated hyphae while the rest were all septate. *Trichoderma, Aspergillus, Fusarium, Phytophthora, Penicillium, Chladosporium*, and *Oedecephalum* showed raised elevation except for *Sporidesmium* species. Among 43 fungal isolates obtained, *Aspergillus* sp. (30.2%), *Trichoderma* sp. (18.6%), *Fusarium* sp. (18.6%), and *Phytophthora sp.* (11.6%) were the most dominant fungal isolates obtained after screening rhizosphere soils from the four counties. *Oedecephalum* sp., (6.9%) *Sporidesmium* sp. (4.7%), *Chladosporium* sp. (4.7%), and *Penicilium* sp. (4.7%) were the least dominant fungal isolates obtained (Table 4.3).



Trichoderma sp.



Aspergillus sp.

Aspergillus sp.

Phytophthora sp.

Plate 4.1: Morphological and Microscopic Description of Colonies of Some Fungal Isolates Obtained During Rhizosphere Screening.

(a) Whitish green with smooth, hairy edge (b) Compact white-yellowish with dense of black colonies (c) Spherical vesicle with conidia arising from the vesicle (d) sickle shape microconidia

The dissimilarity of the fungal isolates based on morphological characteristics is shown on the dendrogram drawn using the Euclidean metric in Figure 4.2 below containing three clusters.

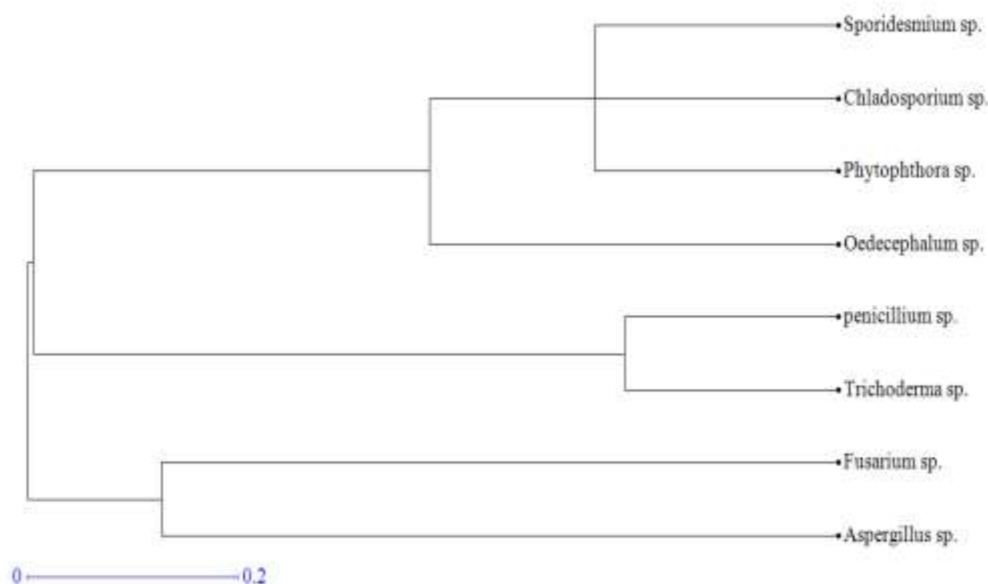


Figure 4.2: Dendrogram Showing how the Fungal Isolates Clustered Morphologically.

Trichoderma and *Penicillium sp.* are closer to each other than to *Phytophthora* and *Sporidemium*. However, *Phytophthora* and *Sporidesmium sp.* are more similar to each other than to *Chladosporium* and *Oedecephalum sp.,* which are far closer to *Trichoderma* and *Penicillium*. *Chladosporium* and *Oedecephalum* are closer to one another than to *Fusarium* and *Aspergillus sp.*, both of which are very different from the rest of the isolates.

4.4.2 Bacterial Isolates

On the other hand, combination of biochemical and microscopic characterization (Table 4.4 and 4.5 respectively) identified bacterial isolates to the 8 genera level as highlighted in Table 4.4. Microscopic observation on the shape of the cells, motile and gram statuses revealed that majority were motile and had rod shapes except *Micrococcus* bacteria which was cocci in shape. Motility tests also revealed that

only two out of 8 genera of bacterial isolates were non-motile. Gram status revealed that 4 genus of bacterial isolates were gram positive and the remaining four were found to be gram negative (Table 4.4). In terms of motility, it was revealed that *Chryseobacterium,* and *Micrococcus sp.,* were not motile while species of *Bacillus Pseudomonas sp*., *Enterobacter sp., Burkholderia sp., Streptomyces sp*., and *Serratia sp*., were motile (Table 4.4).

Table 4.4: Characteristics of Bacterial Colonies Isolated from the Sampled Rhizospheres

No. of Isolate	Cell shape	Motility	Gram reaction	*Genus*
11	Rod	Motile	Positive	*Bacillus sp.*
4	Rod	Motile	Negative	*Pseudomonas sp.*
2	Rod	Non-motile	Positive	*Chryseobacterium sp.*
11	Coccus	Non-motile	Positive	*Micrococcus sp.*
1	Rod	Motile	Negative.	*Enterobacter sp.*
6	Rod	Motile	Negative	*Burkholderia sp.*
3	Rod	Motile	Positive	*Streptomyces sp.*
2	Rod	Motile	Negative	*Serratia sp.*

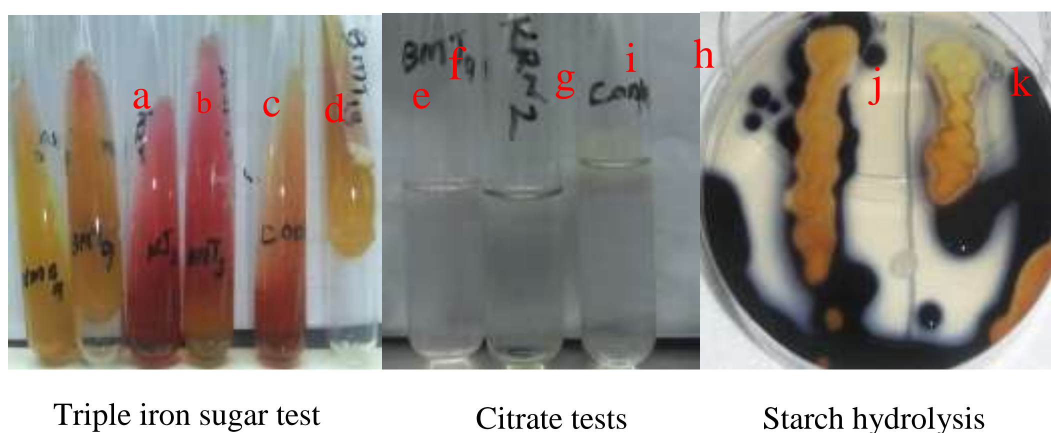
Results of various selected biochemical tests revealed varying observations ranging from positive to negative reactions among all the 40 bacteria isolated. In instances where triple sugar iron tests were positive, the intensity of acidity varied in both slants and butts (Table 4.5). Following the tests, the isolates were identified as *Burkholderia* sp., (15%) *Bacillus* sp. (27.5%) and *Micrococcus* sp., (27.5%). *Pseudomonas* sp. (10%), *Streptomyces* sp. (7.5%), *Serratia* sp. (5%), and *Enterobacter* sp. (2.5%).

Table 4.5: Biochemical Characterizations of Individual Bacterial Isolates

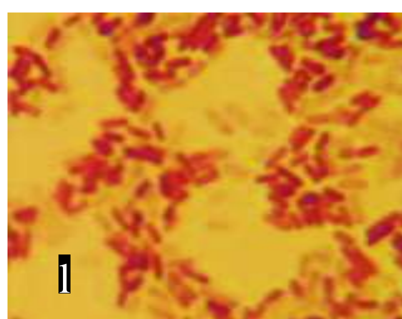
Isolate No.	MR	VP	Catalase	Starch	Citrate	TSI				Organism ID
						Slant	Butt	Gas	H_2S	
BMT1	+	+	+	+	+	-	++	-	-	*Bacillus sp.*
BMT2	-	-	+	-	+	-	+	-	-	*Bacillus sp.*
BMT3	-	-	+	+	+	-	++	-	-	*Pseudomonas sp.*
BMT4	+	+	-	+	-	-	++	-	-	*Bacillus sp.*
BMT5	-	-	+	+	-	++	++	-	-	*Chryseobacterium sp.*
BMT6	-	+	+	+	-	+	++	-	-	*Bacillus sp.*
BMT7	+	+	-	+	+	+	+++	-	-	*Bacillus sp.*
BMT8	-	-	+	-	+	++	+++	-	-	*Micrococcus sp.*
BMT9	-	+	-	+	+	-	+++	+	-	*Enterobactersp.*
BMT10	-	-	-	+	+	-	-	-	-	*Micrococcus sp.*
BMT11	-	-	-	-	+	-	-	-	-	*Micrococcus sp.*
BMT12	-	+	+	-	+	-	-	-	-	*Micrococcus sp.*
BMT13	-	+	+	-	+	-	-	-	-	*Micrococcus sp.*
BMT14	+	-	+	+	+	+	++	-	-	*Burkholderia sp.*
BMT15	-	+	+	-	+	+	+	-	-	*Micrococcus sp.*
BMT16	+	+	+	+	-	-	++	-	-	*Bacillus sp.*
BMT17	+	+	-	+	+	+	++	-	-	*Burkholderia sp.*
BMT20	-	-	+	+	+	-	+	-	-	*Burkholderia sp.*
BMT21	+	-	+	+	+	-	+++	-	-	*Burkholderia sp.*
KRN1	+	+	+	-	-	+	++	-	-	*Bacillus sp.*
KRN2	+	-	+	+	+	+	+	-	-	*Burkholderia sp.*
KRN3	-	-	-	+	-	+	+++	-	-	*Bacillus sp.*
KMB1	-	-	+	-	+	+	+++	-	-	*Streptomyces sp.*
KMB2	-	-	-	-	-	++	++	-	-	*Micrococcus sp.*
KMB3	-	-	+	-	-	+	++	-	-	*Streptomyces sp.*
KMB4	-	-	-	-	+	+	++	-	-	*Micrococcus sp.*
KMB6	-	+	+	-	+	+	+	-	-	*Serratia sp.*
KMB7	-	+	+	-	+	+	+++	-	-	*Serratia sp.*
KMB8	-	-	-	-	+	+	+++	-	-	*Micrococcus sp.*
KMB9	-	-	-	+	+	++	+++	-	-	*Bacillus sp.*
KMB10	-	-	+	+	+	+	+	-	-	*Streptomyces sp.*
KMB11	-	+	+	+	-	+	+++	-	-	*Pseudomonas sp.*
KMB12	-	-	-	-	+	+	++	-	-	*Micrococcus sp.*
KJ2	-	-	+	-	+	+	+	-	-	*Pseudomonas sp.*
KJ4	-	-	+	+	+	+++	+++	-	-	*Bacillus sp.*
KJ5	+	-	+	+	+	+	++	-	-	*Burkholderia sp.*
KJ6	-	-	-	+	-	+	++	-	-	*Bacillus sp.*
KJ7	-	+	+	-	+	+	++	-	-	*Pseudomonas sp.*
KJ8	-	-	+	+	+	+	+	-	-	*Chryseobacterium sp.*
KJ9	-	-	-	+	-	+	++	-	-	*Micrococcus sp.*

Key: (+) Positive, (-) Negative, MR: Methyl Red tests VP: Voges-Proskauer test, and H_2S: Hydrogen Sulphide gas tests, Slants: Acidity level of the slant, Butt: Acidity level of the butt. Plate 4.2 illustrates the appearance of some of the individual's isolates on biochemical tests that included the triple iron sugar test, citrate tests, and starch hydrolysis and the gram reaction of the bacterial isolates observed under a light microscope under oil immersion at X100

Biochemical tests that included triple iron sugar test, citrate tests, and starch hydrolysis performed induced changes in color in some of the bacterial isolates as demonstrated in Plate 4.2. Gram statuses tests on isolated bacteria revealed that *Bacillus sp., Chryseobacterium sp., micrococcus sp*., and *Streptomyces* species were all positive for gram status whereas *Serratia, Burkholderia, Enterobacter* and *Pseudomonas sp*., were found to be negative for gram status.



