PROFILING AND PATHOGENICITY OF *Ralstonia* solanacearum DISEASE OF TOMATO AND IT'S CONTROL USING *Senna didymobotrya* AND *Moringa oleifera* PLANT EXTRACTS IN MASENO (KENYA)

BY

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DECLARATION

This thesis is my own original work and has not been presented for a degree in Maseno Universityor any other University. All sources of information have been specifically acknowledged by means of references.

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ABSTRACT

Tomato (*Lycopersicon esculentum* Mill) is one of the most popular vegetables in the world.Tomato and its products are rich in antioxidants and vitamins C, E and carotenoids. The world consumption of tomato has increased significantly. Despite its importance, its production is below full potential due to the effect of pests and diseases such as bacterial wilt. Bacterial wilt, caused by *Ralstonia solanacearum* is one of the most devastating, and wide-spread bacterial diseases of crops in tropical environments. The disease has limited both commercial and domestic level production of tomato in Maseno region. The disease is one leading cause of great annual losses of tomato globally. It has been difficult to control *R. solanacearum* with chemicals and cultural practices.There is lack of information on *R. solanacearum* strains in Maseno region, it's pathogenicity on tomato plants and use of Botanicals for its management. *Senna didymobotrya* and *Moringa oleifera* plants were chosen because of their wide antimicrobial properties.The objectives the study were; To profile morphologically and biochemically *Ralstonia solanacearum* landraces and biovars from infected tomato plants in Maseno region; to determine the pathogenecity of *R. solanacearum* strains on tomato plants and to determine the inhibitory effect of *Senna didymobotrya,* root extract and *Moringa oleifera seed* extracts on the growth and development of *R. solanacearum.*These study was conducted at MasenoUniversity in the Department of Botany Laboratory and green house. Botanical specimens were collected from Maseno University Botanic Garden and dried under shade for 30 days. They were ground using a heavy duty laboratory mill into a fine powder. 1000 grams of the plant powder was transferred into a conical flask and 250mls methanol was added. Filtration was done and the extracts concentrated. Ten diseased tomato plants from Maseno, Mariwa, Seme and Hollo were collected. The plant sections (0.5–1cm) were then plated onto Triphenyl tetrazolium chloride media. Profilling of the pathogen was done morphologicaly, biochemically and races were determined by pathogenicity test on wide host range. Bacterialstatic activity of the extracts was determined by the disc diffusion method on Mueller Hinton agar. *In vivo* evaluations were conducted in a green house using tomato seedlings. The experimental design was completely randomized design. The data collected was subjected to analysis of variance. All the isolates had fluidal pinkish red centered colonies on Triphenyl tetrazolium chloride media, they were Gram negative, potassium hydroxide solubility positive, produced gas from glucose, ooze test positive and did not hydrolyse starch which is typical of *R. solanacerum*. *R. solanacearum* strains from infected tomato plants in Maseno region were Maseno, Seme and Holo isolates belonged to race3 bioviar III while Mariwa isolate belonged to race 3 biovar 1. All isolates were pathogenic on tomato plants. Both plants extracts had inhibitory activity against *R. solanacearum* pathogen but they had significant difference for the mean widths of clear zones (*M. Oleifera*; Maseno-8.8, Mariwa-7.9, Seme-7.8, Hollo-9.55 and *S. didymobotrya*; Maseno-8.7, Mariwa-8.8, Seme-8.5, Hollo-10.8). Virulence of an isolate can be determined on the basis of colony colour on TZC media. Production of gas may be attributed to the presence of enzyme systems in bacteria. Variation in restricting disease progression between *S. didymobotria* and *M.oleifera* might be due to difference in chemical compositions of the extracts, membrane permeability of the target pathogen, difference in efficacy and durability of extracts in the soil. Botanicals decrease the negative impact of the pathogen.The two plant extracts suppressed bacteria wilt incidence at 15% and had evident control activity against *R. solanacearum* but *S. didymobotrya* which performed better than *M. oleifera* was recommended for further studies inorder to determine cost benefit analysis before actual production as potential botanical agent for control of *R. solanacerum* by tomato farmers in Maseno region.

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ABREVIATIONS AND SYMBOLS

β: beta

Cells/ml: Cells per milliliter

Cfu: Colony forming unit

Cfu/ ml: Colony forming unit per milliliter

Cm: Centimeter

C_4: Carbon4

^oC: Degrees centigrade

CRD: Completely randomized design.

EPS: Exopolysaccharide

Ft: Feet

G: grams

Ha: Hector

H_2O_2: Hydrogen peroxide.

HCDA: Horticultural Crops Development Authority

Hrs: Hours

KOH: Potassium hydroxide

%: percentage

MT: Metric ton

M: meter

Mic: Minimum Inhibitory Concentrations

Mg: Milligram

Mg/ml: Milligram per milliliter

Ml: Milliliter

Min: Minute

Mm: Millimeter

NA: Nutrient agar

Nm : Nanometer

No.: Number

O.D: Optical density

PCR: Polymerace chain reaction

rDNA: Ribosomal deoxyribonucleic acid

Spp: Species

TZC: Triphenyl tetrazolium chloride

µl: Microlitre

µl/ml: Microliter per Milliliter

USA : United States of America

USD: United States dollar

VAM: vescular-arbuscular mycorrhiza

X: Magnification

<: Less than

≤: Equal or less than.

±: Plus or minus

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CHAPTER ONE

INTRODUCTION

1.1 Background to the study

Tomato (*Lycopersicon esculentum* Mill) is one of the most popular vegetables in the world (Salam *et al*., 2011). It is the world's largest vegetable crop after potato and sweet potato but it tops the list of canned vegetables (Olaniyi *et al*., 2010). It is a very versatile plant and it could either be grown for fresh market or for processing in which mechanical processes are involved (Ayandiji *et al*., 2011). The popularity of tomato is because of its taste, colour, high nutritive value and its diversified use. Tomato and its products are rich in antioxidants and considered to be a good source of vitamins C, E and carotenoids, particularly lycopene and β-carotene and other phenolic compounds that protect the body against diseases (Sen and Chakraborty, 2016; Ilahy *et al*., 2011). Despite the importance of tomato in many developing and developed countries, this crop has often been neglected in the making of several agricultural policies (Weinberger and Lumpkin, 2007). This coupled with several constraints like poor soils, use of unimproved local varieties, land tenure system and damage by pests and diseases, has caused the crop to be produced below its full potential. Among the factors, diseases and pests represent major constraints to production (Asgedom *et al.*, 2011). Several authors have reported the contributions of many types of diseases affecting tomato production (Manila and Nelson 2017,Shin *et al*., 2017 and Bona *et al*., 2017); however the effect of Bacterial wilt has been reported to be the most damaging (Nion and Toyota, 2015).

Bacterial wilt is caused by *Ralstonia solanacearum*, which was formerly known as *Pseudomonas solanacearum* E.F. Smith, is one of the most devastating, important and wide-spread bacterial diseases of crops in tropical environments (Mansfield *et al*., 2012 ;Nguyen and Ranamukhaarachchi, 2010). *R. solanacearum* is an aerobic non-sporing Gram negative plant pathogenic bacterium (Sutariati and Ilyas, 2015). It is soil borne and motile with a polar flagellar tuft and sometimes 1 to 4 polar flagella. It colonizes the xylem, causing bacterial wilt in a wide range of potential host plants (Ramesh*et al*., 2014;Yadeta and Thomma, 2014).

Ralstonia solanacearum is a complex species with exceptional diversity among strains regarding host range, geographical distribution, pathogenicity, epidemiological relationships and physiological properties (Siri *et al*., 2011). This complex species has been subdivided into five Landraces on the basis of differences in host range (Murugaiyan *et al*., 2011) and six biovars on the basis of carbohydrate utilization (Ahmed *et al*., 2013). There is no information on the landraces and biovars found in Maseno Region.

The landrace and biovar classification has gained wide acceptance for subdividing *R. solanacearum* (Meng, 2013b). The racial pattern system groups the strains of *R. solanacearum* according to their ability to infect different host plants, viz., landrace 1 is the most widely distributed in the world and comprises of many strains having a wide host range and is pathogenic on different solanaceous plants and weed hosts (Murugaiyan *et al*., 2011); landrace 2 is restricted to triploid banana and Heliconia; while landrace 3 usually occurs at higher altitudes in tropical areas and in those with temperate climate and affects mainly potato and tomato and to an extent, other hosts such as solanaceous weeds and geranium, landrace 4 infects ginger, and landrace 5 is pathogenic to mulberry (Chandrashekara *et al*., 2012). The biovar scheme divides the species into five groups on the ability of the strains to metabolise or oxidise specific hexose sugars and disaccharides (Prasannakumar *et al*., 2013). At present, there is lot of controversy regarding the prevalence of strains in the various parts of the world. In Kenya however, limited information is available on the prevalence of biovars, landraces and strains in various parts of the country especially Westernkenya region because no studies have been done on the same. Therefore, the present investigations on isolates of *R. solanacearum* causing wilt on tomato plants in Maseno region was carried out.

This soil borne vascular pathogen (*R. solanacearum*) is widely distributed in tropical and subtropical climates and affects an unusually broad range of crops including monocot and dicot plants (Mansfield *et al*., 2012). *Ralstonia solanacearum* has a wide host range representing 44 families (Santiago *et al*., 2017). Highly susceptible crops are potato (*Solanum tuberosum*), tomato (*Lycopersicon esculentum* Mill), egg plant (*Solanum*

melongena), chili (*Capsicum annum*), bell pepper (*Capsicum Annuum*) and peanut (*Arachis hypogaea*). Approximately 450 crop species have been reported as hosts of this pathogen (Achari and Ramesh, 2014). The disease has limited both commercial and domestic level production. It has caused economic problems worldwide leading to serious annual losses exceeding USD 950 million (Mansfield *et al*., 2012). The pathogen has caused significant yield and economic loss in tomato production in Kenya. Yield losses caused by bacterial wilt are estimated at 50-100% in traditional tomato production areas in Kenya (Oboo *et al*., 2014). In Kenya the pathogen has been reported at both low and high altitudes (Muthoni *et al*., 2014). Management is difficult due to high variability of the pathogen, limited possibility for chemical management, high capacity of the pathogen to survive in diverse environments and its extremely wide host range (Hassan *et al*., 2016), hence the need to come up with a proper control method in order to minimize the prevalence pathogen.

Disease management of *R.solacearum* is being attempted with use of resistant varieties, crop rotation, intercropping, and organic manuring, Biological management includes vescular-arbuscular mycorrhizae (VAM) (Zadehbagheri *et al*., 2014), avirulent mutants of *R.solacearum*, and genetically engineered antagonist bacteria (Abdel-Monaim *et al*., 2014). Some naturally occurring rhizobacteria such as *Bacillus spp* (Hyakumachi *et al*., 2013) or intergration of these strategies (Abo-Elyousr *et al.*, 2014), but all have a limited success. Furthermore, use of chemicals has its adverse effects on the environment and the non-target organisms. *Ralstonia solanacearum* is a genetically diverse soil-borne plant pathogen (Ramsubhag *et al*., 2012). The genetic diversity of the pathogen often overcomes the resistance of the crop (Lebeau *et al.*, 2011). Crop rotation with non-host plants, although recommended, is not an effiective method, since *R. solanacearum* has its disseminating and survival stages in the soil and it remains viable for long periods of time (Abdel-Monaim *et al*., 2014). As such it is important to identify the strains of the organism from the affected area so as to select and introduce a suitable control measure. This problem can be solved by use of plant extracts which are non specific hence can control the pathogen on all susceptible crops. Use of plant extracts has an important role in the management of bacterial wilt (Deberdt *et al*., 2012). Plant extracts can help

development of alternative management measures or can be integrated with other practices for effective disease management at the field level (Sunder *et al*., 2011). Use of plant extracts for control will not only suppresses the disease and increases the crop yield but will be important in preventing the environmental pollution due to pesticides. Use of natural plant products which contain secondary metabolites**,** have been preferred because most of them are locally available, environmental friendly, have no side effect and development of resistance is rare (Shams *et al*., 2015).

Secondary metabolites produced by plants constitute a major source of bioactive substances. The most important of these phytochemicals are alkaloids, tannins, flavonoids and phenolic compounds (Ara *et al*., 2012). The medicinal values of the plants lie in their component phytochemicals, which produce definite physiological actions and pathological behaviour on infective disease on the plant. Modern medicine are derived from medicinal plants (Kaur *et al*., 2011), which possess bacteriostatic properties. Due to the increasing trend of drug resistance by *R.solanacearum* the study has dwelt on potentially newer antimicrobial compounds of plant origin. Two plants were identified with the properties of antimicrobial activity. These plants are *Senna didymobotrya* and *Moringa oleifera.Senna didymobotyra* is one of the many species in *Senna* genera that has been recorded to have several uses most of which are antimicrobial against the microorganisms. This genus contains many species that have been recorded to be a good source of medicine for bacterial and fungal diseases (Ogunjobi and Abiala, 2013). Several species of this genus have been found to contain anthraquinones and flavonoids and they have been found to be active against *Escherichia coli* and *Staphylococcusaureus* (Mining *et al*., 2014). The Moringa tree is cultivated and used as a vegetable (leaves, pods flowers, roasted seeds), for spice (mainly roots), cooking and cosmetics oil (seeds) and as a medicinal plant (all plant organs)(DS and Chavan, 2015). Important medicinal properties of the plant include antipyretic, antiepileptic, anti-inflamatory, antiulcerative (Mehta *et al*., 2011), antihypertensive (Mbikay, 2012), cholesterol lowering, antioxidant (Farooq *et al.*, 2012), anti diabetic, hepatoprotective (Owolabi and Ogunnaike, 2014), antibacterial and antifungal activities (Biswas *et al*., 2012).The use of plant extracts is an effective way of managing plant diseases compared to synthetic chemicals. Therefore,

there is an urgent need to search for effective, safe and biodegradable alternative pesticides.

As such it is important to identify the strain of the organism from the affected area in order to select and introduce suitable management for the identified pathogen (*R.solanacearum*) so as to suppress its disease manifestation. Very little work has been reported on the use of *Senna didymobotrya* and *Moringa oleifera* plant products to control bacterial wilt and hence this study strives to determine the effectiveness of both plants in bacterial wilt management.

1.2 Statement of the Problem

Bacterial wilt due to *R. solanacearum* has caused economic problems worldwide leading to serious annual losses exceeding USD 950 million (Mansfield *et al.*, 2012). The pathogen has caused significant yield and economic loss in tomato production in Kenya. Yield losses caused by bacterial wilt are estimated at 50-100% in traditional tomato production areas in Kenya (Oboo *et al*., 2014). At present, there is lot of controversy regarding the prevalence of strains in the various parts of the world. In Kenya however, limited information is available on the prevalence of biovars, landraces and strains in various parts of the country. Information on the prevalence of biovars, landraces and strains in Maseno region is missing. Unavailability of information on *R. Solanacearum* strains infecting tomato plants in Maseno region has led to poor diagnosis and management of bacteria wilt disease hence the disease continues to cause huge losses to tomato farmers.

Ralstonia solanacearum affects an unusually broad range of crops including monocot and dicot plants (Mansfield *et al*., 2012). It has a wide host range representing 44 families (Santiago *et al*., 2017). Highly susceptible crops are potato, tomato, egg plant, chili, pepper and peanut. There is lack of information on pathogenicity of *R. solanacearum*on tomato plants in Maseno region.

Ralstonia solanacearum is resistant to drugs, has ability to spread very fast both inter and extra-specific and genetic variability which makes it difficult to manage with chemicals and cultural practices. There is no information on the use of botanicals (*Senna didymobotyra and Moringa oleifera*) to control *R. solanacearum*on tomato plants in in Maseno region. Farmers in Maseno region have inadequate knowledge on how to handle the bacterium thus leading to enormous losses of the produce. It has continued to be a big menace in most tomato producing parts of Kenya and worldwide where it causes drastic poor tomato yields. Thousands of farmers face severe poverty and constraints in pursuing a livelihood through tomato farming making it non lucrative venture despite the favorable climatic condition of the region. Farmers from Maseno region abandoned tomato growing long ago because of the losses resulting from the severity of the disease (Interviews from farmers: key informants). The disease therefore poses a major threat to food security in Kenya, and Maseno region.

