PROSPECTING FOR BIOLOGICAL CONTROL AGENTS AGAINST *RALSTONIA SOLANACEARUM* IN POTATO

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2018

PROSPECTING FOR BIOLOGICAL CONTROL AGENTS AGAINST *RALSTONIA SOLANACEARUM* IN POTATO

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A research thesis submitted in partial fulfilment of the requirements for the award of the degree of Master of Science in Molecular Biology and Biotechnology at the Pan African University Institute of basic Sciences Technology and Innovation

2018

DECLARATION

I, the undersigned, declare that this is my original work and has not been submitted to any other college, institution or university for academic credit.

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DEDICATION

This work is dedicated to the memory of my beloved mother, Mawamba Cecile who passed away almost two decades ago. My uncle Wamba Laurent worked hard to raise me in this turbulent life. Without his enormous personal sacrifice and unconditional love, I would have never become the individual that I am today. To God be the Glory for His faithfulness upon my life.

ACKNOWLEDGMENTS

I express my sincere gratitude to African Union Commission (AUC), for this initiative in Africa for Africans. I also appreciate the administration of Pan African University Institute of basic Sciences Technology and Innovation (PAUISTI), the coordinator of Molecular Biology and Biotechnology department, Prof. Naomi Maina who invested her time to contribute to our training and our success.

I deeply appreciate my supervisors, Prof. Viviene Matiru and Dr. Steven Runo for their active interest and keen guidance during the accomplishment of this project. The overwhelming support and assistance from lecturers in the Department of Molecular Biology and Biotechnology PAUISTI, during the entire period of study is highly acknowledged. I wish to thank the Nakuru Agricultural office (Ministry of Agriculture, Kenya) for their guidance and facilitation put in place during the samples collection; the farmers who invested their times are highly acknowledged.

I express my gratitude to the invaluable support from the following staff at the International Potato Centre (CIP): Dr. Monica Parker and Dr. Kalpana Sharma for their constant help and assistance. In addition, I recognize the technical support and assistance of Mr. Joel Masanga, my colleagues Mr. Tonui Ronald, Mr. Atchou Kodzo, Mrs. Judith Nwabugu and Mrs. Marlene Morombaye; the Plant Transformation Laboratory community at Kenyatta University.

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ABBREVIATIONS AND ACRONYMS

%	Percentage
ADC	Agricultural development corporation
ANN	Anonymous node network
ANOVA	Analysis of variance
BLAST	Basic Local Alignment Search Tool
CPG	Casamino Acid-Peptone-Glucose-agar
cv.	Cultivar
DI	Disease incidence
DNA	Deoxyribonucleic acid
EPA	European Protection Agency
FAO	Food and Agriculture Organization
FAOSTAT	Food and Agriculture Organization Corporate Statistical Database
GIZ-PSDA	Partnership for clean indoor air between deutsche Gesellschaft für Internationale Zusammenarbeit (GIZ) and Population and Sustainable Development Alliance (PSDA) of Kenya
GPE	Growth promotion efficacy
HR	Hypersensitive reaction
KARI	Kenya Agricultural Research Institute

LSD	Least Significant Difference
MDG	Millennium development goal
MEGA7	Molecular Evolutionary Genetics Analysis Version 7.0
MKM	Modified Kelman media
MLSA	Multilocus sequence analysis
MoALF	Ministry of Agriculture, Livestock and Fisheries (Kenyan)
NB	Nutrient broth
NCBI	National Center for Biotechnology Information
PGP	Plant growth promotion
Pmx-PCR	Multiplex Polymerase Chain Reaction
RSSC	Ralstonia solanacearum species complex
Sybr	Asymmetrical cyanine dye
TSA	Tryptic soy agar media
TTC	Triphenyl Tetrazolium Chloride
TZC	2,3,5-triphenyl tetrazolium chloride media
USD	United States Dollar

ABSTRACT

In Africa, cultivation of potato (*Solanum tuberosum*) is an enormous economic activity and represents an important food source. Potato contains essential amino acids and has the capacity to produce more energy and protein per unit land than any other single food crop. Despite the importance of the crop, its productivity is constrained by biotic and abiotic stresses. Key among them is the bacterial wilt disease caused by *Ralstonia solanacearum.* In Kenya, the disease affects 77% of potato farms causing yield losses of up to 100%. Existing management strategies have not been effective owing to the nature of the pathogen which possesses several strains that differ in host range, geographical distribution and pathogenicity. For effective control of the disease, there is need for an integrated management approach encompassing breeding for resistance against multiple strains and biocontrol strategies. This study therefore sought to: generate knowledge on specific *Ralstonia solanacearum* strains causing potato wilt disease in Nakuru county, Kenya and to bio-prospect for the potential of rhizopheral bacteria inoculants as biocontrol agents for potato bacterial wilt disease. Three major potato growing areas of Nakuru County were selected, diseased potato plants and soil samples around plants collected. Fifty-four (54) bacterial isolates of the pathogen were successfully isolated from diseased plants using the Triphenyl Tetrazolium chloride medium and 20 isolates selected for further characterization. The isolates were subjected to cultural and molecular techniques to further confirmed their status as *Ralstonia solanacearum*. Analysis of phylotypes showed that all four phylotypes - type I - IV were present across the isolated strains. Based

on the ability of the pathogen to utilize sugars and alcohols, all the isolates were grouped as biovar III except two (Rs18 and Rs49 whose biovar was not identified). The aggressiveness of isolated pathogen was analyzed using a hypersensitive reaction test on tobacco and further confirmed their virulence using a susceptible potato variety Shangi under greenhouse conditions. All isolates elicited a hypersensitive reaction on tobacco. However, they showed varying levels of pathogenicity with isolate Rs6 being the most virulent. In the course of biocontrol assays, 62 rhizobacterial isolates were obtained from potato rhizosphere by serial dilution methods using nutrient agar medium and 20 were selected for antagonistic tests using the paper disc assay. Results showed that 5 isolates were highly antagonistic against four *R. solanacearum* strains. These antagonists were tested for plant growth promoting traits and disease suppression. The results revealed that *Bacillus cereus, Bacillus subtilis, Paenibacillus sp, Providencia rettgeri* and *Providencia vermicola* were dominantly active in potato rhizosphere causing resistance to bacterial wilt disease. The isolates *Bacillus cereus* and *Bacillus subtilis* were potential isolates possessing antagonistic activity along with several plant growth promoting (PGP) traits. Taken together, these findings provide baseline information for improvement programs targeting host-based resistance to multiple strains of the pathogen; and development of biocontrol strategies with the potential antagonists reported. Implementation of these outcomes to the existing control methods would lead to increased farmer incomes, reduced environmental risks and human exposure to chemicals.

CHAPTER ONE

INTRODUCTION

1.1 General background

With the current global population growth rate, food crop production needs to double by 2050 in order to meet the increased demand. This is however not the case as the current estimates are far below the required threshold (Ray *et al.*, 2013). Plant diseases, insects, and weeds decrease the production of crops worldwide by 36%, and diseases alone have been shown to reduce crop yields by 14% (Holguin and Patten, 1999). To ensure food security, most agricultural systems presently are dependent on the use of chemical fertilizers and pesticides (Ward, 2016). These fertilizers are rich in nitrogen, phosphorous, and potassium. Repeated use of such agrochemicals leads to pollution of soil, air, and groundwater (Santos *et al.,* 2012). Given these limitations, beneficial agricultural microorganisms used as biological control agents (BCAs) will be an important focus in pursuing sustainable agriculture while preserving natural resources for the future generations (Youssef and Eissa, 2014).

Among the world food crops, potato is an important food security crop with great potential for poverty alleviation and combating malnutrition in the developing world (FAO, 2008; Devaux *et al.,* 2014). It contains a better balance of essential amino acids and has the capacity to produce more energy and proteins per unit land than any other single food crop (Abong *et al.,* 2009). It is primarily consumed as a source of carbohydrates in place of rice

or wheat, as a vegetable, or in the form of French fries, wafers, chips, mashed and boiled potato, and in soups (FAO, 2008). Potatoes serves as food as well as a source of income in densely populated highlands of sub-Saharan Africa. The crop plays an important role in the rural livelihood system and the commodity could be a good starting point for rural development in sub-Saharan Africa especially under the current conditions of increased cereal prices in the international markets.

Globally, more than 380 million tons of potatoes were cultivated on 20 million hectares of land in 2016 (FAOSTAT, 2018). This ranks potato at fourth position among the worlds' most important staple food crops. In Kenya, potato is the second most important food crop after maize and plays a major role in national food security (Muthoni *et al.*, 2010). The annual potato production in the country is estimated at 3 million tonnes with productivity of 8-15 t/ha, which is low compared to the optimal 25 t/ha that should be attained under rain-fed conditions (Nyaga, 2008).

Despite potato being a strategic food-producing crop, the plant is susceptible to numerous pathogens for which control methods are not efficient. In addition to the different pests (such as insects and nematodes), microbial pathogens including *R. solanacearum* and *Phytophthora infestans* are responsible for annual loss of up to 25% in worldwide production of potatoes (Priou and Jouan, 1996; FAO, 2008). Fungal pathogens of potatoes of economic significance range from slime molds to smuts and rusts. The potato is also susceptible to several viruses belonging to yellow and mosaic families. The plant is also affected by abiotic factors such as nutrient deficiency resulting in diseases such as black

heart and sunscald freezing injury. It is also affected by several nematode diseases (Priou and Jouan, 1996).

The major diseases of potatoes include the most historically significant crop disease, the late blight (caused by *Phytophthora infestans*) followed by bacterial wilt (caused by *R. solanacearum)* which is still the most important bacterial disease of potato and Solanaceae family as whole contributing to significant losses in yields (Kaguongo *et al.*, 2008). Control and management of bacterial wilt disease faces challenges since the causative agent is soil-borne in nature and can persist in soil for a long time (Hayward, 1991). No single management strategy effectively prevents losses caused by bacterial wilt and there is no cure once a potato plant or tuber is infected (Muthoni *et al.*, 2010). This means that control measures should focus on prevention of initial infection as well as the spread from infected plants.

1.2 Statement of the problem

Potato is an important food security crop with great potential for poverty alleviation and represent a major source of household income for smallholder farmers in Kenya. Potato production in the country is however characterized by extremely low yields compared to developed countries. North America and Western Europe for instance have an average of 40 t/ha in terms of yields compared to Kenya's 20 t/ha (FAO, 2008). Furthermore, Kenya's potato sector is still underdeveloped and is faced with low productivity of 8-15 t/ha, against the attainable yield of 30-40 t/ha under normal field conditions (Muthoni *et al*., 2013; Gitari *et al*., 2018). This decline in yield is attributed to high incidences of

diseases, particularly the bacterial wilt disease which remains an economically significant problem for smallholder farmers with losses estimated at about 50-75% (Felix *et al.*, 2011).

No single control method has been found to be 100% effective in controlling the disease, although in locations where the pathogen is established, some level of bacterial wilt control has been possible through use of a combination of diverse methods (Champoiseau *et al.*, 2010). In addition, phytosanitary methods such as imposing a quarantine is either expensive or difficult to apply. Cultural methods such as crop rotations are largely impractical because the farms are too small to allow effective rotation, the pathogen has a wide host range and its soil-borne nature (Muthoni *et al.*, 2010). The use of tolerant/resistant varieties is the most viable strategy. However, this approach is ineffective in that the genetic diversity of the causative pathogen often enables it to overcome crop resistance (Wang *et al.*, 1998). Moreover, strains of *R. solanacearum* differ in host range, geographical distribution, pathogenicity, epidemiological relationships, and physiological properties (Fegan and Prior, 2005). Hence, a better understanding of the population structure and geographical distribution of the pathogen is vital in the management of potato wilt disease and for regional risk preparedness and possible control of the disease, resulting in reduced losses and poverty.

1.3 Justification and significance of the study

Increase in production has mainly been due to increase in the use of chemicals and the areas under potato cultivation with severe environmental degradation since this is done at

the cost of clearing natural forests. The repeated use of pesticides contributes to the pollution of the environment and the degradation of the soil. With increased global population pressure, land is becoming increasingly limited and the world's demand for food will have to be met by intensifying agriculture on land already in cultivation. Therefore, to remain competitive, the potato industry needs to embrace innovative strategies in adapting to these two challenges (climate change and global population growth).

There is a need to improve food production without necessarily using high dosages of chemicals. Prospecting and identification of potential biocontrol agent (s) against *R. solanacearum*, derived from soil microbiome in proximity to healthy potatoes can reduce the devastating effect of the disease and decrease the need to use agrochemicals as a control strategy. This relies on the information on the pathogen's characteristics, geographic distribution, host availability and suitability of climatic conditions; screening and use of environmentally risk-free bacteria strains, which are able to colonize the potato plant and express antagonism under every cultivation condition. Such information gives to the relevant stakeholders a better focus in improving the farmers' practices to increase potato yield. However, little information is available on the geographic distribution of potato bacterial wilt in Nakuru County despite being a major potato production zone in Kenya (Kaguongo *et al.*, 2010).

This study provides comprehensive information on the pathogen structure and distribution. It points out a promising control strategy with the use of soil borne bacteria

that are antagonist to *R. solanacearum* and save for the environment. This knowledge is useful to implement sustainability in control of bacterial wilt disease in the County, by developing pathogen-targeted and possibly, geographically targeted management practices.

1.4 Research Hypotheses

1- The population structure of the pathogen in Nakuru county is not uniform.

2- There is no significant difference in the antagonistic activities of microbes found in rhizosphere of a healthy and infected potato.

3- There is no significant difference in the response of plant treated with the identified potential antagonists compared to non-treated plant.

1.5 General objective

To identify potential biological control agents (BCAs) for effective management of potato bacterial wilt disease.

1.6 Specific objectives

1. To isolate and characterize *R. solanacearum* strains present in major potato growing areas in Nakuru County, Kenya.
2. To bio-prospect for microbial antagonists of *R. solanacearum* isolated from non-infected soils of potato growing farms in Nakuru.
3. To evaluate effect of the identified biocontrol agents on plant responses under *R. solanacearum* infection *in vivo*.

1.7 Scope of the study

This study was limited geographically to Nakuru County of Kenya. Sample collection was done in three major potato growing areas in the county namely, Kuresoi North, Mau Narok and Njoro. The study began with collection of infected potato plants in the mentioned regions from 19th to 24th of February 2018 followed by isolation of *R. solanacearum* in plant transformation laboratory (Biosafety level 2) at Kenyatta University. For identification of the pathogen, both cultural and molecular methods were used. The isolates were characterization based on phylotype, biovars, aggressiveness on tobacco leaves and their virulence was confirmed on a susceptible potato cultivar Shangi. Further, a study was conducted on screening and identification of potential antagonists against four *R. solanacearum* strains followed by the evaluation for disease suppression and growth promotion of five bacteria with significant antagonistic activity. This evaluation was done using one the most virulent isolate of *R. solanacearum* strain. Certified potato seeds free of disease were purchased from KALRO Tigoni and ADC Molo. The greenhouse experiments were carry out at Kenyatta University.

CHAPTER TWO

LITERATURE REVIEW

2.1 Potato and potato farming in Kenya

2.1.1 Potato

Potato (*Solanum tuberosum* L.), is a starchy edible tuber belonging to the Solanaceae family (nightshades family) which also includes eggplant, peppers and tomato among others. It is grown in many parts of the world (Figure 2.1). Potato is a cool-season vegetable that despite its looks is not a root but a "tuber", a specialized underground plant energy storage system (FOA, 2008). The potato is an annual plant of a height of about 30-100 cm depending on the variety. It is vegetative propagated through tubers which bear the buds, commonly known as the "eyes" which sprout on germination and grow into new plants (Appendix 1).

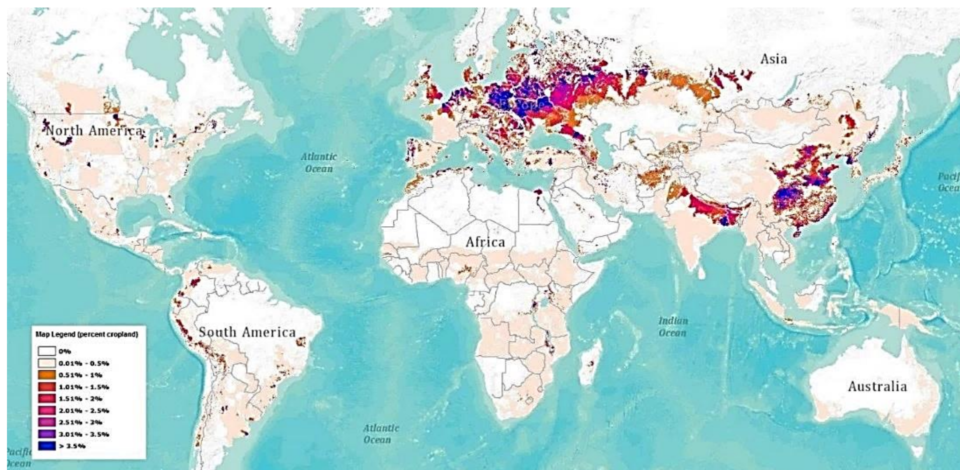

Figure 2.1: Geographical distribution of potato cultivation around the world (Source: RTB Maps). https://www.potatopro.com/world/potato-statistics

Worldwide more than 380 million tons of potatoes are being cultivated annually on 20 million hectares of land in 2016 (FAOSTAT, 2018). This ranks the potato at number four among the worlds' most important staple food crops, after rice, wheat and corn. Among the plant food sources, potato contains a better balance of essential amino acid, particularly lysine. Additionally, potato has the capacity to produce more energy and protein per unit land than any other single food crop (Abong *et al.*, 2009). The potato has high nutritive value (Figure 2.2) and can supply considerable amounts of energy, minerals and vitamins (Ahuja *et al.*, 2013). The tubers consist of 80% water on average and the remaining 20% is the solid matter. The minerals that are present include sodium, iron, calcium, sulfur, potassium, phosphorous and magnesium (Woolfe and Poats, 1987). It has high quality protein and provides high amount of starch. There are also some important vitamins that are found in potatoes which include thiamine, riboflavin, niacin and vitamin C (Burlingame *et al.*, 2009). Potato is useful in many ways with 30-72% being used for food (FAO, 2008). It is primarily consumed as a source of carbohydrates in place of rice or wheat, as a vegetable, or in the form of French fries, wafers, chips, mashed and boiled potato, and in soups.

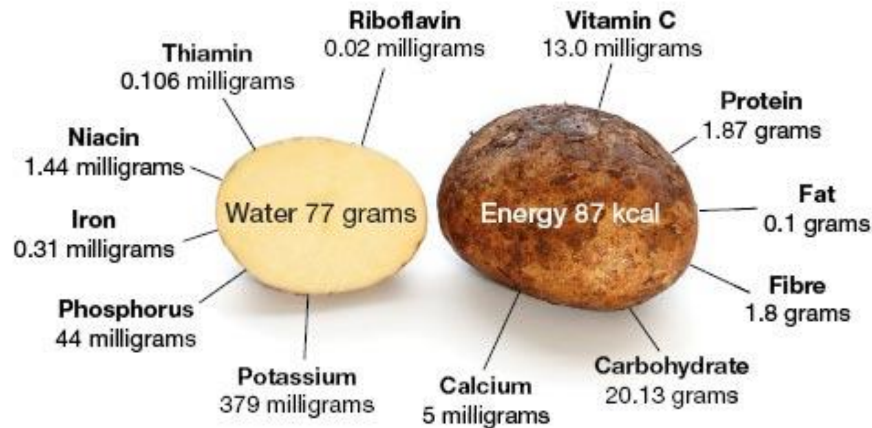

Figure 2.2: Nutrient content of Potatoes (Per 100 g, after boiling in skin and peeling before consumption), Source: United States Department of Agriculture, National Nutrient Database (2013).

2.1.2 Potato farming in Kenya

In Kenya, Potatoes are grown by about 800,000 farmers cultivating about 161, 000 hectares per season with an annual production of about 3 million tonnes in two growing season (GIZ-PSDA, 2011; MoALF, 2016). The annual potato production is valued at KSh. 50 billion (USD 500 million) at farm gate prices (GIZ-PSDA, 2011; MoALF, 2016). Several cultivars are present in the sub-sector and Shangi is the most common potato cultivar with an early maturity of 3−4 months and an attainable yield of 30−40 t/ha under normal conditions (Gitari *et al.*, 2018). Beyond the farm, the industry employs about 3.3 million people as market agents, transporters, processors, vendors and exporters (ANN, 2009; MoALF, 2016). Potato has become the second most important food crop in Kenya after maize and plays a major role in national food security (Muthoni *et al.*, 2010). The crop also generates income for a lot of people who include farmers' laborers, traders and

processors, and hence contributing to the country's effort of attaining millennium development goal (MDG) of alleviating hunger and reducing poverty.

Kenya's production constitutes 0.3% of the world's total and 6.5% of Africa's production (Muthoni *et al.*, 2010). It is cultivated both as a subsistence and commercial crop. Potato production in the country, is concentrated in the highlands (1500-3000 masl) under rain-fed conditions. Kenya has five major potato-growing regions namely Mt. Kenya (Embu, Meru, Kirinyaga); Central (Kiambu, Nyandarua, Murang'a, Njabini and Nyeri); Central Rift (Bomet, Mau Narok, and Molo areas); North Rift region (Marakwet, Uasin Ngishu, Keiyo, Mt. Elgon and Cherangani Hills) and Coast (Taita Taveta and Wundanyi) (MoA, 2008; FAO, 2013). Based on geographic location, production practices and variety preferences, the traditional major potato growing areas are divided into five regions (Figure 2.3): Mt. Kenya, Aberdares and Eastern Rift Valley Mau region, Mt. Elgon and other highlands, such as Taita hills in Taita Taveta county in the southern border of Kenya and Tanzania (Kaguongo *et al.*, 2010). Other emerging potato growing areas include Nandi, Baringo, Laikipia, Nyamira and Kisii counties. Due to increased demand, potato production has expanded to non-traditional potato growing areas such as Kirinyaga, Naivasha and Tana River. This is possibly due to availability of irrigation facilities (MoALF, 2016). In Central province, Nyandarua County is the largest potato producing area (MoA, 2008). In Eastern province, the main potato growing county is Meru and in the Rift Valley province where potatoes are grown in Kericho, Bomet, and Uasin Gishu counties (MoA, 2008).