Triple iron sugar test Citrate tests Starch hydrolysis



Gram negative rod

Gram positive rod

Plate 4.2: Appearance of the Individual's Isolates on Selected Biochemical Tests and Under a Light Microscope

Key: (a) Moderate acid slant, intense acid butt, no H_2S, and no gas (**b**) Alkaline slant, intense acid butt, positive for gas, no H_2S **(c)** Partial acid slant, partial acid butt, no gas, no H_2S (**d**) alkaline slant, moderate acid butt, no gas, no H_2S (**e**) Control, no reaction took place (**f**) moderate acid slant, intense acid, Butt, positive for gas, no H_2S (**g**) positive for citrate utilization (**h**) negative for citrate utilization (**i**) control (**j**) negative for starch hydrolysis (**k**) positive for starch hydrolysis. (**l**) Gram-negative rod (**m**) gram-positive rod.

In Table 4.5, results pointed that *Pseudomonas, Enterobacter, Streptomyc*es, and *Burkholderia sp*., were all positive for citrate utilization. *Bacillus* and *Micrococcus sp*. on the other hand, were found to be negative for citrate utilization. Further, 58% of the bacterial isolates obtained were found to be positive for starch hydrolysis and the remaining 42% were negative. However, it was noted that some isolates of *Bacillus* were either negative or positive for starch hydrolysis. *Micrococcus* KJ9 and *Micrococcus* BMT 10 were able to hydrolyze starch whereas the remaining were negative for starch hydrolysis. Tests conducted to determine the production of hydrogen sulphide and gas revealed that only *Enterobacter* BMT9 was the only bacteria that produced gas, while the rest did not produce both hydrogen sulphide and gas. MR tests showed that all bacterial isolates except *Bacillus* and *Burkholderia sp*. were found to be negative for the MR tests.

Finally, VP tests showed that few isolates of *Bacillus, Pseudomonas, Micrococcus*, and *Burkholderia* gave positive results during the VP tests while the majority were negative. Those isolates that were positive for both acids slants and acid butts demonstrated varying levels of acidity ranging from intense, moderate, and finally, slight acidity, as was the case for few isolates of *Micrococcus*, *Bacillus*, and *Chryseobacterium*. Observations on slant acidity revealed that some isolates of *Bacillus*, *Pseudomonas*, *Burkholderia*, and *Micrococcus* were negative while the rest were positive. Results of clustering and the dissimilarity of bacterial isolates based on their shared morphology and biochemical characteristics were presented in a dendrogram as shown in Figure 4.2 below.

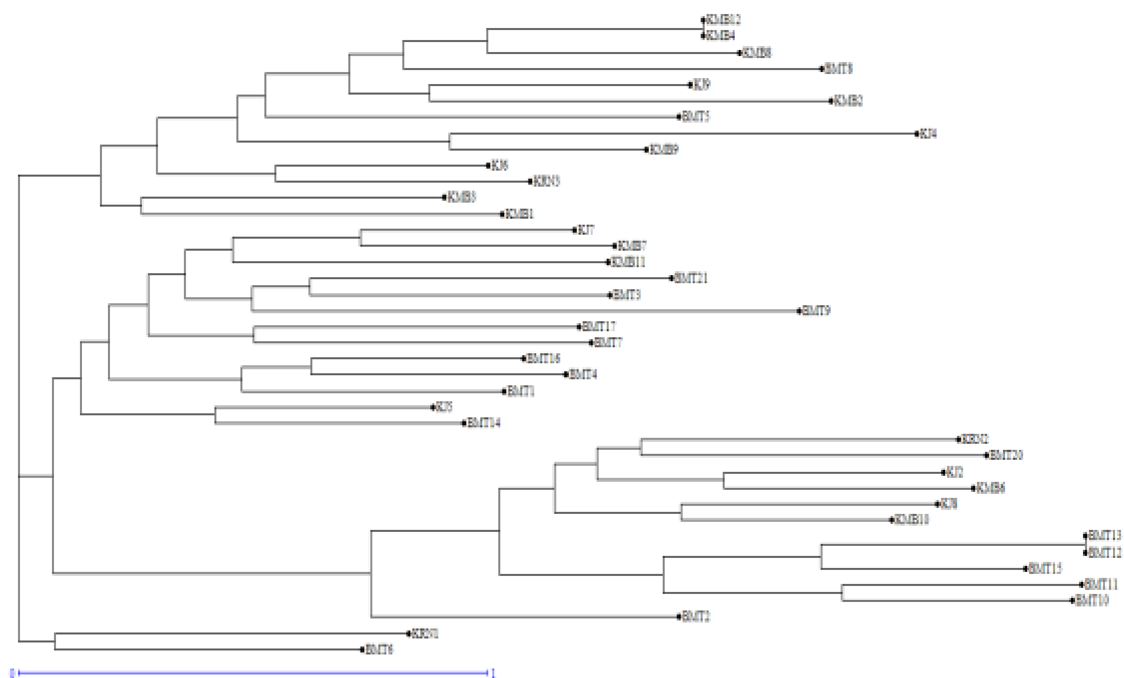


Figure 4.3: Dendrogram Showing Bacterial Isolates Clustered Based on Biochemical Characteristics.

Key: BMT1-*Bacillus sp*., BMT2-*Bacillus sp*., BMT3-*Pseudomonas sp*., BMT4-*Bacillus sp*., BMT5-*Chryseobacterium sp*., BMT6-*Bacillus sp.,* BMT7-*Bacillus sp*., BMT8-*Micrococcus sp*., BMT9-*Enterobacter sp*., BMT10-*Micrococcus sp*.,BMT11-*Micrococcus sp*., BMT12-*Micrococcus sp*., BMT13-*Micrococcus sp*., BMT14-*Burkholderia sp*., BMT15-*Micrococcus sp*., BMT16-*Bacillus sp*., BMT17-*Burkholderia sp*.,BMT20-*Burkholderia sp.,* BMT21-*Burkholderia sp*., KRN1-*Bacillus sp.,* KRN2-*Burkholderia sp*., KRN3-*Bacillus sp*., KMB1-*Streptomyces sp*., KMB2-*Micrococcus sp*., KMB3-*Streptomyces sp.,* KMB4-*Micrococcus sp*., KMB6-*Serratia sp*., KMB7-*Serratia sp.,* KMB8-*Micrococcus sp*., KMB9-*Bacillus sp*., KMB10-*Streptomyces sp*., KMB11-*Pseudomonas sp*., KMB12-*Micrococcus sp*., KJ2-*Pseudomonas sp.,* KJ4- *Bacillus sp.,* KJ5-*Burkholderia sp*., KJ6-*Bacillus sp.,* KJ7-*Pseudomonas sp*., KJ8-*Chryseobacterium sp*., KJ9-*Micrococcus sp.*

The distance between characters were compared using Euclidean metric after drawing a dendrogram using the WPMGA method. As shown in Figure 4.2, it can

be noted that isolates BMT10 and BMT11 are closer to one another as than they are to BMT15, BMT12, BMT13, KRN2, BMT20, KJ2, KMB6, KJ8, and KMB10. Similarly, bacterial isolates KJ7 and KMB7 were also found to be closer to one another, but not to KMB11, whereas BMT16 and BMT4 were closer to each other than they were to bacterial isolate BMT1. BMT9 and BMT21 isolates were closer to one another but dissimilar to BMT3 and BMT21. Similarly, BMT14 and KJ5 isolates were found to be closer to one another, but morphologically different to all bacterial isolates in other clusters. Bacterial isolates KMB3 and KMB1 were closer to one another but found to be morphologically different from other bacterial isolates under the first cluster. Finally, BMT6 and KRN1 found in the fourth cluster were significantly dissimilar to all other isolates falling under the first three clusters but the two isolates were closer to one another. The dissimilarity and how close the microbes were to each other can be attributed to the likelihood that some could have been of the same genera. Even though bacterial isolates, for instance, BMT10 and BMT11 were found to belong to the same genera of *Micrococcus,* the isolates could belong to different species, which can only be affirmed with the use of molecular techniques. Further, their relationship could have been based on their ability to use diverse substrates as energy sources in the soil. KMB6 and KJ2 belonged to the genera of *Serratia sp.* and *Pseudomonas sp.* respectively, but were hierarchically placed as closer to each other because they showed similarity in terms of citrate utilization, catalase reaction and acidic butts and slants. Additionally, both did not utilize starch and were negative for MR tests, gas, and hydrogen sulphide production. On the other hand, it was found out that despite some isolates belonging to the same genera, for instance, KJ5 and KRN2 belonged to *Burkholderia,* and were clustered together, they were not closer to one another. This was due to the ability of isolates to demonstrate varying levels of acid slants, where KJ5 showed slightly acidic slants, contrary to KJ5 that showed moderate acidic slants. Based on this, it was concluded that the two isolates belonged to different species of *Burkholderia.*

Chemical changes within the rhizosphere takes place as consequence of interaction between plant roots and the rhizosphere soils. These interactions, patterns of root exudation, soil pH, nutrients and rhizode-position have demonstrated to have a huge impact on nutrient solubility and uptake (Brahmaprakash *et al.,* 2017) by plants.

This result in diverse behavior of individual plants in the fields and their selectivity of rhizosphere microflora and thus making it difficult to exactly know the types of dominant microbes that actively influence plant growth and health status. However, in numerous studies, this challenge have been widely addressed by combining other tests with biochemical testing processes, for instance, using the 3% gram KOH method and gram staining tests (Hardiansyah *et al.,* 2021). Similarly, under this study, combination of microscopic, biochemical and macroscopic description were used to identify dominant microbes in the healthy tomato rhizosphere.

The ever changing soil parameters have an impact on the survival and growth of the soil microbes (Timmusk *et al.,* 2011). Majority of microbes obtained from screening the rhizosphere of healthy tomato included fungi like *Penicillium, Trichoderma*, *Fusarium* and *Aspergillus,* whereas bacterial isolates were dominantly *Bacillus*, *Pseudomonas*, *Burkholderia*. *Streptomyces* and *Enterobacter* bacteria were also available but least in the population. In a similar study by Zhang *et al*. (2020b), 55 bacterial strains possessing distinct antagonistic activities against *R. solanacearum* were realized during the screening of healthy tomato rhizosphere soils (conducive soils) as compared to bacterial wilt infected soils. These two findings points out that the bacterial diversity and their interaction networks is distinct between healthy and non-healthy soils. It is revealed that *R. solanacearum* is highly competitive and exhibits phenotypic changes as a response to changing environments (Perrier *et al.,* 2019), therefore, could outcompete incompetent microbes that could infer resistance to plants.

MR-VP tests which determines the mixed acid fermenting bacteria through the detection of their ability of the isolate to use glucose and produce adequate acid end products (Harold, 2002) were evaluated based on the color change of the media. Mixed acids are typical end products of specific fermentation pathway, which makes the medium to be acidic ($pH<4.5$) can be revealed by the change in color when methyl red indicator is added (Cappuccino and Sherman, 2002) to the medium. On the other hand, the VP test detects the intermediate products or the neutral final products from organic acids resulting from fermentation pathway which yield 2, 3-butanediol (acetoin) when Barret's reagent is added. The results obtained concurs

with the views of Hashem *et al*. (2019) that *Bacillus* and *Pseudomonas* species are among the predominant genera of PGPR, with bacillus being the major rhizobacteria that harsh soil environment for a longer period of time. This is because *Bacillus* species are capable of forming spores that can survive such harsh conditions. These beneficial microbes promotes plant growth and health using diverse mechanisms. For the microbes to positively impact plant growth and health, most of them must first colonize the roots to establish sufficient population densities (Manivannan *et al*., 2012). Although morphological, biochemical and microscopic methods have been heavily relied to identify microbes to genus level, the method should be used with caution as some microbes share similar biochemical and morphological characteristics.

The compositions of bacterial and fungal species in the rhizosphere differ according to the root zone, plant species, plant phenological phase, stress, and the pathological events (Naylor *et al.,* 2017). Under this study, results found in terms of the population of *Trichoderma*, *Aspergillus*, and *Fusarium* species concurs with that of Thormann and Rice (2007), which identifies *Trichoderma* as the most frequently occurring fungi within the rhizosphere. As pointed out by Marschner *et al*. (2011), microorganisms can take up and utilize diverse siderophores produced by other microbial organisms as source of carbon instead of utilizing their own. This creates a scenario where some microbes benefit from others. The implication of this one sided interaction is that those microbes that use distinct types of siderophores possess competitive ability (Mirleau *et al*., 2000) over others. This competitive ability demonstrated by some microbes elucidates the dominance of bacterial microbes particularly *Bacillus*, *Micrococcus*, and *Burkholderia* in the sampled rhizosphere soils. According to Weisskopf and his colleagues (2011), citrate utilization aids rhizosphere bacteria to maintain competitive roots colonization. Citrate positive microbes can utilize citrate as their carbon source (Harold, 2002). Depending only biochemical and morphological characteristics to determine the diversity of rhizosphere microbes may be not be completely reliable because organisms belonging to different species may demonstrate some similarities despite having no genetic relations. This method should be combined with other approaches to enhance microflora identification and recognition of their diversity. The study further reveals

that microorganisms exists as a consortium in the occupying the same habitat. Despite isolating *R. solanacearum* from the soil in bacterial wilt infested area, the beneficial microbial isolates that demonstrated varying level of inhibition against *R. solanacearum* confirms the co-existence of both beneficial and non-beneficial microorganisms within the plant rhizosphere.