1.3 Justification of the study

Tomato is one of the most popular vegetables in the world (Javanmardi and Moradiani, 2017). It is the world's largest vegetable crop after potato and sweet potato but it tops the list of canned vegetables (Verma *et al*., 2015). Tomato and its products are rich in antioxidants and considered to be a good source of vitamins C, E and carotenoids, particularly lycopene and β-carotene and other phenolic compounds that protect the body against diseases (Ilahy *et al*., 2011.The world consumption of tomato has increased significantly over the past 25 years (Perveen *et al*., 2015). Productivity of tomato in Maseno region has been limited by bacterial wilt disease which has caused enomours losses to farmers. Green house tomatoes have picked up with farmers in Maseno region but the main challenge is the spread of bacterial wilt that has caused some green houses to be abandoned due to wide spreadout distribution of the bacteria in the region. The disease therefore poses a major threat to food security in Kenya, and Maseno region.

Successful management of bacterial wilt of tomato is hinged on accurate diagnosis of causative agent. This study is very important because it intends to profile *R. Solanacearum* species in Maseno region and search for new interventions inform of

botanicals which are environmentally friendly in order to overcome the problem of pesticide resistance. Farmers will benefit from the research by getting a suitable alternative to synthetic remedies for management of bacteria wilt. Tomato production in Maseno region will be increased once bacterial wilt is controlled and this will in turn improve the economy of the country by increasing per capita income. Results from this work may be used by policy makers in the country to come up with a more precise intervention programme to reduce the menace of bacterial wilt in tomatoes.

Profiling of *Ralstonia solanacearum* will offer information on strains found in Maseno region which can be the starting point for plant pathologist to design control measures specific to Maseno region. By identifying the strains of *R. solanacearum* it will be possible to get the right antibiotics for its management.

Documentation of the effects of plant extracts from these species on the growth and disease-causing ability of *Ralstonia solanacearum* will provide potential sources of widely sought solution to the losses incurred in tomato production due to bacterial wilt infection. The study will also provide background information on possible plant extracts that can serve as sources of constituents for development of remedies for management of bacteria wilt.

1.4 Objective of the study

1.4.1 General objective

To profile and determine the pathogenecity of *Ralstonia solanacearum* from infected tomato plants in Maseno region and evaluate antibacterial activity of *Senna didymobotrya* and *Moringa oleifera* plant extracts against *R. solanacearum.*

Specific objectives

1. To profile morphologically and biochemically landraces and biovarsof *Ralstonia solanacearum* strains from infected tomato plants in Maseno region.
2. To determine the pathogenicity of *R. solanacearum* strains on tomato plants.
3. To determine the inhibitory effect of *Senna didymobotrya,* root extract and *Moringa oleifera seed* extract on the *in vitro* and *in vivo* growth and development of *Ralstonia solanacea.*

1.5 Hypotheses

The hypotheses of this study were:

1. There are no morphologically and biochemically different landraces and biovars of *Ralstonia solanacearum* strains on infected tomato plants in Maseno region.
2. There is no difference in pathogenicity of *Ralstonia solanacearum* strains to tomato plants.
3. *Senna didymobotrya* and *Moringa oleifera* plant extracts do not inhibit *in vitro* and *in vivo* growth and development of *Ralstonia solanacearum.*

CHAPTER TWO
LITERATURE REVIEW

2.1 Tomato (*Lycopersicon esculentum* Mill.)

2.1.1 Botanical description of tomato

Tomato plant belongs to the nightshade family, *Solanaceae* (Singh *et al*., 2014). The plants typically grow to 1–3 meters (3–10 ft) in height and have a weak stem that often sprawls over the ground and vines over other plants. It is a perennial plant in its native habitat, although often grown outdoors in temperate climates as an annual.

2.1.2. Importance of Tomato

Tomato is the most widely grown vegetable in Kenya. It can be produced in window box gardens or in single pots (Anastacia *et al*., 2011). Commercially, it is of equally great importance, from processing to fresh market and from beefsteak to grape tomatoes, they can be processed into various products such as soup, catsup, sauce, salsa and prepared foods, the variety and usefulness of the fruit is virtually boundless (Tusiime, 2014). Use of the crop has expanded rapidly over the past 100 years (Zhang *et al*., 2017).

Tomatoes have significant nutritional value. In recent years, tomatoes have become known as an important source of lycopene, which is a powerful antioxidant that acts as an anticarcinogen. They also provide vitamins and minerals (Aghajanpour *et al*., 2017). One medium ripe tomato (~145 grams) can provide up to 40 percent of the recommended daily allowance of vitamin C and 20 percent of vitamin A. They also contribute B vitamins, potassium, iron and calcium to the diet (Mwaura *et al*., 2013).

2.1.2 World production of tomato

Global production of tomatoes exceeds 133 million metric tons per year (Al-Shadiadeh *et al*., 2012). Tomato ranks third in priority after potato and onion in India but ranks second after potato in the world (Priya and Patel, 2016). Today more than 400,000 acres of tomatoes are produced in the United States. The yearly production exceeds 14 million tons (12.7 million metric tons)(MacDonald *et al*., 2013), India ranks second in the area as well as in production of tomato. The major tomato growing countries are China, USA, Italy, Turkey, India and Egypt. Total area under tomato is 4582438 thousand ha with

production of 150,513,813 thousand tons and with productivity of 32.8 tons/ha (Ramappa and Manjunatha, 2016).

2.1.3 Tomato production in Kenya

During the year 2012/2013, the area under tomatoes was estimated to be 18,612 ha in Kenya. The total production for the country was 397,000 MT with a value of Ksh 12.8 billion according to Horticultural Crops Development Authority (HCDA) 2012-2013 report. There was no difference in the area grown while the quantity and value reduced by 3 per cent and 27 per cent as compared to 2011 due to the price offered in Meru County (Rao *et al*., 2015). Tomato was majorly produced in Kirinyaga (24%), Kajiando (9%) and Taita Taveta (7%) (Wachira *et al*., 2014). In Maseno region tomato production is mainly in few green houses and small farms (key informants).

2.2 Bacterial wilt of tomatoes

This is one of the most pathological and destructive plant diseases (Mansfield *et al*., 2012). The disease has a very wide host range (Sapna and Ramesh, 2014). Hosts include economically important crops such as tobacco, pepper, banana, beans, potato, tomato and eggplant (Sarkar and Chaudhuri, 2016). Thorn apple and nightshade are two common weed hosts that are attacked by the disease (Seleim *et al*., 2014).

The spread of the causative agent has been mostly favoured by dissemination in latently infected planting materials. The disease is the second most important constrain to tomato production in tropical and subtropical regions of the world (Prasath *et al*., 2014). Breeding programmes have not been successful in developing varieties with stable resistance to the disease (Huet, 2014).

Bacterial wilt has spread to most tomato producing countries. Its occurrence in Australia and in the South eastern United States has resulted in concentration of Bacterial wilt research in these areas (Abdel-Monaim *et al.*, 2014). This is the case in Latin America where the disease has spread to all tomato producing countries. The disease is also common in Asia, particularly in the midhills of the Himalayas in India, Nepal, Bhutan and Pakistan (Bové, J. 2014). In East and Southern Asia, this bacterial disease is common

in Indonesia, the Philippines, Southern Vietnam, Laos, Japan and Southern China. In the early 1990s bacterial wilt became a serious threat to tomato production in European countries including Belgium, England, France, Spain, Italy and Portugal (Toth *et al*., 2011).

Apart from the above-mentioned areas, the disease is also present in the Central, Eastern and Southern Africa. It has been reported in countries such as Uganda, Ethiopia, Kenya, Madagascar, Rwanda, Burundi, Nigeria and Cameroon (Muthoni *et al*., 2012).

In Kenya it was first reported in 1945 around the Embu area, from where it spread to other parts of the country (Muthoni *et al.*, 2014). Currently there is no documentation of the disease in Western and Nyanza regions but the presence of the disease is being felt by tomato farmers in the region.

2.2.1. Taxonomy and diversity of *Ralstonia solanacearum*

Scientific name; *Ralstonia solanacearum*

Synonym; *Pseudomonas solanacearum*

Kingdom: Bacteria

Phylum: Proteobacteria

Class: Betaproteobacteria

Order: Burkholderiales

Family: Burkholderiaceae

Genus: Ralstonia

Species: solanacearum

Ralstonia solanacearum has complex taxonomic properties with a great degree of diversity at all levels, including physiological, phenotypic, genotypic, and host range (Remigi *et al*., 2016:Wicker *et al.*, 2012). Traditionally the bacterium has been subdivided into six biovars on the basis of carbohydrate catabolism (Hyakumachi *et al*., 2013) and five landraces designated by host range (Horita *et al.*, 2004).

Buddenhagen *et al*. (1962) distinguished five races on the basis of pathogenecity.
Landrace 1: Affects tobacco, potatoes, tomatoes, diploid bananas and other solanaceae crops and weeds; with high optimum growth temperature of 35 – 37ºC. It is also known as solanaceae landrace (Popoola *et al.*, 2015). Landrace 2: Affects the triploid bananas and *Heliconia spp* with high optimum temp of 35 – 37ºC. It is also called Musa landrace. Landrace 3: Affects potato, tomato and to lesser extent, other hosts such as solanaceous weeds and geranium. The landrace grows well at lower temperature of 27ºC (Tahat and Sijam, 2010). Landrace 4 infects ginger while Landrace 5 is pathogenic on mulberry (Rodrigues *et al*., 2011).

Ralstonia Solanacearum species are divided into five different biovars based on how the bacterial isolate utilize and/or oxidize three hexose alcohols and three disaccharides (Meng, 2013b; Ahmed *et al*., 2013) (cellobiose, lactose, and maltose) and hexose achohols (dulcitol, mannitol, and sorbitol). *Ralstonia solanacearum* strains were originally divided into five biovars (Prior *et al*., 2016). Biovar 1 strains metabolize none of the above mentioned sugars; biovar 2 strains only metabolize disaccharides (cellobiose, lactose, and maltose); biovar 3 strains metabolize all of the above mentioned sugars ; biovar 4 strains metabolize only hexose alcohols (dulcitol, mannitol, and sorbitol); biovar 5 strains metabolize all the above mentioned sugars except dulcitol and sorbitol (Álvarez *et al*., 2010). Later, a new group of *R. solanacearum* isolates from the Amazon basin was differentiated from original biovar 2 using ribose and trehalose (Meng, 2013b). This group is named biovar 2-T or biovar N2 and the original biovar 2 strains are now referred to as 2-A. Except for biovar 2-A, which almost always corresponds to landrace 3, and biovar 5, which is identical to landrace 5, there is no correlation between biovars and landraces (Wang *et al*., 2017).

2.2.2 Symptoms of Bacterial wilt

There are several symptoms characterizing the bacterial wilt disease. The most frequent external symptoms of the infected plants are wilting, stunting and yellowing of the

foliage (Suhaimi *et al*., 2016). Other symptoms are bending of the leaves downward showing leaf epinasty, adventitious roots growing in the stems, and the observance of narrow dark stripes corresponding to the infected vascular bundles beneath the epidermis (Nguyen and Ranamukhaarachchi, 2010). Although the disease usually progresses until complete wilting and collapse of the plant, expression of the symptoms and rate of disease development may vary depending on host susceptibility and the aggressiveness of the pathogenic strain. In the cases where an infected plant does not show wilting, characteristic external symptoms may be dwarfing and stunting of the plant (Hernández-Romano *et al*., 2012).

The most frequent internal symptoms are progressive discoloration of the vascular tissue, mainly the xylem, at early stages of infection, and of portions of the pith and cortex, as disease develops, until complete necrosis (Jyothi and Santhosha, 2012). Slimy viscous ooze typically appears on transverse-sectioned stems at the points corresponding to the vascular bundles. As a result, collapse and death of the plant take place because of the degradation of occluded xylem vessels and the destruction of surrounding tissues (Álvarez *et al*., 2010).

2.2.3 Life cycle, epidemiology and dissemination of *Ralstonia solanacearum*

The bacterium (*R. solanacearum*) is primarily a soilborne and waterborne pathogen (Sutariati and Ilyas, 2015). It infects host plants primarily through roots, entering through wounds formed by lateral root emergence or by damage caused by soilborne organisms (Bittner *et al*., 2016). The bacterium can also enter plants by way of stem injuries caused by insects, handling or from mechanical damage. Once inside roots or stems, the bacterium colonizes the plant through the xylem in the vascular bundles (Pandey, 2015).

The race 3 biovar 2 (R3b2) of the bacterium is most severe on plants when temperatures are between 25 and 35°C and decreases in aggressiveness when temperatures exceed 35°C or fall below 18°C (El Jarroudi *et al*., 2010). Active disease at temperatures below 18°C is rare (Stevens *et al*., 2011; Bocsanczy *et al*., 2014). The pathogen could be spread from infested to healthy fields by soil transfer on machinery and surface runoff water and

it could be disseminated from infested surface water to uninfested fields by flooding or irrigation (Fajinmi, A. and Fajinmi, O. 2010). Plant to plant infection can occur when bacteria shed from infected roots move to roots of nearby healthy plant. Onduso, (2014) reported that long distance spread of the pathogen can occur with transportation of latently infected transplants. The bacterium could survive for days and up to years in infested water, wet soils or in soil layers >75 cm (Muthoni *et al*., 2012) from where it can be dispersed and only antagonist microorganisms and environmental factors, mainly temperature, soil type and soil moisture can affect *R. solanacearum* survival.

2.2.4 Phenotypic characteristics of *Ralstonia solanacearum*

The single cell is a small rod with rounded ends, with an average size of 0.5 to 0.7 by 1.5 to 2.5 μm (Jyothi and Santhosha, 2012). Cell wall structure is that of Gram-negative bacteria, and flagella are polar (Plate 1). The bacterium has an oxidative metabolism and is generally considered a strict aerobe. However, under some circumstances, it is able to limit, slow growth when cells are not in direct contact with the air. *Ralstonia solanacearum* produces poly-β-hydroxybutyrate granules as cell energetic reserve(Beeby *et al*., 2012).

Ralstonia solanacearum strains from tropical areas all over the world have a high temperature optimum (35°C), whereas that of strains occurring at higher altitudes in the tropics and in subtropical and temperate areas is lower (27°C); no growth has been observed at 4°C or 40°C (Wei *et al*., 2017). Approximate minimal and maximal growth temperature values would be 8-10°C and 37-39°C respectively (Álvarez *et al*., 2010).

Two morphological types of *R. solanacearum* colonies can be typically observed on agar plates fluidal or mucoid and afluidal or non-mucoid. The mucoid substance is produced by the accumulation of an exopolysaccharide (EPS), which causes these mucoid colonies to exhibit a typical irregularity of their surfaces, often with characteristic whorls in the centre (Pawaskar *et al*., 2014). All *R. solanacearum* colonies are non-fluorescent (Shahbaz *et al*., 2015).

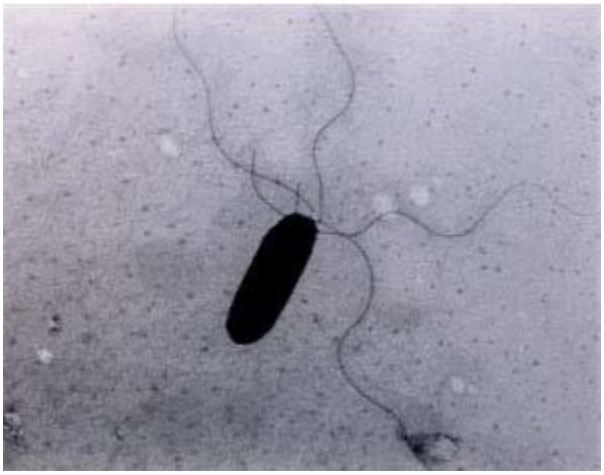

Plate 1. Electron microscopic image of *Ralstonia solanacearum*Mgx 10,000 (photo courtesy of Digonnet*et al*.(2012).