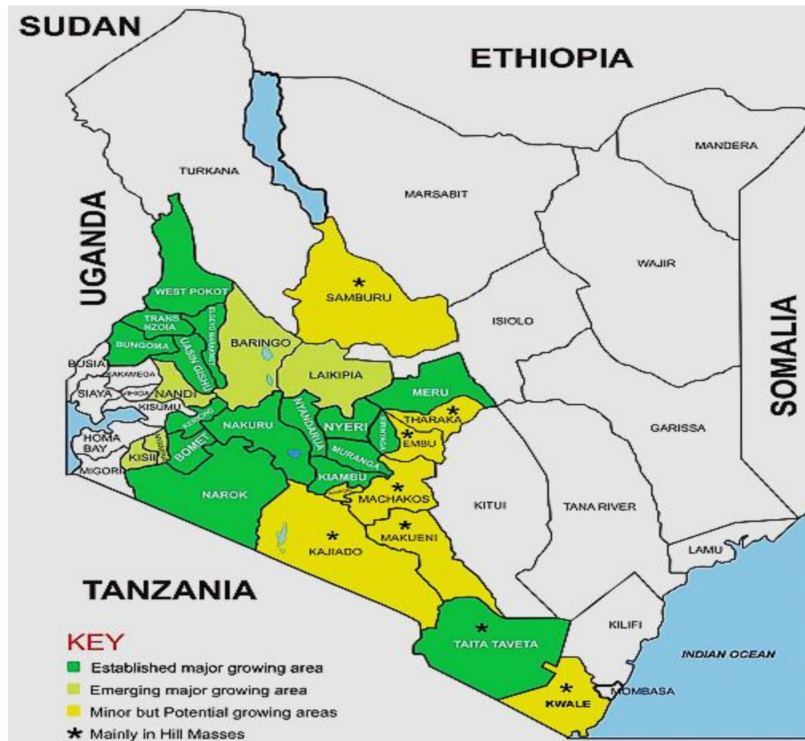

Figure 2.3: Potato growing counties in Kenya (MoALF, 2016)

Despite the importance of potato in Kenya, its production has been declining in recent years (Appendix 2.2); this is mainly due to lack of good quality planting material, pests and diseases (Muthoni and Nyamongo, 2009; MoALF, 2016). Among the disease, bacterial wilt is the common disease in all potato growing areas of the country, affecting 77% of potato farms followed by late blight (67%) and viral disease (12%) (Kaguongo *et al.*, 2010). Potato research in Kenya has focused on development and dissemination of high yielding and disease resistant varieties (MoA, 2007). Although efforts have been directed to improve potato production over the years, low productivity remains a major challenge in the sub-sector. This lead to the average national farm level yields of 7.3

metric tonnes per hectare (mt/ha), which is low compare to the on-station research yields of 25-35 mt/ha and the potential of 14.5-20 mt/ha under farm level conditions (KARI, 2005).

2.2 Constraints in potato production

The qualities of the potato define it as a strategic food-producing crop. However, this Solanaceous plant is susceptible to numerous pathogens for which control methods are ineffective. In addition to the different pests (i.e. insects, nematodes), microbial pathogens are annually responsible for the loss of 25% of worldwide production (Priou and Jouan, 1996; FAO, 2008). The fungi that might attack it ranges from the slime molds to the smuts and rusts. It is susceptible to several viruses of the yellow and mosaic groups. It is also susceptible to some nonparasitic diseases as black heart and sunscald freezing injury. Similarly, potato is susceptible to several nematode diseases. Malnutrition caused by deficiency in magnesium, potash and boron can also cause serious damage on the plant. The major diseases of potatoes include the most historically significant crop disease, late blight (caused by *Phytophthora infestans*) and bacterial wilt caused by *R. solanacearum* which is still the most important bacteria causing disease to the Solanaceae family and contributing to significant potato yield losses (Kaguongo *et al.*, 2008).

2.3 Potato bacterial wilt disease

2.3.1 Disease importance and distribution

Among the diseases affecting potato, bacterial wilt caused by *R. solanacearum* (Yabuuchi *et al.*, 1995) is a major problem worldwide. This pathogen is one of the most devastating,

important and wide-spread bacterial diseases of crops in tropical environments (Hayward, 1991). *R. solanacearum* is a soil borne vascular pathogen that is distributed worldwide, attacking over 50 botanical families (Strange and Scott, 2005). Highly susceptible crops are potato, tomato, eggplant, chili, bell pepper and peanut.

Potato bacterial wilt disease or brown root of potatoes is caused mainly by race 3 biovar 2 of *R. solanacearum*. It ranks as second most important potato disease after late blight (Felix *et al.*, 2011). This disease has been estimated to affect about 1.7 million hectares of potatoes in around 80 countries (Champoiseau *et al.*, 2009). Currently, global damages exceed USD 950 million annually (Elphinstone, 2005; Nion and Toyota, 2015). In Kenya, bacterial wilt was first reported in 1940s and has since spread to most potato growing regions (Michieka, 1993). Several surveys that have been undertaken in Kenya, on the prevalence and incidence of bacterial wilt in a number of potato producing zones have shown bacterial wilt to be an important potato disease in the Country (Ateka *et al.*, 2001; Kwambai *et al.*, 2011; Mwaniki *et al.,* 2016). According to some recent studies, the disease is found in all the potato growing areas of the country affecting 77% of potato farms and causing yield losses of 50 to 100% (Kaguongo *et al.,* 2010; Muthoni *et al.*, 2012).

Andes is thought to be the origin of the potato wilt disease pathogens (Patrice G Champoiseau et al., 2009). The pathogen has been then spread through the tropical highlands and subtropical warm-temperate areas throughout the world with cuttings or seeds. Today, *R. solanacearum* strains are select agent under the United State Agricultural

Bioterrorism Protection Act of 2002 and are considered as quarantine pathogen in Europe (Janse, 2012). Although many plant pathogens are narrowly adapted to one or a few related plant hosts, *R. solanacearum* has an unusually broad host range that includes monocotyledonous and dicotyledonous hosts (Hayward, 1991). Its extensive host range, together with a wide geographic distribution, makes it one of the world's most destructive crop pathogens (Denny, 2007).

2.3.2 Disease cycle and epidemiology

R. solanacearum primarily infects host plants through their roots, entering through wounds formed by lateral root emergence or by root damage caused by soil borne organisms (such as nematodes), transplanting, cultivation or insects. The bacterium can also enter plants by way of stem injuries from insects, handling, or tools. Once the bacteria infect the roots or stems, they colonize the plant through the xylem in the vascular bundles (Figure 2.4). This leads to a condition in which infected plants start wilting irreversibly (Mansfield *et al.*, 2012). Vegetative propagation can play a major role in dissemination of this pathogen particularly via latently infected potato seed tubers and infected cuttings. Plant-to-plant infection can also occur when bacteria shed from infected roots move to roots of nearby healthy plants. The pathogen can be spread from infested to healthy fields by soil transfer on machinery and surface runoff water after irrigation or rainfall. It also can be disseminated from infested ponds or rivers to healthy fields by flooding or irrigation. *R. solanacearum* is reported to survive in soil temperatures as low as 4˚C and is considered a worrisome pathogen (Milling *et al.*, 2009). *R. solanacearum* survives in

infected debris, soil, water, weed hosts, seeds and vegetative propagated material. The pathogen spreads through movement of soil, irrigation with contaminated water, use of infected vegetative planting material, and mechanically via workers, tools, insects and equipment. Geographic distributions of pathogens are highly influenced by factors such as availability, susceptibility and abundance of the host and suitability of the climatic conditions (Shaw and Osborne, 2011).

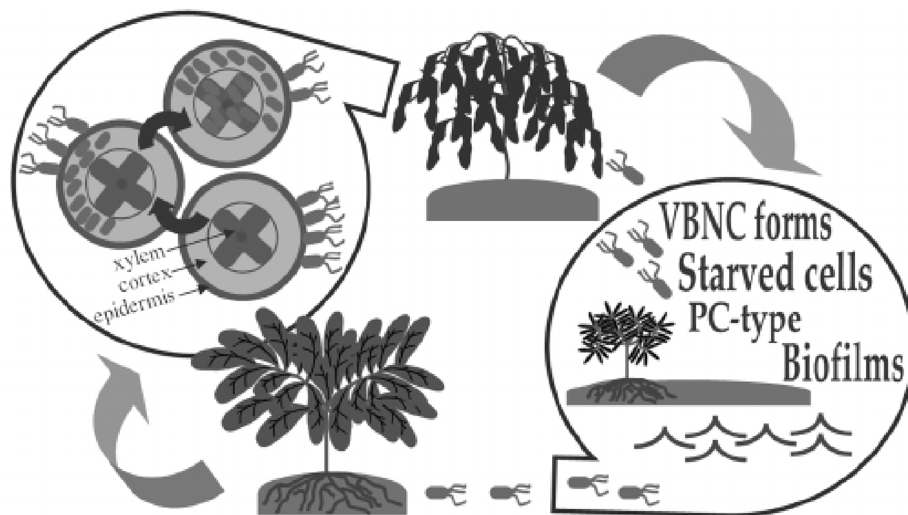

Figure 2.4: Life cycle of *R. solanacearum*: life inside and outside the host. The bacterium moves to the host roots, attaches to the epidermis, infects the cortex, and colonizes the xylem, resulting in host wilting. After death of the plant, the bacterium is released into the environment, where it seems to survive in reservoir plants, soil and/or water, through diverse strategies, such as the VBNC state, the starvation survival response, the PC process, or the biofilm formation, until contact with a new host (Álvarez *et al.*, 2010).

Under natural conditions, the initial symptom in mature plants is wilting of upper leaves during hot days followed by recovery throughout the evening and early hours of the morning (Figure 2.5-1A). The wilted leaves maintain their green color as the disease

progresses. Under hot and humid conditions, complete wilting occurs and eventually the plant dies (Figure 2.5-1B). Massive invasion of the cortex might result in the appearance of water-soaked lesions on the external surface of infected stems. If an infected stem is cut crosswise, tiny drops of dirty white or yellowish viscous ooze exude from several vascular bundles (Champoiseau *et al.*, 2009). In addition to the above symptoms, infected potato plants produce tubers that may rot and a brownish discoloration of the vascular ring could be seen in the cross-section of the tuber (Figure 2.5-2A). Slimy, sticky pus may exude from the ring when the tuber is squeezed (Figure 2.5-2B and D). Pale ooze may exude from eyes and heel end of potato tubers (Figure 2.5-2C). Soil will adhere to the oozing eyes (Figure 2.5-2C). A plant infected with *R. solanacearum* may express all or none of the symptoms outlined above, even under environmental conditions that are ideal for the pathogen. If symptoms are not evident on an infected susceptible host, the condition is known as latency (CIP, 2017).

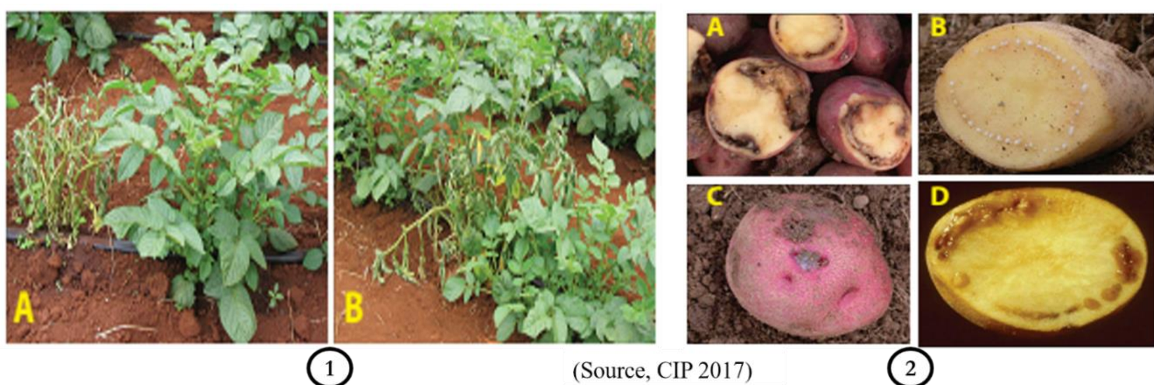

Figure 2.5: (1) wilt symptoms, (1A) whole potato plant wilting, (1B) partial wilting; (2) Oozing symptoms, (2A) rotting of the tubers, (2B) ooze coming from the vascular ring, (2C) oozing eyes and soil sticking to the eyes and (2D) vascular browning.

2.3.3 Biology of *Ralstonia solanacearum*

2.3.3.1 Identification

R. solanacearum is gram-negative, rod-shaped bacterium measuring 0.5-0.7 ×1.5-2.0 µm in size. It grows well at 28 to 32°C strictly in aerobic conditions (Hayward, 1991; Schaad *et al.*, 2001). Individual colonies of normal or virulent isolates are usually visible after 36 to 48 hours, appearing as opaque white or cream-colored colonies that are irregularly shaped and highly fluidal on a cultivation medium such as Casamino Acid-Peptone-Glucose-agar (CPG). On tetrazolium chloride (TZC) medium, these colonies are white with pink centers (Kelman, 1954). Mutant or non-virulent type colonies of *R. solanacearum* are uniformly round and dark red, smaller in size and butyrous or dry on TZC. *R. solanacearum* is a genetically diverse (Poussier *et al.*, 1999) soil-borne plant pathogen (Hayward, 1994).

Much effort has been devoted to develop quick and reliable methods to detect *R. solanacearum* (Hayward, 1991; Elphinstone *et al.*, 1996; Glick *et al.*, 2002; Fegan and Prior, 2005; Prior and Fegan, 2005). These assays include culture-based methods using semi-selective media or enrichment culture; serological methods such as lateral-flow devices (ImmunoStrip) and enzyme-linked immunosorbent assay (ELISA); nucleic acid-based methods including direct polymerase chain reaction (PCR), real time PCR, and loop-mediated isothermal amplification (LAMP); and various combinations of these. Culture-based methods rely on the isolation and growth of the target pathogen (Elphinstone *et al.*, 1996). These methods can be highly sensitive and they have the important advantage

of yielding a living culture that can be used for additional confirmatory analyses such as pathogenicity tests. However, their usefulness is limited by the fact that *R. solanacearum* is relatively slow growing, has an undistinctive colony morphology, is significantly out-numbered by saprophytic microbes present in field samples (Poussier *et al.*, 2002), and can become viable but non culturable (Elsas *et al.*, 2002). Further, culturing bacteria from plant materials may not yield pathogen colonies because *R. solanacearum* populations are not evenly distributed in plants (Swanson *et al.*, 2005). Finally, the bacterium can undergo phenotypic conversion in culture, which results in an atypical colony morphology that is hard to recognize (Denny, 2007).

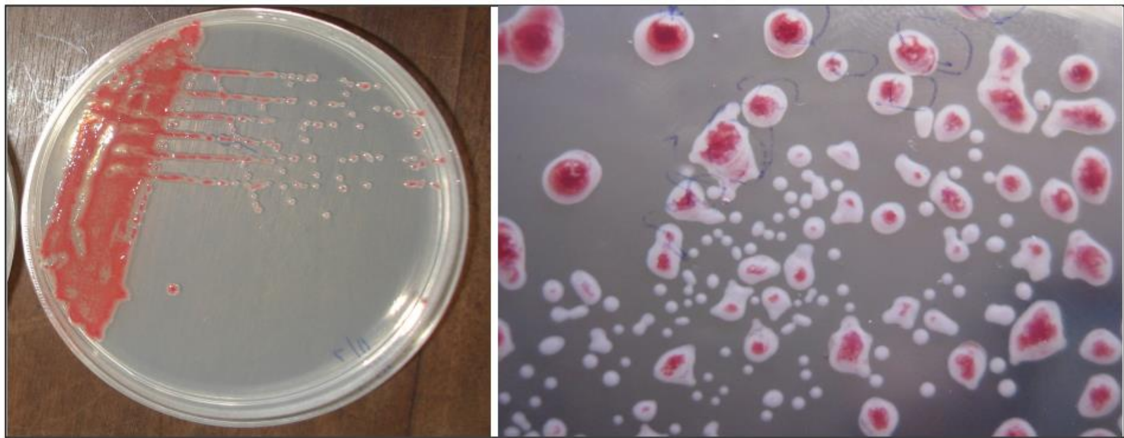

Figure 2.6: Bacterial growth on streaked medium (left) and characteristic colonies of *R. solanacearum* on SMSA (right).

Appearance on modified tetrazolium chloride (TZC) medium, *R. solanacearum* colonies are characteristically mucoid. Often there is a brown discoloration of the medium around

the colonies. Growth also occurs more quickly on TZC than on SMSA (Kinyua *et al*, 2014).

2.3.3.2 Characterization

R. solanacearum is a species complex with considerable diversity. It attacks over 450 plant species and contains many strains that differ in host range, geographical distribution, pathogenicity and biochemical properties (Fegan and Prior, 2005). This genetic diversity of the pathogen often overcomes the resistance of the crop (Wang *et al.*, 1998). Strains of *R. solanacearum* have previously been grouped into five races based on susceptible host plants and biovar classification, which is determined by utilization of a panel of five to eight carbohydrate substrates (Table 2.1) (Schaad *et al.*, 2001).

Table 2.1: Races and biovars of *R. solanacearum*

Race	Host Range	Geographic distribution	Biovar
1	Wide	Asia, Australia, Americas	1,3,4
2	Banana and others Musa spp.	Caribbean, Brazil Philippines	1
3	Potato, some other Solanaceae, Geranium; plus a few other species	Worldwide except US and Canada	2
4	Ginger	Asia	3,4
5	Mulberry	China	5

The five races and biovars do not correspond except that in general, race 3 is equivalent to biovar 2. Race 3 biovar 2 (r3b2) causes highly destructive brown rot of potato and bacterial wilt of geranium. Compared to other strains of *R. solanacearum*, r3b2 is more adapted to cooler temperatures found in temperate climates and at higher elevations and latitudes in the tropics. *R. solanacearum* r3b2 is a quarantined pathogen in Europe and Canada (Williamson *et al.*, 2002). Determination of the five biovars of *R. solanacearum* is done on the basis of carbon utilization in disaccharides and hexose alcohols (Table 2.2) (Hayward, 1964; Denny and Hayward, 2001). The disaccharides used are cellobiose, lactose and maltose, while the hexose alcohols are dulcitol, mannitol and sorbitol.

Table 2.2: Differentiation of *R. solanacearum* biovars based on utilization of various carbon sources

	Biovars				
Test	**1**	**2***	**3**	**4**	**5**
Mannitol	-	-	+	+	+
Sorbitol	-	-	+	+	-
Dulcitol	-	-	+	+	-
Lactose	-	+	+	-	+
Maltose	-	+	+	-	+
Cellobiose	-	+	+	-	+
Dextrose	+	+	+	+	+

*In order to differentiate between the sub-phenotypes 2A and 2T in biovar 2, D (+) trehalose, L (-) tryptophan and D-ribose are included in the panel of test carbon sources; strain 2A gives a negative result while strain 2T gives a positive result (as is the case with all the other biovars) (Kinyua *et al.*, 2014).

Since host ranges of strains are broad and often overlap and tests to define races are cumbersome, it is preferable to designate isolate biovars and determine their phylotype. Classifications based on molecular techniques have been reported, as phylotypes or monophyletic clusters of strains and these have resulted into four phylotypes I, II, III and IV originated from different part of the world (Fegan and Prior, 2005; Prior *et al.*, 2016). Phylotype I strain originated in Asia; phylotype II strains originated in the Americas; phylotype III strains in Africa; and phylotype IV strains in Indonesia. Phylotypes can further be subdivided into sequevars based on the sequence of the endoglucanase (*egl*) gene (Fegan and Prior, 2005). *R. solanacearum* strains within each phylotype can be further sub-classified into sequevars based on the similarity of a 750-bp fragment of the endoglucanases (*egl*) gene (Fegan *et al.*, 1998). So far, 55 sequevars of *R. solanacearum* have been identified (Li *et al.*, 2016; Liu *et al.*, 2017).

2.4 Management strategies

Bacterial wilt is difficult to control or eradicate and there is no cure once a potato plant or tuber is infected (Muthoni *et al.*, 2010). This results in huge economic losses each year in many tropical and subtropical areas. Even though, some level of control of bacterial wilt have been achieved, no effective control measures have been developed yet. Difficulties are associated with controlling the pathogen of bacteria wilt due to its abilities to grow endophytically, survive in soil for a long periode, especially in the deeper layers, travel along water, and its relationship with weeds (Wang and Lin, 2005). This means that control measures should be applied to prevent initial infection.

If an infection has already occurred, then measures are needed to contain the spread of the disease. Therefore, the containment strategy for bacterial wilt should follow the system approach that incorporates specific operational practices to reduce the likelihood of incursion, establishment and growth of *R. solanacearum* in potato crops (CIP, 2017). In addition to causing yield losses in field crops and during storage, management efforts for prevention, eradication, and general control of this pathogen are extremely costly, and contribute heavily to economic losses. Several control methods or strategies exit although none of them is 100% effective (Figure 2.7) (Champoiseau *et al.*, 2010).

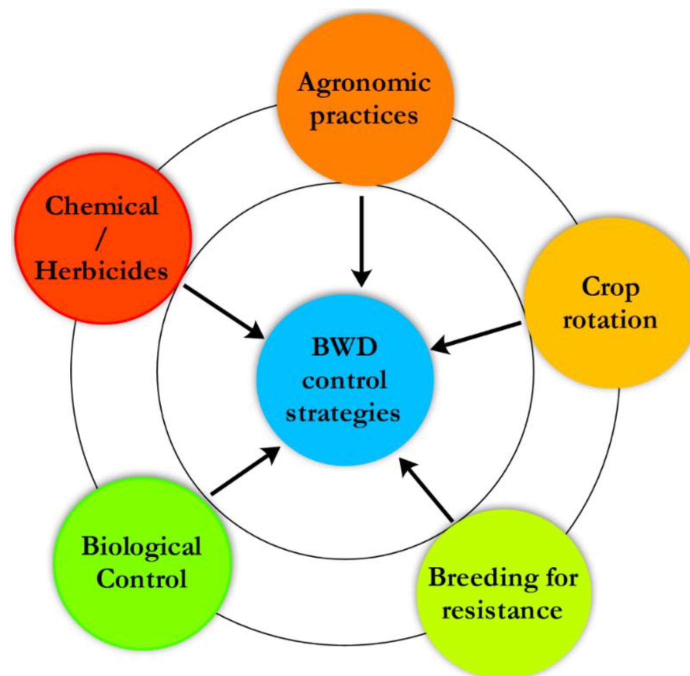

Figure 2.7: Bacterial wilt control strategies

Methods used in the management of bacterial wilt disease. The color classifies the control strategies from the most expensive with negative impact on the environment (Red) to the environmentally friendly and cost-effective methods (Green).