4.5 Pathogenicity Tests of Isolated R. Solanacearum

A set up to conduct pathogenicity tests of isolated bacteria on tomato plants indicated that wilting was associated to *R. solanacearum* isolated in the rhizosphere of infected plants. All the plants demonstrated typical symptoms of bacterial wilt on host plants, confirming the pathogenicity of all the four isolates obtained from the four counties (Plate 4.3). Wilting of tomato plants started on the third day and progressed gradually to the 7th day after inoculating with inoculum containing suspensions of *R. solanacearum*. Plants inoculated with *R. solanacearum* showed varied levels of bacterial wilt symptoms which included wilting of newly formed leaves followed by older leaves. Eventually leaves turned yellow, and long, narrow and dark brown streaks were seen on the stems. Plants inoculated with *R. solanacearum* isolated from Bomet had severe infection as compared with those inoculated with *R. solanacearum* isolated from Kiambu, Kirinyaga and Kajiado (Table 4.6). This indicate the possibility of diverse biotypes among the four counties where sampling was done. Control experiment inoculated with sterile distilled water did not show any symptoms of bacterial wilt disease.

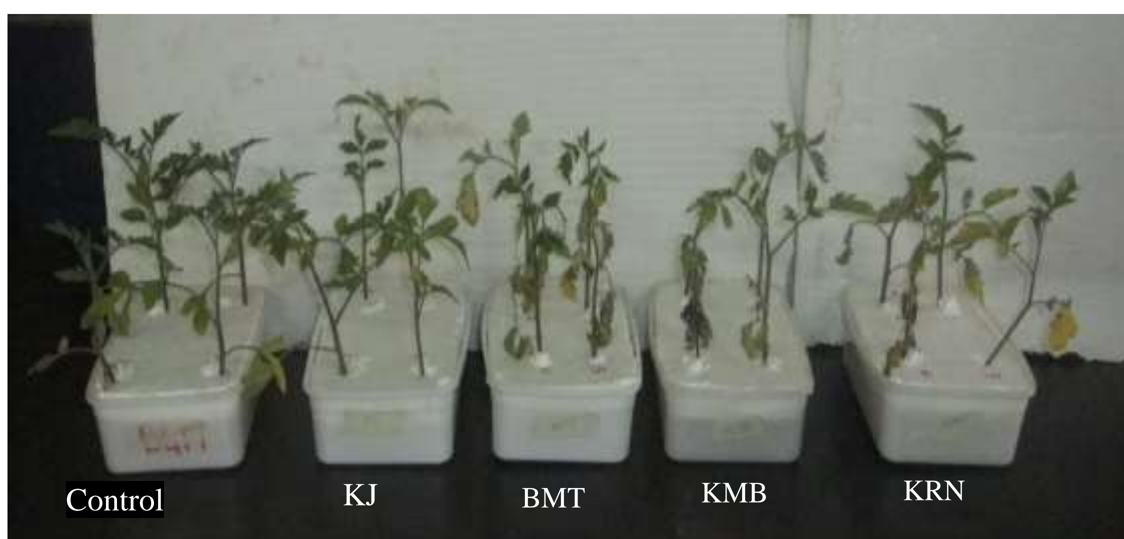


Plate 4.3: Pathogenicity Experiment Where Tomato Plants Were Inoculated with *R. Solanacearum*.

The incidence of infection varied among the isolates where BMTR.s exhibited the highest incidence (>75%), implying that it was very pathogenic (Table 4.6). KRNR.s and KMBR.s followed as moderately pathogenic (50%) whereas KJR.s induced weak infection on the tomato plants (<25%) as shown below.

Table 4.6: Variations in Pathogenicity of *R. Solanacearum* Isolates on the Rio-Grande Tomato Variety

Isolate	Infection (%)	Scale	Pathogenicity
BMTR.s	75%	4	Very pathogenic
KRNR.s	50%	2	Moderately pathogenic
KMBR.s	50%	3	Moderately pathogenic
KJR.s	25%	1	Weakly pathogenic

The pathogenicity study demonstrated that *R. solanacearum* is significant disease of economic importance in Kenya, with the four counties being part of those encountering huge losses as a result of the disease. Ooze plated on TZC media revealed mucoid, pink centered and irregular colonies confirming the virulence of *R. solanacearum* that was used to inoculate tomatoes during pathogenicity study were able to cause disease (Plate 4.4).

Plate 4.4: Typical Colonies of *R. Solanacearum* Obtained from the Ooze. Pink Centered, Mucoid and Irregular Colonies of Virulent *R. Solanacearum* Obtained from Ooze.

Similar results were found by Shashitu (2021), where four strains of *R. solanacearumm* induced 25 to 75% wilting on hosts tomatoes. Sikirou *et al.* (2017) also recorded up to 71% wilting on tomatoes found. Seleim *et al*. (2011) also found related results where seven isolates tested showed bacterial wilt incidence ranging from 40-96%. The varying level of virulence of *R. solanacearum* is associated with pathogenic machinery and how they are expressed in the pathogen. The varying level of pathogenicity of *R. solanacearum* isolates from four counties concurs with the results of (Abadi & Djauhari, 2020) who tested the virulence of the pathogen

obtained from five different districts, where all the pathogens caused bacterial wilt disease in plantain bananas between 60-66 days. It also echoes the results of Abo-Elyousr and Asran (2009) who obtained varying levels of pathogenicity of *R. solanacearum* against tomato plants under similar study. Popoola *et al.* (2015) also obtained a varying level of pathogenicity among different strains of *R. solanacearum.* The varying levels of virulence of *R. solanacearum* isolated from the four counties could be attributed to changes in environmental factors known to cause emergence of genetically diverse types among strains (Gashaw *et al*., 2022). Based on their level of pathogenicity, *R. solanacearum* isolated from Bomet was selected for further experimental challenge against antagonists believed to confer resistance to those tomato plants that were healthy, yet growing in bacterial wilt infected fields. Additionally, interaction existing between the host plants and rhizosphere microbes, particularly potential beneficial rhizomicrobes, greatly impact the pathogenicity expression of *R. solanacearum*.

4.6 *In Vitro* Assays of Fungal and Microbial Isolates against *R. Solanacearum*

Out of 40 bacterial isolates screened, only 10% demonstrated inhibition above 11mm. Significantly ($p<0.05$) varying level of inhibition of *R. solanacearum* (16mm, 13.67mm, 13.33mm, and 12mm) were observed from *Bacillus* sp. BMT16, *Pseudomonas* sp.KJ2, *Burkholderia* sp.KRN2, *Bacillus* sp.KJ4 respectively (Table 4.7). Inhibition distance for *Bacillus* sp. BMT16 was significantly ($p < 0.05$) different from that of *Burkholderia* sp.KRN2 and *Bacillus* sp.KJ4 but not from that of *Pseudomonas* sp.KJ2. However, inhibition from distance of *Pseudomonas* sp.KJ2 was not significantly ($P< 0.05$) different from *Burkholderia* sp.KRN2 and *Bacillus* sp.KJ4. Among the four regions where rhizosphere sampling was done, Kajiado produced two effective anti-pathogenic isolates; *Pseudomonas* sp.KJ2 and *Bacillus* sp.KJ4 with both recording inhibition zones distances above threshold (>11mm). The second trial were repeated for the four bacterial isolates that recorded highest inhibition zones diameter whereas those that were below minimum recommended threshold (<11mm) were not included because they are considered ineffective in the greenhouse. The four best isolates which represented 10% of isolated bacteria, *Bacillus* species accounted for 50%. The remaining 90% of the bacterial isolates

significantly (P<0.05) differed on their levels of antagonistic activity against *R. solanacearum* as it produced inhibitions zones that ranged from 2.97 mm to 10.0 mm. Under this category of the isolates, majority were *Microccocus* species while the least were *Serratia*, *Streptomyce*s and *Enterobacter* species.

Significant (P<0.05) variations were observed for fungal antagonists tested against *R. solanacearum*. Only 11.6% of the fungal isolates screened for their efficacy against *R. solanacearum* exhibited inhibition zones diameter ranging from 9.67mm to 6.33mm (Table 4.8). The remaining 89.4% did not demonstrate any inhibition at all. Significantly ($p < 0.05$) *Trichoderma sp*. KJ4 recorded higher inhibition distance (8.667mm) as compared to *Penicillium sp.*KMB2 which recorded inhibition distance of 6.33mm. However, inhibition distance among *Aspergillus sp*.KRN2, *Trichoderma sp*.KJ2, *Penicillium sp*.BMT11 and *Penicilium sp*.KMB2was no significantly different (p<0.005) from that of *Trichoderma sp*.KJ4. This trend was also witnessed in the second trial. Those fungal isolates that did not inhibit the growth of *R. solanacearum* were not repeated in the second experiment. It is important to note that despite exhibiting inhibition of *R. solanacearum* growth, none of them exhibited inhibition zone distance above the minimum threshold of 11mm. The efficacy of fungal isolates and bacterial isolates against *R. solanacearum* revealed that bacterial isolates were more effective against pathogenic bacteria causing wilt diseases. This was witnessed among the best four bacterial isolates recording a higher inhibition zones diameter as compared to fungal isolates that showed antagonistic activity against *R. solanacearum*. The most effective bacterial isolates recorded an inhibition zone diameter of 16mm whereas the most effective fungal isolates recorded the highest inhibition zone diameter of 9.67m. Plate 4.5 below demonstrate the appearance of clear halos resulting from antagonistic bacterial and fungal isolates limiting the growth of *R. solanacearum*.

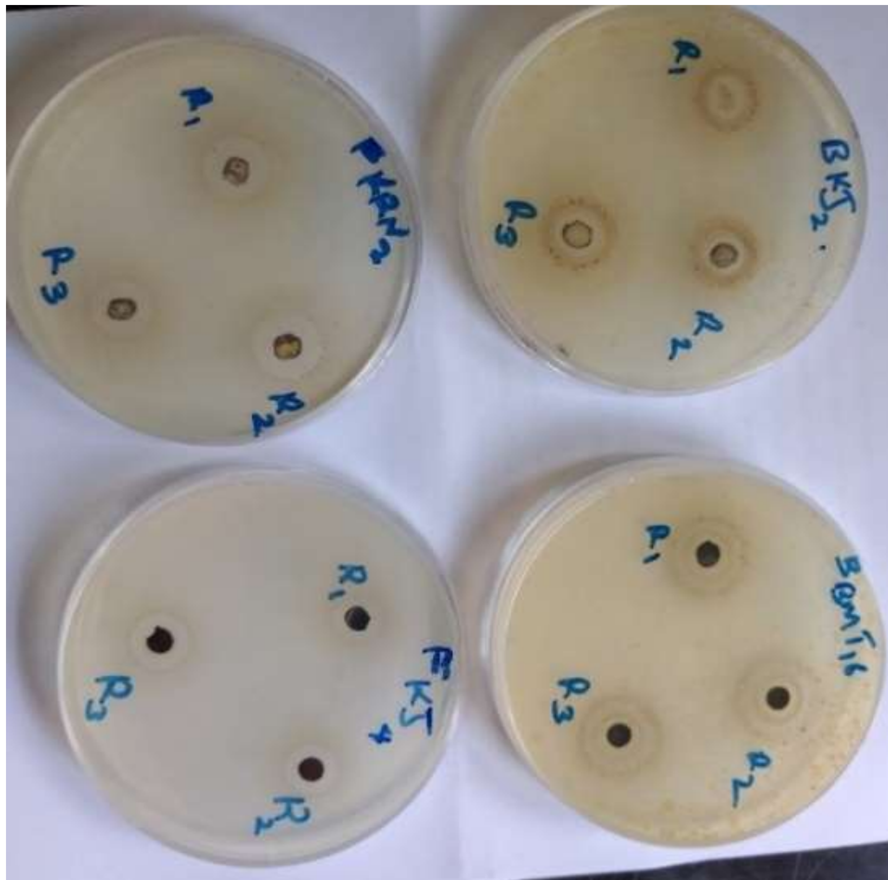


Plate 4.5: Inhibition Zones as was Depicted by Individual Bacterial Antagonists

Table 4.7: Inhibition Distances (Mm) by Bacterial Isolates against *R. Solanacearum*