2.2.5 Virulence Factors of *Ralstonia solanacearum*

One of the most important virulence factors of *R.solanacearum* is a heterogeneous polymer of N-acetylated extracellular polysaccharide 1 (EPS 1)(Milling *et al*., 2011). Extracellular polysaccharide 1 is more than 90% of the total *R.solanacearum* extracellular polysaccharide produced, and approximately 85% appears as a released, cell-free slime, whereas 15% has a cellsurface-bound capsular form (Murthy and Srinivas, 2015). Extracellular polysaccharide 1-deficient mutants are nearly avirulent and do not colonize plant xylem vessels as efficient as wild type (Meng, 2013a). Extracellular polysaccharide 1 directly cause wilting by physically blocking the vascular system and thereby alters water movement (Dalsing and Allen, 2014). Extracellular polysaccharide 1 also protects *R. solanacearum* from plant antimicrobial defenses by blocking bacterial surface features (proteins, lipopolysaccharides and peptidoglycans) that could be recognized by hosts (Meng *et al*., 2011). In susceptible tomato plants, the wild-type and EPS 1-deficient mutant induced generally similar defense responses; but in resistant tomato plants, the wild-type induced significantly greater defense responses than the EPS 1-deficient mutants, suggesting that the EPS 1 itself is a specific elicitor of plant defense responses (Milling *et al*., 2011).

The Type III Secretion System (T3SS) has a central role in pathogenesis of many bacterial pathogens of plants. In *Ralstonia solanacearum* the T3SS is encoded by the hypersensitive reaction and pathogenicity (*hrp*) genes (Lohou *et al*., 2013). *Ralstonia solanacearum hrp* genes are key determinants for disease development on compatible hosts and for induction of the defensive hypersensitive response (HR) on resistant plants. *Ralstonia solanacearum* is estimated to produce 70–80 type III effectors. The T3SS of *R. solanacearum* contributes greatly to pathogenesisof *Ralstonia solanacearum* (Poueymiro *et al*., 2014).

Phytopathogenic bacteria have often developed enzymes to hydrolyze plant cell wall components to obtain nutrients and energy, which are further involved in early stages of the infective process, favouring the entry and advance of the pathogenic agent in host tissues (Dangl, 2013). *Ralstonia solanacearum* produces several plant cell wall-degrading enzymes, secreted via the type two secretion system (T2SS) (Wei *et al*., 2017). These include one β-1,4-cellobiohydrolase (CbhA) and some pectinases whose activities have been identified respectively as one β-1,4-endoglucanase (Egl), one endopolygalacturonase (PehA), two exopolygalacturonases (PehB and PehC) , and one pectin methyl esterase (Pme) . *Ralstonia solanacearum* Egl is a 43-kDa protein that has proved to be involved in pathogenicity (Álvarez *et al*., 2010).

Ralstonia solanacearum possesses flagella-driven swimming motility and type IV pili-driven twitching motility that are important to its virulence (Burdman *et al*., 2011). Both nonmotile and nontactic mutants are significantly reduced in virulence on soil-drench inoculated tomato plants but exhibit normal virulence when directly inoculated into plant xylem, indicating that *R. solanacearum* needs directed motility and that swimming motility contributes to virulence in the early stage of host colonization and invasion (Yao and Allen, 2007).

2.2.6 Control of Bacterial wilt in Tomato

According to Aliye *et al.*(2015) bacterial wilt is a major problem to farmers of tomatoes worldwide particularly because its management is difficult. Integrated management

approaches are usually advocated for inorder to reduce the spread of the disease but these approaches are based on appropriate combinations of management measures that suit specific circumstances (Aliye *et al*., 2015). The measures, which have individual practical, technological and economic limitations, include; use of clean seeds (Tahat and Sijam, 2010), but this has the shortcomings when the soils used for planting are already infected by *R. solanacearum* hence infection of the seedlings. Another measure is use of fields that have not yet become contaminated by the disease (Czajkowski *et al*., 2011). It is very difficult for most farmers to differentiate infected soils from uninfected soils unless laboratory tests are carried out which are very expensive in terms of the cost. This is because the disease stays in the soil for a very long time infecting any susceptible crops planted.

Crop rotation with non-host plants e.g Maize, cassava and sorghum, although recommended (Zanon *et al*., 2011) is not an efficient method, since *R. solanacearum* has its disseminating and survival stages in the soil and it remains viable for long periods of time, it has also a wide host range. Rotation with cereals or gramineous pastures can be implemented to eliminate soil inocula (Abdel-Monaim *et al*., 2014) this in most cases is not practical because of the intensive fragmentations of land due to increase in population. Farmers tend to continuously plant tomatoes on the same plot. *Ralstonia solanacearum* can also be controlled by Pesticides such as algicide (3-[3-indolyl] butanoic acid), fumigants (metam sodium, 1, 3-dichloropropene, and chloropicrin),(Yang *et al*., 2012), and plant activators generating systemic resistance on the tomato (validamycin A and validoxylamine) (Abo-Elyousr*et al*., 2012) have been used to manage bacterial wilt. The combination of methyl bromide, 1, 3-dichloropropene, or metam sodium with chloropicrin significantly reduced bacterial wilt in the field from 72% to 100% and increased the yield of tobacco and the tomato. Edwards-Jones (2008) reported that pesticides offered greater net benefits than other control methods, but this has not always been the case. For example, 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), become a contaminant in soil and/or groundwater, and become poisonous to farmers; it also has the disadvantage of destroying even useful

microorganisms in the soil. Use of resistant varieties is an effective measure of managing the disease (Algam *et al.*, 2010) but the landrace and strain diversity of the pathogen has made breeding for resistant cultivars ineffective in the management of bacterial wilt. The genetic diversity of the pathogen often overcomes the resistance of the crop (Maji and Chakrabartty, 2014). Furthermore the development of disease resistant varieties is an expensive venture that takes a long time to develop a single variety. It is also agreed that breeding for resistance is not complete, producing only modest gains and often lacking stability and/or durability (Kurabachew, 2015). As such it is recommended to identify the strain of the organism from the affected area to in order to select and introduce a suitable management.

Removal of rotten plants from the field is also a measure advocated in management of bacterial wilt spread. The rotten plants should be buried deep or burned (Czajkowski *et al*., 2011). This method is labour intensive and is not practicable on large farms. Another recommended measure is to weed before planting tomatoes since weeds and other crops harbors the pathogen. If the incidence of bacterial wilt in a field is low the wilted tomato plants must be removed as soon as they are observed to avoid contamination of healthy neighboring plants (Paret *et al*., 2010). This is just a temporary measure since *R. salanacearum* is a soil dwelling bacteria and some inoculums will remain in the soil and infect other crops.

Tools used in farming should be decontaminated to prevent movement of soil from an infested to a disease free field (Nion and Toyota, 2015). The decontamination process can be accomplished by washing with water and calcium hypochloride or other available bactericide or sterilized by flame. It is not easy to decontaminate large machinery and also most synthetic chemicals are non degradable thus has negative effects on the environment.

Due to the limited effectiveness of the current integrated management strategies, bacterial wilt continues to be an economically serious problem for field-grown crops in many tropical, subtropical, and warmer areas of the world including Kenya and specifically

Maseno region. There is need to come up with a lasting solution for the control of *R. solanacearum* hence the need for Botanicals which are locally available, environmental friendly, have no side effect and development of resistance is rare.

2.3 Taxonomy and biology of *Senna didymobotyra*

Scientific classification

Kingdom: Plantae

Division: Spermatophyta/ Angiospermae

Class: Magniolopsida

Order: Magniolopsidales/ Fabales

Family: Caesalpiniaceae/ Fabaceae

Sub-family: Caesalpiniodeae

Genus: *Senna*

Species: *Senna didymobotyra* (Fresen.) H. S. Irwin & Barneby

Synonyms: *Cassia didymobotrya* Fresen. (1839), *Cassia nairobensis* L. H. Bailey (1941)

Plate 2. *Senna didymobotrya* plant (Photo taken by Buyela Daniel)**.**

2.3.1 Uses of Senna didymobotrya

In medicine, *Sennas* have for millennia played a major role in herbalism and folk medicine (Sen *et al*., 2011). Alexandrian *Senna* (*S. alexandrina*) was and still is a significant item of trans-national trade for example by the Ababdeh people and grown commercially, traditionally along the middle Nile but more generally in many regions around the north western Indian Ocean (Mazumder *et al*., 2008).

Senna is a large genus with around 500 species of flowering plants in family Leguminosae (Singh*et al.,* 2017). *Senna* species are used extensively in various parts of the world against a wide range of ailments, the synergistic action of its metabolite being probably responsible for the plants beneficial effects (Singh *et al*., 2011). These plants contain alkaloids, site sterols, anthraquinones, glycosides, alkaloids, tannins and terpenoids (Howlader *et al*., 2016).The phytochemicals found in this plant can be attributed to its medicinal value (Nyamwamu *et al*., 2015). *Casia occidentalis* is used for treatment of mycosis (skin infection). The flowers, roots, and stems have both antibacterial and antifungal activity (Kitonde *et al*., 2014).

The flowers, roots, and stems have both antibacterial activity against *Escherichia coli*, *Salmonella typhi*, *Enterobacter aerogenes*, *Klebsiella sp*., *Streptococcus pyogenes*, *Proteusvulgaris* and *Bacillus cereus* (Ngule and Swamy, 2013) and antifungal activity against *Trichophyton tonsurans* and *Candida albicans* (Korir *et al*., 2012). An aqueous extract of *Senna didymoboytra* have antifungal activity by inhibition of mycelia growth and aflatoxin formation (Kuri *et al*., 2011). It is also used to treat worm infestation, ringworm and eruptive skin conditions (Nguedia and Shey, 2014).

Senna didymobotyra is one of the many species in *Senna* genera that has been recorded to have several uses most of which are antimicrobial against the microorganisms mentioned herein. This genus contains many species that have been recorded to be a good source of medicine for bacterial and fungal diseases (Ogunjobi and Abiala, 2013). Several species of this genus have been found to contain anthraquinones and flavonoids and they have been found to be active against *Escherichia coli* and *Staphylococcusaureus* (Mining *et al*., 2014). *Escherichia coli* a human pathogenis a Gram –negative bacteria (Makarova *et al*., 2011), *Ralstonia solanacearum* is also a Gram- negative bacteria (Zhang *et al*., 2015), hence the mechanism of action of the plant extract is the same on *Escherichia coli* and *Ralstonia solanacearum*. There is limited literature on the use of plant extracts against plant pathogens such as *R. solanacearum*, most researches have concentrated on

pathogens of medical importance. This research sought to come out with a remedy to bacteria wilt of tomatoes.

The study byAnthoney Swamy *et al*., (2014) showed that the plant root extract of *S.ditymobotra* have immense pharmacological value against both Gram negative and Gram positive human pathogenic organisms.The aqueous fraction of *S. didymobotrya* root inhibited *Bacillus cereus* giving the best results followed by *Salmonella typhi*, *P.vulgaris*, *S.liquefaciens* and *E. coli* respectively. Crude extract of *S. didymobotrya* root inhibited all the above mentioned microbes. Whereas Anthoney Swamy *et al*., (2014 experiment used water extraction, other studies including this one used methanol extracts because it can extract various polar and non polar compounds (Mokgotho *et al*., 2013), it can also extract hydrophobic compounds.This ensures all bioactive compounds are extracted. His research did not vary the concentrations of the extract, other researches including this one varied concentration; 15%, 10%, 5% and 2.5% and came up with the best concentration that inhibited *R. solanacearum.* His experiment used wells to dispense the extract while other researches including this one used paper discs to dispense the extract.

Owoseni and Sangoyomi (2014) evaluated the efficacy of different solvent extracts (chloroform, ethanol, methanol and hexane) of ten plants on *R. solanacearum* the causal organism of bacterial wilt of tomato. The study used leaf extracts of *Ocimum gratissimum*, *Vernonia amygdalina, Allium sativum, Zingiber officinale, Azadirachta indica, Jatropha curcas, Senna obtusifolia, Senna occidentalis* and *Senna alata.* It was concluded that the solvent extracts of the above mentioned plants had inhibitory effect on the growth of*R. solanacearum*(Owoseni and Sangoyomi, 2014). *Senna alata* and *Senna occidentalis* belong to the same family with *Senna ditymobotria*, hence the high possibility of the latter having inhibitory effect on the growth of *R .solanacearum.*

Senna alata and *Senna occidentalis* leaf extracts were used to test their efficacy against *R. ralstonia*. In other experiments root extract of *senna ditymobotria* has been used against *R. solanacearum* since they have been found to have higher efficacy against most

microorganisms as compared to other plant parts (Mining *et al*., 2014). In their experiment they did not vary the concentrations of the crude extracts. However varying the concentrations of the crude extracts may helb in establishing the optimum inhibitory percentage.

In another study by Swamy *et al*., (2014) *Senna didymobotrya* was found to contain secondary metabolites, saponins, flavonoids, tannins, phenols, steroids, steroidal ring (glycone portions of the glycoside), steroidal nucleus (glycone portion of the glycoside) and cardiacglycosides. The bioactivity of the plant extract observed in the above study was attributed to the presence of these phytochemicals. Results from the bioassay of *S.didymobotrya* extract showed inhibition against *Escherichia coli, Salmonella typhi, Enterobacter aerogenes, Klebsiella sp*., *Streptococcus pyogenes, Proteusvulgaris* and *Bacillus cereus,* (Swamy *et al*., 2014).

The above experiment used dimethylsulfoxide (DMSO) to prepare the leaf extracts. Other experiments including this one have used methanol to prepare root extracts. Root extracts has been shown to have more inhibitory power against microorganism as compared to leaf extracts because they contain more secondary metabolites as compared to the secondary metabolites in leaves (Kitonde *et al*., 2014). Since very no research has been conducted on the inhibition of *Senna didymobotrya root* extracts on *R. solanacearum*, this study sought to determine the inhibitory effect of *Senna didymobotrya*, root extract on the *in vitro and in vivo* growth and development of *R. solanacearum*.

2.4 Taxonomy and biology of *Moringa oleifera*

Scientific classification

Domain: Eukaryota

Kingdom: Plantae

Phylum: Spermatophyta

Subphylum: Angiospermae

Class: Dicotyledonae

Order: Capparida

Family: Moringaceae

Genus: Moringa

Species: *Moringa oleifera*

Plate 3.*Moringa oleifera* plant with seed pods (photo taken by Buyela Daniel).

Moringa oleifera Lam (Moringaceae) is native to the western and sub- Hima-layan region, India, Pakistan, Asia Minor, Africa and Arabia (Bukar *et al*., 2010). The Moringa tree is cultivated and used as a vegetable (leaves, pods flowers, roasted seeds), for spice (mainly roots), cooking and cosmetics oil (seeds) and as a medicinal plant (all plant organs)(DS and Chavan, 2015). Important medicinal properties of the plant include antipyretic, antiepileptic, anti-inflamatory, antiulcerative (Mehta *et al*., 2011), antihypertensive (Mbikay, 2012), cholesterol lowering, antioxidant (Farooq *et al.*, 2012), anti diabetic, hepatoprotective (Owolabi and Ogunnaike, 2014), antibacterial and antifungal activities (Biswas *et al*., 2012). In addition, *M. oleifera* seed possesses water purifying powers and therefore helps in reducing the incidence of water borne diseases (Daniyan *et al*., 2011). They are known to be anti-helminthic, antibiotic, detoxifiers, immune builders and have been used to treat malaria (Thilza *et al*., 2010) and it can also be used as a less expensive bio-absorbent for the removal of heavy metals (Abalaka *et al*., 2012). *Moringa oleifera* is a tropical tree whose numerous economic applications and facility of propagation are arousing growing international interests.

Rahman *et al*., (2009) determined antibacterial activity of leaf juice extracts of *Moringa oleifera* Lam, *in vitro,* using disc diffusion and minimum inhibitory concentration (MIC) determination method against human pathogenic bacteria. The fresh leaf juice (10 µl $disc^{-1}$), powder from fresh leaf juice, cold water extract of fresh leaf, each of 1175 µg $disc^{-1}$, displayed a potential antibacterial activity against all the tested four Gram negative bacteria: *Shigella shinga, Pseudomonas aeruginosa, Shigella sonnei* and *Pseudomonas spp*. and six Gram-positive bacteria*: Staphylococcus aureus, Bacillus cereus, Streptococcus-B- haemolytica, Bacillus subtilis, Sarcina lutea and Bacillus megaterium.*

Rahman *et al*., (2009) experiment was only based on *in vitro* studies on human pathogenic bacteria. Positive *in vitro* studies results can only be confirmed by *in vivo* studies. In their experiment they only used aqueous extracts, other researchers have used methanol extract, which has got advantage over aqueous extracts in that polar, nonpolar and hydrophobic compounds are extracted.