2.4.1 Agronomic practices

The growth of potato cultivars that are resistant to bacterial wilt is considered to be the most economical, environmentally friendly, and effective method of disease control. However, breeding for resistance to bacterial wilt has been influenced by several factors such as the availability of resistance sources, the diversity of the pathogen, genetic linkage between resistance, and other agronomic traits, differentiation and variability in pathogenic strains, the mechanism of plant-pathogen interactions, and breeding or selection methodology (Hanson *et al.*, 1996; Boshou, 2005; Elphinstone, 2005). Moreover, resistant plants are subjected to latent infection with high density of *R. solanacearum* without displaying wilt symptoms and thus contribute to the spread of the disease (Hayward, 1991; Prior *et al.*, 1996). In addition, resistance to bacterial wilt in many crops has been negatively correlated with yield and quality. Thus, the release of resistant cultivars may be poor because they are not widely accepted by farmers or consumers.

Crop rotation and multiple-cropping with non-susceptible plants are other important cultural practices. The benefits of crop rotation are maintenance of the soil structure and organic matter, and a reduction in soil erosion that is often associated with continuous row crops (Janvier *et al.*, 2007). But these methods are not effective at 100% because of the soil borne nature of the pathogen (Hayward, 1991). Several studies have also revealed that the application of fertilizers can reduce the incidence of bacterial wilt. For example, Calcium (Ca) is the most well-known fertilizer to reduce the severity of bacterial wilt as

well as the population of *R. solanacearum* in the stems of tomato (Yamazaki and Hoshina, 1995; Yamazaki *et al.*, 2000).

Integrated pest management (IPM) can also reduce bacterial wilt disease by 20–100% in the field or under laboratory conditions. This typically combines two or three methods among cultural practices, chemical and biological methods (Yuliar *et al.*, 2015). The main goals of an integrated plant disease control program, regarded as integrated pest management (IPM), are to: (i) eliminate or reduce the initial inoculums, (ii) reduce the effectiveness of initial inoculums, (iii) increase the resistance of the host, (iv) delay the onset of disease, and (v) slow secondary cycles (Agrios, 2005). Currently, biocontrol methods represent a significant complement to other control methods, which are based on prophylactic measures, chemical treatments or genetic approaches to make a perfect combination.

2.4.2 Genetics and breeding for resistance

The growth of cultivars that are resistant to bacterial wilt is considered to be the most economical, environmentally friendly, and sustainable strategy to control bacterial wilt *(Prior et al.*, 1994). However, Prior *et al.*, (1996) showed that resistant plants can be heavily invaded by *R. solanacearum* without displaying wilt symptoms. This is shown to be an important source of dissemination of the pathogen. No high level of resistance to bacterial wilt exists in potato cultivars, although breeding for resistance has resulted in some levels of resistance to bacterial wilt. In addition, the high frequency of latent

infection in tubers exhibiting some resistance is still a problem (Priou *et al.*, 2001; Priou *et al.*, 2005).

In order to control bacterial wilt disease, much effort has gone into developing transgenic bacteria and fungi expressing genes that provide enhanced biocontrol activity, and in transgenic plants expressing genes that provide disease resistance, while also allowing a greater understanding of the mechanisms operating in the rhizosphere. However, with the environmental concerns and existing legislation, it remains to be seen whether transgenic micro-organisms and plants for disease control become universally accepted both as research tools and as commercial products. Moreover, the genetic complexity of potato combined with its small number of resistance genes limits genetic control methods to a few cryptogamic or viral diseases (Priou and Jouan, 1996; Gebhardt and Valkonen, 2001). Consequently, up to this point the potato's prosperity relies mainly on sanitary and phytosanitary measures, and the development of microbiological control which is a worthy and challenging alternative (Latour *et al.*, 2008).

2.4.3 Chemicals

Currently, bactericide application is one of the most important strategies for the management of bacterial wilt, and a variety of chemical bactericides are available (Li *et al.*, 2015; Nion *et al.*, 2015). However, the use of chemical pesticides is associated with phytotoxicity, toxic residues, and environmental pollution. Edwards-Jones, (2008) reported that pesticides offered greater net benefits than other control methods, but this has not always been the case. Because, if farmers use pesticides carelessly or without

proper knowledge, a percentage of the pesticide may remain in the environment for many years (Gadeva and Dimitrov, 2008), becoming a contaminant in soil and/or groundwater and can be poisonous to farmers (Bardin *et al.*, 2015). Pesticides such as algicide (3-[3-indolyl] butanoic acid), fumigants (metam sodium, 1,3-dichloropropene, and chloropicrin), and plant activators generating systemic resistance on tomato (validamycin A and validoxylamine) have been used to control bacterial wilt. Chemical control such as bactericides, in addition to being potentially harmful to the environment, has not proved to be efficient in controlling *R. solanacearum*. Alternatively, biological control has been proposed to be an effective, safe and eco-friendly approach in plant disease management (Almoneafy *et al.*, 2014). In addition, food, health and environmental concerns have attracted increasing consumer attention and concern. Therefore, biological control is a non-hazardous alternative for integrated pest management.

2.4.4 Biological control

Biological control or biocontrol is the use of the disease-suppressive Rhizobacteria with a beneficial agronomic effect on plant growth, commonly referred to as plant growth-promoting rhizobacteria (PGPR) and fungi to keep the level of deleterious microorganisms under control or below a threshold limit (Pal and Gardener, 2006). This suggests the introduction of biological control agents (BCAs) from outside in the rhizosphere of a plant to achieve disease suppression. Biological control is an economical and eco-friendly disease suppression method. Interspecies competition causes reduction in growth, productiveness and other activities of the competing organisms. Biological

control arises when pathogenic and nonpathogenic organisms compete for space and nutrients around the host plant (Pal and Gardener, 2006).

Biological Control provides an alternative safe method of disease control instead of chemicals which are hazardous to the environment. Moreover, the durability of biological control is often considered to be higher than that of chemical control (Holt and Hochberg, 1997). This may be related to specific traits of the plant pathogen such as genetic diversity and ability to evolve in response to a selection pressure; and can be affected by population genetic processes *viz.* mutation, population size, recombination, gene flow and selection (McDonald and Linde, 2002; McDonald, 2014). Biological control agents (BCAs) have been dominated by bacteria (90%) and fungi (10%) and Montesinos (2003) reported that most patented BCAs are made of bacteria.

Interest in biological control has increased recently fueled by public concerns over the use of chemicals in the environment in general, and the need to find alternatives to the use of chemicals for disease control. The search for an environmental-friendly pest management approach has led to study the use of microbial antagonists to control diseases spread by soil borne plant pathogenic bacteria and fungi, in an attempt to replace chemical control and avoid extensive use of fungicides, which often lead to resistance in plant pathogens. Historically, the first potential biocontrol agents were investigated in relation to the potato rhizosphere (Burr *et al.*, 1978; Kloepper *et al,* 1980). Recently, numerous studies on the potato rhizosphere, mycorrhizosphere and endorhiza revealed the presence of a diverse and dense microbial community where various candidate of antagonistic bacteria and

fungi can potentially be used. Thus, potato roots host a microbial community that constitutes a rich source for plant growth-promoting rhizobacteria and biocontrol agents. Furthermore, antibiotic production by biocontrol agents can not only affect the pathogen but also plant development (Brazelton *et al.*, 2008).

The mode of action of biocontrol agents is still partial. However, it is generally considered that there are three main ways for a biocontrol agent to control a plant pathogen (Jacobsen, 2006; Alabouvette *et al.*, 2009): first, by acting directly on the plant pathogen, through antibiosis, competition for nutrient or space, or parasitism; secondly by interfering with the mechanisms of pathogenesis of the plant pathogen, and thirdly by modifying the interaction of the pathogen with its plant host for instance through the induction of local or systemic acquired resistance. None of the mechanisms are necessarily mutually exclusive and frequently several modes of action are exhibited by a single biocontrol agent (Janisiewicz and Korsten, 2002).

The benefits of BCAs are 1) potentially self-sustaining, 2) spread on their own after initial establishment, 3) reduced input of non-renewable resources, and 4) long-term disease suppression in an environmentally friendly manner (Quimby *et al.*, 2002; Whipps, 2007). Besides the benefits, there are some disadvantages associated to BCAs. The biggest obstacle is their poor performance due to inconsistent colonization. Suppression by BCAs has been observed in a narrow range of host plants or restricted to a single pathogen or disease (Whipps, 2007). The degree of suppression is sometimes too low to be commercially acceptable or requires uneconomically high rates of inoculums to be applied

(Whipps, 2007). Difficulties have also been associated with producing, storing, and subsequently applying BCAs. While there are multiple factors that can influence the effectiveness of biological control agents, increased attention should be paid to: (1) characterizing natural enemy candidates and target hosts using morphological taxonomy and molecular techniques at the onset of a program; (2) utilizing climatic matching models to determine accurately the most likely areas to find and successfully establish candidate agents; (3) understanding biological control agent host-finding behavior and attack rates; (4) elucidating the most relevant habitat characteristics of biological control agents in their place of origin for better prediction of the rates of colonization and spread in the invaded range (Nowierski *et al.*, 2002; Hoelmer and Kirk, 2005).

Topics regarding biocontrol agents for bacterial wilt have been separated into the following categories: isolation, screening and identification of BCAs, application methods of BCAs, improved BCAs, suppression mechanisms of BCAs, and effects of BCAs on the environment. Currently, biocontrol formulations are an expanding market as they represent 1% of the overall pesticides sales (Diallo *et al.*, 2011). Fravel, (2005) and Montesinos, (2003) have drawn up lists of biocontrol products and strains registered by the United States Environmental Protection Agency (USEPA) and the European Protection Agency (EPA). These strains mainly belong to *Bacillus* and *Pseudomonas* bacterial genera and *Aspergillus* and *Trichoderma* fungal genera.

2.5 Potato rhizosphere as an important source of biocontrol agent

Potato cultivation has a strategic role as a food source for the human population. Its promising future development relies on improving the control of the numerous microbial diseases that affect its growth. Recent studies on the potato rhizosphere, mycorrhizosphere and endorhiza reveal the presence of a diverse and dense microbial community. This microbial community constitutes a rich source for plant growth-promoting rhizobacteria and biocontrol agents. So far, the beneficial effects achieved are related to microbial siderophores, antibiotics, biosynthesis of surfactants and phytohormones, nutrient and spatial competition, mycoparasitism, induced systemic resistance, phage therapy, quorum quenching and construction of transgenic lines (Diallo *et al.*, 2011).

Potato roots host a highly diverse and dense microbial community where various candidate antagonistic bacteria and fungi can potentially be used. The structural analysis of potato rhizosphere microbial communities has been detailed by describing spatial and temporal colonization of underground organs by rhizobacteria (Loper *et al.*, 1985; Bahme and Schroth, 1987; Frommel *et al.*, 1993). The fungal genera *Alternaria, Clonostachys, Fusarium, Penicillium* and *Rhizoctonia* are cited to be commonly encountered in both the rhizosphere and the geocaulosphere of potato (Pieta and Patkowska, 2003; Fiers *et al.*, 2010), and more than 60 bacterial genera have been identified by culture dependent methods in different potato cultivars. Three genera, known to mainly contain rhizobacteria species, were consistently identified in each microenvironment: *Agrobacterium, Bacillus* and *Pseudomonas.* Denser populations are found near the seed piece, which constitutes

the main nutrient reservoir and often carries the microbial inoculum or contaminants promoting the plant and daughter tubers' infestation (De Boer *et al.*, 1978; Helias *et al.*, 2000).

The potato mycorrhizosphere was also investigated in two different soils (Cesaro *et al.*, 2008). The roots were preferentially colonized by mycorrhizal fungi belonging to the *Glomus intraradices* species and to a lesser degree to the *Glomus mosseae* species whereas in bulk soil a markedly greater diversity was shown (Cesaro *et al.*, 2008). This mycorrhizosphere compartment is even extended to saprophytic fungi that have close associations with roots (Buée *et al.*, 2009). Among them, *Trichoderma* and *Penicillium* communities have been described and detected at concentration levels ranging from 10^4 to 10^5 cfu/g of potato cultivated soil (Larkin and Honeycutt, 2006; Meincke *et al.*, 2010).

Some rhizospheric bacteria have a beneficial agronomic effect on plant growth. These bacteria are commonly referred to as plant growth-promoting rhizobacteria (PGPR) (Compant *et al.*, 2005). PGPR can directly stimulate plant growth by synthesizing hormones (phyto-stimulators) or by supplying the plants with nutrients (bio-fertilizing). Growth stimulation can also be indirectly achieved by suppressing or preventing the deleterious effects of pathogens. It has been reported that, potato seeds treated with fluorescent pseudomonad strains were shown to improve the yields by about 10% compared with non-inoculated pseudomonads. This result was obtained through trials conducted on different field sites over several years (Burr *et al.*, 1978; Kloepper *et al.*, 1980).

PGPR showing biological control activities are referred to as biocontrol agents or biopesticides. Because of their proximity to telluric pathogens and the host to be protected, rhizosphere and endorhiza are privileged microenvironments in biocontrol agent research. Sturz *et al.*, (2005) have estimated that endophytic growth-promoting bacteria ranged from 10% to 20% of the total bacterial population, and a similar proportion of the total bacterial population were shown to be growth inhibiting. The effectiveness of these endophytic PGPR has been evaluated in a multidisciplinary study combining *in planta, in vitro* and molecular analysis (Sessitsch *et al.*, 2004).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Identification and characterization of *Ralstonia solanacearum* strains infecting potato in Nakuru

3.1.1 Survey site and sample collection

Nakuru County lies within the Great Rift Valley, bordering eight other Counties: Kericho and Bomet to the West, Baringo and Laikipia to the North, Nyandarua to the East, Narok to the South-west and Kajiado and Kiambu to the south. The County covers an area of 7,495.1 square kilometers (Km^2) (GoK, 2013). The County has fairly moderate temperature throughout, and ranges from moister in the north and western portions to drier in the south. The northwest and northeast receive over 1250 mm precipitation and a temperature of 15°C on average, whereas the entire central part of the County running from the north to the south receives less than 750 mm of precipitation and is around 17-21 °C on average annually. Potato is a food security and income-generating crop for farmers in the county and its production largely takes place in Molo, Kuresoi North and South, Njoro and Bahati sub-counties. About 40% of the population in the County are engaged in potato value chain and the average acreage for potato production is 1.5 acres. The common potato varieties include Shangi, Nyayo, Rudolf, Jerry and Carruso with shangi being the most preferred by farmers and buyers for its quality in French fries and crisps processing (Mwaniki *et al.*, 2016).

Plants exhibiting symptoms of bacterial wilt were collected from three major potato growing areas in Nakuru county namely, Kuresoi North, Mau Narok and Njoro. This county was selected because farmers rank potatoes there as their most important commercial crop (Kaguongo *et al.*, 2010) and it has been reported that bacterial wilt disease is a problem in the area. In each sub-county, sampling was done at several levels: three divisions in each sub-county where potato is a major crop were selected. Plant samples were randomly collected together with soil under healthy and diseased plants. Five samples each (infected plants, infected soil and soil underneath of healthy plant) were collected from each surveyed field. The samples were labelled properly then brought into the plant transformation laboratory at Kenyatta University and kept under refrigeration at 4°C before further analysis.

3.1.2 Isolation and purification of *Ralstonia solanacearum*

R. solanacearum was isolated from the collected samples. The procedure for isolation involved disinfection of the plant material, preparation of a bacterial suspension from the target material and culturing on agar medium as described by Kinyua *et al.*, (2014). Infected plant material from each farm was washed with running tap water, immersed in sodium hypochlorite (0.5% NaCl) solutions for 3 minutes, then transferred to ethanol (95%) solution for 2 minutes. They were then rinsed with distilled sterile water, to remove any saprophytic or epiphytic bacteria from the plant surfaces. The initial isolation was then achieved by cutting the surface-sterilized plant material with sterile scalpels into small pieces of 0.5 cm and placed on tetrazolium chloride (TZC) agar plates (peptone 10g,

glucose 2.5 g, Casamino-acid 1 g, agar 18 g, TZC 50 mg in 1 L of distilled water) (Kelman, 1954). The agar plates were then sealed and incubated in an inverted position at 28°C for 48 hours. Separately growing colonies were picked and sub-cultured onto fresh media to obtain pure cultures. After isolation, one colony from each sample was re-streaked onto new TZC medium for further purification and bacterial species validation. The isolates were classified into three groups based on the growing areas from where they were obtained (Kuresoi North, Njoro and Mau Narok) and were preserved in 25% glycerol solution at -20℃ refrigerator for further study.

3.1.3 Virulence assessment and species validation

Cultural and morphological characteristics were used for identification of the isolated bacteria as follows: virulent strains (milky, white, flat, irregular, fluidal colonies with pink or red color center and whitish margin) and avirulent strains (smaller, off-white and non-fluidal or less fluidal colonies) strains of *R. solanacearum* were differentiated in Triphenyl Tetrazolium Chloride (TTC) medium containing 0.005% TTC (Kelman, 1954). Based on these virulence characteristics on TTC media, 20 isolates out of 54 were selected for further cultural, morphological and molecular characterization.

For molecular identification of the isolates, genomic DNA was extracted and the common region to *R. solanacearum* genome was amplified. To extract genomic DNA, bacterial cells grown on TZC agar medium were inoculated into Nutrient broth (peptone 5 g, sodium chloride 5 g, beef extract 1.5g, yeast extract 1.5 g, in 1L distilled water) and placed in a 28°C shaking incubator for 24 hours. Genomic DNA of all selected isolates were

extracted using DNA extraction Kit (Qiagen) according to the manufacturer's instructions. The *R. solanacearum* specie was determined by PCR using universal primers of *R. solanacearum* species: 759/760 which amplified, 281bp amplicons of the specie genome common region (Fegan and Prior, 2005). Amplification was carried out in a total volume of 25 µl reaction containing: One Taq 2X Master mix with standard buffer (New England Biolabs), 0.2 µM of each primer and 1 µl DNA template. Amplifications were done in an Eppendorf AG thermocycler using the following conditions: initial denaturation at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 30 s, and a final extension step at 72°C for 5 min. PCR products (10 µl) were checked by electrophoresis using 1.5% (w/v) agarose gels, stained with Sybr green, and visualized on a UV trans-illuminator.

3.1.4 Phylotype determination

The isolated *R. solanacearum* were classified into phylotypes, based on phylotype-specific multiplex PCR (Pmx-PCR) as previously described (Fegan and Prior, 2005; *Sagar et al.*, 2014). Phylotype have been determined using specific primers (Table 3.1). PCR reactions were carried out in a total volume of 25µl with One Taq 2X Master Mix (containing a standard buffer), primer sets and genomic DNA in an automated thermocycler (Eppendorf AG, 22331 Hamburg, Germany) as follows: initial denaturation at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, and extension at 72°C for 30 sec, with a final extension at 72°C for 7 min. Ten microliters of each PCR product was examined by electrophoresis through 1.5% agarose gel, stained with Sybr green and visualized on a UV trans-illuminator.

Table 3.1: Primers used for species validation, phylotypes determination and endoglucanase gene sequencing

Primer name	Sequences	Amplicon size	Tm	Remark
759R	5'-GTCGCCGTCAACTCACTTTCC-3'	280bp	55˚C	Species validation
760F	5'-GTCGCCGTCAGCAATGCGGAATCG-3'			
Nmult:21:1F	5'-CGTTGATGAGGCGCGCAATTT-3'	144bp	55˚C	Phylotype I
Nmult:21:2F	5'-AAGTTATGGACGGTGGAAGTC-3'	372bp	55˚C	Phylotype II
Nmult:23:AF	5'-ATTACGAGAGCAATCGAAAGATT-3'	91bp	55˚C	Phylotype III
Nmult:22:InF	5'-ATTGCCAAGACGAGAGAAGTA-3'	213bp	55˚C	Phylotype IV
Nmult:22:RR	5'-TCGCTTGACCCTATAACGAGTA-3'	-	-	-
Endo-F	5′-ATGCATGCCGCTGGTCGCCGC-3′	~750bp	62˚C	*egl* gene
Endo-R	5′-GCGTTGCCCGGCACGAACACC-3′			

Tm: melting temperature

3.1.5 Endoglucanase (*egl*) gene sequencing

A 750-bp partial endoglucanase (*egl*) gene was amplified by PCR with primer pairs of Endo-F / Endo-R (Poussier *et al.*, 2000). The primer sequences are indicated in Table 3.1. After the PCR, amplicons of endoglucanase gene were confirmed by electrophoresis and their size was estimated by comparison with a 1 Kb Plus DNA marker. The PCR products were purified using PCR purification kit (Qiagen) following the manufacturer's instructions and then sent for sequencing using Endo-F and Endo-R primers at InqabaBiotech, South Africa. The DNA sequences of the isolates were deposited in the NCBI sequence database. The phylogenetic analysis was done based on partial endoglucanase sequences using MEGA7 (Kumar *et al.*, 2016). Phylogenetic tree was constructed from the genetic distance data by the Neighbor-Joining (NJ) and Maximum

Likelihood (ML) method using the algorithm of Jukes–Cantor with 1000 bootstrap resampling of the data to test the tree topologies.