Isolate source	Treatments	Zones of Inhibition (mm)	
		Trial I	**Trial II**
Bomet	*Bacillus sp.*BMT16	16.00 c	16.13 c
Kajiado	*Pseudomonas sp.*KJ2	13.67 bc	13.77 bc
Kirinyaga	*Burkholderia sp.*KRN2	13.33 b	13.43 b
Kajiado	*Bacillus sp.*KJ4	12.00 b	12.30 b
Bomet	*Bacillus sp*. BMT2	10.33	*
Bomet	*Burkholderia* sp. BMT14	10.00	*
Bomet	*Bacillus sp*. BMT7	9.83	*
Kajiado	*Pseudomonas* sp.KJ7	9.77	*
Bomet	*Bacillus sp*. BMT4	9.67	*
Kajiado	*Bacillus sp*. KJ6	9.33	*
Bomet	*Pseudomonas sp*. BMT3	9.17	*
Bomet	*Bacillus sp*. BMT6	9.00	*
Kajiado	*Burkholderia sp*. KJ5	8.93	*
Kiambu	*Bacillus sp*. KMB9	8.80	*
Bomet	*Burkholderia* sp. BMT20	8.80	*
Bomet	*Burkholderia sp*. BMT17	8.73	*
Bomet	*Burkholderia sp*. BMT21	8.30	*
Kirinyaga	*Bacillus sp*. KRN1	8.17	*
Kiambu	*Streptomyces sp.* KMB1	8.00	*
Kirinyaga	*Bacillus sp*. KRN3	7.73	*
Kiambu	*Streptomyces* sp. KMB10	7.50	*
Kiambu	*Streptomyces* sp. KMB3	7.10	*
Kiambu	*Pseudomonas* sp. KMB11	6.93	*
Bomet	*Bacillus sp*. BMT1	6.67	*
Bomet	*Micrococcus* sp. BMT11	6.50	*
Bomet	*Enterobacter sp*. BMT9	6.43	*
Bomet	*Micrococcus sp*. BMT12	5.33	*
Bomet	*Micrococcus sp*. BMT10	5.23	*
Kiambu	*Micrococcus sp*. KMB4	4.57	*
Kiambu	*Serratia sp*. KMB6	4.27	*
Bomet	*Micrococcus sp*. BMT15	4.10	*
Kiambu	*Serratia sp.* KMB7	4.03	*
Kiambu	*Micrococcus sp*. KMB8	4.00	*
Bomet	*Micrococcus sp*. BMT13	3.97	*
Kiambu	*Micrococcus sp*. KMB12	3.93	*
Kiambu	*Micrococcus sp*. KJ9	3.93	*
Kiambu	*Micrococcus sp*. KMB2	3.90	*
Bomet	*Micrococcus sp*. BMT8	3.53	*
Bomet	*Chryseobacterium sp*. BMT5	3.23	*
Kajiado	*Chryseobacterium sp*. KJ8	2.97	*
	Control	0.00 a	0.00 a

Values within the same column followed by similar letter are not significantly different at 0.05 level of probability

Beneficial fungi isolated from the rhizosphere of healthy tomato plants revealed the presence of commonly known plant pathogenic antagonists including *Trichoderma, Penicillium* and *Aspergiullus*. Despite the presence of *Trichoderma* and *Penicillium* in most of rhizosphere soil samples obtained, their efficacy were limited to origin of isolation, depicting a likelihood of diversity of *Trichoderma* and *Penicillium* strains which should be identified based on molecular techniques. As shown on the Table 4.8 below, all fungal isolates obtained from the four counties demonstrated varying levels of efficacy in limiting the growth of *R. solanacearum* but all were below the minimum threshold.

Table 4.8: Showing Inhibition Distances (Mm) among Fungal Antagonists against *R. Solanacearum*

		Zones of Inhibition (mm)	
Isolate source	**Fungal Isolate**	**Trial 1**	**Trial II**
Kajiado	*Trichoderma sp*.KJ4	9.67 c	9.27 c
Kirinyaga	*Aspergillus sp*.KRN2	8.87 bc	8.83 bc
Kajido	*Trichoderma sp*.KJ2	8.67 bc	8.53 bc
Bomet	*Penicillium sp*.BMT11	7.33 bc	7.37 bc
Kiambu	*Penicilium sp*.KMB2	6.33 b	7.30 b
Kirinyaga	*Aspergillus sp*. KRN1	0.00	*
Bomet	*Aspergillus sp*.BMT12	0.00	*
Bomet	*Aspergillus sp*.BMT7	0.00	*
Bomet	*Aspergillus sp*.BMT8	0.00	*
Bomet	*Aspergillus sp*.BMT9	0.00	*
Kajiado	*Aspergillus sp*.KJ3	0.00	*
Kiambu	*Aspergillus sp*.KJ5	0.00	*
Kiambu	*Aspergillus sp*.KMB1	0.00	*
Kiambu	*Aspergillus sp*.KMB2	0.00	*
Kiambu	*Aspergillus sp*.KMB5	0.00	*
Kiambu	*Aspergillus sp*.KMB7	0.00	*
Kiambu	*Aspergillus sp*.KMB8	0.00	*
Kajiado	*Chladosporium sp*.KJ8	0.00	*
Kirinyaga	*Chladosporium sp*.KRN8	0.00	*
Bomet	*Fusarium sp*. BMT2	0.00	*
Bomet	*Fusarium sp*. BMT3	0.00	*
Kajiado	*Fusarium sp*.KJ1	0.00	*
Kajiado	*Fusarium sp*.KJ6	0.00	*
Kajiado	*Fusarium sp*.KJ7	0.00	*
Kiambu	*Fusarium sp*. KMB3	0.00	*
Kiambu	*Fusarium sp*. KMB9	0.00	*
Kirinyaga	*Fusarium sp*. KRN3	0.00	*
Kiambu	*Oedecephalum sp*. KMB11	0.00	*
Kirinyaga	*Oedecephalum sp*. KRN5	0.00	*
Kirinyaga	*Oedecephalum sp*. KRN7	0.00	*
Bomet	*Penicilin sp*. BMT6	0.00	*
Bomet	*Phytophtora sp*. BMT1	0.00	*
Kajiado	*Phytopthora sp.* KJ9	0.00	*
Kiambu	*Phytopthora sp*. KMB12	0.00	*
Kirinyaga	*phytopthora sp*. KRN4	0.00	*
Kirinyaga	*Phytopthora sp.* KRN6	0.00	*
Kajiado	*Sporidesmium sp.* KJ10	0.00	*
Kiambu	*Sporidesmium sp.* KMB10	0.00	*
Bomet	*Trichoderma sp*. BMT13	0.00	*
Bomet	*Trichoderma sp. BMT4*	0.00	*
Bomet	*Trichoderma sp*.BMT5	0.00	*
Kiambu	*Trichoderma sp*.KMB4	0.00	*
Kiambu	*Trichoderma sp*.KMB6	0.00	*
	Control	0.00 a	0 a

Values within the same column followed by similar letter are not significantly different at 0.05 level of probability

Since the inhibition distance were below the minimum threshold of 11mm, the fungal isolates were therefore, considered not viable for further experiment. In that regard, all fungal isolates were left out in subsequent greenhouse trials.

4.7 Efficacy of Bacterial Isolates against *R. Solanacearum* in the Greenhouse

Prequalification of both bacterial and fungal isolates was done in the lab by subjecting them into a challenge tests with *R. solanacearum*. None out of 43 fungal isolates demonstrated viability against the pathogen. On the other hand, based on the level of inhibition of *R. solanacearum*, only four bacterial isolates were concluded to be potentially viable against the pathogen in the greenhouse. The four isolates were screened to determine their efficacy against the *R.solanacearum* in Rio-grande tomatoes in the greenhouse (Plate 4.6). Studies conducted to affirm the efficacy of bacterial antagonists against *R. solanacearum* in the greenhouse revealed a very significant reduction in disease incidence and severity among tests plants, particularly by *Pseudomonas* and *Bacillus* species. Additionally, *Burkholderia* and *Bacillus* isolates obtained from rhizosphere soils sampled from Kajiado reduced disease severity and incidence as compared to negative control that were not treated with antagonists.

Plate 4.6: Greenhouse Test to Determine Efficacy of Bacterial Antagonists against *R. Solanacerum*.

Wilt symptoms appeared seven days after inoculation in those plants treated with antagonists whereas those with *R. solanacearum* only (negative control) took only three days to manifest, but ten days post inoculation period, the plants had wilted completely. Results among the treatments varied significantly between the antagonists and the control experiment in both percentages of disease severity and incidence. Significant differences ($p<0.05$) were observed in the disease incidence and severity between control and tests organism. The difference between *Bacillus sp*.Kj4 and *Bacillus sp*. KRN2 was however not significant. Plants that were inoculated with *R. solanacearum* only without any antagonists recorded the highest disease incidence (49.03%) as compared to those treated with *Bacillus* sp.KMB16 (0%), *Pseudomonas* sp.KJ2 (0%), *Bacillus* sp.KJ4 (16.99%), and *Burkholderi*a sp.KRN2 (7.87%) (Fig 4.3). The highest disease severity was recorded on those plants inoculated with *R. solanacearum* only without any antagonists (33.33%) followed by *Bacillus* sp.KJ4 (13.9%) and *Burkholderia* sp.KRN2 (8.33%). Plants inoculated with *Bacillus* sp.KMB16 and *Pseudomonas* sp.KJ2 did not show any bacterial wilt symptoms throughout the experiment. Determination of biocontrol efficacy were highest among the treatment that did not record any wilt symptoms i.e *Bacillus* sp.KMB16 and *Pseudomonas* sp.KJ2 recorded 100% biocontrol efficacy against *R. solanacearum*. *Bacillus* sp.KJ4 and *Burkholderia* sp.KRN2 recorded 58% and 75% biocontrol efficacy respectively (Table 7). The results revealed that those plants that were not inoculated with antagonists lacked inferred resistance to bacterial wilt and therefore could not demonstrate tolerance to *R. solanacearum*. Antagonists that recorded 100 % bio control efficacy did not show any wilt symptoms associated with *R. solanacearum* and therefore no disease incidence and severity were recorded from the plants treated with these microbial isolates as antagonists.

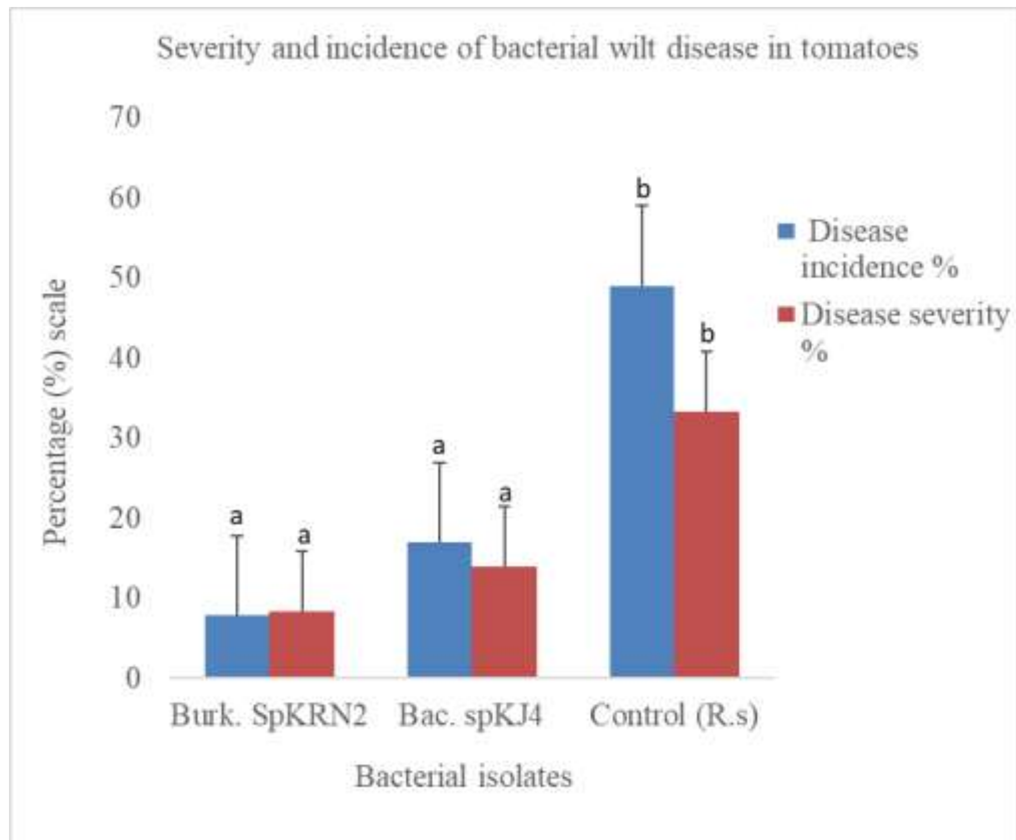


Figure 4.4: Level of Disease Incidence and Severity (%) among Treatments with *Burkholderia* Sp. KRN2, *Bacillus* Sp. KJ4 Isolates and Control Experiment