Sumathi *et al*., (2014) investigated *in vitro* antimicrobial activity of aqueous methanol extract of *Moringa oleifera petals* against *S. aureus, E. coli, B. subtilis, P. aeruginosa* and *P. vulgaris* using the disc diffusion method. The methanol extract inhibited the growth of all investigated bacteria with zone of inhibition between 12.4 – 23.4 mm at 20µl/ml whereas the minimum inhibitory effect of the methanol extract of *M. Oleifera* petals were effective at the highest concentration (20µl/ml) against pathogenic microorganism. *In vitro* experiments can only be confirmed through *in vivo* experiments and thus Sumathi *et al*. (2014) failed to perform *in vivo* experiment to back up their results. Use of petals is un-environmental friendly procedure since it inhibits plant propagation through seeds and can end up destroying many trees. Other researchers including this one used seeds which are environmentaly friendly and some seeds can be re-planted. Their experiment only concentrated on pathogens of medical importance such as *S. aureus, E. coli, B.*subtilis, *P. aeruginosa* and *P. vulgaris*, other experiments involving plant pathogens (*R. solanacearum*) should be done.

Although *Moringa oleifera* has got antibacterial activities, no studies have been done on its effect on *R. solanacearum* the causative agent of bacteria wilt of tomato in Maseno region. This study therefore aimed at establishing inhibitory effect of *M. oleifera* on *R. solanacearum* and its potential to manage tomato wilt caused by *R. solanacearum.*

CHAPTER THREE
MATERIALS AND METHODS

3.1 Study site

This study was conducted at MasenoUniversity in the Department of Botany Microbiology Laboratory and under polythene coverd green house (Day Temperatures 25^0C-40^0C and Night Temperatures 20^0C-30^0C, 14/10h photoperiod and humidity 70-90%) located at University Research farm. Maseno is situated in western Kenya, its geographical coordinates are 0° 10' 0" South, 34° 36' 0" Eastandthe altitude is 1,503 metres or 4,934 feet above sea level (KNBS, report, 2013). Maseno receives both short and long rains averaging 1750mm per anum with mean temperature of 28.7^0C.The study was carried out between October 2015 and April 2016.The study consisted of three parts: (i) isolation and profilling of the pathogen, (ii) *in vitro* screening of the plant extracts against *Ralstonia solanacearum,* and (iii) *in vivo* evaluation of selected plant extracts for the control of bacterial wilt disease of tomatoes.

3.2 Collection, preparation and preservation of plants parts

Senna didymobotrya and *Moringa oleifera* plants were identified in the field with reference to taxonomic keys (Cowan, 1999). The healthy, infection free mature root bark of *S. didymobotrya* and seeds of *M. oleifera* were collected from MasenoUniversity, Botanic Garden, Kenya. For standardization, all the samples were transported and air-dried in the shade until completely dry for thirty (30) days, according to the procedure by Jeyaseelan *et al*., (2012). They were ground using a heavy duty laboratory mill into a fine powder, and stored in airtight plastic containers at room temperature (25-30^{o}C) for extractions. This prevented absorption of moisture from the atmosphere.

3.3 Plant extraction using organic solvent

1000 grams of the powdered root bark of *S. didymobotrya* and seeds of *M. oleifera* were weighed using a top loading balance (Denver instrument) and transferred to two five liter conical flasks (Pyrex) according to the procedure of McCloud, (2010). Then 2.5 liters of 99% methanol was added to cover the plant material under fume hood and left to soak in the solvent at room temperature for 5 days with shaking on rotary shaker (Digisystem laboratory Instrument incl) with a speed of 20 revolutions per minute. Extracts were

filtered through No. 1 Whatman filter paper on a Buchner funnel under vacuum pump (Vacuubrand GMBH). The filtrate was then rotar vapoured using a Rotary vapour pump (Eyela SB-1000) to concentrate the extracts (appendix11).

Plate 4. Extraction on rotary shaker (photo by Buyela Daniel).

3.4 *Ralstonia solanacearum* infected plant sample collection

A total of 10 diseased tomato plants were collected from four sites that is Maseno, Holo, Seme and Mariwa, where they were selected on the basis of tomato production and representative of Maseno division. From each plant, 5 samples were prepared. Field diagnosis of infected plant samples was done by critically observing the bacterial wilt symptoms which included wilting, yellowing of leaves, stunting of growth and observance of narrow dark stripes corresponding to the infected vascular bundles beneath the epidermis (Ahmed *et al*., 2013). Simple random sampling technique was used for collection of samples so as to eliminate selection biasandforaccuracy of representation.

Plate 5. *R.solanacearum* infected plant partshowing leaf epinasty (photo by Buyela Daniel)

3.5 Ralstonia solanacearum

3.5.1 Isolation of Ralstonia solanacearum

Collected tomato plant materials were surface sterilized with 1% Sodium Hypochlorite (NaOCl) solution for 1 to 2 min, followed by three repeated washings with distilled water and blot dried according to procedure by Singh *et al*.(2014). The plant sections (0.5–1 cm) were then plated onto 2, 3, 5 triphenyl tetrazolium chloride (Kelman's TZC agar) medium (glucose 10 g, peptone 10 g, casein hydrolysate 1 g, agar 18 g, distilled water 1000 ml). 5 ml of TZC solution filter sterilized was added to the autoclaved medium to give final concentration of 0.005%) according to the procedure by Seleim *et al*.(2014). The plates were incubated at 28^{o}C ± 2°C for 24–48 hr. The virulent colonies in the medium characterized by dull white colour, fluidal, irregularly round with light pink centres were further streaked on TZC medium to get pure colonies of the bacterium (Plate 6).

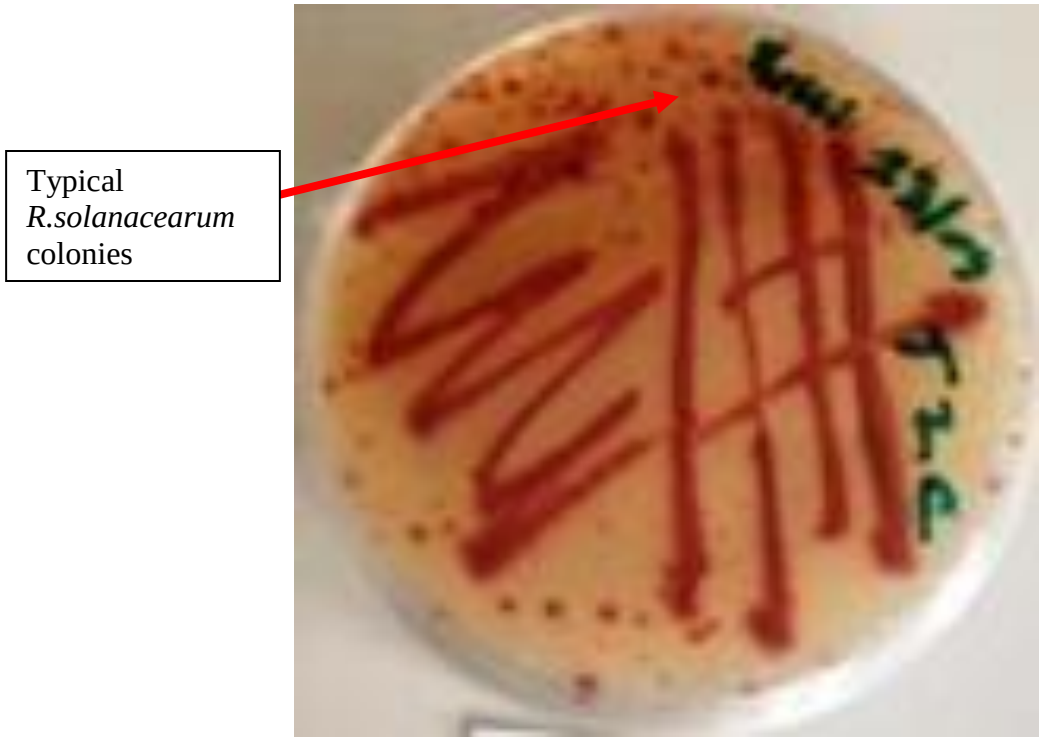

Plate 6. Appearance of *R.solanacearum* on TZC media.

3.5.2 Preservation of *Ralstonia solanacearum*

Two loopfuls of bacterium from 48 hr old colonies grown on Kelman's TZC Agar was transferred to 5 mL of sterile double distilled water in screw capped vials according to procedure by Grover *et al*.(2012). They were stored under refrigeration at 20^{o}C for maintenance of virulence. To revive an isolate, the stored bacteria were streaked on TZC Agar medium and well separated fluidal colonies were selected.

3.5.3 Preparation of *Ralstonia solanacearum*

A bacterial suspension was prepared by pouring sterile distilled water over 24 hour old bacterial growths on Nutrient agar slants.The suspension was then poured into a test tube and adjusted to optical density (O.D) 0.5 in Spectrophotometer (Novaspec II) in blue filter (425nm) to obtain a bacterial population of 1 x 10^{8} colony forming unit per millilter of the suspension according to the procedure by Mushore and Matuvhunye, (2013).

3.6.0 Profiling of Ralstonia solanacearum

3.6. 1 Morphological profiling of *Ralstonia solanacearum*

3.6.1.1 Simple staining of *Ralstonia solanacearum*

Fresh pure culture of the bacterium were stained by Ziehl's carbolfuchsin according to the procedure byWeller *et al*.(2015). Bacteria cells were spread over the central area of the slide by use of an inoculating loop. The slides were placed on a dryer with smeared surface upwards, and air dried for 30 minutes. The dried smear was then heat fixed. The smear was then covered with carbol fuchsin stain and heated until vapour just began to rise (about 60degree Celsius). The heated stain was allowed to remain on the slide for 5 minutes and then washed off with clean water. The smear was then covered with 3% v/v acid alcohol for 2-5 minutes. It was then washed well with clean water. The cells were then stained with methylene blue and viewed on a microscope (Nikon) under oil immersion for determination of size, shape and arrangement of cells.

3.6.1.2 Cultural studies of *Ralstonia solanacearum*

Cultural characteristics of the bacterium such as size and shape of colonies, colour of colonies, surface margin of colonies, and growth on nutrient agar (NA) and in nutrient broth were studied according to the procedure by Pawaskar *et al*.(2014).

3.6.2 Biochemical profiling of *Ralstonia solanacearum*

3.6.2.1 Gram stainingtest of *Ralstonia solanacearum*

Gram staining of *R.solanacearum* was done according to the procedure by Chaudhry and Rashid, (2011). A loop full of the bacterium was spread on a glass slide and fixed by heating on a very low flame. Aqueous crystal violet solution (0.5%) was spread over the smear for 30 seconds and then washed with running tap water for one minute. It was then flooded with iodine for one minute, rinsed in tap water and decolorized with 95% ethanol until colorless runoff. After washing, the specimen was counter-stained with safranin for approximately 10 seconds, washed with water, dried and observed microscopically at MgX10, MgX 40 and MgX100 using oil.

3.6.2.2 Potassium hydroxide test for *Ralstonia solanacearum*

Bacteria was aseptically removed from petri plates with an inoculating wire loop, placed on glass slide in a drop of 3% Potassium hydroxide (KOH) solution, stirred for 10 seconds and observed for the formation of slime threads according to the procedure by Antony, (2014). The colony's solubility in 3% KOH solution eliminated any possible confusion of the organism with other wilt causing pathogen of tomato.

3.6.2.3 Catalase oxidase test of *Ralstonia solanacearum*

Young agar cultures (18-24 hrs) and 3% hydrogen peroxide (H_2O_2) were used to observe production of gas bubbles according to the procedure by Chaudhry and Rashid, (2011). A loop full of bacterial culture was mixed with a drop of H_2O_2 on a glass slide and observed for the production of gas bubbles with unaided eye and under a dissecting magnification of 25X.

3.6.2.4 Gas production test for *Ralstonia solanacearum*

The ability of the culture to produce gas was tested by growing the organism in nutrient broth containing 2 per cent glucose, according to the procedure by Pawaskar *et al*., (2014). The medium was distributed in test tube containing inverted Durham's tube. These were sterilized by autoclaving at 15 lbs psi for 20 minutes. The tubes were inoculated with 0.5 ml of bacterial suspension and incubated at room temperature (28 ± 1 ^{0}C) for seven days. Gas production was indicated by air bubbles in the inverted Durham's tube.

3.6.2.5 Starch hydrolysis test for *Ralstonia solanacearum*

The ability of bacterium to hydrolyze starch was studied by growing on nutrient agar containing one per cent soluble starch according to the procedure by Kumar and Khare,(2015).The sterilized liquefied nutrient agar was poured to sterilized Petri plates and allowed to solidify. The culture was inoculated in the center of the plates and incubated for seven days at room temperature (28 ± 1 ^{0}C). The plates were then flooded

with Lugol'siodine (Iodine 1g, potassium iodide 2gm and distilled water 300 ml). Clear zone around bacterial culture was an indication of positive test.

3.6.2.6 Profiling of *R. solanacearum* isolates into biovars

Biovars of *R. solanacearum* strains were determined by standard procedure according to Grover *et al*. (2012). The following basal medium was used for biovar identification: $NH_4H_2PO_4$, 1.0 g; KCl,0.2 g; $MgSO_4.7H_2O$, 0.2 g; Peptone, 1.0 g; 1% (wv^{-1}) aqueous solution of bromothymol blue, 0.3 ml; agar, 1.5 g; distilled water, 1litre. The pH of the medium was adjusted to 7.1 with 40% (wv^{-1}) NaOH solution before addition of the agar. 5ml of a 10% (wv^{-1}) pre-sterilized solution of the sugars (lactose, maltose and cellobiose) and sugar alcohols (mannitol, sorbitol and dulcitol) was added to 45 ml of molten cooled basal medium separately. 200µl of these media was then dispensed into each test tube. Hayward's medium without a carbon source and un-inoculated test tubes served as control. Each test tube was inoculated with 3 µl of a 2 x 10^9 CFU ml^{-1} cell suspension prepared from overnight Kelman's TZC broth culture. The cultures were incubated at 28±1^{o}C and examined at 3, 7 and 14 days for change of pH (yellow colour). Positive cultures changed the culture medium from green to yellow. Each test was replicated three times. The arrangement was in completely randomized design.

3.6.3 Landrace Identification

The landraces of *Ralstonia solanacearum* were identified by pathogenicity test on wide host range (Dhital *et al*., 2001). Seedlings of eggplant, tomato, potato, tobacco, peanut and pepper were raised in tray. One month (30 days) old seedlingswere inoculated by soil inoculation method. The incubated plants were then kept in the greenhouse until symptoms development.

3.7 Effects of plant extract on *R. solanacearum*

Bacteriostatic activity of the extracts was determined by the disc diffusion method on Mueller Hinton agar according to the procedure by Mushore and Matuvhunye (2013).The experimental design was complete randomized design (CRD) with three replications. An overnight culture of the bacterium was diluted to 10^5 cells/ml using a spectrophotometer

(Novespec II) at a wavelength of 625nm. One milliliter of the bacterial suspension was introduced into sterile petri plate and 20 ml of Mueller Hinton agar at 40ºC was poured into the inoculated plates. The plates were allowed to cool and solidify. A sterile filter disc (Whatmann No. 9) soaked in the different crude extracts with a concentration of 15%, 10%, 5% and 2.5% respectively were picked with sterile forceps and placed on the surface of a solid inoculated agar plates. The plates were incubated at 37ºC for 24hr. This was carried out in triplicates. The petri plates were then assessed for bacteriostatic activities. The control consisted of the water alone and served as the negative control.

3.8 Pathogenicity test of *Ralstonia solanacearum* on tomato seedlings

Pathogenecity test was done according to the procedure by Chandrashekara *et al*.(2012). A sufficient volume of soil autoclaved at 121°C for 30 min was used to fill pots 18cm tall and 30×13 cm diameter to a height of 15 cm. Five tomato seeds were then planted in each pot. Four isolates from all sites were replicated three times with a control which consisted of only water. The tomato seedlings were allowed to grow for 20 days under natural greenhouse conditions (Day Temperatures 25^0C-40^0C and Night Temperatures 20^0C-30^0C, 14/10h photoperiod and humidity 70-90%) and watered twice a day (morning and late afternoon) using micro-sprinklers.