3.1.6 Biovar determination

The isolates were differentiated into biovars based on their ability to oxidize three disaccharides (maltose, lactose, and cellobiose) and three hexose alcohols (mannitol, sorbitol, and dulcitol) as previously described (Denny and Hayward, 2001). The standard biovar test medium (basal medium) was prepared by adding 1.0g $NH_4H_2PO_4$, 0.2g KCl, 0.2g $MgSO_4 .7H_2O$, 1.0g Difco bacto peptone, 3.0g Agar and 80.0 mg bromothymol blue into a final volume of 1L of distilled water according to Denny and Hayward (2001). The pH was adjusted to 7.0, by drop wise addition of 3 N of NaOH. Filter sterilized solutions (10% W/V) of cellobiose, lactose, maltose, dulcitol, mannitol and sorbitol were prepared and 1 part of each carbon solution was mixed with 9 parts of the basal medium to obtain a final concentration of 1% of the carbohydrate. The medium was autoclaved at 121℃ and 15 psi pressure for 15 minutes. After cooling, 150µl of the mineral medium containing 1% of carbon sources was dispensed into the 96 wells micro-titration plates. All isolates were inoculated into individual wells with 50µl of bacterial suspensions (adjusted to OD_{600} = 0.1 ~10^8 cfu/ml), with two replicates per isolate. The plates were incubated at 28°C and were observed daily for change in pH by a color change (Schaad *et al.*, 2001). The test was repeated twice.

3.1.7 Hypersensitivity reaction test

The isolates of *R. solanacearum* collected from the wilted potato plant were tested for hypersensitive reaction in tobacco by cell infiltration. Tobacco plants (*Nicotiana tabacum*) were grown under natural conditions in a glass house at 18-22°C. Fully expanded leaves from 54-days old plants were infiltrated with a suspension of *R. solanacearum* in distilled water, using the injection technique of Klement (1963). Bacterial cell suspension (adjusted to $OD_{600} = 0.1 \sim 10^8$ cfu/ml) of each isolate was inoculated into the intracellular space of the leaves of tobacco plants with a syringe. Distilled sterile water was used as a negative control. A total of 4 plants were inoculated with each isolate. Two leaves randomly selected were inoculated per plant with three pecs per leaf. Hypersensitive reaction (necrotic or yellowing areas in the region surrounding an infection) was monitored daily and continued up to five days after infiltration (Ahmed, 2013). The experiment was repeated twice.

3.1.8 Pathogenicity assays

To confirm the virulence of the *R. solanacearum* isolates, pathogenicity test was performed on one-month old potato seedlings of variety Shangi by root irrigation method according to Rado *et al*., (2015). To prepare the inoculum, one *R. solanacearum* isolate for each survey's area was grown on TZC plates for 48 hours at 28°C. A single colony of *R. solanacearum* showing virulence (fluidal, irregular and creamy white with pink at the center) was randomly selected and transferred into a 10ml tube containing Modified Kelman media (French *et al.*, 1995) then incubated at 28°C for 24 hours in a shaker. After

centrifugation, bacterial cells were suspended in sterile distilled water and the concentration was adjusted to $OD_{600} = 0.1$ (~10^8 cfu/ml) using a spectrophotometer. The root of each plant was wounded by stabbing with a sterile 1ml tips and 10ml of bacterial suspension was poured at the base of each plant. Ten plants were inoculated with each identified *Ralstonia* isolate. Sterile distilled water was used as a negative control. The experiment was repeated three times in a greenhouse under natural light conditions. Disease incidence (DI) was monitored every week for 4-week post inoculation. Plants with visible symptoms (wilted leaves) were recorded as diseased plants. The disease incidence was calculated as DI (%) = 100 × (number of disease plants/10 inoculated plants). The pathogen was re-isolated from diseased plants for confirmation.

3.2 *In vitro* screening and identification of potential bio-control agents (BCAs) against *Ralstonia solanacearum*

3.2.1 Bacteria isolation and purification

Bacteria were isolated from soils collected around healthy and infected potato plants using serial dilution method. One gram of each soil samples was suspended in 9ml sterile distilled water and diluted up to 10^{-4}. Aliquots (100µl) of each suspension were spread on nutrient agar (NA) plates in triplicates and incubated at 28°C in an incubator. After 2 days of incubation, individual isolated bacteria colonies were sub-cultured onto fresh NA plates. A total of 62 bacteria were isolated and purified. To avoid the harmful effects of the use of biological control agents (BCAs) to plant, screening assay for bacteria present only around healthy plant was performed on our core collection. This was done based on the color, shape and texture. Bacteria from non-infected and infected soil were compared

and 20 bacteria were selected (for their present in the non-infected soil and absence in the infected soil) for antagonistic activity screening against four *R. solanacearum* strains. Each isolate was preserved in 25% glycerol stock at -20°C.

3.2.2 Inoculum preparations

Pre-cultures of bacteria were prepared by growing them in 10 ml of liquid nutrient broth (NB) for 24 hours at 28°C with agitation (200 rpm). *R. solanacearum* cells were cultured in Casamino Acid-Peptone-Glucose-agar (CPG) medium containing 0.1% Casamino Acids, 1% peptone, and 0.5% glucose at 28°C with shaking at 200 rpm. After growth, the medium was centrifuged at 5,000 g for 10 min at room temperature and bacterial cells were re-suspended in distilled sterile water. The optical density of samples from each tube was measured at 600 nm using a spectrophotometer (JENWAY 6300, Dunmow, UK) and the optical density (OD) was adjusted to 0.1 by adding more bacteria cells if the suspension was too light or diluting with sterile distilled water if the suspension was too heavy. Aliquots (100µl) of the dilutions were spread on nutrient agar plates and the colonies were counted after 24 hours of incubation at 28°C.

3.2.3 *In vitro* interactions of the identified *Ralstonia solanacearum* with the selected bacteria

The antagonistic activity of the selected bacteria against four *R. solanacearum* strains was assayed by dual culture (disc diffusion method) (Balouiri *et al.*, 2016). Paper discs of 6 mm diameter prepared from filter paper were autoclaved and impregnated with bacteria by soaking them in bacteria suspension and drying for 2-3 minutes. Discs impregnated

with sterile distilled water were used as negative control. While the positive control constituted discs with gentamycin (10µg) and imipenem (10µg). Aliquots (100ul) of *R. solanacearum* suspensions (approximately 10^8 CFU/ml) was streaked on tryptic soy agar (TSA) plates and 9 discs of each bacteria isolates placed on top of the plates in triplicates. Plates were incubated for 48 hours in an incubator set at 28°C. The interaction between *R. solanacearum* and the test isolates monitored and a zone of inhibition around the paper disc was measured. The inhibition zone's diameter (including that of the disc) were measured at day 2, 3 and 4 after incubation. The experiment was conducted two times.

3.2.5 Bacterial isolates identification

3.2.5.1 Api 20 E test

The potential antagonists showing highly significant *in vitro* activity were subjected to identification test using Api 20E kit. Bacteria were culture on nutrient agar plates. A single isolated colony (from a pure culture) was picked and a suspension was made in 5ml of NaCl 0.85% prepared with sterile distilled water. A Pasteur pipette was used to fill up (up to the brim) the compartments of API 20E Biochemical Test Strip with the bacterial suspension adjusted to $OD_{600} = 0.1$ (~10^8 cfu/ml). For the tests ADH, LDC, ODC, H2S and URE, an anaerobiosis was created by overlaying the cupules with sterile mineral oil. Some drops of water were put in the tray to create humidity, the API Test strip was placed and the tray was closed. The tray was marked and incubated at 37°C for 18 to 24 hours. After the incubation period, the strips were read by referring to the reading table of the

kit. The identification was obtained with the numerical number using the API catalog and apiweb (https://apiweb.biomerieux.com).

3.2.5.2 DNA sequencing of 16S gene

Bacterial identification was further performed through 16S rRNA gene sequencing. For this, bacterial cells grown for 1 day on nutrient agar (NA) were sub-cultured in Nutrient broth (peptone 5 g, sodium chloride 5 g, beef extract 1.5g, yeast extract 1.5 g, in 1L distilled water) and placed in a 28°C shaking incubator for 24 hours. Genomic DNA of all isolates was extracted using DNA extraction Kit (Qiagen) according to the manufacturer's instructions. PCR reactions were performed using the primers pair 27F (AGAGTTTGATCCTGGCTCAG) and 1492F (GGTTACCTTGTTACGACTT); and OneTaq 2X Master Mix with standard buffer (New England BioLabs) following the manufacturer's recommendations. Thermal cycling parameters were as follows: a denaturation step at 94°C for 5 min followed by 35 cycles at 94°C for 30 s, 55°C for 30 s and 72°C for 90 s, with a final elongation step at 72°C for 7 min. PCR amplicons were verified by gel electrophoresis, purified using the GenElute PCR cleanup kit (Qigen) and sequenced in both orientations at Inqaba biotech (Inqaba, SA). For each bacterial isolate, nucleotide sequences were trimmed, aligned and compared with the BLASTn search available in GenBank database. Sequences were deposited in the NCBI database. Phylogenetic relationships based on partial 16S rRNA gene sequences were determined with MEGA 7.0 software iterations (Kumar *et al.*, 2016) using maximum likelihood (ML)

method with the General Time-Reversible plus gamma model of nucleotide substitution and bootstrap values of 1,000 iterations.

3.3 Evaluation of the effects of BCAs on plant responses under *Ralstonia solanacearum* infection

The effect of the identified antagonists in inhibiting the growth of *R. solanacearum* cells *in vitro* leads to evaluate their effects *in planta*. The greenhouse experiments were performed in order to evaluate the antagonistic effect of selected bacteria against bacteria wilt and their effects on plant response under bacterial wilt infection.

3.3.1 Plant growth

Certified tubers of *S. tuberosum*, “Shangi” variety from ADC (Molo, Kenya) were used in greenhouse experiments. This variety was chosen based on its agronomic traits and farmers preferences. It is the most cultivated variety in the area where sampling was conducted. The tubers were surface-sterilized with 0.5% sodium hypochlorite (NaOCl) and grown in pots containing twice-sterilized combination of 4:1 soil and sand. Plants were watered daily and, maintained under normal conditions with a photoperiod of 14:10 hours at 13-25°C.

3.3.2 Inoculum preparation

Assays were conducted with five potential antagonists showing significant *in vitro* antagonistic activities against different strains of *R. solanacearum*. Bacteria were grown on nutrient agar (NA) plates and a single colony was sub-culture in 10 ml of liquid nutrient broth (NB) for 48 hours at 28°C with agitation (200 rpm). *R. solanacearum* strain (Rs6)

with high severity during the pathogenicity assay was used and cultured in CPG medium containing 0.1% Casamino Acids, 1% peptone, and 0.5% glucose at 28°C with shaking at 200 rpm. After growth, bacterial cultures with the medium (NB) were adjusted to an OD_{600} of 1 using a spectrophotometer (JENWAY 6300, Dunmow, UK). The OD was adjusted by adding more culture if the suspension was too light or diluting with sterile medium if the suspension was too heavy.

3.3.3 Bio-Control Assays Against Potato wilt in Greenhouse

Pot experiments in a greenhouse were carried out following a randomized complete block design with 10 plants per treatment and replicated twice. The experimental treatments were as follows: (1) control 1, containing plants with no treatment, no infection; (2) control 2, pots with no treatment but infected; (3) A2 inoculation, in which the plants were treated with A2 alone (10^8 cfu/ml); (4) A3 inoculation, in which the plants were treated with A3 alone (10^8 cfu/ml); (5) A4A inoculation, in which the plants were treated with A4A alone (10^8 cfu/ml); (6) A5 inoculation, in which the plants were treated with A5 alone (10^8 cfu/ml); (7) A15 inoculation, in which the plants were treated with A15 alone (10^8 cfu/ml); (8) co-inoculation1, in which the plants were treated with A2 combined with A3 (two bacteria which shown inhibitory effect *in vitro*); (9) co-inoculation2, in which the plants were treated with A5 combined with A15 (two bacteria which shown competition for space and nutrient *in vitro*) and (10) co-inoculation3, in which the plants were treated with A2 combined with A15 (two bacteria which shown different antagonistic activities *in vitro*) (Figure 3.1).

Treatments were applied via root irrigation seed treatment (Rado *et al.*, 2015). Ten pots were used for each treatment (total 100 pots for all treatments), with each pot exposed to 50 ml of bacteria culture prepared as described above. All pots except that of control 1 were inoculated 24 hours before treatment, with 50 ml suspension of *Rs6* (the most virulent strains). The pots were well labeled and arranged in a complete randomized block design in a greenhouse at plant transformation laboratory (Kenyatta University). Plants were observed weekly and the wilt incidence was recorded weekly after the first wilting symptoms began to appear (17 days). Stems of those plants exhibiting no wilt were also analyzed, to detect latent infections by isolating the bacteria on MKM (French *et al.*, 1995). The experiment was performed twice.

The treated plants were monitored for disease development for 30 days and disease index was scored on a scale of 0–4: where 0 = no wilting, 1 = 1–25% wilted or dead plants, 2 = 26–50%, 3 = 51–75% and 4 = 76–100% (Park *et al.*, 2007). The disease incidence (DI) was calculated as DI (%) = 100 × (number of disease plants/10 inoculated plants). Biocontrol efficacy was calculated as [(disease incidence of control − disease incidence of treated plants) / disease incidence of control] ×100% (Xue *et al.*, 2009). Moreover, the synergistic or antagonistic responses of two bacteria in co-inoculation treatments were calculated using the method described by Colby (Colby, 1967): $E = If + Ib - If*Ib / 100$ where If is the observed control efficacy of bacteria1, Ib denotes the observed control efficacy of bacteria 2 and E represents the expected control efficacy of the combined biological control agents. When the observed response was greater than expected, the

combination was considered synergistic and when it was less than expected, it was deemed antagonistic.

After treatment, the effect of potential antagonists on plant growth was assessed in terms of germination percentage, plant height, fresh and dry biomass weight and water content. The growth promotion efficacy (GPE) was calculated to show the relative effect of antagonistic isolates on plant growth compared with control treatments by the following formula (Almoneafy *et al.*, 2012): Growth promotion efficacy (%) = [(Growth parameter in antagonist-treated group -Growth parameter in control group) / (Growth parameter in control group)] x 100.

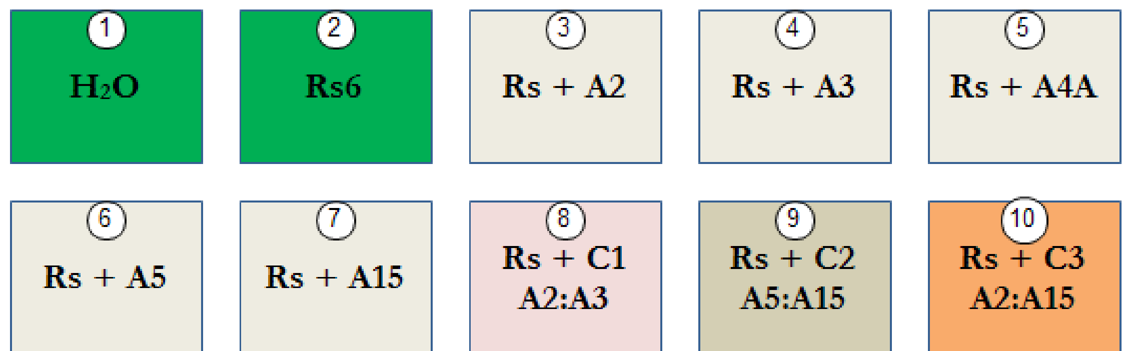

Figure 3.1: Factorial design used in green house experiment for the application and evaluation of the identified biocontrol agents.

3.3.4 Data analysis

During the dual culture assays, zone of inhibition was measured to define the interaction between the tested bacteria and the *R. solanacearum* strains used. In the greenhouse experiments, the identified potential antagonists were tested for plant growth promotion where germination percentage, plant height, fresh and dry biomass were measured. Disease suppression by the BCAs were also evaluated by measuring the disease incidence and water content, since bacterial wilt development is known to restrict the absorption of water and minerals causing wilting. Data from both *in vitro* assays and greenhouse experiments were subjected to ANOVA analysis using R software and when the ANOVA was significant ($P<0.05$), the Tukey HSD multiple-comparison test was used for comparison of the means, with confidence interval specified through p-value. The graphical presentation of data was done using graphpad prism 6 software.

CHAPTER FOUR

RESULTS

4.1 Identification and characterization of *Ralstonia solanacearum* strains infecting potato in Nakuru

4.1.1 Isolation, virulence and species validation of the pathogen

In this study, the geographical distribution of potato bacterial wilt was surveyed and samples were collected from three major sub-counties *viz*., Kuresoi North, Mau Narok and Njoro. A total of fifty-four (54) *R. solanacearum* strains causing potato wilt were obtained and subjected to various tests. This pathogen was found in all the surveyed areas. The Kelman Tetrazolium Chloride (TZC) agar differentiation test gave pink or light red color colonies or colonies with characteristic red center and whitish margin for the virulent isolates while the avirulent isolates produced smaller, off-white and non-fluidal or dry colonies on TZC medium after 48 hours of incubation (Plate 4.1). The virulent isolates (Plate 4.2), were selected for further characterization. Out of the 20 isolates selected, 7 were from Kuresoi North, 6 from Mau Narok and 7 from Njoro (Table 4.1). The results from DNA-based analysis with 759/760 primers showed a PCR product of approximately 281bp (Plate 4.3). This confirmed that the 20 isolates belonged to *R. solanacearum* species.

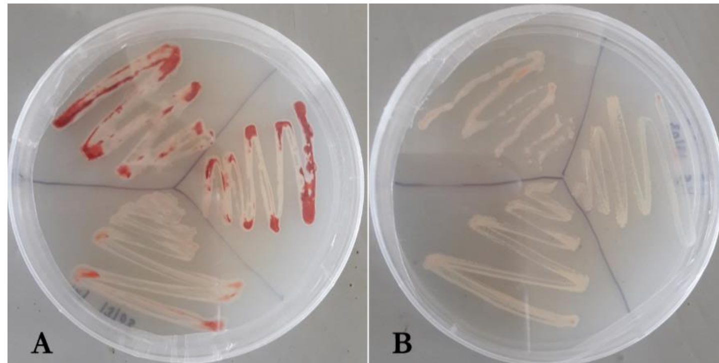

Plate 4. 1: Profile of *Ralstonia* isolation from potato tissues on TZC medium A. virulent *Ralstonia* colonies and B. Avirulent *Ralstonia* colonies.

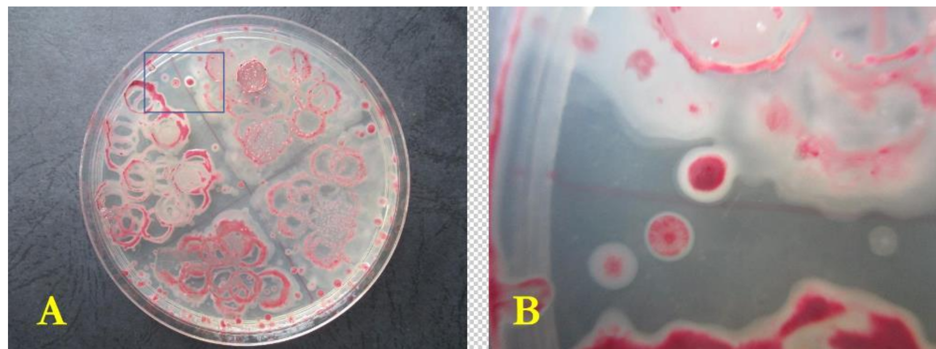

Plate 4. 2: A. Pure culture of *R. solanacearum* on TZC media, B. Zoom on *R. solanacearum* colonies under microscope.

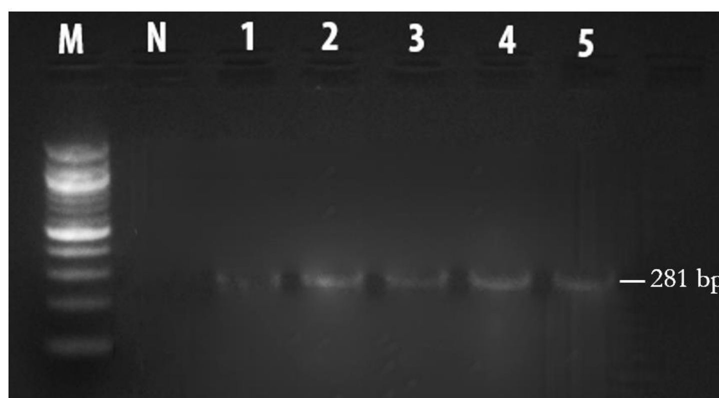

Plate 4. 3: *R. solanacearum* species validation with 759/760 primers; M-1kb DNA ladder, N-negative control, 1-5 bacterial isolates

Table 4.1: Characteristics of *R. solanacearum* isolates collected from wilted potato plants.

№	Isolates	Origin	Virulence	HR	Phylotypes
1	Rs1A	Muthera farm1	+	+	III
2	Rs2A	Mau Narok farm2	+	+	I
3	Rs4A2	Mau Narok farm1	+	+	I
4	Rs6	Kuresol North farm6	+	++	IV
5	Rs8	Mau Narok farm2	+	+	I
6	Rs9A	Kuresol North farm2	+	+++	IV
7	Rs13	Muthera farm2	+	+	I
8	Rs13B	Muthera farm2	+	++	I
9	Rs15	Kuresol North farm3	+	++	II
10	Rs18	Kuresol North farm6	+	+	II
11	Rs19	Muthera farm1	+	++	III
12	Rs21	Kuresol North farm5	+	+	III
13	Rs26	Mau Narok farm2	+	++	I
14	Rs34	Muthera farm1	+	+	IV
15	Rs35	Muthera farm1	+	+	I
16	Rs36	Kuresol north farm1	+	++	I
17	Rs37	Kuresol north farm2	+	+++	IV
18	Rs49	Mau Narok farm2	+	+	I
19	Rs56	Mau Narok farm4	+	++	I
20	Rs57	Muthera farm1	+	++	III
21	Reference	CIP, Kenya	+	++	IV

Hypersensitive reaction (HR) positive isolates score: slight localized chlorosis (+) followed by necrosis (++) and collapse of whole tissue (+++) (Shahbaz *et al.* 2015).