The results obtained in this study point out that rhizosphere microbes associated with tolerant plants can biologically reduce disease infection in crops. *In vitro* data conforms to the findings of Mohammed *et al*. (2020) where *Pseudomonas* species demonstrated the inhibition of *R. solanacearum* that ranged from 0.0 mm to 33.0 mm under *in vitro* tests. The results of greenhouse experiment under this study also conforms to their findings where diseases incidence under treatments with *Pseudomonas* species was reduced to 0% (100% biocontrol efficacy) as compared to the control treatment *(R. solanacearum)* where they recorded highest disease incidence (67%). Further, under greenhouse experiment, the highest control efficacy achieved in this study under treatment with *Bacillus* sp.KMB16 and *Pseudomonas sp*.KJ2 are in tandem with what Mohammed *et al*. (2020) achieved where they recorded control efficacy of 100% under treatment with *Pseudomonas aeruginosa.*

Bacteria use diverse mechanisms including production of bacterial allelochemicals like lytic enzymes, iron-chelating siderophores and antibiotics for their antagonistic activity and environment colonization (Mohammed *et al.,* 2020). Screening for the presence of these compounds among rhizosphere microbes is critical in finding beneficial microbes that can effectively colonize the roots and provide deleterious effect on non-beneficial microbes.

4.7.1 Population of *R. Solanacearum* in the Soils

The population of *R. solanacearum* in the soil were determined after the completion of the experiment. The results revealed antagonistic interactions among the treatments except control where there was an increase in *R. solanacearum* in comparison with the population during inoculation. Bacterial antagonists that demonstrated significant inhibitions diameter negatively impacted the multiplication of *R. solanacearum* in the soil. Soils or pots treated with *Baccilus* sp. BMT16 recorded the lowest population density of *R. solanacearum* as compared to *Pseudomonas* sp. KJ2 which recorded the highest number of *R. solanacearum* population density followed by *Burkholderia* KRN2 and *Bacillus* sp. KJ4 (Table 4.9). Similar to *Bacillus sp.*BMT16, the other three isolates followed a similar pattern in terms of their population during inoculation and after the experiment. Their effects on the population density of *R. solanacearum* was similar to that demonstrated by *Bacillus sp.*BMT16. As demonstrated in Table 4.9, bacterial antagonists that produced the highest inhibition zones diameter during *in vitro* assays recorded the lowest number of *R. solanacearum* colonies per unit weight of soil (gram).

Table 4.9: Population of Bacterial Antagonists against *R. Solanacearum* in the Soil Media

Population of *R. solanacearum* (Log10 CFU/gram of soil)	
Treatment	***R. solanacearum***
Control (*R. solanacearum*)	4.10 e
Pse. KJ2+Rs	2.93 d
Bac. KJ4+Rs	2.70 c
Burk. KRN2+Rs	2.50 b
Bac. BMT16+Rs	2.07 a

Values within the same column followed by similar letter are not significantly different at 0.05 level of probability

4.7.2 Population of R. *Solanacearum* in the Roots

All the roots systems of tomatoes used in the experiment were analyzed for the population of *R. solanacearum*. As was witnessed in the change of population densities of *R. solanacearum* in the soils, results achieved under fresh roots followed a similar pattern. As presented in Table 4.10, bacterial antagonists that were prequalified to be used in the greenhouse lowered the population density of *R. solanacearum*. *Bacillus* sp.BMT16 significantly reduced the population of *R. solanacearum* as compared to *Burkolderia* sp.KRN2, *Bacillus* sp.KJ4 and *Pseudomonas* sp.KJ2 but there was no significant difference in the population *of R. solanacearum* among the three isolates.

Table 4.10: Population of Bacterial Antagonists against *R. Solanacearum* in the Root Fresh Weight

Population of *R. solanacearum* (Log10 CFU/gram of roots)	
Treatment	***R. solanacearum***
Control	3.30 d
Pse. KJ2+RS	2.63 c
Bac. KJ4+RS	2.60 c
Burk. KRN2+Rs	2.37 b
Bac. BMT16+Rs	1.70 a

Values within the same column followed by similar letter are not significantly different at 0.05 level of probability

As witnessed above, interaction between beneficial and non-beneficial microbes takes place within the rhizosphere and inside plants organs, including the roots systems. The results on Table 4.9 and 4.10 demonstrate that some microbes are superior over the others and their effect significantly affect diverse physiological status of the plant, including plant health. Effective application of these biocontrol agents in the field needs a comprehensive understanding of their diversity. Bacterial and fungal isolates obtained from the four selected regions conferred varying levels of antagonistic activity against *R. solanacearum*, with bacterial isolates showing better antagonistic activity than fungal isolates. These observations are in line with the findings of Yuliar *et al*. (2015) who found out that bio-control organisms are dominated by bacteria (90%) as compared to fungi (10%). The superiority of bacterial isolates over fungal isolates can be attributed to their rapid growth rate and cell multiplication through binary fission.

In this study, inhibitory ability against the pathogens varied considerably according to the genera of both fungal and bacterial isolates. The results showed that only four out of 40 potent bacterial antagonists were able to inhibit growth of *R. solanacearum* by over 11mm. Similarly, four fungal isolates also showed inhibition of *R. solanacearum* though below the minimum threshold of 11mm. The results concurs

with those found by Priya *et al.* (2020) where 5 out of 30 strains of *Bacillus* strains tested against *R. solanacearum* were found to be effective. Similarly, Mao *et al*. (2017), tested 20 rhizobacteria species against *R. solanacearum in vitro* and found 5 species to be highly effective against the pathogen. Among the bacterial isolates obtained, *Bacillus* sp. demonstrated higher efficacy in inhibiting growth of *R. solanacearum* under *in vitro* test as compared to other bacterial species. In this study, *Trichoderma* sp., *Penicillium* sp., and *Aspergillus* sp. were the only isolates that inhibited growth of *R. solanacearum,* but not above the required minimum threshold *(>11 mm).* Murthy and Srinivas (2012) reported domination of *Trichoderma asperellum* over other strains in growth inhibition of *R. solanacearum.* Under this study, identification of fungal isolates were limited to the genus level and there is a possibility that *Trichoderma sp.* obtained and tested *in vitro*, against *R. solanacearum* were not *Trichoderma asperellum,* and hence low growth inhibition. In a different experiment *Trichoderma hamatum* was found to exhibit higher growth inhibition of *R. solanacearum* as compared to *T. virens* and *T. asperellum* (Cheng *et al.,* 2015). However, the data obtained in this study contradicts those of Shashitu (2021) who identified *Trichoderma*, as effective against *R. solanacearum*. Further, the results obtained in this study further contradicts their report that *Streptomyces*, *Trichoderma,* and *Penicillium* are useful abundant microorganisms in the rhizosphere. The discrepancy of the results obtained could be tied to change in environment or the fungal isolates obtained are those species that are ineffective against pathogens.

Soil organic matter, soil pH, levels of nutrients available and the amount of moisture affects efficacy of biocontrol agents including *Streptomyces* (Vurukonda *et al.,* 2018). The results on varying levels of growth inhibition by *Bacillus* species against *R. solanacearum* are consistent with several studies done previously, demonstrating varying levels of inhibition against *R. solanacearum* by different strains of *Bacillus* sp. (Priya *et al*., 2020, Hasinu *et al*., 2021; Huang *et al*., 2014; Li *et al*., 2021). Further, two other bacterial isolates; *Pseudomonas*, and *Burkholderia* species also had significant inhibitory effect on the growth of *R. solanacearum*. The result obtained on the efficacy of *Pseudomonas* species on antimicrobial effect on *R. solanacearum* is similar to those of Murthy and Srinivas (2012) who established

antagonistic effects of different strains of *Pseudomon*as strains against *R. solanacearum*. This trend was also witnessed among fungal isolates where some genera showed higher inhibition to *R. solanacearum* than the others, despite all of them showing inhibitions zones of below 11mm. For instance, *Trichoderma* sp.KJ4 and *Trichoderma* sp.KJ2 showed significant difference in their antibacterial activity. Similar, *Penicilium* sp.BMT11 significantly differed in its antibacterial activity with *Penicillium* sp.KMB2.

These observations are consistent with those of Guo *et al*. (2021) which point out that *Trichoderma* sp. were superior in its antibacterial activity against *R. solanacearum* than other fungal genera under *In vitro* conditions. The *In vitro* activity of *Burkholderia* isolate also demonstrated strong inhibition of *R. solanacearum* growth. This results confirms reports by Elshafie and Camele (2021), which pointed out *Burkholderia* as an important bacterial species with different benefits to plants, including production of antibiotics that inhibits pathogens infection. Previous research done have pointed out varying inhibitory efficacy of diverse species of *Streptomyces, Micrococcus, Serratia* and *Chryseobacterium* against various pathogens including *R. solanacearum*. Boukaew *et al*. (2010), tested 14 isolates of *Streptomyces* and found out that only 3 isolates were effective against *R. solanacearum*. Though other bacterial isolates that included *Streptomyces, Micrococcus, Serratia and Chryseobacterium* had inhibitory effect on growth of *R. solanacearum*, their efficacy were minimal and, therefore, were not viable to be repeated in the second trial. These four bacterial isolates, with other species from *Bacillus, Burkholderia,* and *Pseudomonas*, did not effectively inhibited growth of *R. solanacearum.* The appearance of clear halos observed after the incubation of dual culture plates (Plate 4.5) seeded with *R. solonacearum* and impregnated with antagonists points out the possibility of production of antibacterial substances into the growth media, which have a negative effect on the growth of the pathogen.

Some previous studies have attributed the presence of clear halos to antibacterial substances, which could include antibiotics and enzymes released by the antagonists (Cheng *et al*., 2015). These substances could compromise the pathogenic machinery of the *R. solanacearum,* thus hampering its virulent activity. According to Krishna

and Gardener (2006), antibiotic produced by the various microbes are known to be particularly effective in controlling specific plant pathogens. The rhizosphere microbes under this study, despite some falling in the same genera, demonstrated different level of inhibition against *R. solanacearum*. This implies that these microbes could have produced diverse enzymes and antibiotics that are pathogens specific. Plants antagonists significantly decreased disease incidence and severity depending on the isolate. For instance, Bacillus sp. KMB16 and *Pseudomonas* sp.KJ2 completely suppressed the pathogenic effect (0% in disease incidence and severity) *R. solanacearum* in tomato plants. This recorded the highest decrease in disease severity and incidence (100%) where there was not a single wilting symptom on the host plants, as compared to *Burkholderia* sp. KRN2 and *Bacillus* sp.KJ4 and control treatment. These results demonstrating reduction in disease incidence and severity are in line with those found by Chandrasekaran *et al.* (2016) who found that biocontrol agents including Pseudomonas (*P. fluorescens, P. putida*) and Bacillus (*B. cereus, B. subtilis* and *B. amyloliquefaciens*) were effective in decreasing disease incidence and severity on average by 53.7% *and* 49.3% respectively.

The efficacy of *Bacillus* species in reduction disease incidence and its high biocontrol efficacy confirms the results found by Ding *et al*. (2012) where bacterial wilt incidence of potted potatoes was significantly reduced to 8.9% and 11.1 % by bio fertilizers formulated from *Bacillus amyloliquefaciens* and *Bacillus subtilis* respectively, as compared to control (57.7%). Further, the biocontrol efficacy of the bio-fertilizers from these respective BCAs was 84.6% and 80.8%. Similar to the results of this study, biocontrol efficacy of tested antagonists ranged from 58%, and 75% *Bacillus* sp.KJ4, *Burkholderia* sp.KRN2 respectively. *Bacillus* BMT16 and *Pseudomonas* sp. KJ2 both recorded 100% biocontrol efficacy. Lack of symptoms associated with bacterial wilt caused by *R. solanacearum* can be attributed to interactions between the antagonists, hosts plants, environment and virulence of the disease causing organisms. Interaction of *R. solanacearum* with both antagonists could have altered pathogenicity machinery, leading to its loss of DNA integrity, and thus leading to a loss of virulence. This likelihood was further affirmed by the presence of *R. solanacearum* in both the root systems and rhizospheric soils of the tests plants, yet plants did not reveal any symptoms of bacterial wilt disease despite

its presence. Loss of virulence of *R. solanacearum*, due to its ability to metamorphose into avirulent strain and possibility of disintegration of DNA as a result of antagonists have been pointed to positively impact on the plant. This explains the healthy appearance of tests plants treated with *Bacillus* sp.KMB16 and *Pseudomonas* sp.KJ2, unlike the rest that recorded different levels of incidence and severity.