The bacterial suspension was adjusted to a concentration of $5x10^8$colony forming unit per milliliter (cfu/ml) using spectrophotometer (Novespec II) readings at a wave length equivalent to A600 nm = 0.8 to 1.0, which was about 10^8 colony forming units (cfu) per ml. Twenty-day-old seedlings of tomato were pulled out gently, washed free of soil and a few tertiary roots were clipped with sterilized scissors and dipped in the bacterial culture for 10 minutes (Plate 7). Three inoculated seedlings were transplanted to each of the pots containing sterilized soil. Each treatment was replicated three times and arranged in a completely randomized design. Inoculated plants were observed after every seven days for evaluation of pathogenicity for four weeks.

Plat 7. Tomato seedlings with clipped roots dipped in the *R.solanacearum* bacterial culture to initiate infection (photo by Buyela Daniel).

Disease severity was assessed at weekly interval for four weeks following the scale of Nguyen and Ranamukhaarachchi, **(**2010)

1- No symptoms

2-Two leaves wilted

3- Three leaves wilted

4- Four or more leaves wilted

5- Plant dead.

Disease index (%) = $\lceil \sum (ni\ Xvi) / (V \times N) \rfloor \times 100$.

Where the *ni* =number of plants with the respective disease rating;

vi=disease rating;

V=the highest disease rating (5); and

N=the number of plants observed.

The most virulent strains were used to test the effect of plant extracts on *Ralstonia solanacearum.*

3.9. Effects of plant extract on tomato wilt

In vivo evaluations were conducted in a green house to assess the screened plant extracts (plate 8) using tomato seedlings, according to the procedure by Almoneafy *et al*. (2012), using susceptible tomato variety Rio Grande. The soil that was used in these studies was obtained from the top layer (first 15 cm, corresponding to the area of the roots of tomato plants). A sufficient volume of soil was autoclaved at 121°C for 30 min which was then used to fill pots 18cm tall and 30×13 cm diameter to a height of 15 cm, to which 20ml of pathogen suspension was added as Inoculum. One week after inoculation, 20-day-old seedlings of wilt-susceptible tomato was transplanted into the pots, grown at 28°C and watered twice daily (morning and late afternoon) using micro-sprinklers. When these plants senescenced due to bacterial wilt disease, a set of new plants was re-transplanted into the same pots. When the second set of plants died, the soils were considered to be sufficiently infested by the pathogen. These pots were then used for testing the plant extracts capacity of the two plants to control the disease.

To test the selected extracts, three 20-day-old tomato seedlings were planted into each of the pots and grown under the same conditions described herein. Selected plant extracts 15% were applied individually to each pot seven days after transplanting, and arranged in a completely randomized design with three replicates for each treatment. These extracts

were again appliedafter a week. In addition, two control treatments were included: one with no pathogen nor extract, and the other with pathogen, but without an extract.

Plate 8.Tomato plants showing *R.solanacearum* symptoms; wilting, leaf epinasty and yellowing of leaves (photo by Buyela Daniel).

Plants were examined for disease incidence starting from one week after transplanting and continuing until the end of harvesting time. Disease incidence was assessed using the 1-5 (0-5) scolding scale with modifications suggested for bacterial wilt by Nguyen and Ranamukhaarachchi, **(**2010):

1. No visible symptoms; 1-10

2.1-25% of the plants showing wilting; 11-30

3. 26-50% wilting; 31-60

4. 51-75% wilting; more than 60 but less than 100

5. More than 75% wilting.

Disease index (%) = $\lceil \sum (ni\ Xvi) / (V \times N) \rfloor \times 100$.

Where the *ni*=number of plants with the respective disease rating;

vi=disease rating;

V=the highest disease rating (5); and

N=the number of plants observed.

Based on disease index collected data, two parameters; disease incidence and control efficacy of extracts was estimated according to the formulae of Xu*et al*.(2009):

%Disease incidence= Disease index × number of diseased plants in this index x 100%

Total number of plants investigated × the highest disease index

%Extract control efficacy = D.I of control – D.I of extract treated groupx 100%

Disease incidence of control

Where D.I= Disease index.

3.10 Determination of plant growth parameters

3.10.1 Root and Shoot length

Tomato plants were removed from the greenhouse, and gently washed to remove all soil, according to the procedure by Riaz *et al*.(2013). The plants were then spread on paper for measurement of root and shoot length (cm) using a ruler.

3.10.2 Fresh and dry shoot and root weight

Plants were cut into roots and shoots, and fresh root weight (gm) and fresh shoot weight (gm) was taken using a weighing balance (Denver instrument) and recorded, according to procedure by Riaz *et al*. (2013). The roots and shoots were then dried for 72 h at 60°C, and the dry weight was determined using weighing balance (Denver instrument) and recorded. The plants were evaluated for root dry weight and shoot dry weight.

3.10.3 Determination of number of fruits

To obtain total yield, the number of fruits per plant were counted and average yield per plant was determined according to procedure by Lolaei.(2012).The fruits were gathered as soon as they started to ripe. Collection of fruits continued until the end of the harvesting period (three months).

3.11 Data analysis

Statistical analysis of data was conducted using SAS 9.1 package. Data on effect of plant extracts on in *vitro* growth of *R.solanacearum*, effect of *R.solanacearum* on tomato plants, disease severity, plant height, and yields in the field were analyzed using analysis of variance (ANOVA). Means that were considered significantly different ($P \leq 0.05$) were separated using Turkeys LSD.

CHAPTER FOUR
RESULTS

4.1 Isolation, identification and profiling of *Ralstonia solanacearum*

4.1.1 Isolation of *Ralstonia Solanacearum*

Isolation of the bacterium was done from tomato (*S. lycopersicum*), plants showing typical symptoms of bacterial wilt. Such signs were: lower leaves turning pale yellow, loss of leaf turgidity followed by drooping of leaves and sudden wilt of the plants (plate 9). The vascular bundles of the infected plants showed brown discoloration (plate 10). Infected plants showed milky white bacterial streaming from diseased stem and thus were ooze test positive (plate11, Table 4.1).

Plate 9. Tomato plant showing symptoms of *R.solanacearum* infection

Plate 10. Longitudinal section of *R.solanacearum* infected tomato stem with vascular bundles showing brown discoloration.

Plate 11. Milky white bacteria streaming from diseased stem suspended in water.

4.1.2 **Morphological Profiling** of *Ralstonia Solanacearum*

Fluidal pinkish red centered colonies of typical *R. solanacerum* were observed on TZC media (Plate 12, 13 and14).Virulent colonies appeard white with pink colour at the centre. The different colony morphology was observed, colonies were irregular, white and fluidal (plate 15 and 16) on nutrient agar media.

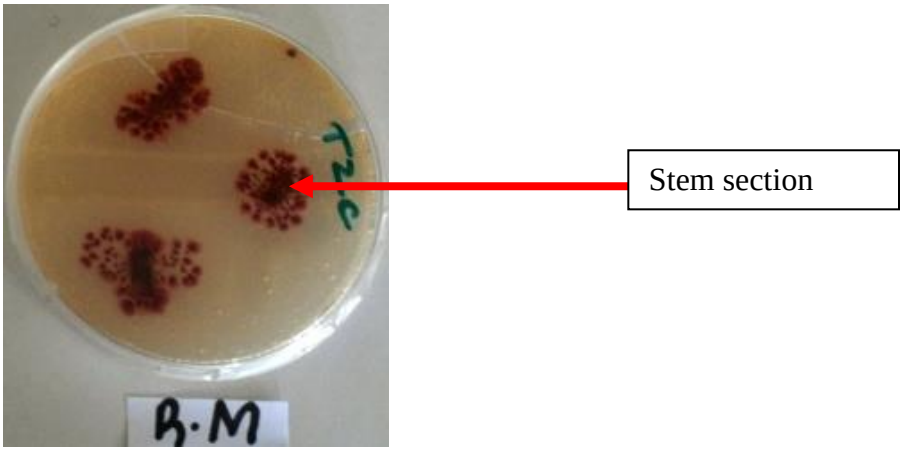

Plate 12. Tomato stem sections isolation on TZC medium.

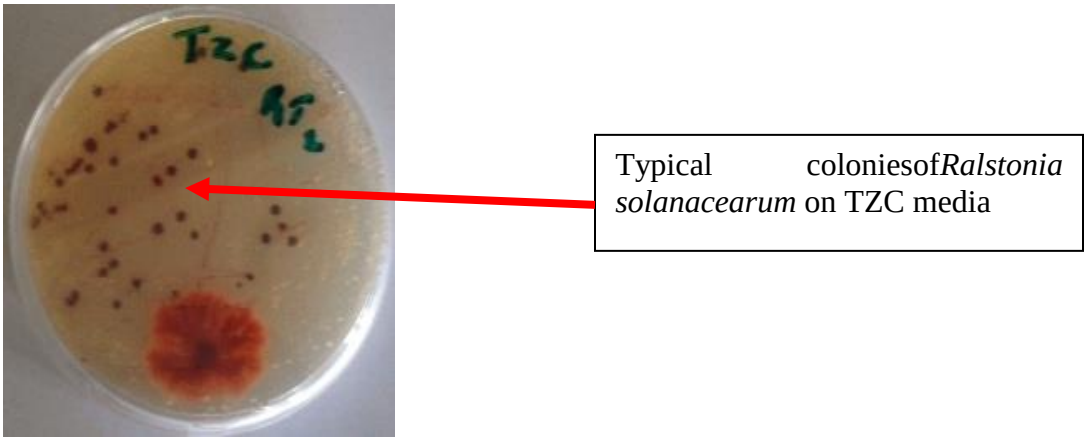

Plate 13. Typical colonies of *R.solanacearum* on TZC medium**.**

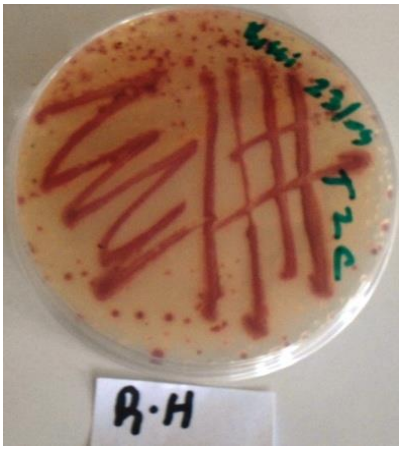

Plate 14. Confirmation of *Ralstonia solanacearum* onTZC medium

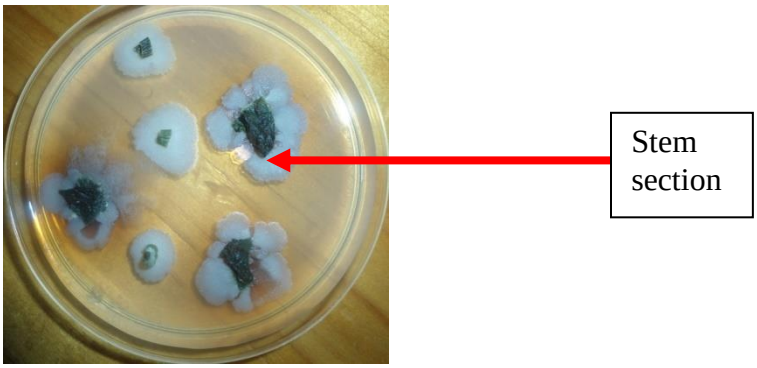

Plate 15. Tomato stem sections isolation on Nutrient Agar medium.

Plate 16. Appearance of of *Ralstonia solanacearum* on Nutrient Agar medium showing whirling pattern.

Table 4.1. Morphological profiling of four isolates of *R. Solanacearum*on TZC media

Sno.	Isolate	Morphology on TZC medium
1	Maseno	irregular with smooth margin, slimy dull white colonies with pink to red center
2	Mariwa	moderately fluidal, irregularly shaped, convex, dull whitecolonies with pink colored center and bluish margin
3	Seme	irregular with smooth margin, slimy dull white colonies with pink to red center
4	Hollo	irregular with smooth margin, slimy dull white colonies with pink to red center

4.1.3 Biochemical profilingof *Ralstonia Solanacearum*

4.1.3.1 Gram's Stain of *R.solanacearum*

The microscopic results showed that all the isolates of *R.solanacearum* did not retain violet colour that is the isolates retained counter stain (pink colour) (plate 17, table 4.2). This was an indication that all isolates of *R. solanacearum* representing each group are gram negative and straight or curved rod shaped.

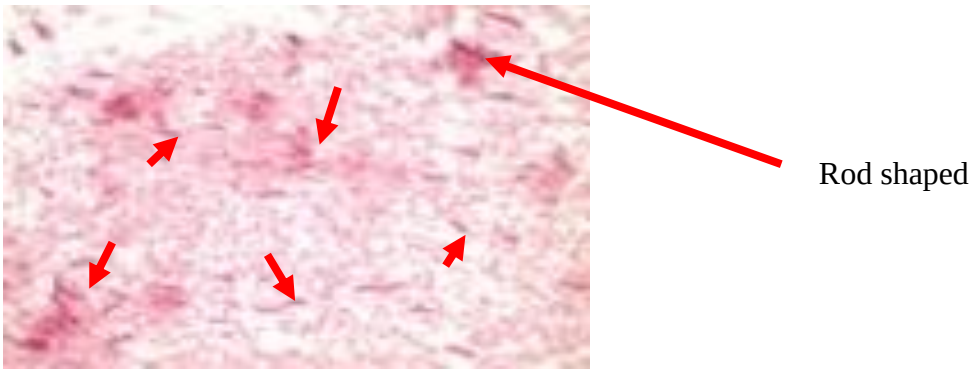

Plate 17. Appearance of *Ralstonia solanacearum* cells after gram staining (Mgx100)

4.1.3.2 Potassium Hydroxide solubility test

The Gram negative test of *R.solanacearum* was also confirmed by Potassium hydroxide solubility test.An elastic thread or viscous thread was observed when the loop was raised from the bacterial solution by toothpick, a few centimeters from glass slides for all the isolates from the four sites indicating that all groups of *R.solanacearum*isolates were Gram negative (Table 4.2).

4.1.3.3 Catalase oxidase test

All the isolates tested produced gas bubbles during these tests (Table 4.2), indicating that these might be *Ralstonia solanacearum*.

4.1.3.4 Gas production

All the isolates tested produced gas from glucose within eighteen hours of incubation (Plate18, Table 4.2).

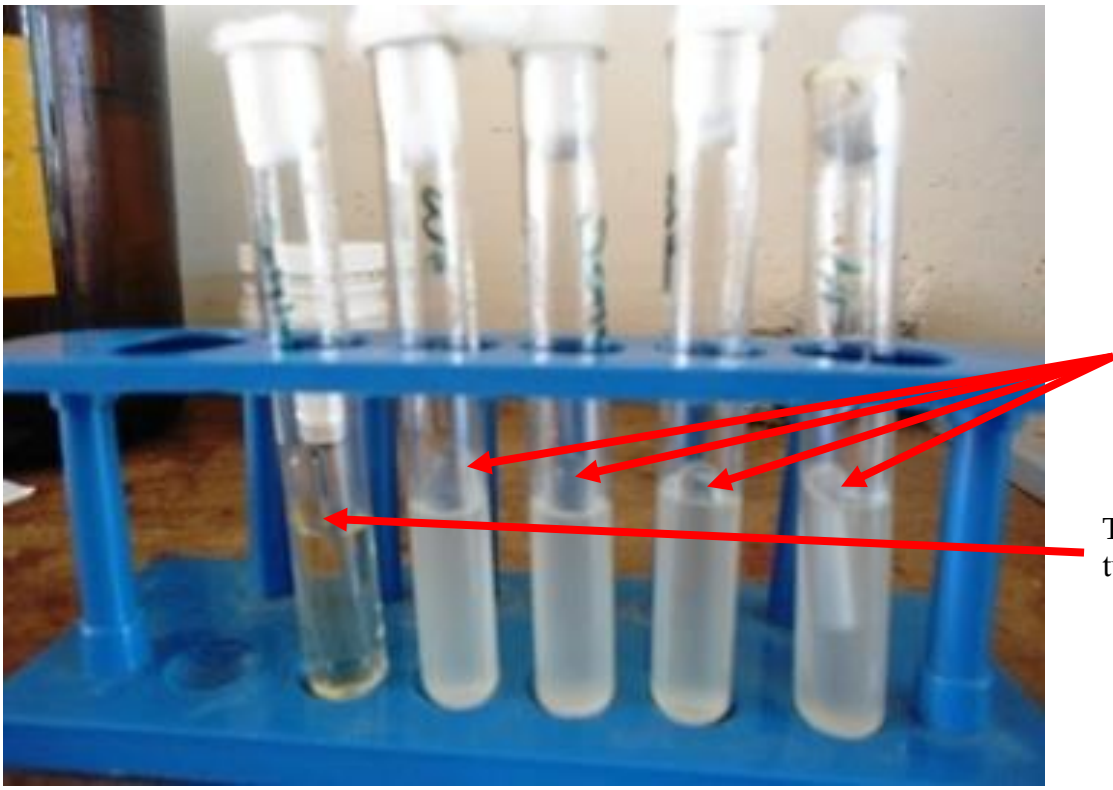

Plate 18.Gas production by *R.solanacearum* isolates.