4.1.2 Phylotype analyses

From this study, it was found that the prevalence and distribution of the four phylotypes appeared to be variable throughout the potato growing areas surveyed (Nakuru). Phylotype I (n = 10) was the most prevalent, representing 50% of the collection compared to Phylotype II (n = 2), III (n = 4), and IV (n = 4), which represented 10, 20, and 20% of the collection, respectively. The greatest diversity was found in Kuresoi North, where the four phylotypes of *R. solanacearum* were identified. The second highest diversity was found in Njoro with three different phylotypes, except phylotype II. In contrast, Mau Narok was dominated with phylotype I, Showing a homogenous distribution of *R. solanacearum* phylotype in the area.

4.1.3 Characterization of partial endoglucanase (*egl*) gene sequences

Partial *egl* gene sequences were obtained from 4 isolates from different area and phylotype. To analyze the evolutionary relationship among the isolates from this study, 16 additional strains of *R. solanacearum* from previous studies were retrieved from NCBI-Genebank database. These sequences were compared with those obtained from this study, using the ClustalW program. The sequences analysis allowed molecular confirmation of the *Ralstonia* cultures and further affirmed their status as belonging to the *solanacearum* species. The sequences have been deposited in the NCBI database awaiting accession numbers. Multiple sequence alignment and phylogenetic analysis resulted in generation of a phylogenetic tree indicating the evolutionary relationships between the identified isolates and other known sequences in NCBI (Figure 4.1). According to the Maximum

Likelihood algorithm, the analyzed *R. solanacearum* isolates were clustered into 3 groups with our isolates found in group I and III. Isolate Rs15 was clustered in group I in a short evolutionary distance with *R. syzygii* and *R. solanacearum* isolated from banana. The remaining 3 isolates were clustered in group III (Figure 4.1). This partial endoglucanase gene (*egl*) sequences analysis further confirm the identity of the isolates to be *R. solanacearum*.

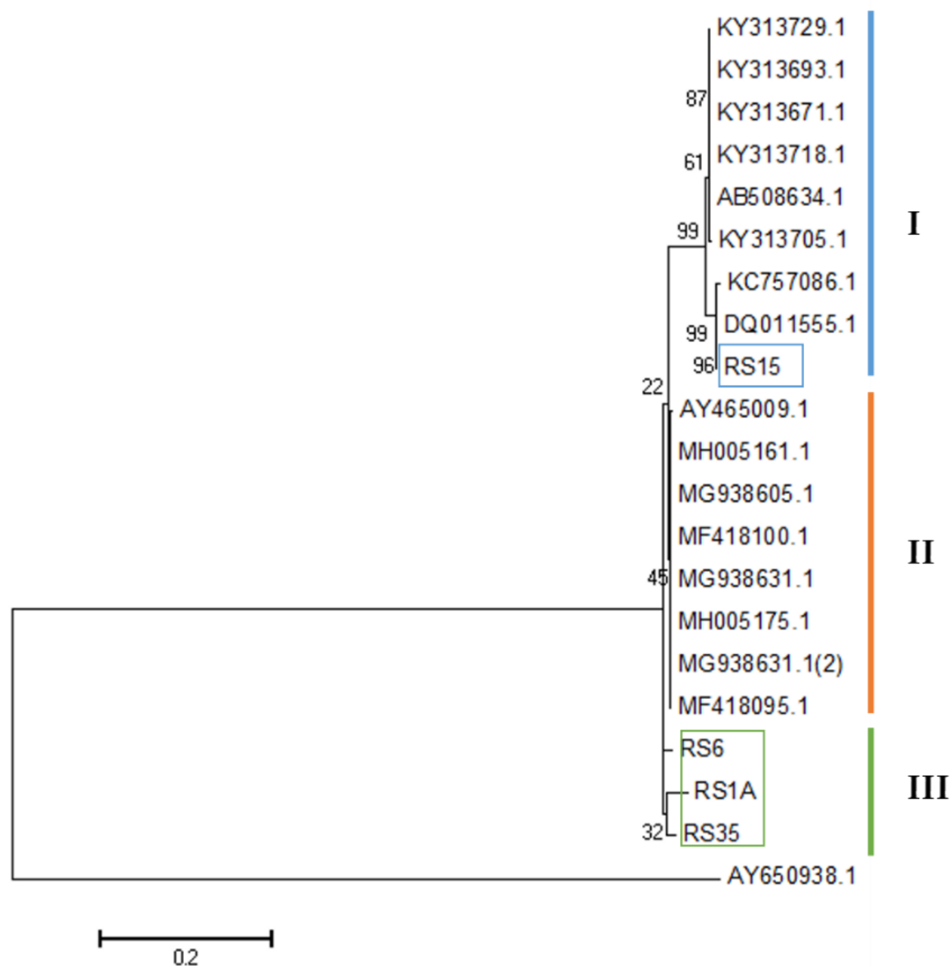

Figure 4. 1: Molecular Phylogenetic analysis of the evolutionary relationships between the identified *R. solanacearum* isolates and other known sequences from NCBI. The phylogenetic tree was generated using the Maximum Likelihood algorithm in MEGA 7 following a multiple sequence alignment.

4.1.4 Biovars characteristics

The result of the biovar test showed that *R. solanacearum* isolates identified in this study were able to oxidize, disaccharides (cellobiose, lactose, and maltose) and sugar alcohols (mannitol, sorbitol and dulcitol) within 3-5 days at different rates, except isolates Rs18

and Rs49 whose biovar was not identified (Table 4.2). The reaction was presented by the color change from blue green to yellow which was an indication of an oxidation reaction of sugar/alcohol by bacterial isolates (Table 4.2). Eighteen (18) isolates analyzed were identified as biovar III and isolate 18 and 49 were not identified. Mannitol and sorbitol were used faster, completely causing color changes in the test medium by day 2 compared to dulcitol which completely changed color at day 4 after incubation. All the three disaccharides (cellobiose, lactose and maltose) were also utilized at a similar rate, causing complete color changes by day 4. The control plates with sterile distilled water remained unchanged (Plate 4.4).

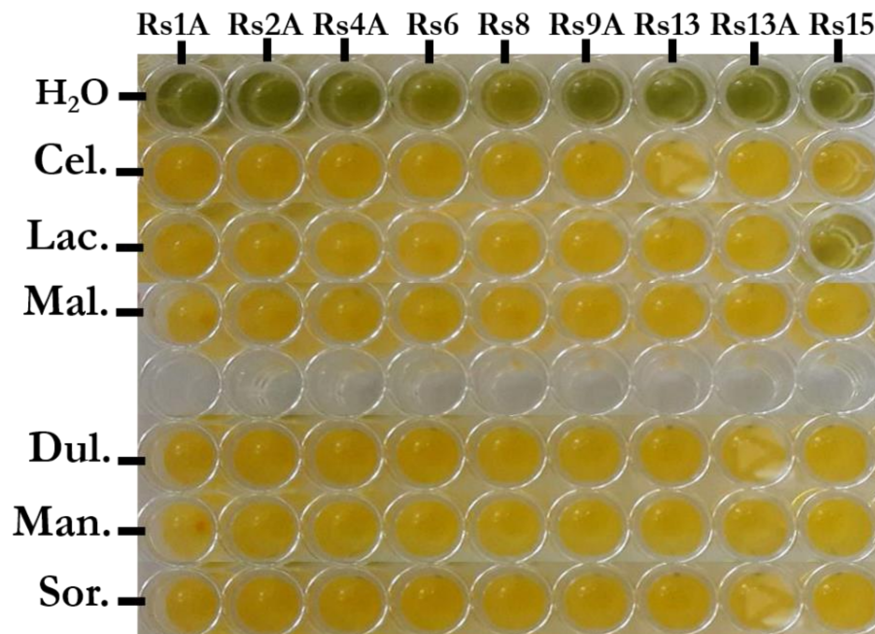

Plate 4. 4: Ability of the identified *R. solanacearum* isolates to oxidize certain carbohydrates using specific basal medium as describe by Hayward (1954). H_2O- basal medium without carbohydrate was used as negative control, Cel.-Cellobiose, Lac.-Lactose, Mal.-Maltose, Dul.-Dulcitol, Man.-Mannose, Sor.-Sorbitol, and Rs1A-Rs15 were different *R. solanacearum* strains tested.

Table 4.2: Biovar differentiation of *R. solanacearum* strains isolated from different location.

Isolate	Cellobiose	Maltose	Lactose	Mannitol	Sorbitol	Dulcitol	Control	Biovar
Rs1A	+	+	+	+	+	+	-	III
Rs2A	+	+	+	+	+	+	-	III
Rs4A2	+	+	+	+	+	+	-	III
Rs6	+	+	+	+	+	+	-	III
Rs8	+	+	+	+	+	+	-	III
Rs9A	+	+	+	+	+	+	-	III
Rs13	+	+	+	+	+	+	-	III
Rs13B	+	+	+	+	+	+	-	III
Rs15	+	+	+	+	+	+	-	III
Rs18	+	+	-	+	+	-	-	*
Rs19	+	+	+	+	+	+	-	III
Rs21	+	+	+	+	+	+	-	III
Rs26	+	+	+	+	+	+	-	III
Rs34	+	+	+	+	+	+	-	III
Rs35	+	+	+	+	+	+	-	III
Rs36	+	+	+	+	+	+	-	III
Rs37	+	+	+	+	+	+	-	III
Rs49	-	-	+	+	+	-	-	*
Rs56	+	+	+	+	+	+	-	III
Rs57	+	+	+	+	+	+	-	III
Reference	+	+	+	+	+	+	-	III

+ = positive reaction; - = negative reaction; *= not identified

4.1.5 Hypersensitivity response

A specific interaction between *R. solanacearum* isolates and plant cell wall receptors was examined by bacterial cells infiltration into the intracellular space of tobacco leaves. This was visualized as slight localized chlorosis followed by necrosis in the plant tissues infected (Plate 4.5). All the isolates were able to cause localized lesions, although at different levels. Tobacco leaves inoculated with sterile water were unaffected.

Plate 4. 5: Hypersensitivity responses induced on tobacco plants by tested isolates: A-Original leave, B-No reaction with distilled sterile water, C- Slight localized chlorosis (+), D- followed by necrosis (++) and collapse of whole tissue (+++).

4.1.6 Pathogenicity determination

For breeding and epidemiological purposes, it is very important to analyze the variability of aggressiveness of the isolates on the most cultivated cultivars. Among the 20 strains characterized from potato, a set of four representatives was randomly selected for pathogenicity testing by inoculating the most cultivated potato cultivar "Shangi" in the surveyed areas. The 4 strains in pathogenicity assay were distributed among all phylotypes and surveyed sub-counties (Table 4.1). The first symptom was observed a week after

inoculation, as slight wilting found in the unilateral stems. As for virulence of the four tested *R. solanacearum* isolates, results exhibited different levels of disease severity on potato plants (cv. Shangi) after 15 days of incubation period (Figure. 4.2). Rs6 isolate from Kuresoi North was the most virulent strain followed by Rs15 also from the same region. Plants inoculated with isolates from Mau Narok and Njoro did not showed typical signs of wilting even though yellowing of leaves coupled with senescence reaction was observed. The result indicated that there is some slight variation among the isolates tested (Plate 4.6). Also, positive results were obtained with TZC test to confirm the infection and/or latent infection.

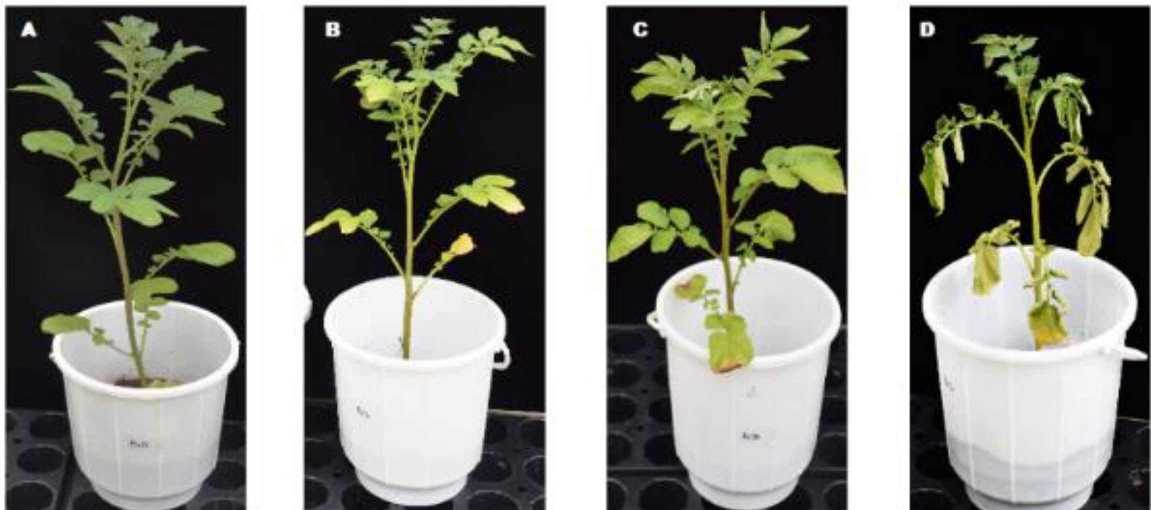

Plate 4. 6: Scoring scheme; different types of symptoms induced in potatoes (cv. Shangi) by the tested *R. solanacearum* strains. A-No visible symptoms (control with H_2O), B-Yellowing or chlorotic spots on the leaves, C- Black streak on the stem and wilting of the upper leaves, and D-Wilting of all leaves and plant death.

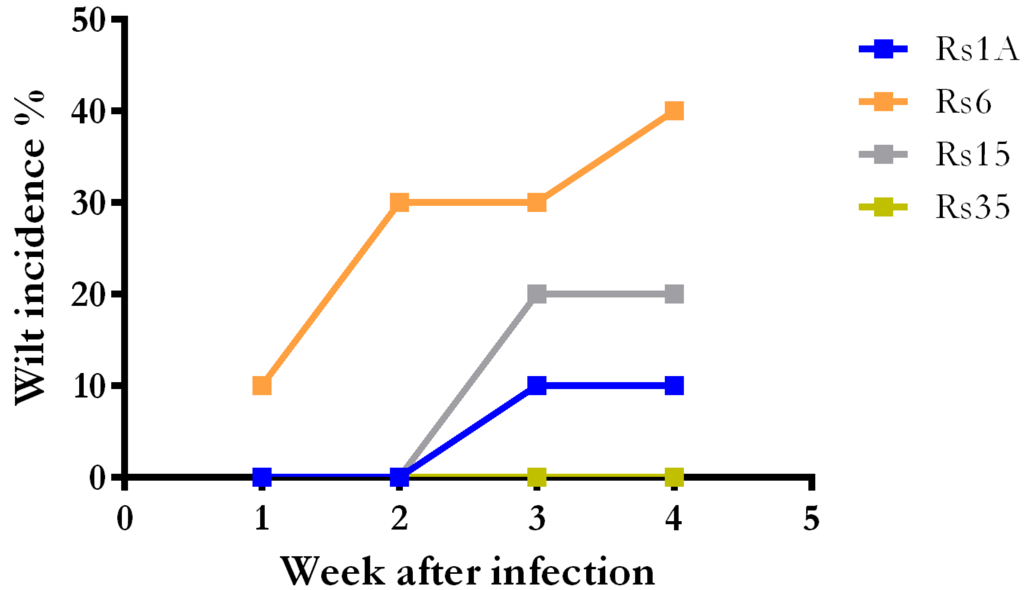

Figure 4. 2: Variation in the virulence of *R. solanacearum* on potato (cv. Shangi). Four isolates induced symptoms development on potato at different times and different levels.

4.2 *In vitro* screening and identification of potential bio-control agents (BCAs) against *Ralstonia solanacearum*

4.2.1 Isolation and screening of antagonists

From the isolation of rhizobacteria associated with potato plants, several isolates were obtained which were differentiated on the basis of their color, colony structure, elevation and the colonies appearing different were streaked separately to get purified isolates. A collection of sixty-two (62) bacterial strains was isolated from the collected soil samples. For *in vitro* screening for potential antagonists, it was hypothesized that soil microbiome around healthy plants growing in an infested field, have an antagonistic influence on *R. solanacearum* and/or help the plant to withstand the disease. Isolated microbes from this

micro-environment were cultured and resulting color, structure and elevation of their colonies compared. Twenty (20) bacteria were selected based on their presence in non-infected soils and their absence in infected soil. This was also done, in order to avoid the harmful effects of potential antagonists on plant development.

Screening of rhizobacterial isolates with dual culture technique revealed 8 potential isolates (zone of inhibition > 8 mm in radius and space competition > 12 mm in radius) antagonistic to four *R. solanacearum* strains (Table 4.3). The tested bacteria had two types of interaction with the pathogen. Most of antagonists inhibited growth of the pathogen and produced a zone of inhibition while other inhibited and overgrew on top of the pathogen colony (Nutrient and spatial competition) (Plate 4.7). In screening against Rs35 (I), the highest antagonistic activity of 23.00 ± 1.53 mm was produced by A5 exhibiting competition for space and nutrient. The other isolates A3, A10C, A11, and A17A also showed significant inhibition results against Rs35 (I) with highest inhibition zone of 15.33 ± 0.58 mm by A3 having antibiosis as mode of interaction. Similarly, screening against Rs15 (II), A2 was the best with highest inhibition zone of 9.00 ± 0.00 mm followed by A3, A5 and A10B with inhibition zone of 8.33 ± 0.58 mm. Antagonism against Rs1A (III) was recorded highest with isolate A3 having an inhibition zone of 14.33 ± 0.58 mm similar to that of gentamycin, isolate A15 also produced reasonable antagonistic activity of 10.33 ± 0.58 mm. Against isolate Rs6 (IV), A4A was found best antagonizing agent with mean zone of inhibition of 17.00 ± 0.00 mm and other isolates (A6 and A15) were also effective in competing for space and nutrient. Interestingly, some antagonists showed

greater inhibition zone compare to the positive control (Gentamycin) as shown on Table 4.3. Rs35 was the most sensitive among all the pathogens tested since all antagonists inhibited its growth. On the other hand, Rs15 showed highest tolerance to the antagonistic effects of microorganisms since only 13 antagonists were able to inhibit its growth.

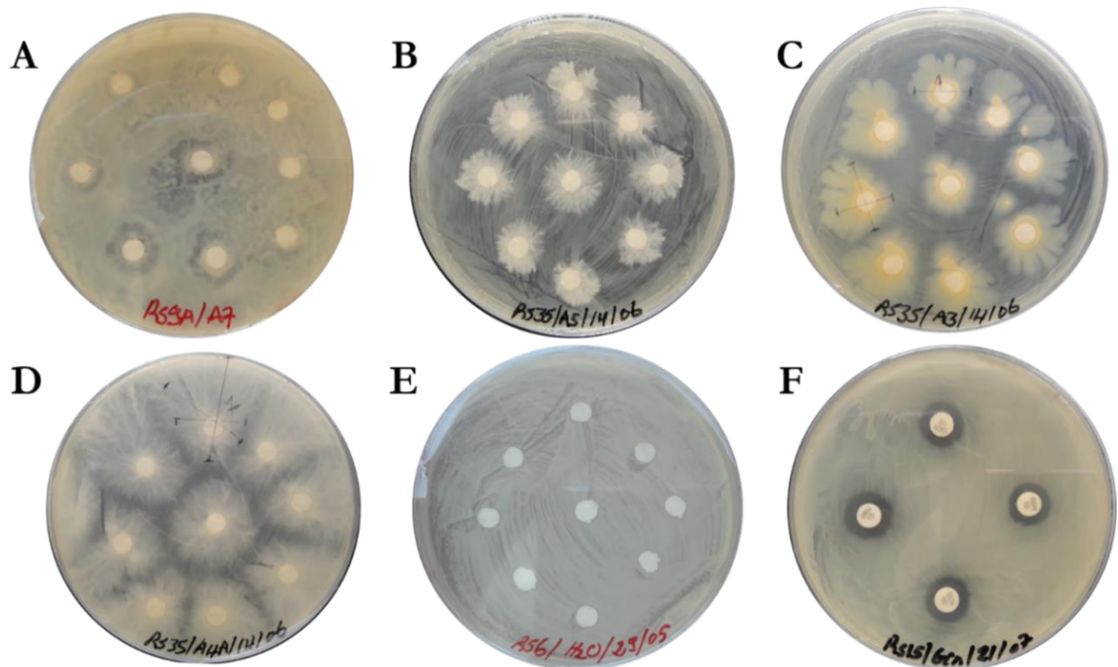

Plate 4. 7: Antagonistic strategies used by potential BCA against four *R. solanacearum* phylotypes. A-Inhibition, B, C and D-Competition for nutrient and space, E- No interaction (H_2O, negative control), F-Inhibition by a Positive control.

Table 4.3: Antagonistic activity of the selected bacteria against four virulent strains of *R. solanacearum*.