The efficacy of microbes in their antagonistic activity against plant pathogens are affected by several factors. Padder and Sharma (2011), identified some of the factors to include environmental factors, time of treatment, application season, the frequency of the application and the technique of the treatment. The growing conditions for the microbial bio-control agents in plants, plant physiology, cultivar genetics and conditions during germination and infection of the pathogens on hosts' plants impact the final outcome of microbial bio-control mechanisms (Köhl *et al*., 2019). According to Cook (1993), numerous strategies are utilized by those microbes regarded as effective biocontrol agents in antagonizing plant pathogens. Pseudomonas, for instance, can secrete 2, 4-diacetylphloroglucocinol (DAPG) (Silva *et al.,* 2004), while at the same time inducing host defense mechanisms against plant pathogens (Silva *et al*., 2004). Though the conclusive evidence of the role of antibiosis in the control of plant pathogens was questionable (Abrudan *et al.,* 2015), overtime, there have been new evaluations methods providing new and sufficient evidence indicating antibiosis as a biocontrol mechanism among biocontrol agents (Fravel, 1988). Currently, antiobiosis is considered as a superior method used by biocontrol agents in suppressing plant pathogens. However, latest insights have pointed out the possibility of other mechanisms that include root colonization, which allows these beneficial organisms to outcompete pathogens in nutrients utilization (Heydari & Pessarakli, 2010). Sequence of events starting from the establishment of the biocontrol organisms, release of signaling complex and the induction of a series of metabolic events inducing defense systems of the plant and how the pathogen reacts to the plant's defense mechanisms is another key factor that impacts biocontrol agents' efficacy (Köhl *et al*., 2019).

Understanding the interactions between the pathogens and microbial antagonists in the soil or in the infection site is critical in the prediction of the success of biocontrol organism. Critical study of the changes in population densities over time can inform the impact of the biocontrol agents and pathogens on the population dynamics of each organisms, and the role that the physical and biological environment play in the interaction (Paulitz, 2000). In this study, results pointed out that biological antagonists that demonstrated higher confrontation with *R. solanacearum* during dual culture assays had a negative impact on the population of the pathogen causing organisms in the soil and the roots of the hosts' plants (Table 4.9 and Table 4.10). This study confirms the reports of Yuliar and Toyota (2015) who reported a reduction in the population of *R. solanacearum* in the rhizosphere, roots surfaces and xylem of tomato plants by 26.7%, 79.3% and 81.7% respectively. *Bacillus* sp. BMT16 was the most effective bacterial antagonists among the four isolates that considerably reduced the population of *R. solanacearum* in both the rhizophere soils and roots of the hosts' plants. However, its reduction ability was greater in the roots than in the rhizophere. Similarly, this trend was also witnessed among the remaining three bacterial isolates; *Pseudomonas* sp. KJ2, *Bacillus* sp.KJ4 and *Burkholderia* sp.KRN2. Multiple interactions among plants, disease causing organisms and biological antagonists mediate biological control process. Production of bacterial allelochemicals that include lytic enzymes, antibiotics and iron-chelating siderophores enables bacterial colonization and inhibitory activity in the environment (Mohammed *et al*., 2020). *Bacillus cereus* AR156, for instance, works as an elicitor of plant immune reactions (Wang *et al*., 2019). Plants natural defense systems, in addition, to diverse mechanisms as a results of bacterial isolates used during inoculation explains the reduced *R. solanacearum* populations in the roots systems of tomato hosts plants. The effective isolates can be attributed to tolerance of tomato plants to bacterial wilt disease caused by *R. solanacearum* (Elsayed *et al.,* 2020).

CHAPTER FIVE

SUMMARY, CONCLUSION AND RECOMMENDATIONS

5.1 Summary

Rhizospheric environment, together with processes that are mediated by microbes are crucial in the functioning and productivity of the ecosystem. This study presents the screening and use of beneficial rhizosphere microbes to manage *Ralstonia solanacearum* responsible for causing bacterial wilt in tomatoes in the greenhouse. Beneficial microbes within the tolerant tomato plant rhizosphere is responsible for inducing resistance against pathogen including bacterial wilt disease (Elsayed *et al*., 2020). In this regards, this study was devoted to purposively sampling rhizosphere soils of tolerant tomatoes and infected tomato plants from Kajiado, Bomet, Kirinyaga and Kiambu. The counties are known to be bacterial wilt endemic regions in Kenya. The study involved isolating both fungi and bacteria using general media. Isolated fungi were characterized based on their morphology while bacteria were characterized using both microscopic and biochemical observations. However, molecular research should be done to ascertain the molecular interaction between the antagonists, hosts plants and plant pathogens with an aim to understand strategies utilized by the antagonists for more efficient control of the pathogens. As witnessed from the results, the population of microbes varied across the regions where sampling was done. Even though the rhizosphere harbors a wide range of microbes, the diversity vary depending on the various factors including the root zone, species of the hosts plants, phenological phase of plants, stress, and disease events (Marschner *et al*., 2011). Soil properties, for instance, structure, p^H and nutrient status by selectively creating conducive environment favoring certain types of microorga.nisms and regulating available root exudates, thus affecting the selection of microbes by plants (Igiehon & Babalola, 2018). The study also included testing the efficacy of isolated microbes in controlling the growth of *R. solanacearum* under *in vitro* conditions and control of bacterial wilt disease in tomatoes in the greenhouse. Further details revealed the impact of the bacterial antagonists on the population of *R. solanacearum* in the roots and rhizosphere soils.

5.2 Conclusion

The thesis presents the isolation, characterization and use of beneficial rhizosphere microbes associated to bacterial wilt tolerant tomatoes sampled from bacterial wilt endemic fields. General purpose media (PDA for isolation of fungal isolates and NA for isolation of bacterial isolates) were relied for isolation and purification instead of selective media since there was no predetermined microbes targeted. Macroscopic and microscopic characteristics were used to characterize fungi whereas bacteria were characterized using biochemical reactions and microscopic methods. The approach used in this work demonstrated the reliability of culture-dependent screening method to isolate beneficial microbes existing as a community within the rhizospheres of healthy plants. Additionally, it demonstrated the alternative method of characterizing isolated microbes, which oftenly requires timely preparation of the sample and use of expensive standards and equipment commonly out of reach to many labs or plants pathologist. In the wake of increased demand to develop pathogen control solutions that are reliable, affordable and friendly to environment, current research into biological control agents adapted to particular environments offer viable solutions. These alternative methods can a restore a balance in the soil. Antagonistic tests of isolated fungi and bacteria against *R. solanacearum* during *in vitro* and *in vivo* screening demonstrated varying levels of efficacy. However, the *in vitro* screening method was limiting because it could only pick out those effective by antibiosis or competing on the culture media. Likely isolates with potential were discarded. Future research can screen the discarded isolates to determine their efficacy *in vivo*. Effective fungal and microbial isolates from rhizosphere soils can provide alternative or additional component to existing integrated pests and disease management tools. Harnessing the potential of these effective microbes will reduce overreliance of synthetic chemicals which leads to the development of resistance by the pathogen and unfavorable shift in the population of rhizosphere microbes as a result of altered environment. Additionally, the method if harnessed well will increase tomato production and allow farmers to harness prime markets commonly characterized by organically produced products. This will increase households' income among smallholder farmers normally considered as resource deficient.

5.3 Recommendations

The following recommendations are made according to this study to enhancing efforts towards achieving a more sustainable and effective control of bacterial wilt disease of tomato in Kenya

i. Further research using molecular tools should be used to accurately identify characterized isolates inhabiting healthy rhizosphere, up to the species level for better understanding of their relation and mode of action
ii. More study on the specific effect of changes in soil chemical composition and climatic factors on the efficacy of the rhizosphere microbes effective under *in vitro* conditions, and formulate them as products easily usable by farmers.

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APPENDICES

Appendix I: ANOVA of Inhibition of Growth of R. *Solanacearum* by Bacterial Antagonists in 1st Experiment

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Treatments	40	1207.2929	30.1823	35.67	<.001
Residual	80	67.6933	0.8462		
Total	120	1274.9863			

Appendix II: ANOVA of Inhibition of Growth of *R. Solanacearum* by Bacterial Antagonists in 2nd Experiment

Source of variation	**d.f.**	**s.s.**	**m.s.**	**v.r**	**F pr.**
Treatments	40	2114.4383	52.861	314.65	<.001
Residual	80	13.44	0.168		
Total	120	2127.8783			

Appendix III: ANOVA of Inhibition of Growth of *R. Solanacearum* by Fungal Antagonists in 1st Experiment

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Treatments	43	907.565	21.1062	84.14	<.001
Residual	86	21.5733	0.2509		
Total	129	929.139			

Appendix IV: ANOVA of Inhibition of Growth of *R. Solanacearum* by Fungal Antagonists in 2nd Experiment

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Treatments	43	914.737	21.273	68.81	<.001
Residual	86	26.5867	0.3091		
Total	129	941.324			

Appendix V: ANOVA of *R. Solanacearum* Population in Unit Gram of Soil

Source of variation	**d.f**	**s.s**	**m.s.**	**v.r.**	**F pr.**
Treatment	4	3.434359	0.85859	128.79	<.001
Residual	8	0.053333	0.00667		
Total	12	3.487692			

Appendix VI: ANOVA of *R. Solanacearum* Population in Roots of Tomato Test Plants

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Treatment	4	2.56667	0.64167	33.48	<.001
Residual	8	0.15333	0.01917		
Total	12	2.72			

Appendix VII: Output Data Analysis on Inhibition of *R. Solanacearum* by Bacterial Isolates

GenStat Release 14.1 (PC/Windows) 17 November 2010 09:05:20

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GenStat Procedure Library Release PL22.1

```
1 %CD 'C:/Users/pc/Documents'
2 "Data taken from File: C:/Users/pc/Desktop/Final Datas.xlsx"
3 DELETE [REDEFINE=yes] _stitle_: TEXT _stitle_
4 READ [PRINT=*; SETNVALUES=yes] _stitle_
8 PRINT [IPRINT=*] _stitle_; JUST=left
```

Data imported from Excel file: C:\Users\pc\Desktop\Final Datas.xlsx

on: 17-Nov-2010 10:11:05

taken from sheet "Bacterial Isolates (mm)", cells A2:L16

```
 9    DELETE   [REDEFINE=yes]   Treatments,Experiment_1,Experiment_2
10                                  UNITS                    [NVALUES=*]
11   FACTOR  [MODIFY=yes;  NVALUES=15;  LEVELS=5;  LABELS=!t('Bacillus
sp.BMT16',\
12                            'Bacillus            sp.KJ4','Burkholderia
sp.KRN2','Control','Pseudomonas                                sp.KJ2')\
13                        ;            REFERENCE=1]            Treatments
14              READ       Treatments;       FREPRESENTATION=ordinal
```

Identifier	Values	Missing	Levels
Treatments	15	0	5

```
16                VARIATE          [NVALUES=15]          Experiment_1
17                                 READ                  Experiment_1
```

Identifier	Minimum	Mean	Maximum	Values	Missing
Experiment_1	0.0000	11.00	17.00	15	0

```
19                VARIATE          [NVALUES=15]          Experiment_2
20                                 READ                  Experiment_2
```

Identifier	Minimum	Mean	Maximum	Values	Missing
Experiment_2	0.0000	11.13	17.20	15	0

```
  22
  23   %PostMessage  1129;  0;  86439688  "Sheet  Update  Completed"
  24                                "One-way                design"
  25                  DELETE         [REDEFINE=yes]          _ibalance
  26                 A2WAY         [PRINT=aovtable,information,means;
TREATMENTS=Treatments;              FPROB=yes;              PSE=diff,\
  27   lsd,means,alldiff,alllsd;  LSDLEVEL=5;  PLOT=*;  EXIT=_ibalance]
Experiment_1;  SAVE=_a2save
```

Analysis of variance

Variate: Experiment_1

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Treatments	4	478.667	119.667	89.75	<.001
Residual	10	13.333	1.333		
Total	14	492.000			

Information summary

All terms orthogonal, none aliased.