4.1.3.5 Starch hydrolysis

Starch hydrolysis test of the bacterium showed that all the isolates from the four sites were unable to hydrolyse starch (Plate 19, Table 4.2); no clear zone surrounding the bacteria growth was observed when plates were flooded with Lugol'siodine (IKI) solution.

Plate 19. Starch hydrolysis

Table 4.2 Profiling of *Ralstonia solanacearum* strains isolated from bacterial wilt infected tomato plants in Maseno region.

TEST	Maseno isolate	Mariwa isolate	Seme isolate	Holo isolate
Gram's Stain	–	–	–	–
Potassium Hydroxide Solubility Test	+	+	+	+
Catalase oxidase test	+	+	+	+
Gas production	+	+	+	+
Starch hydrolysis	–	–	–	–
Ooze test	+	+	+	+
Growth on Tzc	+	+	+	+

Legend: += Positive reaction;- =Negative reaction

4.1.3.6 Profiling of *R. solanacearum* isolates into biovars

The oxidation reaction was indicated by the change of the colour of Hayward's medium. The results revealed a change of color from green to yellow color indicating the oxidization of sugars by bacterial isolates (plate 20). All tested strains utilized glucose and fructose invariably. However marked differences were observed in the ability of the strains to oxidize three dissacharides (Lactose, Celiobiose and Maltose) and three sugar alcohols (Mannitol, Sorbitol and Ducitol). Three strains were classified as biovar III and one strain as biovar I based on Haywards classification scheme (Hayward 1964). Biovar III strains (Maseno, Seme and Holo) oxidized all of the sugar and sugar alcohols while biovar I strain (Mariwa) didn't oxidize any of the disaccharides or sugar alcohols even after five weeks of incubation (Table 4.3). No reaction was produced in inoculated media without carbohydrate source.

Table 4.3 Biochemical differentiation of *Ralstonia solanacearum* into biovars

ISOLATE	Glucose	Fructose	Lactose	Celiobiose	Maltose	Mannitol	Sorbitol	Ducitol	Biovar
Distilled water(control)	-	-	-	-	-	-	-	-	-
Maseno	+	+	+	+	+	+	+	+	III
Mariwa	+	+	-	-	-	-	-	-	I
Seme	+	+	+	+	+	+	+	+	III
Holo	+	+	+	+	+	+	+	+	III

Legend: += Positive reaction or change of colour;

-= Negative reaction or no change of colour

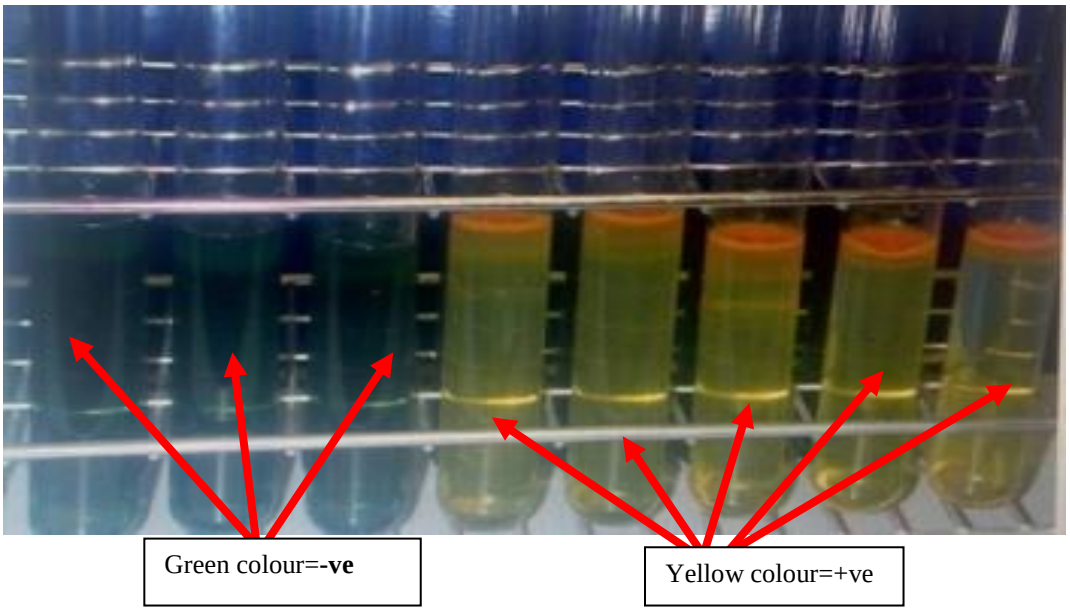

Plate 20. Carbohydrate utilization test for strains *of R.solanacearum*.

4.1.4 Designation of landraces of *R.solanacearum* in Maseno region

The landraces of *R. solanacearum* were identified by pathogenicity tests in wide host which included; tomato, Potato, Pepper tobacco, peanut and eggplant (Bock *et al*., 2014). Initial symptoms of wilting in succeptible hosts appeared 3-4 days afterstem inoculation.The symptoms consisted of wilting of the inoculated leaf and eventually the whole plant wilted.The result of the pathogenicity test showed that all of the groups of *R.solanacearum* isolates tested in the study were able to cause wilt symptom in inoculated tomato, Potato, Pepper, eggplant, tobacco and peanut plants.The four strains (Maseno, Mariwa,Seme and Holo) were highly virulent on potato and tomato plants but were moderate to slight virulent on eggplant and Pepper after four weeks of inoculation (Table 4.4).

The four strains (Maseno, Mariwa, Seme and Holo) were highly virulent on potato and tomato plants but were moderate to slight virulent on pepper and low virulent on eggplant after four weeks of inoculation. But other hosts such as tobacco and peanut did not show wilting symptoms.Therefore, all the four strains from Maseno region had characteristic of race 3 with a limited host range on potato, tomato and a few other hosts (Table 4.4). The

limited host range is the characteristic of landrace 3 of *R.solanacearum* (Buddenhagen *et al*., 1962).

Table 4.4. Pathogenicity test on potato, tomato, pepper and egg plant and classification of *Ralstonia solanacearum* strains in Maseno region

ISOLATE	TOMATO	POTATO	PEPPER	EGGPLANT	TOBACCO	PEANUT
Distilled water	0	0	0	0	0	0
MASENO	H	H	M	L	0	0
MARIWA	H	H	M	L	0	0
SEME	H	H	M	L	0	0
HOLO	H	H	M	L	0	0

Legend: Average disease indices of 9 plants at 28 days after inoculation and rating scales (Dhital*et al*., 2001) were as followed: H, High (disease index 4.1 to 5.0); M, Moderate (2.6 to 4.0); L, Low (1.1 to 2.5); and, 0, none (1.0)

4.2 Incidence of Ralstonia Solanacearum

4.2.1 Response of tomato to infection by *Ralstonia solanacearum*

Typical bacterial wilt symptoms were observed one week after transplanting. The cause of wilt was confirmed to be *R. solanacearum* by looking for bacterial ooze (Plate 11) and isolating the bacterium from wilted plants followed by culture on TZC medium. The tomato cultivar used, Rio Grande, was very susceptible to bacterial wilt. Disease incidence progressed rapidly beginningweek one after transplanting.

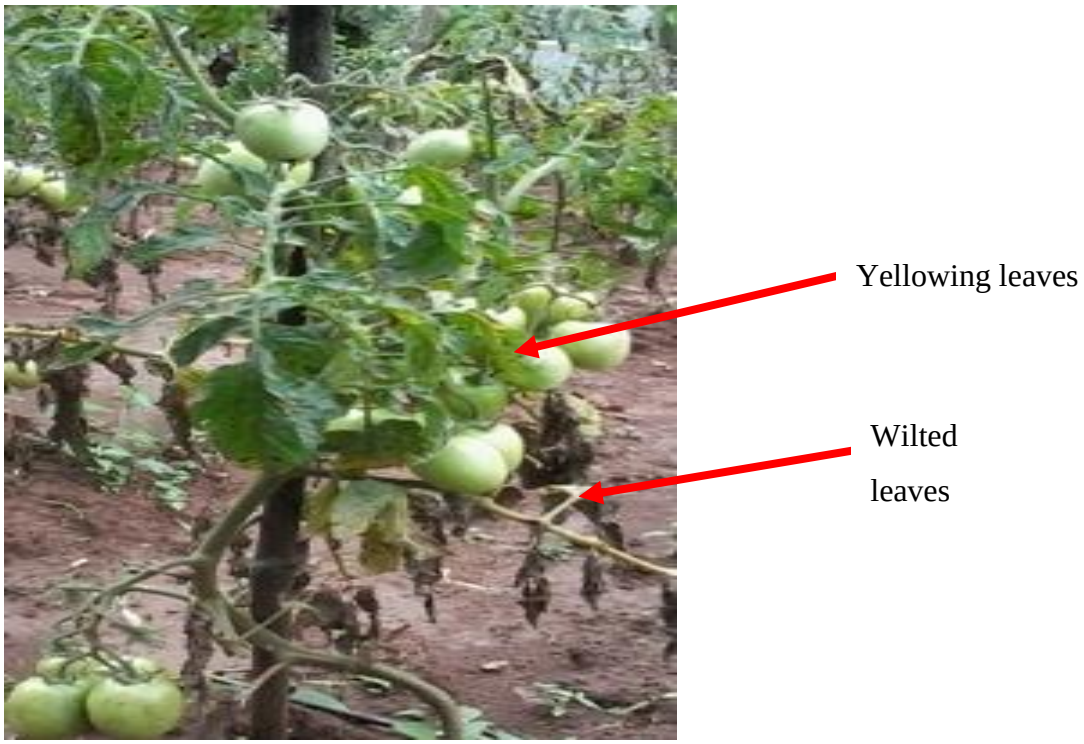

Plate 21. Amature tomato plantgrown under greenhouse conditions showing symptoms of bacteria wilt (photo by Buyela Daniel).

Four isolates of *Ralstonia solanacearum* were tested with tomato plants under greenhouse conditions. All isolates were pathogenic on tomato plants and produced typical symptoms of bacteria wilt (plate 21). Tomato crops responded differently to infection by *R. Solanacearum* from different locations that is Maseno, Seme, Mariwa and Holo (Fig 41). A significant difference ($P \leq 0.05$) in the incidence of bacteria wilt disease as per isolate was established from the control (Table 4.5). Holo isolate was the most virulent as observed from the mean disease index with amean of 56.65, followed by Maseno isolate with a mean of 52.175, Mariwa isolate with a mean of 48.225 and Seme isolate had a mean of 34.375. Disease index increased from week one to week four in all the isolates. A significant increase ($P \leq 0.05$) in the incidence of bacteria wilt disease on weekly basis was established between week one and week two but there was no significance difference ($P \geq 0.05$) between week three and four (Table4.6). On Overall, weekly disease incidence for the entire isolates week one had the least incidence with a

mean of 16.54, week two had a mean of 33.34, week three had a mean of 45.70 and week four had a mean of 57.56.

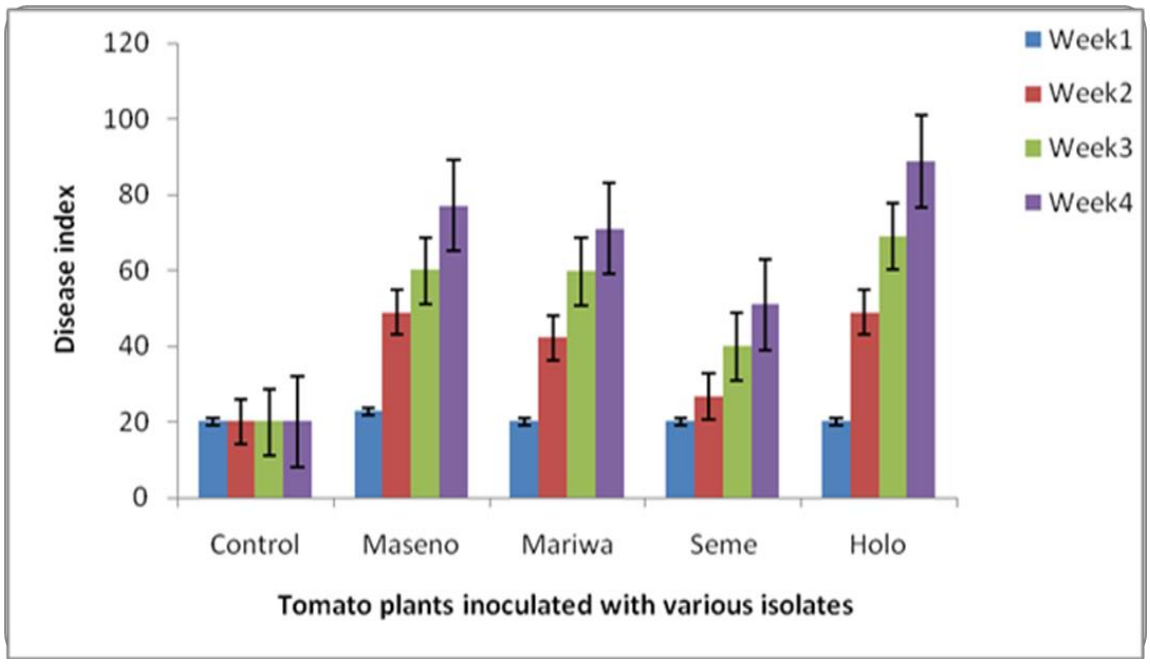

Figure 4.1 Trend of severity of tomato plants to bacteria wilt,error bars indicate standard mean error of three replicates

Table 4.5 Mean disease index as per isolates

Isolate	**Mean disease index**
Maseno	52.18 a
Mariwa	48.23 a,b
Seme	34.38b
Holo	56.65 b
Distilled water (Control)	0.00c
LSD	17.69
P.Value	0.0001
%CV	29.99

Legend: Means followed by different letterdown the column are statistically different at P≤0.05 by Fisher's protected least significant difference test.

Table 4.6 Weekly mean disease index

Week	Mean disease index (%)
1	16.54c
2	33.34b
3	45.70ab
4	57.56a
LSD	15.82
P.Value	0.0001
%CV	30

Legend: Means followed by different letter down the column are statistically different at P≤ 0.05 by Fisher's protected least significant difference test

4.3. Effects of plant extract on test bacteria

4.3.1 Inhibitory effect of different concentrations of *Moringa oleifera* and *Senna ditymobotria* plants extracts on *Ralstonia solanacearum*

Bacteriastatic activity results (Table 4.7) showed that *R. solanacearum* were sensitive to *Moringa oleifera* and *Senna ditymobotria* plant extracts at 15%. All the extracts showed antimicrobial activity by developing clear zones of inhibition (plate 22 and 23). The presence of inhibition zones depicted the antibacterial activity of *Moringa oleifera* and *Senna ditymobotria* plant extracts. There were no inhibition zones in the controls which consisted of sterile distilled water (plates 24 and 25).Based on the four *R. solanacearum* isolates *M. oleifera* extract had a higher zone of inhibition for Maseno isolate at (8.8) as compared to *S. ditymobotria*at (8.7) (table 4.7). *Senna ditymobotria* had higher means of inhibition for all the other three isolates that is Mariwa, Seme and Holo (table 4.7)

Table 4.7 Mean diameterof zones of inhibitionof *M.oleifera* and *S.didymobotria* for the four isolates

	Senna didymobotria	***Moringa oleifera***
ISOLATE	**INHIBITION means(mm)**	**INHIBITION means(mm)**
Maseno	8.71b	8.82b
Mariwa	8.78b	7.91c
Seme	8.49b	7.80c
Holo	10.84a	9.55a
LSD	0.6203	0.6598
P.value	0.0001	0.0001
%C.V	16.14784	18.55546

Legend: Means followed by different letter down the column are statistically different at P≤ 0.05 by Fisher's protected least significant difference test.