Antagonists	Antagonistic activity (mm radius)				Type of interaction
	Rs35 (I)	**Rs15 (II)**	**Rs1A (III)**	**Rs6 (IV)**	
A1A	7.33 ± 0.58^{ijk}	7.67 ± 0.58^{de}	9.33 ± 0.58^{c}	6.33 ± 0.58^{fg}	A
A1C	6.33 ± 0.58^{jk}	6.67 ± 0.58^{ef}	7.33 ± 0.58^{d}	7.33 ± 0.58^{efg}	A
A2	6.67 ± 0.58^{jk}	9.00 ± 0.00^{c}	9.67 ± 0.58^{c}	8.33 ± 0.58^{e}	A
A3	15.33 ± 0.58^{c}	8.33 ± 0.58^{cd}	14.33 ± 0.58^{b}	8.00 ± 0.00^{ef}	A
A4A	15.33 ± 0.61^{c}	7.33 ± 0.58^{de}	7.33 ± 0.58^{d}	17.00 ± 0.00^{b}	B
A4B	6.67 ± 0.58^{jk}	7.33 ± 0.58^{de}	7.33 ± 0.58^{d}	7.67 ± 0.59^{efg}	A
A5	23.00 ± 1.53^{a}	8.33 ± 0.58^{cd}	7.33 ± 0.58^{d}	8.00 ± 0.00^{ef}	B
A6	6.33 ± 0.58^{jk}	6.00 ± 0.00^{f}	6.67 ± 0.58^{d}	23.00 ± 1.52^{a}	B
A7	8.33 ± 0.70^{jk}	8.00 ± 0.00^{cd}	6.67 ± 0.58^{d}	7.00 ± 0.00^{efg}	A
A8	8.33 ± 0.55^{ghi}	8.00 ± 0.00^{cd}	7.00 ± 0.00	8.00 ± 0.00^{ef}	A
A9	6.67 ± 0.58^{ghi}	6.00 ± 0.00^{f}	6.00 ± 0.00^{d}	7.33 ± 0.49^{efg}	A
A10B	12.33 ± 0.58^{d}	8.33 ± 0.58^{cd}	9.00 ± 0.00^{c}	13.33 ± 0.58^{c}	B
A10C	12.00 ± 0.00^{d}	6.67 ± 0.58^{ef}	6.67 ± 0.58^{d}	6.00 ± 0.00^{g}	A
A11	11.00 ± 0.00^{de}	6.00 ± 0.00^{f}	6.33 ± 0.58^{d}	7.00 ± 0.00^{efg}	A
A12	9.33 ± 0.58^{fg}	6.00 ± 0.00^{f}	6.00 ± 0.00^{d}	11.33 ± 0.58^{d}	A
A13	9.00 ± 0.00^{fgh}	6.00 ± 0.00^{f}	6.00 ± 0.00^{d}	7.00 ± 0.00^{efg}	A
A14	7.67 ± 0.58^{hij}	6.00 ± 0.00^{f}	6.00 ± 0.00^{d}	6.67 ± 0.58^{efg}	A
A15	12.00 ± 1.00^{d}	6.00 ± 0.00^{f}	10.33 ± 0.58^{c}	22.00 ± 1.00^{a}	B
A16	9.00 ± 0.00^{fgh}	6.00 ± 0.00^{f}	6.00 ± 0.00^{d}	6.33 ± 0.58^{fg}	A
A17A	10.00 ± 0.00^{ef}	6.00 ± 0.00^{f}	10.00 ± 0.00^{c}	6.67 ± 0.58^{efg}	A
DSW	6.00 ± 0.00^{k}	6.00 ± 0.00^{f}	6.00 ± 0.00^{d}	6.00 ± 0.00^{g}	C
Gen	10.00 ± 0.00^{ef}	10.67 ± 0.58^{b}	14.33 ± 0.58^{b}	12.33 ± 0.58^{cd}	A
Imi	19.67 ± 0.58^{b}	20.00 ± 0.00^{a}	22.00 ± 0.00^{a}	22.00 ± 0.00^{a}	A
	P<0.001	P<0.001	P<0.001	P<0.001	

a, b, c, d, e, f, g: means in the same Colum not sharing a common superscript are different (P<0.05)

Interaction: A-Test microorganism inhibited growth of the pathogen and produced a zone of inhibition, B-Test microorganism inhibited and overgrew on top of the pathogen colony (Nutrient and spatial competition) and C-No interaction; DSW: distilled sterile water

4.2.2 Identification of the Antagonistic Bacteria

4.2.2.1 Api 20 E test

The biochemical identification of the bacteria with significant *in vitro* activity was done using Api 20 E kit. Positive reaction was shown by color changes in Api test strips (Plate 4.8). Only *Providenccia rettgeri* was identified with the biochemical pattern obtained in comparison with the Api 20E catalogue. Other bacteria could not be identified as their result patterns did not match with any bacteria in the catalogue (Table 4.4).

Table 4.4: API 20E Test Strip reading, numbering and the corresponding identity from the API catalogue

Biochemical test results										
Isolates	ONPG	ADH	LDC	ODC	CIT	H2S	URE	TDA	IND	VP
A2	+	-	-	-	+	-	-	-	-	-
A3	+	+	-	+	+	+	+	+	-	-
A4A	+	+	-	+	+	-	-	-	-	-
A5	-	+	-	-	+	+	+	+	-	-
A15	-	+	-	+	+	+	+	+	-	-
Isolates	GEL	GLU	MAN	INO	SOR	RHA	SAC	MEL	AMY	ARA
A2	+	-	-	-	-	-	-	-	-	-
A3	+	+	+	+	+	+	+	+	+	+
A4A	+	+	-	+	+	-	+	+	+	+
A5	-	-	-	+	-	+	-	-	-	-
A15	+	+	-	+	-	+	-	-	-	-

Numerical number and corresponding bacteria		
Isolates	Numerical number	Identity
A2	1202000	not identified
A3	3736773	*Providencia rettgeri*
A4A	3306667	not identified
A5	0630210	not identified
A15	0736210	not identified

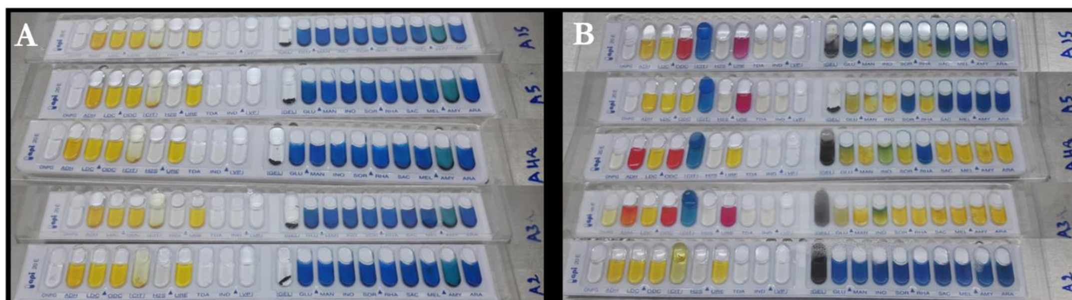

Plate 4. 8: API test strips, A-before incubation, b-After 24 hours of incubation

4.2.2.2 DNA sequences analysis based on 16S gene

Basic Local Alignment and Search Tool (BLAST) and phylogenetic analysis based on 16S rRNA gene sequences revealed that the potential antagonist with significant antagonistic activity belong to the species state in Table 4.5. The species were selected based on the lowest E. value and the highest query cover and identity percentage. Phylogenetic analysis grouped the bacteria into two cluster where *Bacillus cereus, Bacillus subtilis* and *Paenibacillus sp.* were found in the first cluster and the second cluster was constituted of *Providencia rettgeri* and *Providencia vermicola* (Figure 4.3).

Table 4.5: MegaBLAST results generated by the 16S DNA sequences of the BCAs with significant antagonistic activity against *R. solanacearum*

Isolates	Identified Specie	E Value	Query cover	Identity
A2	*Bacillus subtilis*	0.0	77%	99%
A3	*Providencia rettgeri*	0.0	99%	95%
A4A	*Bacillus cereus*	1e-120	95%	76%
A5	*Paenibacillus sp.*	0.0	73%	86%
A15	*Providencia vermicola*	0.0	98%	95%

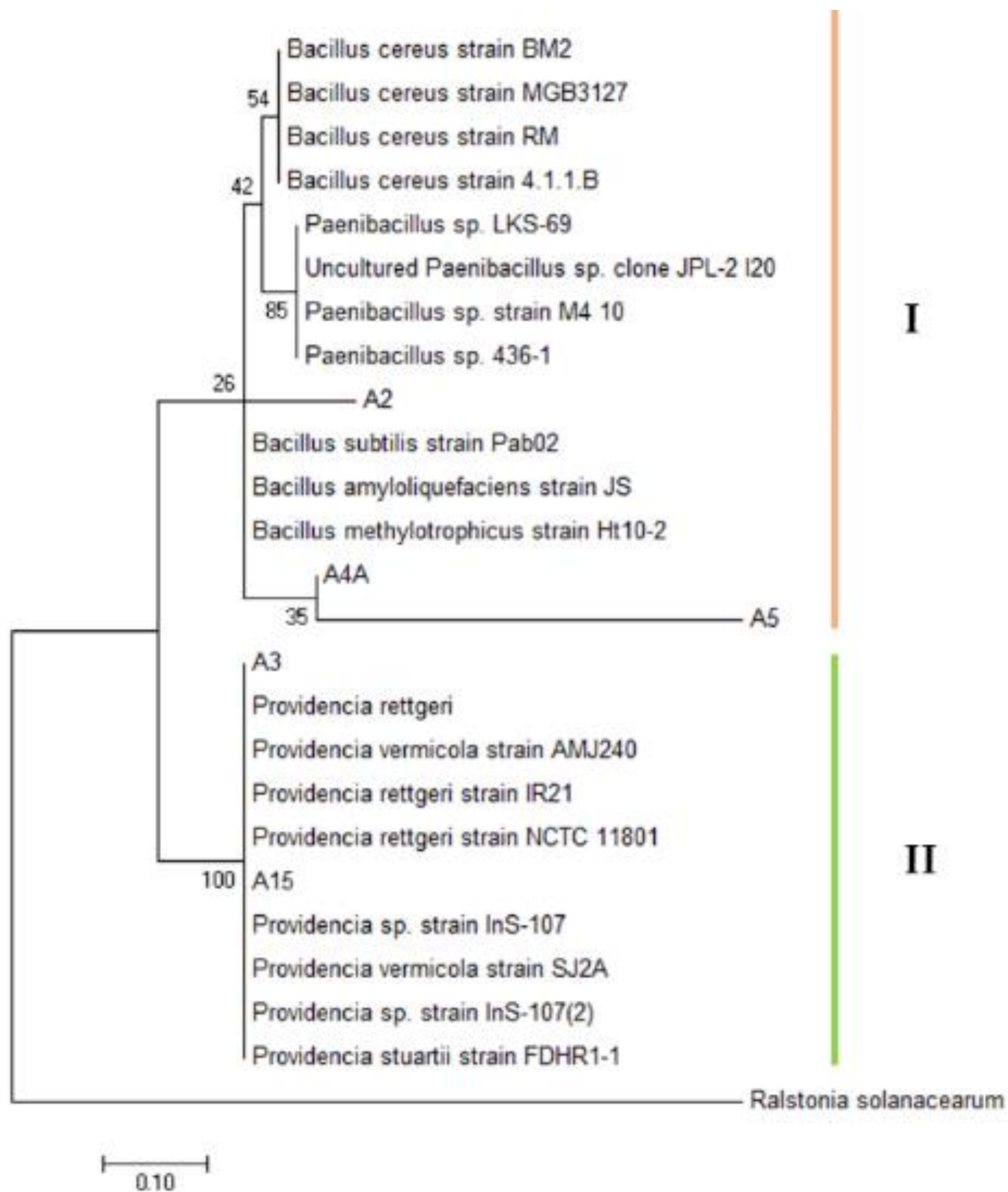

Figure 4. 3: Molecular Phylogenetic analysis of the evolutionary relationship between the identified potential antagonists. The phylogenetic tree was generated using the Maximum Likelihood algorithm in MEGA 7 following a multiple sequence alignment.

4.3 Evaluation of the effects of BCAs on plant responses under *Ralstonia solanacearum* infection

From the *in vitro* assays, 5 potential antagonists were selected for green house experiment based on the type of interaction displayed by the potential antagonists and their effects against all tested *R. solanacearum* strains. The *in vivo* assays conducted under controlled greenhouse condition were designed and tested beforehand to ensure a well-established and reliable pathosystem to allow the pathogen (Rs6) to grow and perform a complete infection of inoculated plants. The selected antagonists were evaluated for growth promotion and disease suppression and further for their efficacy (Plate 4.9).

4.3.1 Growth promotion effects by the BCAs on germination percentage

Growth promotion was observed on treated plants. The germination percentage recorded during the experiment showed, that the treated plants emerged from the soil at early age as compared with the negative control (Rs6). All tested BCAs showed effects on the germination of the plants (Figure 4.4). *Bacillus cereus* (A4A) showed similar results as compared with the positive treatment (H_2O), where the seeds were not treated but were sown in double sterile soil.

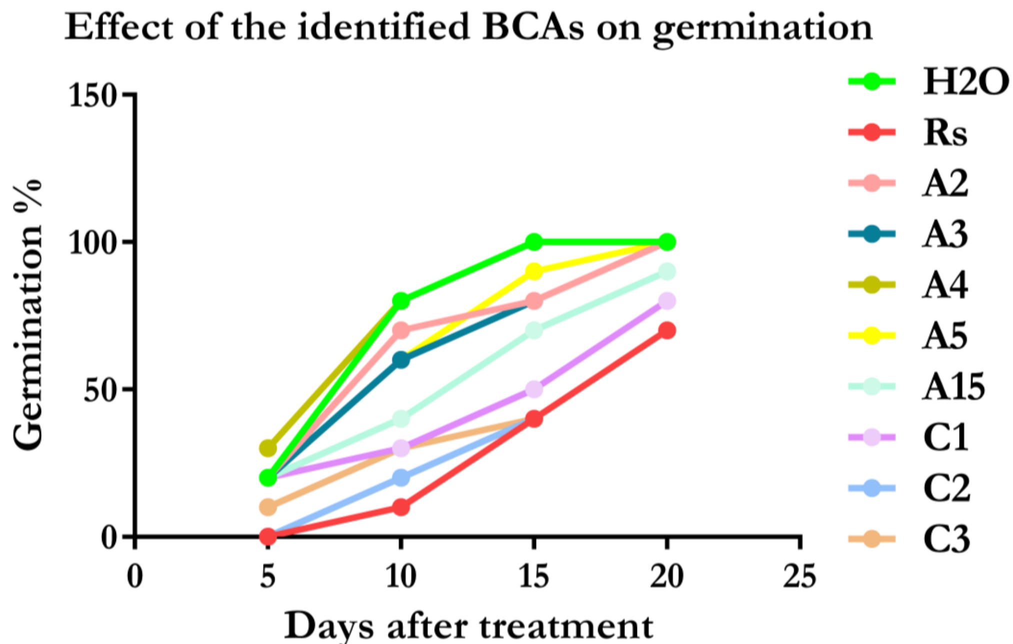

Figure 4.4: Evaluation of plant growth promotion by the identified biocontrol agents through germination percentage.

H_2O-positve control, Rs: negative control infected with Rs6, A2: seeds treated with *Bacillus subtilis,* A3: seeds treated with *Providencia rettgeri,* A4A: seeds treated with *Bacillus cereus,* A5: seeds treated with *Paenibacillus sp.,* C1: seeds treated with co-inoculation1*,* C2: seeds treated with co-inoculation 2*,* C3: seeds treated with co-inoculation 3.

4.3.2 Growth promotion effects by the BCAs on Plant height

The growth promotion activity of the tested bacteria was also evaluated by measurement of plant height at four time points. The results from statistical analysis (ANOVA) performed on 20 replicas for each tested biocontrol strain, distributed among two assays, confirmed the strong antagonistic activities of *Bacillus cereus, Bacillus subtilis, Paenibacillus sp, Providencia rettgeri* and *Providencia vermicola* against *R. solanacearum* on the sensitive potato variety "Shangi". These bacteria significantly promoted the plant growth as compared to the negative control (Rs) where the bacteria

were not applied. Interestingly, *Bacillus cereus* (A4A) promoted the growth the plant to similar level than that of positive control (Table 4.6).

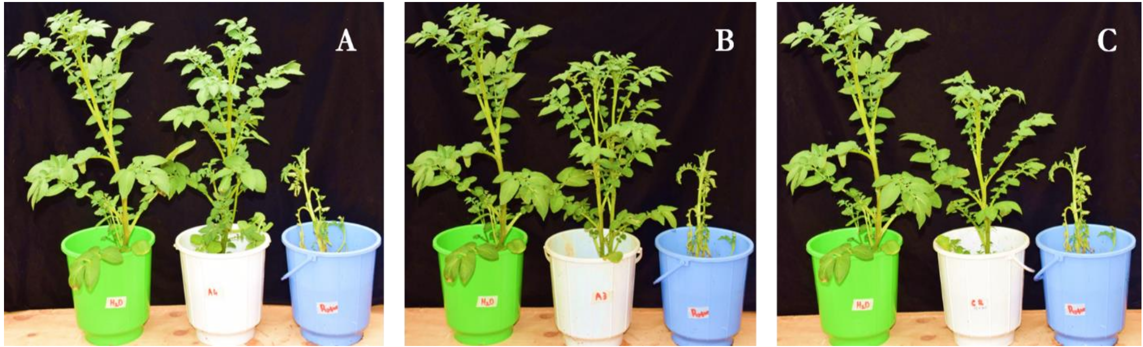

Plate 4. 9: Plant response to bacterial wilt under treatments and disease infection. Green pot positive control with water and no infection; white pot seeds treated with BCAs on an infected soil; blue pot negative control with infected soil but no BCAs. A-Single BCA (A4A) exhibiting competition for space and nutrient; B-Single BCA (A3) with inhibition characteristic and C- Co-inoculation 2 (C2).

Table 4.6: Effects of the identified BCAs on plant growth promotion (Plant height).

	Plant height Mean and standard error (cm)										
Plant age	**H_2O**	**Rs**	**A2**	**A3**	**A4A**	**A5**	**A15**	**C1**	**C2**	**C3**	**P-value**
3 weeks	32.00 ± 3.86a	13.71 ± 2.75d	26.20 ± 4.47b	25.10 ± 3.45b	29.90 ± 3.81a	26.00 ± 3.40b	23.78 ± 2.33b	16.00 ± 2.51cd	16.86 ± 4.34cd	18.86 ± 3.02c	<0.001***
4 weeks	44.80 ± 4.29 a	27.44 ± 3.94cd	35.70 ± 5.03b	36.80 ± 6.01b	44.50 ± 2.01a	37.90 ± 4.75ab	32.33 ± 8.40bc	30.44 ± 4.13bc	25.25 ± 3.77d	26.25 ± 7.32cd	<0.001***
5 weeks	50.10 ± 3.81a	32.22 ± 2.95d	42.00 ± 2.75b	41.70 ± 4.79b	47.40 ± 2.22a	43.40 ± 2.32b	37.22 ± 5.12c	34.44 ± 4.19d	32.25 ± 4.13cd	36.50 ± 4.75c	<0.001***
6 weeks	54.4 ± 4.50a	36.0 ± 6.76e	46.4 ± 4.60cd	46.3 ± 5.25cd	50.9 ± 4.20ab	49.4 ± 2.27bc	43.8 ± 6.00d	43.9 ± 4.23d	37.3 ± 4.88e	42.8 ± 6.16d	<0.001***

a, b, c, d, e: means in the same row not sharing a common superscript are significantly different (P<0.05)

H_2O-control 1, Rs-control 2 with the Pathogen only, A2-seeds treated with *Bacillus subtilis*, A3- seeds treated with *Providencia rettgeri*, A4A- seeds treated with *Bacillus cereus*, A5-seeds treated with *Paenibacillus sp.*, A15- seeds treated with *Providencia vermicola*, C1- seeds treated with co-inoculation of *Bacillus subtilis* and *Providencia rettgeri*, C2- seeds treated with co-inoculation of *Paenibacillus sp* and *Providencia vermicola,* C3- seeds treated with co-inoculation of *Bacillus subtilis* and *Providencia vermicola*

4.3.3 Disease suppression activity of the BCAs

In pot experiments, the tested BCAs showed some level of disease suppression as shown on Figure 4.5. Bacteria wilt symptoms such as wilting appeared at 17 days after treatments and were only observed on the negative control treatment with infected soil and not treated. The observed disease incidence of 20% was obtained in the negative control treatment. Characteristics of bacterial wilt were observed on diseased potato plants including yellowing, stem streaks and wilting. None on these were observed on treated plants (Plate 4.9).

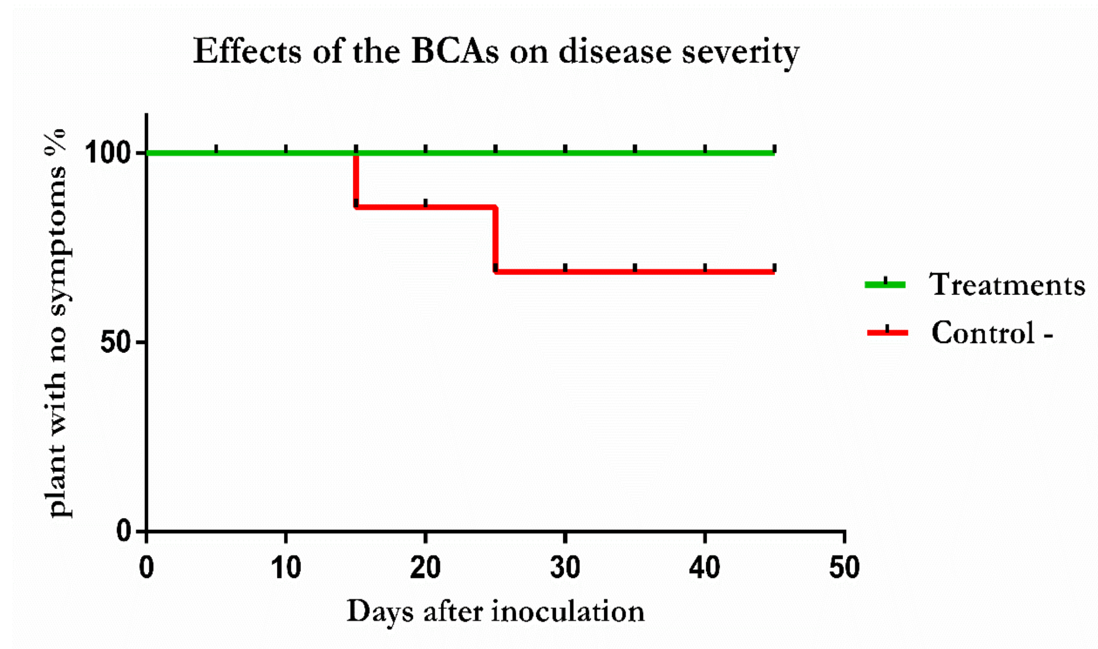

Figure 4.5: Evaluation of the identified BCAs potentials in disease suppression.

Treatments represent all plant treated with potential biocontrol agents including the combinations and the positive control where soil were not infected and no biocontrol was applied. Negative control in red showed disease progression on non-treated pots.