Tables of means

Variate: Experiment_1

Grand mean 11.00

Treatments	Bacillus sp.BMT16	Bacillus sp.KJ4	Burkholderia sp.KRN2
	16.00	12.00	13.33

Treatments	Control	Pseudomonas sp.KJ2
	0.00	13.67

Standard errors of means

Table	Treatments
rep.	3
d.f.	10
e.s.e.	0.667

Standard errors of differences of means

Table	Treatments
rep.	3
d.f.	10
s.e.d.	0.943

Least significant differences of means (5% level)

Table	Treatments
rep.	3
d.f.	10
l.s.d.	2.101

```
  28              IF       _ibalance.eq.0       .OR.       _ibalance.eq.1
  29             DELETE    [REDEFINE=yes]    _mean,    _rep,    _var,    _rdf
  30       AKEEP  [SAVE=_a2save[2]]  Treatments;  MEAN=_mean;  REP=_rep;
VARIANCE=_var;                                                   RTERM=_resid
  31                  AKEEP     [SAVE=_a2save[2]]     #_resid;     DF=_rdf
  32      AMCOMPARISON  [METHOD=fplsd;  DIRECTION=ascending;  PROB=0.05]
Treatments
```

Fisher's protected least significant difference test

Treatments

	Mean	
Control	0.00	a
Bacillus sp.KJ4	12.00	b
Burkholderia sp.KRN2	13.33	b
Pseudomonas sp.KJ2	13.67	b
Bacillus sp.BMT16	16.00	c

```
  33                                                              ENDIF
  34                                          SET                 [IN=*]
  40                                       "One-way              design"
  41              DELETE          [REDEFINE=yes]            _ibalance
  42              A2WAY       [PRINT=aovtable,information,means;
TREATMENTS=Treatments;          FPROB=yes;              PSE=diff,\
  43  lsd,means,alldiff,alllsd; LSDLEVEL=5; PLOT=*; EXIT=_ibalance]
Experiment_2; SAVE=_a2save
```

Analysis of variance

Variate: Experiment_2

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Treatments	4	487.609	121.902	90.70	<.001
Residual	10	13.440	1.344		
Total	14	501.049			

Information summary

All terms orthogonal, none aliased.

Tables of means

Variate: Experiment_2

Grand mean 11.13

Treatments	Bacillus sp.BMT16	Bacillus sp.KJ4	Burkholderia sp.KRN2
	16.13	12.30	13.43

Treatments	Control	Pseudomonas sp.KJ2
	0.00	13.77

Standard errors of means

Table	Treatments
rep.	3
d.f.	10
e.s.e.	0.669

Standard errors of differences of means

Table	Treatments
rep.	3
d.f.	10
s.e.d.	0.947

Least significant differences of means (5% level)

Table	Treatments
rep.	3
d.f.	10
l.s.d.	2.109

```
  44    IF    _ibalance.eq.0    .OR.    _ibalance.eq.1
  45    DELETE    [REDEFINE=yes]    _mean,    _rep,    _var,    _rdf
  46    AKEEP  [SAVE=_a2save[2]]  Treatments;  MEAN=_mean;  REP=_rep;
VARIANCE=_var;                                     RTERM=_resid
  47    AKEEP    [SAVE=_a2save[2]]    #_resid;    DF=_rdf
  48    AMCOMPARISON  [METHOD=fplsd;  DIRECTION=ascending;  PROB=0.05]
Treatments
```

Fisher's protected least significant difference test

Treatments

	Mean	
Control	0.00	a
Bacillus sp.KJ4	12.30	b
Burkholderia sp.KRN2	13.43	b
Pseudomonas sp.KJ2	13.77	b
Bacillus sp.BMT16	16.13	c

```
49                                                                  ENDIF
50  SET [IN=*]
```

Appendix VIII: Output of Data Analysis on Inhibition of *R. Solanacearum* by Fungal Isolates

GenStat Release 14.1 (PC/Windows) 17 November 2010 10:15:22

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GenStat Procedure Library Release PL22.1

```
1                       %CD            'C:/Users/pc/Documents'
2   "Data taken from File: C:/Users/pc/Desktop/Final Datas.xlsx"
3      DELETE    [REDEFINE=yes]    _stitle_:    TEXT    _stitle_
4         READ      [PRINT=*;      SETNVALUES=yes]      _stitle_
8          PRINT      [IPRINT=*]       _stitle_;       JUST=left
```

Data imported from Excel file: C:\Users\pc\Desktop\Final Datas.xlsx

on: 17-Nov-2010 10:16:21

taken from sheet "Fungal Isolates (mm)", cells A2:M19

```
 9    DELETE   [REDEFINE=yes]   Treatments,Experiment_1,Experiment_2
10                              UNITS                   [NVALUES=*]
11           FACTOR      [MODIFY=yes;      NVALUES=18;      LEVELS=6;
LABELS=!t('Aspergillus                                          sp.KRN2',\
12               'Control','Penicilium        sp.KMB2','Penicillium
sp.BMT11','Trichoderma                                            sp.KJ2',\
13         'Trichoderma     sp.KJ4');     REFERENCE=1]     Treatments
14               READ       Treatments;       FREPRESENTATION=ordinal
```

Identifier	Values	Missing	Levels
Treatments	18	0	6

```
16                 VARIATE          [NVALUES=18]          Experiment_1
17                                  READ                  Experiment_1
```

Identifier	Minimum	Mean	Maximum	Values	Missing
Experiment_1	0.0000	6.811	11.00	18	0

```
19                 VARIATE          [NVALUES=18]          Experiment_2
20                                  READ                  Experiment_2
```

Identifier	Minimum	Mean	Maximum	Values	Missing
Experiment_2	0.0000	6.883	10.10	18	0

```
 22
 23  %PostMessage 1129; 0; 86006736 "Sheet Update Completed"
 24                              "One-way design"
 25          DELETE          [REDEFINE=yes]          _ibalance
 26          A2WAY      [PRINT=aovtable,information,means;
TREATMENTS=Treatments;         FPROB=yes;         PSE=diff,\
 27  lsd,means,alldiff,alllsd; LSDLEVEL=5; PLOT=*; EXIT=_ibalance]
Experiment_1; SAVE=_a2save
```

Analysis of variance

Variate: Experiment_1

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Treatments	5	188.144	37.629	20.93	<.001
Residual	12	21.573	1.798		
Total	17	209.718			

Information summary

All terms orthogonal, none aliased.

Tables of means

Variate: Experiment_1

Grand mean 6.81

Treatments	Aspergillus sp.KRN2	Control	Penicilium sp.KMB2
	8.87	0.00	6.33

Treatments	Penicillium sp.BMT11	Trichoderma sp.KJ2	Trichoderma sp.KJ4
	7.33	8.67	9.67

Standard errors of means

Table	Treatments
rep.	3
d.f.	12
e.s.e.	0.774

Standard errors of differences of means

Table	Treatments
rep.	3
d.f.	12
s.e.d.	1.095

Least significant differences of means (5% level)

Table	Treatments
rep.	3
d.f.	12
l.s.d.	2.385

```
  28   IF _ibalance.eq.0 .OR. _ibalance.eq.1
  29   DELETE [REDEFINE=yes] _mean, _rep, _var, _rdf
  30   AKEEP [SAVE=_a2save[2]] Treatments; MEAN=_mean; REP=_rep;
VARIANCE=_var; RTERM=_resid
  31   AKEEP [SAVE=_a2save[2]] #_resid; DF=_rdf
  32   AMCOMPARISON [METHOD=fplsd; DIRECTION=ascending; PROB=0.05]
Treatments
```

Fisher's protected least significant difference test

Treatments

	Mean	
Control	0.000	a
Penicilium sp.KMB2	6.333	b
Penicillium sp.BMT11	7.333	bc
Trichoderma sp.KJ2	8.667	bc
Aspergillus sp.KRN2	8.867	c
Trichoderma sp.KJ4	9.667	c

```
  33 ENDIF
  34 SET [IN=*]
  40 "One-way design"
  41 DELETE [REDEFINE=yes] _ibalance
  42 A2WAY [PRINT=aovtable,information,means; TREATMENTS=Treatments; FPROB=yes; PSE=diff,\
  43 lsd,means,alldiff,alllsd; LSDLEVEL=5; PLOT=*; EXIT=_ibalance] Experiment_2; SAVE=_a2save
```

Analysis of variance

Variate: Experiment_2

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Treatments	5	179.978	35.996	16.25	<.001
Residual	12	26.587	2.216		
Total	17	206.565			

Information summary

All terms orthogonal, none aliased.

Message: the following units have large residuals.

units 9	2.73	s.e. 1.22

Tables of means

Variate: Experiment_2

Grand mean 6.88

Treatments	Aspergillus sp.KRN2	Control	Penicilium sp.KMB2
	8.53	0.00	7.30

Treatments	Penicillium sp.BMT11	Trichoderma sp.KJ2	Trichoderma sp.KJ4
	7.37	8.83	9.27

Standard errors of means

Table	Treatments
rep.	3
d.f.	12
e.s.e.	0.859

Standard errors of differences of means

Table	Treatments
rep.	3
d.f.	12

s.e.d.	1.215

Least significant differences of means (5% level)

Table	Treatments
rep.	3
d.f.	12
l.s.d.	2.648

```
  44        IF     _ibalance.eq.0     .OR.     _ibalance.eq.1
  45        DELETE    [REDEFINE=yes]   _mean,   _rep,   _var,   _rdf
  46     AKEEP  [SAVE=_a2save[2]]  Treatments;  MEAN=_mean;  REP=_rep;
VARIANCE=_var;                                        RTERM=_resid
  47        AKEEP    [SAVE=_a2save[2]]    #_resid;    DF=_rdf
  48    AMCOMPARISON  [METHOD=fplsd;  DIRECTION=ascending;  PROB=0.05]
Treatments
```

Fisher's protected least significant difference test

Treatments

	Mean	
Control	0.000	a
Penicilium sp.KMB2	7.300	b
Penicillium sp.BMT11	7.367	b
Aspergillus sp.KRN2	8.533	b
Trichoderma sp.KJ2	8.833	b
Trichoderma sp.KJ4	9.267	b

```
49                                                                    ENDIF
50  SET [IN=*]
```

Appendix IX: Output of Data Analysis on *R. Solanacearum* Population on Soil

GenStat Release 14.1 (PC/Windows) 17 November 2010 10:19:07

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GenStat Procedure Library Release PL22.1

```
   1  %CD 'C:/Users/pc/Documents'
   2  "Data taken from File: C:/Users/pc/Desktop/Final Datas.xlsx"
   3  DELETE [REDEFINE=yes] _stitle_: TEXT _stitle_
   4  READ [PRINT=*; SETNVALUES=yes] _stitle_
   8  PRINT [IPRINT=*] _stitle_; JUST=left
```

Data imported from Excel file: C:\Users\pc\Desktop\Final Datas.xlsx

on: 17-Nov-2010 10:19:34

taken from sheet "Soil weight", cells A2:I14

```
   9  DELETE [REDEFINE=yes] Treatment,R_solanacearum
  10  UNITS [NVALUES=*]
  11  FACTOR [MODIFY=yes; NVALUES=13; LEVELS=5; LABELS=!t('Bac.
BMT16+Rs',\
  12  'Bac. KJ4+RS','Burk. KRN2+Rs','Control','Pse. KJ2+RS')\
  13  ; REFERENCE=1] Treatment
  14  READ Treatment; FREPRESENTATION=ordinal
```

Identifier Values Missing Levels

Treatment	13	0	5

```
  16              VARIATE    [NVALUES=13]    R_solanacearum
  17                         READ            R_solanacearum
```

Identifier	Minimum	Mean	Maximum	Values	Missing
R_solanacearum	2.000	2.669	4.100	13	0

```
  19
  20   %PostMessage  1129;  0;  85614304  "Sheet  Update  Completed"
  21                          "One-way                       design"
  22                 DELETE         [REDEFINE=yes]          _ibalance
  23  A2WAY [PRINT=aovtable,information,means; TREATMENTS=Treatment;
FPROB=yes;                                                 PSE=diff,\
  24   lsd,means,alldiff,alllsd; LSDLEVEL=5; PLOT=*; EXIT=_ibalance]
R_solanacearum; SAVE=_a2save
```

Analysis of variance

Variate: R_solanacearum

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Treatment	4	3.434359	0.858590	128.79	<.001
Residual	8	0.053333	0.006667		
Total	12	3.487692			

Information summary

All terms orthogonal, none aliased.