Senna ditymobotria root extract did not have a significant difference (P≥0.05) for Maseno, Mariwa and Seme isolates but there was a sigficance different (P≤ 0.05) for Holo isolate (Table4.7). For *Moringa oleifera* seed extract there was a significant difference (P≥0.05 between Maseno and Mariwa, Maseno and Seme, and Maseno and Holo isolates, but did not show significance difference (P≥0.05) between Mariwa and Seme isolates (Table4.7). The overall results showed that *Senna ditymobotria* performed better than *Moringa oleifera* in inhibiting the growth of the four *R.solanacearum* isolates (Table 4.8). There wassignificance difference (P≤0.05) between *M.oleifera* and *S.didymobotria.*

Table 4.8 Comparative effect of *M.oleifera* and *S.didymobotria* on *R.solanacearum*

Treatment	**Inhibition zones (mm)**
M.oleifera seed extract	8.5222b
S.didymobotria root extract	9.2056a
LSD	0.3206
P.value	0.0001

Legend: Values in the same column not sharing the same letter differ significantly at P≤0.05

Plate 22 *S.ditymobotria* inhibition Zone at 15% of the extract

Plate23 *M.oleifera* inhibition zones at 15% of the extract

No clear zone of inhibition around disc

Plate 24 *S.ditymobotria* control

No clear zone of inhibition around disc

Plate 25 *M.oleifera* control

Analysis of variance showed that the four different concertrations (2.5%, 5%, 10% and 15%) of *S.didymobotria* and *M.oleifera* plant extracts exhibited highly significant (P≤0.05) difference on their effect against growth of *R.solanacearum* (Table4.9). In both cases, antibacterial activity of the test materials increased as their concentration increased but *S.didymobotria* was more effective than *M.oleifera*.

Table 4.9 Comparative effect of Mean diameter of zones of inhibition of *M.oleifera* and *S.didymobotria* on *R.solanacearum*

Treatment	**Inhibition means(mm)**				
	0%	**2.5%**	**5%**	**10%**	**15%**
M.oleifera root extract	0.0000a	8.28b	10.11a	12.03b	12.19b
S.didymobotria seed extract	0.0000a	9.78a	10.61a	12.53a	13.11a
LSD	0.5069	0.5069	0.5069	0.5069	0.5069
P.value	0. 0001	0. 0001	0. 0001	0. 0001	0. 0001
%C.V	17.38336	17.38336	17.38336	17.38336	17.38336

Legend: Means followed by different letter in the same column are statistically different at P≤0.05 by Fisher's protected least significant difference test.

4.4. Effects of plant extract on tomato wilt

4.4.1 Effect of *S.didymobotria* root extract and *M.oleifera* seed extract on disease incidence

At the beginning of the experiment (week one to week four), the effect of *S.didymobotria* and *M.oleifera* treatments did not differ significantly (P≥0.05) on the incidence of bacterial wilt (BW) among the different test isolates. However, the treatments effectively (P≤0.05) reduced BW incidence towards the middle (week five and six) and end of the experiment (Table 4.10). At the middle of the experiment (week five) however, the two treatments significantly (P≤0.05) reduced BW incidence relative to control as indicated in table 4.10.Throughout the experiment, the interaction effect between the crops and the treatmentswas significant (P≤0.05).There was a significant difference (P≤0.05) on the incidence of BW between the treatments and the control during the same period as shown in table 4.10. *Moringa oleifera*seed extract treatments recorded higher disease incidence as compared with *Senna didymobotria* root extract.

The tomato cultivar used, Rio Grande, was very susceptible to bacterial wilt. Disease incidence progressed rapidly beginning week one after transplanting. Final disease incidence reached 100% after five weeks time in pots without plant extracts, indicating high inoculum pressure (Table 4.10). Disease incidence stabilized five and continued until harvest.

Senna didymobotria application provided effective protection against bacterial wilt, with only 32.9% of plants wilting, which was significantly lower than the untreated control (82.5%) (Table 4.11). *Moringa oleifera*extract also reduced bacterial wilt incidence significantly (P≤0.05) compared with the untreated pots; however, it was less effective than *Senna didymobotria*, with approximately half of the plants wilting in *Moringa oleifera* seed extract treated pots (49.5)(Table 4.11). Both *Senna didymobotria* root extract and *Moringa oleifera* seed extract treatments significantly protected tomato plants from severe yield loss as a result of the disease.

Table 4.10 Weekly disease incidences in tomato plants after treatment with *senna didymobotria* and *Moringa oleifera* plant extracts

Week	1	2	3	4	5	6	7	8	9	10	11	12
Treatment												
Senna didymobotria	0d	20b	20c	34.75b	40c	40c	40c	40c	40c	40c	40c	40c
Moringa oleifera	0d	24b	38.25b	51.25c	60b	60b	60b	60b	60b	60b	60b	60b
Distilled water (-Ve control)	0d	0d	0d	0d	0 d	0d	0d	0d	0d	0d	0d	0d
R.solanacearum (+ve control)	0d	53a	56.50a	80a	100a	100a	100a	100a	100a	100a	100a	100a
LSD	8.033	8.033	8.033	8.033	8.033	8.033	8.033	8.033	8.033	8.033	8.033	8.033
P.value	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001
%C.V	27.9	27.9	27.9	27.9	27.9	27.9	27.9	27.9	27.9	27.9	27.9	27.9

Legend: Means followed by different letter in the same column are statistically different at P≤0.05 by Fisher's protected least significant difference test. *S.didymobotria and M.oleifera* are plant extracts in milimetres.

Table 4.11 Comparative effect of control efficacy of *M.oleifera* seed extract and *S.didymobotria* root extract on *R.solanacearum* on tomato plants

Treatment	Disease incidence (%)
Senna didymobotria root extract	32.9 c
Moringa oleifera seed extract	49.5 b
Distilled water(Negative control)	0.0d
R.solanacearum (Positive control)	82.5 a
LSD	4.6265
P.value	0.0001
%C.V	27.86342

Legend: Means followed by different letter down the column are statistically different at P≤0.05 by Fisher's protected least significant difference test.*S.didymobotria and M.oleifera* are plant extracts in milimetres.

4.4.2 Effects of *S.didymobotria* root extract and *M.oleifera* seed extract on plant growth parameters

4.4.2.1 Effect of *S.didymobotria* root extract and *M.oleifera* seed extract on shoot and root biomass

The effects of type of botanicals on above-ground biomass (fresh and dry weight) per plant are presented in Table 4.12.There was no significance difference (P≥0.05) between *S.didymobotria* root extract (62.23b) and *M.oleifera*seed extract (51.73b) at 15% for above-ground fresh weight (AGFW) but there was a significance difference (P≤0.05) for the above-ground dry weight (AGDW) between *S.didymobotria* (12.10a) root extract *and M.oleifera* seed extract (8.95 b). A significance difference (P≤0.05) was established for the below ground fresh weight (BGFW) and below ground dry weight (BGDW) between *S.didymobotria* root extract (fresh weight 14.00a, dry weight 6.32a) and *M.oleifera* seed extract (fresh weight 8.54b, dry weight 2.75b). Plants treated with *S.didymobotria* root

extract had no significance difference (P≥0.05) for AGDW, BGFW and BGDW with healthy control plants. But, plants treated with *M.oleifera*seed extract were less in their shoot fresh and dry weights than healthy control plants (Table4.12).

4.4.2.2 Effect of *S.didymobotria* root extract and *M.oleifera* seed extract on shoot height and root length

There was significant difference (P≤0.05) in shoot height observed among the threetreatments (*S. didymobotria* 78.87a, *M.oleifera* 68.60b and unifected Control 60.00c). Tomato plants treated with *S. didymobotria* plant extracts were the tallest (78.9cm) followed by *M.oleifera* plant extract (68.6cm) and unifected control (60.cm) respectively as indicated in Table 4.12. Significant interaction effect (P≤0.05) on the shoot height was established between the various treatments and the tomato plants.

There was a significant decrease (P≤0.05) in root length with roots of unifected tomato plants (21.7a), *S. didymobotria* root extract treated roots (19.06b) and *M.oleifera*seed extract treated roots (16.45c).

4.4.2.3 Effect of *S.didymobotria* root extract and *M.oleifera* seed extract on the number of fruits

For the number of fruits there was a significance difference between the three treatments (water 27.3a, *Senna didymobotria* 19.9b and *M.oleifera* 17.9c). The results clearly indicated that *R. solanacearum* affected fruit production.

Table 4.12Effect of, *S. didymobotria* and *M.oleifera* plant extracts at 15% on fresh and dry (stem and root weight), shoot height, root length and yield of tomato under greenhouse conditions after plants were inoculated with*R.solanacearum*

Treatment	**Fresh stem weight**	**Dry stem weight**	**Fresh root weight**	**Dry root weight**	**Shoot height**	**Root length**	**Number of fruits**
Senna didymobotria	62.23b	12.10a	14.00a	6.32 a	78.87 a	19.06 b	19.93 b
Moringa oleifera	51.73b	8.95b	8.54 b	2.75b	68.60 b	16.45c	17.93 c
Control	76.89a	12.21a	15.34a	7.23a	60.00c	21.67a	27.33a
LSD	13.41	2.23	5.02	2.45	4.79	2.41	1.83
P.value	0.0751	0.0689	0.7151	0.2925	0.0001	0.0130	0.0001
%C.V	28.3	27.0	53.4	60.4	9.3	16.9	11.3

Legend: Means followed by different letter in the same column are statistically different at P≤ 0.05 by Fisher's protected least significant difference test. *S.didymobotria and M.oleifera* are plant extracts in milimetres.

There was no relationship between *in vitro* inhibition of *Ralstonia* strains as measured by zones of inhibition and control efficiency in tomato plants challenged with *R. solanacearum*. *Senna ditymobotria* was the most antagonistic towards *R. solanacerum*strains *in vitro* and also effective in reducing bacterial wilt when used to treat tomato plants later challenged with the pathogen.

CHAPTER FIVE
DISCUSSIONS

5.1 Isolation, identification and profiling of *Ralstonia solanacearum*

5.1.1 Isolation of *Ralstonia Solanacearum*

Stem pieces of wilted tomato plants when dipped in water showed a continuous white streaming of bacterial ooze.The vascular bundles of the infected plants showed brown discoloration. When fragments were cut from plant samplesin which symptoms of disease were evident, fluidal pinkish red centered colonies, typical of *Ralstoniasolanacearum* were observed on TZC media. The results are similar to those reported by Zubeda and Hamid (2011).Virulence of an isolate can be determined on the basis of colony colour on TZC media. Virulent wild type colonies are usually large, elevated, fluidal and either entirely white or with a pale red center.

5.1.2 Morphological Profiling of *Ralstonia Solanacearum*

Cultural traits on different media are important tools for identification of *Ralstoniasolanacearum*. All four strains of *R. solanacearum*from Maseno region produced fluidal and irregular colonies with pink or light red at centers on TZC medium at 30^{o}C after 48 h of incubation. Present results agreed with those reported by Popoola *et al*., (2015), Rahman *et al*., (2013), Seleim *et al*., (2011) and Maji and Chakrabartty, (2014). This is the characteristic feature of *R. solanacearum*. According to Rohini *et al*. (2017), triphenyl tetrazolium chloride (TZC) medium is used to distinguish *R. solanacearum* among other bacteria during isolation. Also when TZC medium is used with *R. solanacearum*, it shows the difference between avirulent colonies that look dark red from virulent colonies described herein (Rahman *et al*., 2013; Nishat *et al*., 2015).

The findings on cultural studiesare in close conformity with Mahdy *et al.* (2012) who described the colonies of *R. solanacearum* as white, wet, shining, circular, raised and smooth. Pawaskar *et al*.(2014) and Tahat and Sijam, (2010) also recorded similar observations regarding colony characters of *R. solanacearum.*The above results showed that these isolates were *R. solanacearum.*

5.1.3Biochemical profiling of *Ralstonia Solanacearum*

5.1.3.1Gram Stain

All of the plant pathogenic bacteria are usually Gram negative except Clavibacter and Streptomyces (Polkade *et al*., 2016;Tancos *et al*., 2013). All four tomato strains of *R. solanacearum* (Maseno, Mariwa, Seme and Holo) from Maseno region were Gram-negative. The results of this study agreed with Wang *et al*.(2016) who reported that *R. solanacearum* are Gram-negative. Gram reaction by staining is a necessary initial step for the identification and classification of bacteria from any source. The outcome of this test is literally fragile cell walls, which are bound by an outer membrane. Gram-positive bacteria have a thick mesh-like cell wall made of peptidoglycan (50-90% of cell wall), which stain purple and Gram-negative bacteria have a thinner layer (10% of cell wall), which stain pink. Gram-negative bacteria also have an additional outer membrane which contains lipids, and is separated from the cell wall by the periplasmic space.

5.1.3.2 Potassium Hydroxide Solubility Test

An elastic thread or viscous thread was observed when the loop was raised from the bacterial solution by toothpick, a few centimeters from glass slides for all the isolates (Maseno, Mariwa, Seme and Holo). The results are similar to those reported by Rahman *et al*., (2013) who reported that Gram negative bacteria had viscous thread when lifted from glass slide when mixed with 3% KOH. These results could be due to theouter membrane of Gram-negative bacteria being readily disrupted on exposure to 3% KOH releasing the viscous DNA according to Ahmed *et al*.(2013) who performed a similar test on *R. solanacearum* causing bacterial wilt disease of potato in Bangladesh. Solubility test confimed that *R.solanacearum* isolates were Gram negative.

5.1.3.3 Catalase oxidase test

All Gram-negative bacteria produced gas bubbles when they were mixed with a drop of Hydrogen peroxide on glass slide thus they were catalase oxidase positive. Present results agreed with those reported by Mwankemwa (2015) and Teng *et al*.(2016). These results could be attributed to the presence of catalase enzyme in *R. solanacearum*. Ślesak *et al*. (2016) working with Gram-negative concluded that Hydrogen peroxide (H_2O_2) is a byproduct of aerobic respiratory metabolism in aerobic bacteria. Production of gas bubbles gives a clue for presence of aerobic bacteria. Catalase is an enzyme, which is produced by microorganisms that live in oxygenated environments to neutralize toxic forms of oxygen metabolites; H_2O_2 (Jesse and Sreejit, 2016). The catalase enzyme neutralizes the bactericidal effects of hydrogen peroxide and protects them against the toxic effects of H_2O_2 (Jesse and Sreejit, 2016). Anaerobes generally lack the catalase enzyme. Catalase mediates the breakdown of hydrogen peroxide H_2O_2 into oxygen and water. Only aerobic bacteria produce catalase enzyme hence the test bacteria (*Ralstonia solanacearum*) was conrnfirmed to be aerobic in nature.

5.1.3.4 Gas production test

Gas production test indicated that the bacterium (*R. solanacearum*) produced gas from glucose within eighteen hours of incubation. The result are in conformation with Pawaskar *et al*. (2014), Zhou *et al*. (2012) and Houfani *et al*. (2017) who reported that *R. solanacearum* produce gas from dextrose, glucose and salicilin. Production of gas may be attributed to the presence of enzyme systems in bacteria which allow them to oxidize environmental nutrient sources. Many bacteria possess the enzymes system required for the oxidation and utilization of the simple sugar, glucose. Bacterial utilization of different energy sources in the medium depends on the specific enzymes of each bacterium (Shaikhi *et al.*, 2013). The characteristics feature of the enzyme production in the bacteria enables them to use diverse carbohydrates and this in turn aid in the identification of unknown bacteria.