4.3.4 Efficacy of the BCAs

The biocontrol efficacy was also observed on plant growth under infection condition. The height of plants was measured at four ages of the plant, the data showed that the treated plants were taller compared to the non-treated. *Bacillus cereus* (A4A) had the highest effect on plant height compared to others individual and combined biocontrol agents (Table 4.6). Increase in the plant biomass were also observed with the treated plant as shown in Figure 4.6. Water content was also used to evaluate the efficacy of the identified biocontrol agents, as bacterial wilt restricts the absorption of water and mineral. The result showed that, all treated plant had highly significant water content as compared to the negative control (Rs). This showed the ability of the BCAs to reduce infection and their properties to promote nutrient uptake, which are crucial for productivity.

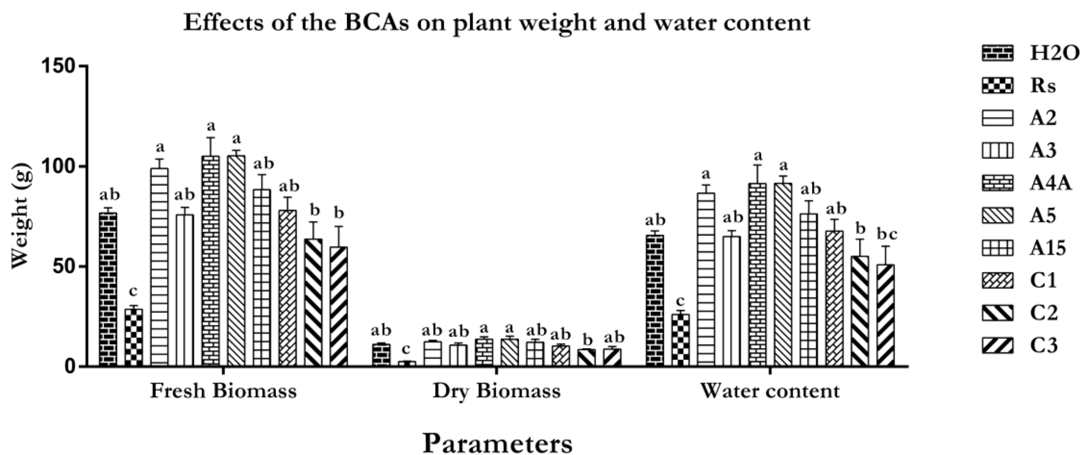

Figure 4.6: Evaluation of the identified biocontrol efficacy.
H_2O-control1, Rs-control2 with the Pathogen only, A2-seeds treated with *Bacillus subtilis*, A3- seeds treated with *Providencia rettgeri*, A4A- seeds treatedwith *Bacillus cereus*, A5-seeds treated with *Paenibacillus sp.*, A15- seeds treated with *Providencia vermicola*, C1- seeds treated with co-inoculation of *Bacillus subtilis* and *Providencia rettgeri*, C2- seeds treated with co-inoculation of *Paenibacillus sp* and *Providencia vermicola,* C3- seeds treated with co-inoculation of *Bacillus subtilis* and *Providencia vermicola.*

CHAPTER FIVE

DISCUSSION

5.1 Identification and characterization of Kenyan potato strains of *Ralstonia solanacearum*

5.1.1 *Ralstonia solanacearum* identification

Potato bacterial wilt caused by *R. solanacearum* is an economically important constraint to the world potato industry. Control methods are limited mainly due to the broad diversity and wide spread of its pathogen. Understanding the population structure and geographical distribution of this pathogen is an important starting point in the development of effective control strategies. In this study, *R. solanacearum* strains affecting potato cultivation in Nakuru county, Kenya were successfully isolated and characterized. The tetrazolium chloride (TZC) medium allowed successful isolation of *R. solanacearum* as revealed by the different pigmentation. Apart from selective isolation, this medium has also been implicated in differentiating virulent from avirulent strains with Kelman (1954) reporting that virulent colonies appear white with pink or light red centers and non-virulent colonies appear as small off-white colonies. On this medium, typical virulent bacterial colonies (fluidal, irregular in shape and white with pink or light red centers) were obtained as also report by Kinyua *et al.* (2014). The isolates were then confirmed to be *R. solanacearum* species, in line with the work by Fegan and Prior (2005) where they also reported the amplification of 281bp of the common region of the specie genome with 759/760 primers. The sequences analysis of endoglucanase (*egl)* gene which is directly related to

pathogenicity further confirmed the identity of the isolates as this was previously used for "sequevar" determination of *R. solanacearum* strains (Fegan and Prior, 2005). In addition, the evolutionary dynamics and the genetic relationship between RSSC have been revealed by phylogenetic and statistical analysis of housekeeping, virulence-related and pathogenicity-related genes including the *egl* gene (Castillo and Greenberg, 2007).

5.1.2 Phylotype determination

Potato strains of *R. solanacearum* from major growing areas in Nakuru County showed different levels of genetic diversity, as reflected by different numbers of phylotypes observed. The prevalence and distribution of the four phylotypes of *R. solanacearum* were variable throughout the three major potato-growing locations. The greatest diversity was observed in Kuresoi North where all the four phylotypes were identified. Three different phylotypes were identified in Njoro while the isolates from Mau Narok were only identified as phylotype I (Table 4.1). In addition, it was found that the most prevalent phylotype was phylotype I, which represented 50% of the total samples collected. The uneven distribution of this pathogen may be due to differences in temperature adaptation and competitive fitness advantage of *R. solanacearum* species (Huerta *et al.*, 2015). The higher prevalence of phylotype I strains may be explained by their higher virulence, since they have the capacity to infect a wide host range that include both herbaceous and woody plants (Hayward, 1994).

Interestingly, similar to the situation in Nakuru, phylotype I was also the most widespread and prevalent phylotype in other Africa countries where extensive RSSC surveys were conducted. For example, three phylotypes (I, II, and III) were identified in Cameroon and the Ivory Coast (Mahbou *et al.,* 2009; N'Guessan *et al.*, 2012), and two phylotypes (I and II) were identified in Ethiopia (Lemessa and Zeller, 2007). Phylotype I have also been previously reported in Madagascar, Kenya and South Africa (Wicker *et al.,* 2012; Ravelomanantsoa *et al.,* 2016; Carstensen *et al.*,2017). Phylotype I strain which infected potato were also reported from highland elevations in India and was known as the lineage with highest evolutionary potential because of its high ability of recombination, pattern of dissemination, large host range, and virulence plasticity (Sagar *et al.* 2014).

When retracing the complex evolutionary history of *R. solanacearum* using multilocus sequence analysis (MLSA), Wicker *et al.* (2012) reported that phylotype I was one of the ongoing diversifying subspecies. Moreover, Phylotype I is distributed worldwide (Hayward, 1991) compared to other phylotypes and it is reported to be highly recombinogenic (Coupat *et al.*, 2008; Wicker *et al.*, 2012). Therefore, this phylotype should be prioritized for monitoring of potato wilt disease in Nakuru county. This study represents the first report on phylotype IV in Kenya. The presence of this Asian phylotype in the country can be attributed to the importation of potato seeds with latent infection (Kaguongo *et al.*, 2010). Therefore, in terms of management programs, control of movement of potato seeds and plant materials from these areas to others areas need to be carefully regulated.

5.1.3 Biovars determination

The ability of the bacteria to utilize sugars and/or alcohols showed that the isolates found in Nakuru, were homogeneous. Since only biovar 3 was identified across the region. Nakuru county climate conditions may be suitable for biovar 3 outbreaks and the cultivation of potatoes throughout the year allows the persistence of this pathogen. Moreover, the high virulence of biovar 3 strains in the region can be justified by their wide host range and compatibility with a number of environmental factors favorable for disease appearance such as temperature, rainfall, soil type, inoculum potential, and other soil biological factors such as wilt complexes formed among nematodes (*Meloioigyne* spp.), Fungi (*Fusarium* spp.) and *R. solanacearum* (Shahbaz *et al.*, 2015). In light of these results, biovar 3 is considered the main and most destructive pathovar in the region; hence, special attention should be put in place to prevent the spread of this potato pathovar to other crops. The differentiation of biovars of *R. solanacearum* based on the utilization of carbohydrates was reported previously by Hayward (1964) and he also observed that biovar 3 oxidizes both disaccharides and hexose alcohols.

5.1.4 Induction of hypersensitive reaction

To evaluate the aggressiveness of the isolates, they were subjected to the hypersensitivity reaction test and all the isolates were able to induce the reaction. The hypersensitive reaction (HR) is a plant defense mechanism preventing the spread of pathogen infection to other parts of the plant. It is associated with plant resistance and characterized by a rapid and programmed plant cell death localized in the region surrounding an infection

(Nimchuk *et al.*, 2003). It has been reported that many but not all pathogenic bacteria can induce hypersensitivity necrosis in leaves of tobacco or other non-host plants (Yabuuchi *et al.*, 2006). Since only the phytopathogenic but not the saprophytic bacteria have this property, the tobacco test is an ideal experiment for quick detection of the pathogenicity of a selected bacterium (Poussier *et al.*, 2003). In *R. solanacearum* species, the hypersensitive reaction and pathogenicity or *hrp* genes control the induction of both disease development and hypersensitive reaction (Boucher *et al.*,1992; Boucher *et al.*, 2001). Therefore, *hrp* mutants are unable to induce symptoms in susceptible host plants (Boucher *et al.*, 1992). Moreover, *R. solanacearum hrp* mutants do not seem to be involved in the infection process and sometimes they show an impaired ability to multiply *in planta* (Trigalet and Demery, 1986). It was found that the isolates from Nakuru County, were able to induce hypersensitive reaction (HR) on tobacco plants with fine necrotic specs (Figure 4.6). Therefore, the twenty (20) isolates were confirmed to have the *hrp* genes involved in the infection process and this was in line with the pathogenicity assays where they induced wilt symptoms of potato plants.

5.1.5 Pathogenicity of the isolates

In the course of pathogenicity assays, the isolates were effectively able to cause disease symptoms to potato plants, although in different manner compared to the symptoms observed from the fields, which might reflect the phenotypic expression of less virulent strains (Huerta *et al.*, 2015). This can be also explained by the fact that, *R. solanacearum* pathogenicity is distinctly regulated in early or late stages of infection in response to environmental conditions, such as soil humidity, temperature and texture and bacterial

population densities (Schell, 2000; Hikichi *et al.*, 2007). In addition, high densities of the pathogen in plant tissues, increase expression of pathogenicity genes, repressed by low bacterial densities in non-host environments (Schell, 2000). This result indicated an association between virulence and geographical distribution of *R. solanacearum* strains, which have been also found in tomato growing areas in Trinidad (Huerta *et al.*, 2015). The results of this study are vital for the development of control methods which are economic and environmentally risk-free, in order to fully support the sustainable agriculture vision which is important to guarantee the next generation needs.

5.2 Potential antagonists against *R. solanacearum*

5.2.1 Screening for antagonists against *R. solanacearum*

The potato rhizosphere revealed the presence of a diverse and dense microbial community where various candidates of antagonistic bacteria which can not only affect the pathogen but also plant development (Brazelton *et al.*, 2008). Dual culture assay is a primary screening of bioactivity *in vitro* by providing information about interactions between a candidate antagonist and a pathogen on an agar plate (Oldenburg *et al*., 1996). This is assessed by measuring the inhibitory and competitive effects of microbes on pathogen growth (Maier *et al.*, 2009). In this study, the microbiome in the rhizosphere of an infected and non-infected soil samples were compared based on their color, shape, elevation and texture. Twenty (20) bacteria were selected and tested against four *R. solanacearum* strains. Most antagonists showed ‘A’ type of interaction with all the pathogens, where they inhibited the pathogen growth by antibiosis and other exhibited ‘B’ type of interaction where they inhibited and overgrew on the colonies of the pathogen showing

competition for space and nutrient (Table 4.3). Significant variation was observed in the susceptibility of the pathogen strains.

In general, five bacteria showed significant antagonistic activity against *R. solanacearum* these included: *Bacillus cereus, Bacillus subtilis, Paenibacillus sp, Providencia rettgeri* and *Providencia vermicola.* The maximum inhibition was observed with *Paenibacillus sp.* when tested against Rs35, *Bacillus subtilis* against Rs15, *Bacillus cereus* against Rs6 and *Providencia rettgeri* when tested against Rs1A. Significant variation among pathogen isolates in their susceptibility to inhibition, was also observed with biological control of potato scab (caused by *Streptomyces scabies*) using antibiotic-producing isolates of *Streptomyces* sp. and the variation was dependent on the isolates of the plant pathogen (Otto-Hanson *et al.*, 2013).

5.2.2 Potential mechanism used by the antagonists to inhibit the pathogen's growth

Bacteria from both *Bacillus* genera are known to be appropriate candidates to be used in a bio-control approach due to their predominance in various environments, resilience and survival ability, but also for the number of bio-active molecules they are potentially able to produce (Kloepper *et al.*, 2004; Haas and Défago, 2005; Raaijmakers *et al.*, 2010). A study by Ashwini and Srividya, (2014) on *B. subtilis* isolated from chilli rhizosphere reported appreciable levels of three mycolytic enzymes: chitinase, glucanase and cellulase which showed broad antagonism spectrum against potent bacterial and fungal phytopathogens. Additionally, *Bacillus* species have a unique ability to replicate rapidly, they are resistant to adverse environmental conditions and have broad spectrum of

biocontrol ability. Moreover, *B. subtilis* and *B. cereus* have been revealed to produce plipastatins to prevent phospholipase A2 which plays an important role in different cytological process in bacteria (Hirata *et al.*, 1980; Vadas *et al.*, 1989). Many strains of *B. subtilis* have been reported for their chitinolytic activities (Das *et al.*, 2010). Podile and Prakash (1996) reported the chitinolytic mechanism of *B. subtilis*. They showed that, *Bacillus* species can produce cell wall hydrolyzing enzymes (chitinases, glucanases and chitosanases) that efficiently hydrolyzed the cell wall of fungal pathogens.

The suppressive activity of the *Paenibacillus* spp. isolate tested in the dual culture assay in this study was associated with the production of an inhibition zone, indicating the production of secondary metabolites that have effects on the pathogen growth. *Paenibacillus* spp. are considered to be promising biocontrol agents of a number of plant diseases because of their wide host plant range, and ability to form endospores and produce various antibiotics (Weid *et al.*, 2003; Timmusk *et al.*, 2005). For example, *Paenibacillus polymyxa* has been previously demonstrated to have strong antagonistic activity against some important plant pathogenic bacteria and fungi (Pichard and Thouvenot, 1999; Beatty and Jensen, 2002; Timmusk, 2003;Timmusk *et al.*, 2009). It is also active against plant pathogenic nematodes such as the root-knot nematode *Meloidogyne incognita* (Khan *et al.*, 2008). Additionally, antibiosis such as competition, root colonization and induced systemic resistance, have been associated with *Paenibacillus spp* and are proposed as the possible modes of action of this bacteria (Haggag and Timmusk, 2008; Mei *et al.*, 2014; Grady *et al.*, 2016).

The observed antagonistic characteristic of *Providencia rettgeri* against *R. solanacearum,* can be related to its capacity to produce acid from D-Adonitol, D-Arabitol, Erythritol, and other metabolic precursors, and reduction of nitrate to nitrite. It has the ability to hydrolyze penicillin G to phenyl acetic acid and 6-aminopenicillanic acid (6-APA) (Ševo *et al.*, 2002). Also, *P. rettgeri* possesses genes coding for a unique penicillin G amidase (PAC), an enzyme heavily used in the industrial synthesis of penicillin (Ljubijankić *et al.*, 1999; Ševo *et al.*, 2002). By producing these enzymes *P. rettgeri* has the ability to inhibit the growth of other bacteria hence protecting the host from infection. The ability of *P. rettgeri* to convert ammonia to nitrogen gas in aerobic conditions have been also revealed (Zhao *et al.*, 2010).

5.3 Efficiency of Biological control agents (BCAs) against *R. solanacearum*

The dual culture assay in screening of rhizobacteria isolates against various *R. solanacearum* strains, provided an insight into the level and range of their bioactivity. However, *in vitro* antagonisms are not always a good indicator of biocontrol activity *in vivo* and the need to evaluate the activity of biological control agents as part of the plant–pathogen interaction is widely recognized (Wulff *et al.*, 2002). This is because, many parameters such as age, temperature, pH, and nutrient composition of the media can affect metabolite production by an organism and can result in different bioactivity outcomes in an *in vitro* study. Moreover, the inhibition zone formation in a dual culture assay is typically due to antibiosis activity which is mediated by metabolites produced by the antagonist due to medium composition (Raaijmakers *et al.*, 2002).

5.3.1 Growth promotion activity of the tested BCAs

In the pot experiment, some treatments indicated significant increase of plant growth parameters and reduction of disease incidence while others did not. The treatment of the seed with the identified antagonists, prevented the development of wilt symptoms and significantly promoted plant growth relative to the non-treated treatment. Kamil *et al.*, (2007) also reported that sunflower seeds coated with *B. licheniformis* induced high reduction in percentage of infection of *Rhizoctonia solani* damping-off (from 60 to 25 %) as compared to the pathogen alone. The increase of plant growth parameters by seed treatment with potential biocontrol agents also is an observation made in previous studies by Jung *et al.*, (2002) and Khan *et al.,* (2008). The reason for the increase may be attributed to synthesis of plant hormones such as cytokinin and auxin (Loper and Schroth, 1986); and facilitation of nutrient availability through nitrogen and phosphate metabolism (Eastman et *al.*, 2014). Rhizobacteria (in particular PGPR) also known to act as biofertilizer can promote plant growth by breaking down soil complexes and suppression of the deleterious effects of abiotic and biotic stresses (Chauhan *et al.*, 2015). Application of different biocontrol agents such as *P. fluorescens, P. lilacinum* and *P. guilliermondii* has been also found to strengthen the growth of plants via production of natural growth hormones and supplying many nutritional elements by breaking down soil complex, induction of systemic resistance in plants and lethal effect on nematodes (Hashem and Abo-Elyousr, 2011).

The reason why rhizobacteria release hydrolase, is to utilize the nutrient stored in substrate by converting it from unavailable form to available form. This also promote plant growth by facilitating nutrient uptake. The application of chemical nitrogen and phosphorus fertilizer could be reduced by inoculating fields with phosphorus-solubilizing microorganisms, such as *Paenibacillus*, *Bacillus* and *Providencia* species (Sharma *et al.*, 2013; Xie *et al.*, 2016; Shafi *et al.*, 2017). Plant-associated species of *Paenibacillus* can directly influence plant growth by solubilizing inaccessible phosphorous into form that can be taken up by plant roots, and some species can also fix atmospheric nitrogen (Weselowski *et al*., 2016). Nain *et al.* (2012) revealed that *Bacillus* species had many valuable features including acetyl-CoA carboxylase (ACC) deamination activity, phosphate solubilization ability, fungicidal, IAA production and ammonia production activity. The studied strains significantly enhanced seed germination, fresh and dry weight, leaf area, root and shoot length and also seed, pods and grain yield compared with untreated control.

Auxins and gibberellic acid (GA3) are hormones that are crucial regulators of gene expression and development throughout a plant's life, participating in cell division, elongation, fruit development and senescence (Srivastava, 2002). These plant growth-promoting hormones also enhance the nutrients uptake ability of plants and help the plant to defend against various biotic and abiotic stresses (Vessey, 2003; Ghanashyam and Jain, 2009). Although plants are able to produce their own phytohormones, they can also utilize foreign sources produced by other organisms including *Paenibacillus, Bacillus* species

and most other plant associated bacteria (Duca *et al.*, 2014; Weselowski *et al.*, 2016). Apart of the antagonistic mechanism of *Bacillus* species, these microbes also have an important role in plant growth promotion by enhancing the biosynthesis of plant hormones gibberellic acid (GA3) and indole-3 acetic acid (IAA) that have a close relation with plant nutrient availability (Presti *et al.*, 2015). Yokoyama and his coworkers, (2004) reported that cultural filtrate of *B. subtilis* strain FZB-G has the ability to trigger phytohormones precursor which plays an important role in signal transduction and activation of defense gene that results in the production of defense-related compounds.

Bacillus species have also been reported to be attractive biological control agents due to their ability to produce hard, resistant endospores and antibiotics which control a broad range of plant pathogens (Cavaglieri *et al.*, 2005). It has been shown by Chowdappa *et al.*, (2013), that tomato seeds treated with spore or cell suspensions of *B. subtilis* OTPB1 considerably enhanced the shoot and root growth, seedling vigor and leaf area of the tomato plant. Higher level of plant growth-promoting hormones (GA3 and IAA) and defense-related enzymes such as peroxidase (PO), polyphenol oxidase (PPO) and superoxide dismutase were detected in treated plants compared with non-treated plants. Furthermore, *B. subtilis* and *B. cereus* can synthesize many potent amphiphilic and surfactant lipopeptides comprising bacillomycins, iturins and mycosubtilin which restrict the pathogen density in the soil (Vadas *et al.*, 1989; Gong *et al.*, 2015).

It has been reported that the plant-growth promoting feature of *Paenibacillus* species comes from their numerous biocontrol capabilities. By producing biocidal substances,

they can induce the plant resistance mechanisms and neutralize a diverse variety of phytopathogens and insect herbivores (Grady *et al.*, 2016). Volatile compounds produced by *Bacillus* species also play an important role in plant growth promotion and activation of plant defense mechanism by triggering the ISR in plants (Compant *et al.*, 2005). *B. subtilis* GB03 and *B. amyloliquefaciens* IN937a have been shown to release volatile compounds including 2,3-butanediol and 3-hydroxy-2butanone (acetoin) abundantly which are related to plant growth (Ryu *et al.*, 2003).

5.3.2 BCAs' ability in suppression of bacterial-wilt disease associated symptoms

Beneficial nonpathogenic microorganisms can induce systemic acquired resistance (SAR), a phenomenon where a non-infected plant acquires an ability to resist the subsequent attack. This is seen when colonization of roots by some nonpathogenic bacteria protects the above-ground plant parts from attack of various pathogenic organisms, which is known as induced systemic resistance (ISR) (Van Loon *et al*, 1998). Many beneficial rhizobacteria and root-associated mutualists, including members of *Bacillus, Paenibacillus,* and *providencia* species can trigger ISR (Van *et al.*, 2008) when present in high enough population densities. Previous studies have investigated the elicitation abilities of *B. subtilis, B. cereus* and other *Bacillus* strains to induce a broad spectrum of resistance against various bacterial and fungal phytopathogens (Kloepper *et al.*, 2004; Akram *et al.*, 2013).