Tables of means

Grand	mean	2.66923

Treatment	Bac. BMT16+Rs	Bac. KJ4+RS	Burk. KRN2+Rs	Control	Pse. KJ2+RS
mean	2.06667	2.70000	2.50000	4.10000	2.93333
rep.	3	3	3	1	3
s.e.	0.04714	0.04714	0.04714	0.08165	0.04714

Standard errors of differences of means

Treatment Bac. BMT16+Rs	1	*			
Treatment Bac. KJ4+RS	2	0.06667	*		
Treatment Burk. KRN2+Rs	3	0.06667	0.06667	*	
Treatment Control	4	0.09428	0.09428	0.09428	*
Treatment Pse. KJ2+RS	5	0.06667	0.06667	0.06667	0.09428
		1	2	3	4

Treatment Pse. KJ2+RS	5	*
		5

Minimum standard error of difference	0.06667
Average standard error of difference	0.07771
Maximum standard error of difference	0.09428

Least significant differences (at 5%)

1	*			
2	0.15373	*		
3	0.15373	0.15373	*	
4	0.21741	0.21741	0.21741	*

5	0.15373	0.15373	0.15373	0.21741	*
	1	2	3	4	5

Minimum least significant difference	0.1537
Average least significant difference	0.1792
Maximum least significant difference	0.2174

```
  25            IF       _ibalance.eq.0       .OR.       _ibalance.eq.1
  26           DELETE    [REDEFINE=yes]    _mean,    _rep,    _var,    _rdf
  27       AKEEP  [SAVE=_a2save[2]]  Treatment;  MEAN=_mean;  REP=_rep;
VARIANCE=_var;                                               RTERM=_resid
  28                AKEEP     [SAVE=_a2save[2]]     #_resid;     DF=_rdf
  29      AMCOMPARISON  [METHOD=fplsd;  DIRECTION=ascending;  PROB=0.05]
Treatment
```

Fisher's protected least significant difference test

Treatment

	Mean	
Bac. BMT16+Rs	2.067	a
Burk. KRN2+Rs	2.500	b
Bac. KJ4+RS	2.700	c
Pse. KJ2+RS	2.933	d
Control	4.100	e

```
30                                                                    ENDIF
31                                        SET                        [IN=*]
```

Appendix X: Output on Data Analysis on the Population of *R. Solanacearum* in the Roots

GenStat Release 14.1 (PC/Windows) 17 November 2010 10:32:11

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GenStat Procedure Library Release PL22.1

```
1 %CD 'C:/Users/pc/Documents'
2 "Data taken from File: C:/Users/pc/Desktop/Final Datas.xlsx"
3 DELETE [REDEFINE=yes] _stitle_: TEXT _stitle_
4 READ [PRINT=*; SETNVALUES=yes] _stitle_
8 PRINT [IPRINT=*] _stitle_; JUST=left
```

Data imported from Excel file: C:\Users\pc\Desktop\Final Datas.xlsx

on: 17-Nov-2010 10:32:32

taken from sheet "Root fresh wt", cells A2:B16

```
  9        DELETE     [REDEFINE=yes]     Treatment,R_solanacearum
  10                          UNITS                    [NVALUES=*]
  11    FACTOR  [MODIFY=yes;  NVALUES=15;  LEVELS=5;  LABELS=!t('Bac.
BMT16+Rs',\
  12      'Bac.   KJ4+RS','Burk.   KRN2+Rs','Control','Pse.   KJ2+RS')\
  13                  ;              REFERENCE=1]            Treatment
  14              READ        Treatment;        FREPRESENTATION=ordinal
```

Identifier	Values	Missing	Levels
Treatment	15	0	5

```
  16                VARIATE         [NVALUES=15]         R_solanacearum
  17                                READ                 R_solanacearum
```

Identifier	Minimum	Mean	Maximum	Values	Missing
R_solanacearum	1.500	2.487	3.300	15	0

```
  19
  20    %PostMessage  1129;  0;  85968952  "Sheet  Update  Completed"
  21                              "One-way                     design"
  22                  DELETE          [REDEFINE=yes]          _ibalance
  23   A2WAY  [PRINT=aovtable,information,means; TREATMENTS=Treatment;
FPROB=yes;                                                PSE=diff,\
  24        means,alllsd;    LSDLEVEL=5;    PLOT=*;    EXIT=_ibalance]
R_solanacearum;                                             SAVE=_a2save
```

Analysis of variance

Variate: R_solanacearum

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Treatment	4	3.25733	0.81433	40.72	<.001

Residual	10	0.20000	0.02000
Total	14	3.45733	

Information summary

All terms orthogonal, none aliased.

Tables of means

Variate: R_solanacearum

Grand mean 2.487

Treatment	Bac. BMT16+Rs	Bac. KJ4+RS	Burk. KRN2+Rs	Control	Pse. KJ2+RS
	1.700	2.600	2.367	3.133	2.633

Standard errors of means

Table	Treatment
rep.	3
d.f.	10
e.s.e.	0.0816

Standard errors of differences of means

Table	Treatment
rep.	3
d.f.	10
s.e.d.	0.1155

Least significant differences of means (5% level)

Table	Treatment
rep.	3
d.f.	10
l.s.d.	0.2573

```
  25            IF      _ibalance.eq.0      .OR.       _ibalance.eq.1
  26           DELETE   [REDEFINE=yes]   _mean,    _rep,    _var,    _rdf
  27       AKEEP  [SAVE=_a2save[2]]  Treatment;  MEAN=_mean;  REP=_rep;
VARIANCE=_var;                                               RTERM=_resid
  28               AKEEP     [SAVE=_a2save[2]]     #_resid;     DF=_rdf
  29      AMCOMPARISON  [METHOD=fplsd;  DIRECTION=ascending;  PROB=0.05]
Treatment
```

Fisher's protected least significant difference test

Treatment

	Mean	
Bac. BMT16+Rs	1.700	a
Burk. KRN2+Rs	2.367	b
Bac. KJ4+RS	2.600	bc
Pse. KJ2+RS	2.633	c
Control	3.133	d

```
  30   ENDIF    31   SET  [IN=*]
```

Appendix XI: Weather Conditions Kapletundo in Bomet

Month	Year 2018											
	Jan	**Feb**	**Mar**	**Apr**	**May**	**Jun**	**Jul**	**Aug**	**Sep**	**Nov**	**Oct**	**Dec**
Record high °C	33.80	33.87	32.74	30.70	28.75	25.58	25.60	27.63	29.70	30.70	28.75	30.70
Average high °C	25.08	26.82	25.90	23.33	22.18	20.92	20.73	24.01	23.36	23.90	23.55	23.53
Daily mean °C	20.20	21.30	20.90	19.23	18.34	17.56	17.20	17.93	18.93	19.30	19.10	19.21
Average low °C	12.72	13.20	13.07	12.92	12.37	11.58	11.28	16.03	11.96	12.30	12.60	12.44
Record low °C	8.20	9.21	10.23	9.21	9.21	9.21	7.16	9.21	9.21	9.21	10.23	9.21
Av. precipitation mm	137.67	126.52	350.21	576.50	535.90	338.78	259.00	385.98	371.63	426.43	418.90	250.50
Av. precipitation days (mm)	15.72	15.35	24.20	28.83	31.06	29.86	30.51	30.51	29.40	29.96	29.40	23.53
Av. relative humidity (%)	66.34	62.70	70.09	84.58	89.72	90.20	87.63	84.90	81.30	80.60	82.95	77.37
Mean monthly sunshine hours	11.61	11.63	11.24	10.92	11.10	10.95	11.15	11.22	11.30	11.40	11.30	11.60

Appendix XII: Weather Conditions Makutano in Kirinyaga

Month	2018											
	Jan	**Feb**	**Mar**	**Apr**	**May**	**Jun**	**Jul**	**Aug**	**Sep**	**Nov**	**Oct**	**Dec**
Record high °C	30.15	32.20	33.20	30.15	28.14	26.13	28.14	27.14	30.15	30.15	28.14	28.14
Average high °C	26.30	27.52	27.62	25.60	23.80	23.16	23.30	24.26	25.75	25.99	24.60	25.03
Daily mean °C	21.81	22.82	23.10	21.60	20.20	19.41	19.25	17.02	21.26	21.80	20.70	20.83
Average low °C	13.90	14.61	15.20	15.41	14.80	13.65	13.28	14.10	14.82	15.72	14.50	13.40
Record low °C	8.04	9.05	10.10	11.10	11.10	10.10	9.05	11.10	10.05	12.06	10.10	8.04
Av. precipitation (mm)	35.83	44.10	127.13	368.60	325.24	180.60	85.20	96.63	79.25	180.50	183.10	78.80
Av. precipitation days	5.70	6.50	12.80	24.50	25.13	19.60	15.81	13.80	14.43	20.84	19.01	10.14
Av. relative humidity (%)	65.18	61.70	65.73	79.91	84.81	81.03	75.30	69.90	67.01	70.20	79.83	75.82
Mean monthly sunshine hours	11.33	11.40	11.10	11.02	11.00	11.10	10.99	11.20	11.23	11.36	11.02	11.34

Appendix XIII: Weather Conditions in Mangu in Kiambu

Month	2018											
	Jan	**Feb**	**Mar**	**Apr**	**May**	**Jun**	**Jul**	**Aug**	**Sep**	**Nov**	**Oct**	**Dec**
Record high °C	31.96	33.96	31.96	29.96	26.96	26.96	27.96	26.43	29.96	30.96	27.96	28.96
Average high °C	25.98	27.22	27.07	24.99	23.27	22.91	23.02	23.81	25.30	25.80	24.52	24.57
Daily mean °C	21.37	22.30	22.54	21.20	19.88	19.25	19.17	19.81	20.97	21.71	20.73	20.52
Average low °C	14.22	14.98	15.87	15.86	14.68	13.53	13.21	14.11	14.91	15.91	15.50	14.34
Record low °C	9.99	10.99	11.98	11.98	9.99	9.99	9.99	11.81	6.99	12.98	11.98	10.99
Av. precipitation mm	61.04	85.93	130.51	285.32	209.02	140.51	81.30	75.00	80.34	121.05	169.11	115.99
Ave. precipitation days (mm)	10.54	11.17	18.80	26.24	25.88	21.06	15.62	12.34	13.43	19.24	23.24	16.34
Av. relative humidity (%)	68.59	64.92	67.19	79.48	83.40	79.80	73.77	69.53	66.29	66.88	76.87	77.07
Mean monthly sunshine hours	11.29	11.34	11.13	10.80	10.87	10.97	11.09	11.28	11.28	11.40	11.07	11.32

Appendix XIV: Weather Conditions in Olchonyori in Kajiado

	2018											
Month	**Jan**	**Feb**	**Mar**	**Apr**	**May**	**Jun**	**Jul**	**Aug**	**Sep**	**Nov**	**Oct**	**Dec**
Record high °C	31.40	34.00	31.40	30.30	27.20	27.20	28.21	33.90	29.26	30.30	28.21	28.21
Average high °C	25.51	26.93	26.93	24.98	23.68	23.33	23.44	24.14	25.71	26.30	24.41	24.20
Daily mean °C	21.80	22.70	23.11	21.90	20.53	19.90	19.60	20.20	21.70	22.93	21.74	21.30
Average low °C	16.30	17.14	18.32	18.00	16.40	14.91	14.30	15.10	16.53	18.19	17.96	17.01
Record low °C	10.00	12.54	13.60	9.40	10.45	9.40	9.40	10.10	11.50	13.60	7.31	9.40
Av. precipitation (mm)	78.80	97.04	148.40	213.70	104.54	38.60	23.23	23.60	24.84	75.80	210.53	173.10
Av. precipitation days (mm)	13.30	12.73	19.18	25.36	22.13	11.21	7.22	7.31	6.65	15.30	26.40	22.13
Av. relative humidity (%)	76.63	72.60	74.94	85.90	86.64	80.86	75.53	71.58	68.70	70.18	84.10	85.95
Mean monthly sunshine hours	11.74	11.68	11.48	11.21	11.39	11.63	11.53	11.79	11.78	11.92	11.26	11.66

Appendix XV: Publication

Kones C., Mwajita M., Kariuki L., Kiirika L. and Kavoo A (2020): Isolation and characterization of rhizospheric microorganisms from bacterial wilt endemic areas in Kenya. *African Journal of Microbiology Research,* 14(7), 349-360