5.1.3.5 Starch hydrolysis test

Starch hydrolysis test of the bacterium showed that the bacterium were unable to hydrolyse starch. These results are similar to those of Pawaskar *et al*. (2014) who reported *R. solanacearum* as negative in starch hydrolysis. Similarly Zhang *et al*. (2006), Nouri *et al*. (2009) and Vaneechoutte *et al*. (2004) also reported *R. solanacearum* to be negative in starch hydrolysis.These results can be attributed to the absence of some exoenzymes (amylases) by *R.solanacearum* (Achari, 2015). Starch agar is a differential medium that tests the ability of an organism to produce certain exoenzymes including amylase and oligo-1, 6-glucosidase that hydrolyze starch. Starch molecules are too large to enter the bacterial cell, so some bacteria secrete exoenzymes to degrade starch into subunits that can then be utilized by the organism. It is on this basis that *R.solanacearum* strains were classified as negative in starch hydrolysis.

5.1.3.6 Profiling of *R. solanacearum* isolates into biovars

Ralstonia solanacearum isolates varied in utilization pattern of different sugars but Biovar I didn't utilize any sugar from sugar test. Biovar III isolates, utilized Mannitol, Sorbitol, Ducitol, Maltose, Lactose and Cellubiose. Biovar III seems more prevalent in Maseno region; it was present in Maseno, Seme and Holo sites. Biovar I is restricted to Mariwa only. These results concurred with those obtained by Hassan *et al*. (2016), who performed similar biochemical test on all bacterial wilt strains from Rawalpindi and Islamabad whose results of the biovar test showed that all seven groups of *R. solanacearum* isolates oxidized disaccharides (Sucrose, lactose, maltose) and sugar alcohols (manitol, sorbitol and dulcitol) within 3-5days.

In different utilization pattern of various sugars; pathogenic variability and virulence pattern of *R. solanacearum* belonging to Biovar I and Biovar III could be due to genetic variability among different strains of pathogen (Grover *et al*., 2012**)**. Genetic variation represents an alternative adaptive strategy, which by providing some degree of diversity ensures survival in specific niches and adaptation to sudden changes in the environment. By responding to specific environmental signals, regulatory genes allow bacteria to adapt to changes in their habitat. High virulence of Biovar 3 strains could be due to their wide

host range and compatibility with 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*.

5.1.4. Designation of landraces of *R.solanacearum* in Maseno region

All groups of *R.solanacearum*isolates causing bacterial wilt of tomato collected from four (Maseno, Mariwa, Seme and Holo) tomato growing areas in Maseno region belong to landrace 3.The strains showed high to moderate virulence on potato, tomato and eggplant. Therefore, all of them had characteristic of landrace 3 with a limited host range on potato, tomato and pepper (Clarke *et al*., 2015). In a host range study, all strains were pathogenic (moderate to high) on potato, tomato and eggplant. But other hosts such as pepper showed low level of pathogenicity but tobacco and peanut did not show any wilt symptoms. The limited host range is the characteristic of landrace 3 of *R.solanacearum* (Lebeau *et al.,* 2011).The findings of this study were in line with those obtained by Tjou-Tam-Sin *et al*. (2016): Manasa *et al*. (2016) and Ahmed *et al*. (2013) who classified *R. solanacearum* into five landraces. Five landraces have been described so far, but they differ in host range, geographical distribution and ability to survive under different environmental conditions. Landace 1 infects many solanaceous plants such as brinjal, tomato, tobacco, pepper and other plants including some weeds. Landace 2 causes wilt of triploid banana (Musa spp.) and Heliconia spp., while landrace 3 infects potato and tomato but it is weakly virulent on other solanaceous crops, landrace 4 infects ginger and landrace 5 infectsmulberry (Lebeau *et al*., 2011**)**. The results of this study primarily indicated that bacterial wilt pathogen of tomato; *R. solanacearum*belongs to landrace 3.

5.2 Pathogenicity of *Ralstonia solanacearum* on tomato plants

Typical symptoms of bacterial wilt on tomato plant began to appear at day five after inoculation. Initial symptoms of bacterial wilt appeared on the leaves and progressed with time after inoculation through injured roots. The wilt reached the entire tomato plant after 21 days. Present results agreed with those reported by Sultana (2016) and Seleim *et al*. (2011). This results can be attributed to the production of extracellular polysaccharide I

by *R. solanacearum* which directly cause wilting by physically blocking the vascular system and thereby alters water movement (Meng, 2013a). EPS I also protect *R. solanacearum* from plant antimicrobial defenses by cloaking bacterial surface features that could be recognized by hosts plant (Meng, 2013b). Results reported herein indicate that the four bacteria isolates obtained from diseased tomato plants collected from different localities of Maseno region proved to be pathogenic and able to infect tomato plants causing wilt symptoms and varied in their pathogenicity.

5.3 *In vitro* evaluation of the plant extracts

5.3.1 Efficacy of treatments in *in vitro* experiment on *R. solanacearum*

The results of *S. didymobotria* root extract inhibition of *R. solanacearum* concurred with studies by Kitonde *et al.* (2014) who also reported that *S. didymobotrya* root had significant inhibition against Gram-negative bacteria. Growth inhibition by *S. didymobotria* could be due to presence of secondary metabolites such as saponins, flavonoids, anthraquinones tannins, alkaloids, phenols, terpenoids, steroids, steroidal nucleus and cardiac glycosides as documented by Ngule and Swamy (2013).

In this study *Moringa oleifera* seed extract was found to significantly $P \leq 0.05$ inhibit the growth of *R. solanacearum*. The results of this study are in agreement with those reported by Kalappurayil and Joseph, (2017) and Surendra *et al*. (2016) who reported that *M. Oleifera* extracts had a significant inhibition effect against both Gram positive and Gram negative bacteria. The antimicrobial activity of *M. oleifera* seed can be atributed to the presence of an array of phytochemiacals (phenolic acids, flavonoids, alkaloids and phytosterols), but most importantly due to the activity of a short polypeptide named 4 (ά–L – rhamnosyloxy) benzyl-isothiocyanate. Bukar *et al*. (2010) and Rasika(2013) working with *M. oleifera* dertermined the presence of the above mentioned phytochemiacals in the plant and associated them with its medicinal properties. The peptide may act directly on microorganisms and result in growth inhibition by disrupting cell membrane synthesis or synthesis of essential enzymes like catalase enzyme

5.4 Effects of plant extract on tomato wilt

5.4.1 Efficacy of treatments on incidence of *Ralstonia solanacearum*

Overall, the results indicated that drenching topsoil with crude methanolic extracts of *S. didymobotria* and *M. oleifera* had the potential to suppress the bacterial wilt incidence and severity. This results are in agreement with those previously reported by other investigators using plant extracts to control tomato bacterial wilt under greenhouse conditions (Nguyen and Ranamukhaarachchi 2010; Lee *et al*., 2012; Tahat *et al*.,2012 and Abo-Elyousr *et al*., 2014).

Application of crude methanolic extract of *S. didymobotria* and *M.oleifera* significantly ($P<0.05$) reduced bacterialwilt incidence. However, varied resultson disease incidence were found based on plant species used (*S. didymobotria* and *M. oleifera*). This variation in restricting disease progression between *S. didymobotria* and *M.oleifera* might be due to difference in chemical compositions of the extracts, membrane permeability of the target pathogen, difference in efficacy and durability of extracts in the soil (Gaggìa *et al*., 2008). This was supported by the work by Hassan *et al.* (2009), who reported that soil drenching of some aqueous plant extracts variably and significantly reduced the disease severity of bacterial wilt, caused by *R. solanacearum*, on potato plants compared with inoculated control under both greenhouse and field conditions.

Plants produce phytochemical compounds by secondary metabolism to protect themselves from pathogen attack, and therefore many plant species possess substantial antimicrobial properties. Mithöfer and Maffei (2016) in a similar study concluded that antimicrobial activities of plant extracts may exist in a variety of different components, including aldehyde and phenolic compounds. Naturally occurring combination of the secondary compounds can be synergistic and often result in crude extracts having greater antimicrobial activity than the purified, individual constituents (Ningombam *et al*., 2017). Cowan, (1999) indicated that substrate deprivation, membrane disruption, bind to adhesives, complex with cell wall, enzyme inhibition and inactivation, metal ion

complexation and intercalate into cell wall and/or DNA are the possible mechanisms of plant secondary metabolites against different microorganisms. Other mechanisms of the crude plant extracts include, direct antimicrobial activity of active compounds contained in the extract or liberated by the extract, indirect antimicrobial activity induced by the stimulation of antagonistic or competitive microorganisms, and induction of systemic resistance in host plants resulting in reduction of disease development (Ammon, 2016).

5.4.2 Effect of plant extracts on plant growth parameters

The results of the present study are in agreement with those reported by Alemu *et al*.(2014), who reported that treated tomato plant with different plant extracts at different application time exhibited lesser shoot fresh and dry weight than healthy plants, uninoculated control.

The results might be due tothe fact that botanicals by themselves do not increase biomass (promote plant growth) rather they decrease the negative impact of the pathogen, stunting nature of the disease, on plant growth, which also affects fresh weight and dry weight of plants. This is in accordance with Alemu *et al*.(2014), who reported that some aqueous plant extracts increased plant growth parameters including plant height at varying degrees over the infected control against *Ralstonia solanacearum* under field experiment.

CHAPTER SIX

CONCLUSION, RECOMMENDATIONS AND SUGGESTIONS FOR FURTHER RESEARCH

6.1 Conclusion

i. *Ralstonia solanacearum* strains from infected tomato plants in Maseno region are; Maseno isolate which belong to landrace3 biovar III, Mariwa isolate which belong to landrace 3 biovar 1, Seme and Holo Isolates which belong to landrace 3 biovar III.

ii. The four bacteria isolates proved to be pathogenic and able to infect tomato plants causing wilt symptoms and varied in their pathogenicity; Holo isolate was the most virulent followed by Maseno isolate, Mariwa isolate and then Seme isolate.

iii. *Senna didymobotria* root extract and*Moringa oleifera*seed extract at 15% were found to inhibit the growth of *Ralstonia solanacearum* both *in vivo* and *in vitro*, but *Senna didymobotria* root extract was better in action as compared to *Moringa oleifera* seed extract.

6.2 Recommendation

i. *Senna didymobotria* at 15% can be used in the integrated management of *Ralstonia solanacearum* disease in tomato because it has a lower disease incidence which in turn increased the yield.

ii. Present study indicates that *Senna didymobotria* plant has some antimicrobial properties that can be further exploited for development of phytomedicine for the management of tomato bacterial wilt infection caused by *R.solanacearum*.

6.3 Suggestions for further research

The following are the Suggestionsfor further research on this study:

i. Profiling of the *Ralstonia solanacearum*was done using isolates from *Lycopersicon esculentum* therefore more studies should be done usingother susceptible plants.

ii. Genetic profiling of *R. solanacearum* should be done using RAPD markers, inorder to establish phylogenetic relationships between different landraces and biovars of *Ralstonia solanacearum.*

iii. Further studies are required to determine the effectiveness of *Senna didymobotria* and *Moringa oleifera* products in the field and to compare different tomato cultivars using different solvents.

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APPENDICES

Appendix 1: Analysis of variance (ANOVA) tableonthe effects of *Senna didymobotria* and *Moringa oleifera*plant extracts against *Ralstonia Solanacearum*

Parameter	**Source**	**DF**	**SS**	**MS**	**F**	**Pr > F**
Effect of plant extracts on *invitro* growth of *R.solanacearum*	Model	187	8751.969444	46.801976	19.71	**<0.0001**
	Error	172	408.361111	2.374193		
	Corrected total	359	9160.330556			
Effect of *S. didymobotria* on *invitro* growth of *R.solanacearum*	Model	55	4517.3	82.2	37.2	**<0.0001**
	Error	124	274	2.2		
	Corrected total	179	4793.4			
Effect of *M. oleifera* on *invitro* growth of *R.solanacearum*	Model	55	4014.	72.9	29.2	**<0.0001**
	Error	124	310.1	2.5		
	Corrected total	179	4324.9			
Effect of plant extracts on disease incidence	Model	7	13059.18250	1865.59750	14.15	**< 0.0001**
	Error	12	1582.40300	131.86692		
	Corrected total	19	14641.58550			
Effect of*S. didymobotria*on disease incidence	Model	88	133376.9	1515.6	151.55	**< 0.0001**
	Error	55	550	10		
	Corrected total	143	133926.9			
Effect of*M. oleifera*on disease incidence	Model	14	16357.3	16448.4	425.26	**< 0.0001**
	Error	33	90.7	2.7		
	Corrected total	47	16448			

Appendix 2: Analysis of variance (ANOVA) table onthe Effect of *R.solanacearum* on tomato plants and effect of *Senna didymobotria* and *Moringa oleifera* plant extracts on growth parameters

Parameter	**Source**	**DF**	**SS**	**MS**	**F**	**Pr > F**
Effect of *R.solanacearum* on tomato plants (Disease incidence).	Model Error Corrected total	17 174 191	213912.7135 22945.5313 236858.2448	12583.1008 131.8709	95.42	**<0.0001**
Effects of *S. didymobotria* and *M. oleifera*plant extracts on shootbiomass, Root biomass, Shoot height, Root length and number of fruits.	Model Error Corrected total	9 20 29	4459.1 5790.7 10249.8	495.5 289.5	1.71	0.1518

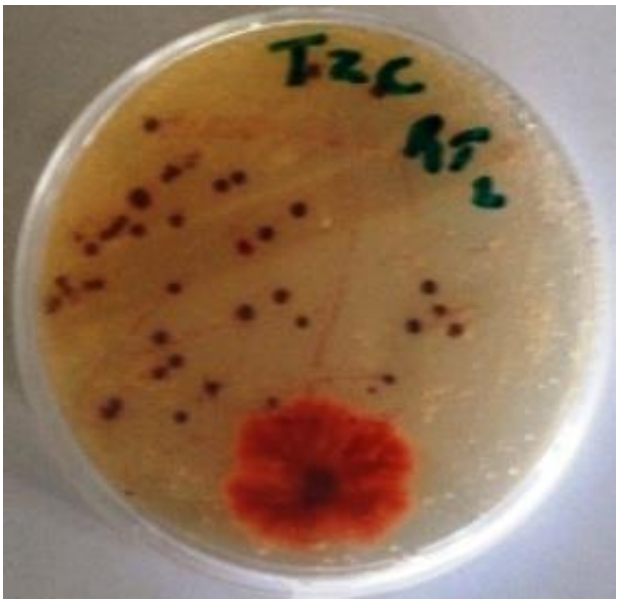
Appendix 3 *Senna didymobtria* powder.

Appendix 4 Rotary vapour pump.

Appendix 5 Disease free tomato plants in greenhouse

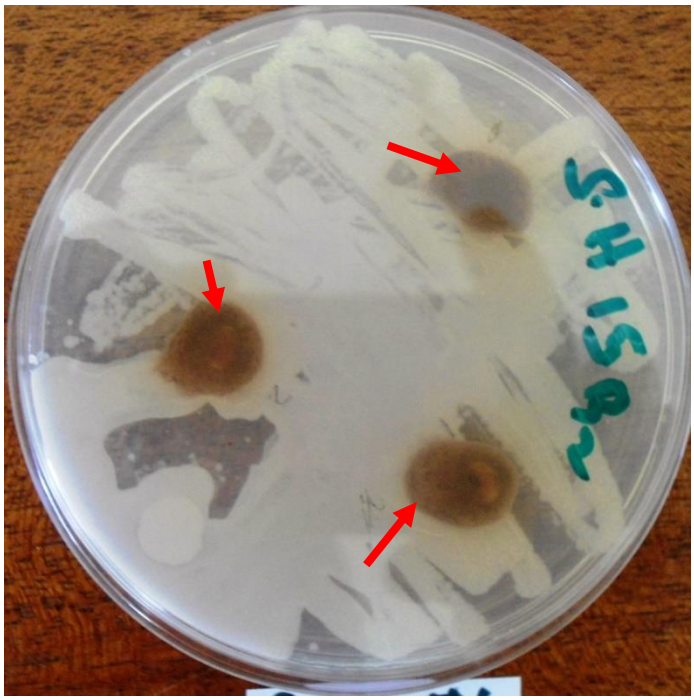
Appendix 6 Diseased tomato plants in greenhouse

Appendix 7 Colonies of *R. solanacearum* on Tzc medium



Appendix 8.Appearance of of *Ralstonia solanacearum* on Nutrient Agar medium

Appendix 9 zones of inhibition by *Senna didymobotria* plant



Appendix 10 Tomato pots arranged in a completely randomized design

Appendix 11concerntration of plant extracts using rotary vapour pump