In this study, the tested BCAs significantly reduce the disease incidence as compared to the non-treated plant. Disease suppression mechanisms have by reported by Wang *et al.*,

(2014), who concluded that treatment with *B. cereus* (strain AR156) enhances defense-related activities such as phenylalanine ammonia-lyase (PAL), chitinase, β-1,3-glucanase, peroxidase (PO), polyphenol oxidase (PPO), and stimulated amassing of Hydrogen peroxide (H_2O_2). β-1,3-Glucanases and chitinases actively contributed in defense of plant against a variety of plant pathogens while PO and PAL actively involved in phenylpropanoid breakdown in plant tissues (Vidhyasekaran *et al.*, 2001). Park and his coworkers (2007) evaluated five strains of *Bacillus* species against *R. solanacearum* and the studied strains were proved to be effective. They also showed that reduction of disease was not due to direct antagonism but as a result of elicitation of host plant resistance genes. *Paenibacillus* have also been reported to control phytopathogens by triggering induced systemic resistance (ISR) and/or producing a variety of biocidal substances (Grady *et al.*, 2016).

Interspecies competition causes reduction in growth, productiveness and other activities of the competing organisms. Biological control can be observed when pathogenic and nonpathogenic organisms compete for space and nutrients around the host plant (Pal and Gardener, 2006). Plant and soil surface have a limited amount of nutrients. Therefore, for effective colonization of plant surface, pathogenic and nonpathogenic microbes must compete to fulfil their nutrient requirements. It is difficult to demonstrate the direct role of competition in plant disease suppression but there are numerous studies that have proved that pathogenic and nonpathogenic microbes compete for nutrients and space and thus play a very crucial role in pathogen severity and incidence reduction. In general, soil-

borne plant pathogens such as *R. solanacearum* are more exposed to competition because they only infect through infiltration of plant tissues in comparison with those pathogenic diseases whose causal organisms can directly germinate on plant surface. Non-pathogenic microbes can protect plants by rapid colonization and exhausting the developmental resources thus making them unavailable for pathogenic microbes (Shafi *et al.*, 2017). Biocontrol can also be established by competing for essential micronutrients, for example iron in rhizosphere, is extremely limited and its availability is highly dependent on the type of soil (Shafi *et al.*, 2017).

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

5.1 CONCLUSIONS

In this study, *R. solanacearum* strains affecting potato production in Nakuru County were successfully isolated and characterized. The findings show that the distribution of the pathogen in the region is not homogeneous. The strains obtained belonged to biovar III with different phylotypes, virulence and aggressivity. This study showed the wealth of naturally occurring indigenous rhizobacteria associated with potato agro-systems which have the potential to be used as biological control agents. The interaction between the identified potential antagonists and the pathogen was significantly different depending on the *R. solanacearum* strains. The result from antagonist screening showed that: *Bacillus cereus, Bacillus subtilis, Paenibacillus sp, Providencia rettgeri* and *Providencia vermicola* were potential antagonists which can be implemented in the development of bacteria-based biocontrol strategies. These BCAs significantly promoted plant growth under disease infection, as they lead to early seeds germination, increased plant height, fresh and dry biomass. They also reduced the disease severity. Consider the increased concern on the use of chemicals, the outcomes of this study showed that the identified BCAs could be used to increase potato productivity under infestation without affecting the stability of the environment and the quality of the production.

5.2 RECOMMENDATIONS

1. Based on the high heterogeneity of the pathogen in Nakuru, special management practices such as quarantine methods need to be put in place to avoid the spread of the disease.
2. Control strategies such as grafting and breeding for resistance to bacterial wilt disease should consider the diversity of the pathogen.
3. Hypersensitivity reaction test can be used for rapid investigation of *R. solanacearum* virulence, before further confirmation on potato plants.
4. The identified bacteria in this study, should be considered in formulation of BCAs against *R. solanacearum*.

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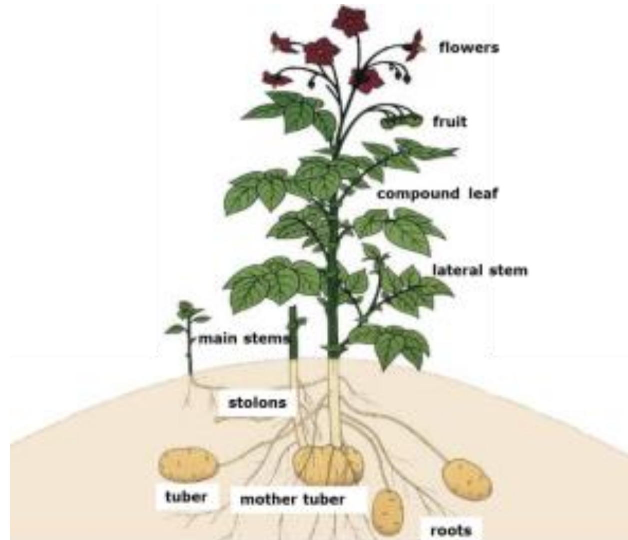
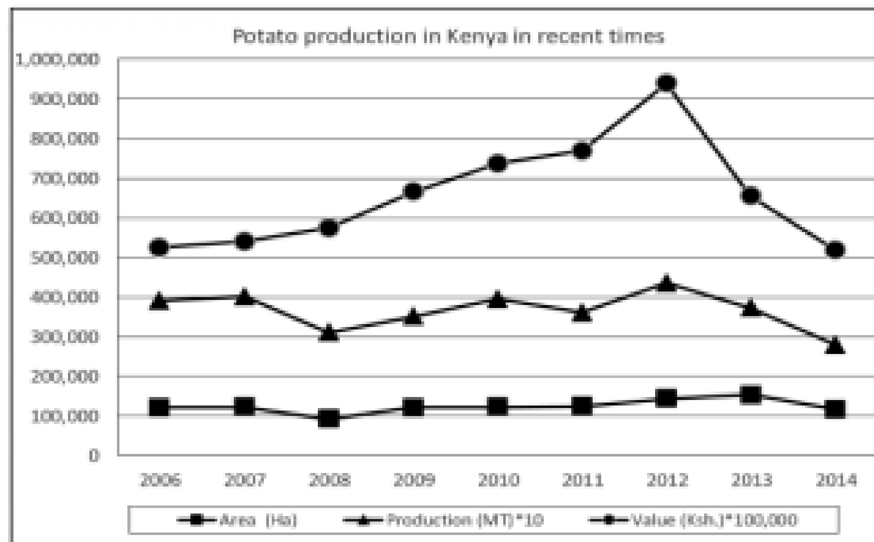
APPENDICES

Appendix 2. 1:Potato plant structure



Appendix 2. 2:Trends in potato production in Kenya



Appendix 2. 3:Diversity of microbial antagonists and mechanisms providing biological control of potato pathogens

Diseases (Pathogen)	Biocontrol agents	Mechanisms involved (hypothesized or demonstrated)	Biocontrol assays	References
Bacterial diseases				
Bacterial wilt/brown rot (*Ralstonia solanacearum*)	*Bacillus polymyxa*	Preemptive colonization	Soil microcosm	Aspiras & de la Cruz (1986)
	Bacillus subtilis and *Paenibacillus macerans*	Induced systemic Resistance	*In vitro* screening and soil microcosm	Naser *et al*. (2008)
	Fluorescent pseudomonads	Induced systemic resistance, preemptive Colonization	Tuber assay, soil microcosm and field trial	Kempe & Sequiera (1983), Aspiras & de la Cruz (1986), Naser *et al*. (2008)
	Nonpathogenic *Ralstonia solanacearum*	Induced systemic Resistance	Tuber assay and field trial	Kempe & Sequiera (1983)
Blackleg and soft-rot (*Dickeya* spp./ *Pectobacterium* spp.)	*Bacillus subtilis*	Antibiosis	*In vitro* screening and tuber assay	Sharga & Lyon (1998)
	Fluorescent pseudomonads	Antibiosis, iron Competition	*In vitro* screening, tuber assay, soil microcosm and field trial	Kloepper (1983), Xu & Gross (1986), Rhodes & Logan (1987), Axelrood *et al*. (1988), Cronin *et al*. (1997), Kastelein *et al*. (1999)
	Pectobacterium spp.	Competition	Tuber assay	Costa & Loper (1994)
Ring rot (*Clavibacter Michiganensis* ssp. *sepedonicus*.)	Fluorescent pseudomonads	Antibiosis, preemptive Colonization	*In vitro* screening and soil microcosm	De la Cruz *et al*. (1992)
Scab (*Streptomyces* spp., mainly *Streptomyces scabiei*)	*Streptomyces* bacteriophage	Cell lysis	Tuber assay	McKenna *et al*. (2001)
	Fluorescent pseudomonads	Not determined	Field trial	Nanri *et al*. (1992)
	Nonpathogenic *Streptomyces*	Antibiosis, competition	*In vitro* screening, tuber assay, soil microcosm and field trial	Liu *et al*. (1996), Neeno-Eckwall *et al*. (2001) Hiltunen *et al*. (2009)
Fungal diseases				
Fusarium dry rot (*Fusarium* spp., mainly *Fusarium roseum* var. *sambucinum* and some *Fusarium oxysporum*)	*Bacillus* spp.	Antagonism	*In vitro* screening and tuber assay	Sadfi *et al*. (2002), Kotan *et al*. (2009)
	Enterobacter cloacae	Antagonism	Tuber assay and storage	Schisler *et al*. (2000)
	Fluorescent pseudomonads	Antagonism	Tuber assay and storage	Schisler *et al*. (2000)
Late blight/Mildew *(Phytophtora infestans)*	Hyphal wall components	Induced systemic Resistance	Soil microcosm	Doke *et al*. (2008)
	Pseudomonas koreensis or its biosurfactant	Antagonism	Greenhouse trial (leaf assay)	Hultberg *et al*. (2010)
	Pseudomonas putida	Antibiosis, competition	Soil microcosm	Andreote *et al*. (2009)
	Phytophthora cryptogea	Induced systemic Resistance	Soil microcosm	Stro¨ mberg & Brishammar (1991)
Rhizoctonia black scurf and stem canker (*Rhizoctonia solani*)	Binucleate *Rhizoctonia*	Competition	Soil microcosm and field trial	Escande & Echandi (1991)
	Rhizoctonia zeae	Competition	Soil microcosm	Brewer & Larkin (2005)
	Verticillium bigutattum	Mycoparasitism	Soil microcosm and field trial	Van den Boogert & Velvis (1992)
	Trichoderma harzianum	Competition, induced systemic resistance	Tuber assay, sand and plantlet microcosms	Wilson *et al*. (2008), Gallou *et al*. (2009)
Verticillium wilt (*Verticillium dahliae)*	*Clonostachys rosea*	Mycoparasitism	Soil microcosm	Keinath *et al*. (1991)
	Pseudomonas fluorescens	Antagonism	Soil microcosm	Leben *et al*. (1987)
	Talaromyces flavum	Mycoparasitism	Soil microcosm	Nagtzaam & Bollen (1997)

Appendix 3. 1:Media for isolation and cultivation of *R. solanacearum*

- **TZC medium**

Tetrazolium chloride (TZC) agar medium (Kelman, 1954)	
Bacto-Peptone (Difco)	10g
Glucose	2.5g
Casamino acids (Difco)	1g
Bacto-Agar (Difco)	8g
Distilled water	1000ml

Autoclave the above ingredients at 121˚C for 15 minutes and cool to about 40-45˚C. Then add 5ml of filter sterilized 1% TZC solution.

- **CPG medium**

CPG Medium for cultivation of *R. solanacearum*	
Casamino acids (Difco)	1.0g
Bacto-Peptone (Difco)	10.0g
Glucose	10.0g
Bacto-Agar (Difco)	18.0g
Distilled water	1000ml

Autoclave the above ingredients at 121˚C for 15 minutes and cool to about 40-45˚C before pouring into sterile Petri dishes.

- **MKM medium**

Modified Kelman medium (French *et al.*, 1995)	
Bacto-Peptone (Difco)	10.0g
Casamino acids (Difco)	1.0g
Sucrose	5.0g
Distilled water	1000ml

Sterilize by autoclaving at 121˚C for 15 minutes and cool to about 40-45˚C before pouring into sterile 10ml tubes.

- **Basal medium for Biovars determination**

Basal Medium preparation	
$NH_4H_2PO_4$	1.0g
KCl	0.2g
$MgSO_4.7H_2O$	0.2g
Bacto-Peptone (Difco)	1.0g
Bromothymol blue	80.0 mg
Distilled water	1000ml

Adjust the pH to 7.0 with 40% NaOH. Then add 3.0g Bacto-Agar (Difco). Sterilize by autoclaving at 121˚C for 15 minutes.

Appendix 3. 2:Media for isolation and Screening for potential antagonists

- TSA media

Tryptone Soy Agar medium preparation (Broad spectrum medium)	
Tryptone	15g
Soya peptone	5g
Sodium chloride	5g
Agar	15g
Distilled water	1000ml

Autoclave the above ingredients at 121˚C for 15 minutes. Final pH 7.3

- Nutrient Agar
- Nutrient Broth

Appendix 3. 3: Formulae

1- Inhibition percentage

$$I= \frac{(C - T)}{C} \times 100$$

I = Percent inhibition, C = Radial growth of the pathogen in control, T = Radial growth of pathogen in treatment

2- Disease incidence (DI)

$$DI=\frac{\text{No. of infected plants}}{\text{Total no.of assessed plants}} \text{ x } 100$$

3-Biocontrol efficacy (BE)

$$BE= \left(\frac{\text{DI control} - \text{DI of treated}}{\text{DI control}}\right) \text{ X } 100$$

4-Growth promotion efficacy (GPE)

$$GPE= \left(\frac{\text{GP in control} - \text{GP in treated}}{\text{GP in control}}\right) \text{ X } 100$$

Appendix 3. 4:DNA sequences

1- Endoglucanase gene sequences from *R. solanacearum* isolates

>Rs1A

accctcatac tacgcacggc ggccgctacc gacaccacga ccctgaagac ggccgccacc 60
acctcgattt cgccgttgtg gctcaccatc gccaaggaca gcgcggcgtt cacggtgagc 120
ggcacgcgca cggtgcgcta tggcgccggc agcgcgtggg tggcgaagag cgtgtccggc 180
acaggccagt gcaccgccgc cttctttggc aaggatccgg cggccggtgt cgccaaggta 240
tgccaggtgg cgcagggcac gggcaccctg ctgtggcgcg gcgtcagcct ggccggcgcc 300
gagttcgggg agggcagcct gccttcacca tgggcaccta cgggagcaac tacatctatc 360
cgtccgccga cagcgcgacc tactacaaga acaagggcat gaacctcgtg cgcctgccgt 420
tccgctggga gcggctgcag cccacgctca accaggcgct cgacgcgaac gagctgtcgc 480
gcctgaccgg gttcgtcaac gccgtgacgg cggccggcca gacggtgctg ctcgatccgc 540
accagcgaac tacgcgcgct actacggcaa cgtgatcggc tcgagcgcgg tgcccaacgt 600
acgccgattt ctggcggcgc gtggccaccc agttcagggg caatgcccgc gtcctcttcg 660
ggctgatgaa cgagcccaat tcgatgccga ccgacgaact aagcagtggc 710

>Rs6

accctcaggc gctgcacggc ggccgccacc gacaccacga ccctgaagac ggccgccacc 60
acctcgatct cgccgttgtg gctcaccgtc gccaaggaca gcgcggcgtt cacggtgagc 120
ggcacgcgca cggtgcgcta tggcgccggc agcgcgtggg tggcgaagag catgtccggc 180
acaggccagt gcaccgccgc cttcttcggt aaggatccgg cggccggtgt cgccaaggta 240
tgccaggtgg cgcagggcac gggcaccctg ctgtggcgcg gcgtcagcct ggccggcgcc 300
gagttcgggg agggcagccc ggcgcaggct gccgggcacc tacgggagca actacatcta 360
tccgtccgcc gacagcgcga cctactacaa gaacaagggc atgaacctcg tgcgcctgcc 420
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gcacaactac gcgcgctact acggcaacgt gatcggctcg agcgcggtgc ccaacagcgc 600
gtacgccgat ttctggccag gcgcacgccg gcgcgtggcc acccagttca agggcaatgc 660
ccgcgtcatc ttcgggctga tgaacgagcc caattcgatg ccgaccgagc agtggc 716

>Rs15

gcttgcggct gcggcggcgg tgacggcgat accagtttga gcaccgcgag cgcttccggc 60
accgacacca cgaccctgaa gacggccgcc accaccccga tctcgccgtt gtggctcacc 120
atcgccaagg acagcgcggc gtttacggtg agcggcacgc gcacggtgcg ctatggcgcc 180
ggcagcacgt gggtgggaaa gagcatgtcc ggcacaggcc agtgcaccgc cgccttcttc 240
ggcaaggatc cggcggccgg tgtcgccaag gtctgccagg tggcgcaggg cacgggcacc 300
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acctacggca ccaactacat ctagcaggtc cgtccgccga cagcgcgacg tactacaaga 420
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accaggcgtt cgatccaaac gaactgttgc gcctgaccgg gtttgtcgac gccgtgacgg 540
cggccggcca gacggtgctg ctcgatccgc acaactacgc gcgctattac ggcaacgtga 600
tcggctcggg cgcggtgccc aacaccgcgt acgcggattt ctggcggcgc ctggcgaccc 660
agttcaaggg taatgcccgc gtcatcttcg ggctgatgaa cgagcccaat tcgatgccga 720
ccgagcaatg gctgtccggt gccaacgccg cgctggc 757

>Rs35

accctcagca cgggcacgtg gcggtcgcta ccgacaccac gaccctgaag acggccgcca 60
ccacctcgat ctcgccgttg tggctcacca tcgccaagga cagcgcggcg ttcacggtga 120
gcggcacgcg cacggtgcgc tatggcgccg gcagcgcgtg ggtggcgaag agcatgtccg 180
gcacaggcca gtgcaccgcc gccttctttg gcaaggatcc ggcggccggt gtcgccaagg 240
tatgccaagg cttcacgcgg taggtggcgc agggcacggg caccctgctg tggcgcggcg 300
tcagcctggc cggcgccgag ttcggggagg gcagcctgcc gggcacctac gggagcaact 360
acatctatcc gtccgccgac agcgcgacct actacaagaa caagggcatg aacctcgtgc 420
gcctgccgtt ccgctgggag cggctgcagc ccacgctcaa ccaggcgctc gacgcgaacg 480
agctgtcgcg cctgaccggg ttcgtcaacg ccgtgacggc ggccggccag acggtgctgc 540
tcgatccgca caactacgcg cgctactacg gcaacgtgat cggctcgagc gcggtgccca 600
acagcgcgta cgccgatttc tggcggcgcg tggccaccca gttcaagggc aatgcccgcg 660
tcctcttcgg gctgatgaac gagcccaatt cgatgccgac cgagccccag ccatcagtgg 720
c 721

16S DNA sequences from potential biological control agents

>A2

ccaccatgca gtcgagcggt aacaggggaa gcttgcttcc gctgacgagc ggcggacggg 60
tgagtaatgt tggggatctg cccgatagag gggataact actggaacgg tggctaatac 120
cgcataatct cttaggagca aagcagggga acttcggtcc ttgcgctatc ggatgaaccc 180
tatgggatta gctagtggtg gggtatggct cacctaggcg acgatcccta gctggtctga 240
gaggatgatc accacactgg gactgagaca cggcccaact cctacgggag gcgcagtggg 300
gaatattgca caatgggcgc aagcctgatg cagccatgcc gcgtgtatga agaaggccct 360
aggttgtaaa gtactttcat cgggaggaag gcgttgatgc taatatcatc cgattgacgt 420
taccgacaga agaagcaccg gctactccgg ccacagccgc ggtaatacgg agggtgcagc 480

gttaatcgaa ttactgggcg taaagcgcac gcaggcggtt gattaagtag atgtgaaatc 540
cccgggctta acctgggaat ggcatctaag actggtcagc tagagtcttg tgaggggggt 600
agaattccat gtgtagcggt gaaatgcgta agatgtggag gaataccggt ggcgaaggcg 660
gccccctgga caaagactga cgctcagtgc gaaagcgtgg ggagcaaaca ggattagata 720
ccctggtagt ccacgctgta aacgatgtcg atttggaggt tgtgcccttg aggcgtggct 780
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acctctcttg catcagagat ttacagagat gctttggtgc cttcgggaac tctgagacag 960
gtgctgcatg gctgtcgtca gctcggttgt gaaatgttgg gtttaattcc cgcacggagc 1020
ccaacctatc ctgtgtccgc gatcggtcgg aactcaagga agactgcggc gaataaaccc 1080
cgtgaggaga aagat 1095

>A3

ccaccatgca gtcgagcggt aacaggggaa gcttgcttcc gctgacgagc ggcggacggg 60
tgagtaatgt tggggatctg cccgatagag gggataact actggaacgg tggctaatac 120
cgcataatct cttaggagca aagcagggga acttcggtcc ttgcgctatc ggatgaaccc 180
tatgggatta gctagtggtg gggtatggct cacctaggcg acgatcccta gctggtctga 240
gaggatgatc accacactgg gactgagaca cggcccaact cctacgggag gcgcagtggg 300
gaatattgca caatgggcgc aagcctgatg cagccatgcc gcgtgtatga agaaggccct 360
aggttgtaaa gtactttcat cgggaggaag gcgttgatgc taatatcatc cgattgacgt 420
taccgacaga agaagcaccg gctactccgg ccacagccgc ggtaatacgg agggtgcagc 480
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agaattccat gtgtagcggt gaaatgcgta agatgtggag gaataccggt ggcgaaggcg 660
gccccctgga caaagactga cgctcagtgc gaaagcgtgg ggagcaaaca ggattagata 720
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cgtgaggaga aagat 1095

>A4A

```
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gagtgacacg tgttgacatg cgccttactg gggtactccg ggaaaaaggg taccttatac   120
ctaaaattgt gaactgcggg ggcgaaatct gagggagctt cggcggtcac ttatgggtgg   180
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>A5

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>A15

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