MANAGEMENT OF CROWN GALL DISEASE IN SELECTED *Rosa hybrida* FARMS IN KENYA USING *Artemisia annua* LEAVES AND *Zingiber officinale* RHIZOME EXTRACTS

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DECLARATION

This thesis is based on my original work except for quotations and citations which have been duly acknowledged. This work has not been presented previously or concurrently for award of a degree or any other award in any other University.

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DEDICATION

This work is dedicated to my wife Jane and my children Bryson, June, Violet, Innocent and Ivy for their profound emotional and psychological support during the entire period of the study.

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ABBREVIATIONS AND ACRONYMS

ANOVA	Analysis of Variance
BC	Before Christ
CFU	Colony Forming Units
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic Acid
EPZ	Export Processing Zone
$FeCl_3$	Iron chloride
MIC	Minimum Inhibitory Concentration
Psi	Pound-force per Square Inch
RPM	Revolution Per Minute
SIM	Sulphide Indole Motility
T-DNA	Transferable Deoxyribonucleic Acid
Ti	Tumor inducing
Vir	Virulence
YEM	Yeast Extract Mannitol
YEMA	Yeast Extract Mannitol Agar

ABSTRACT

Rose flower is the world's most traded cut-flower with 74% of it coming from Kenya. Kenya has potential for higher rose production if challenges of pests and diseases are dealt with. Pests like spidermites, false codling moth, caterpillars, aphids, thrips, nematodes and diseases such as crown gall, downy mildew, powdery mildew and botrytis highly compromise production. Crown gall disease, caused by *Agrobacterium tumefaciens* is the most problematic disease of roses in Kenya and causes a production loss of up to 60% depending on age and variety. This study evaluated the prevalence of crown gall disease and management of the disease using *Zingiber officinale* rhizome and *Artemisia annua* leaves extracts, in an effort to replace conventional chemicals due to their environmental and economic cost. Survey of selected flower farms in Kenyan showed a crown gall prevalence ranging from 0.1% to 65%. *Zingiber officinale* and *Artemisia annua* phytochemical screening indicated presence of terpenoids, flavonoids, alkaloids, saponins, glycosides, phenols and tannins. *Agrobacterium tumefaciens* colonies used in testing antibacterial effect of the extracts were isolated from crushed crown galls and purified through their growth in Congo red YEMA medium and yeast peptone glucose agar. The colonies were authenticated biochemically by Gram staining, motility test, citrate utilization, catalase production, urease production and ketolactose test. Antibacterial effect of the extracts were determined by measuring the diameter of the inhibitory zone around the filter discs soaked in extracts on *Agrobacterium tumefaciens* inoculated media. Micro-dilution technique on microtiter plate was used to determine the minimum inhibitory concentrations of the plant extracts used in soaking the filter discs. *Artemisia annua*, *Zingiber officinale*, mixture of *Zingiber officinale* and *Artemisia annua* had Minimum inhibitory concentration of 125mg/ml, 62.5mg/ml and 31.25mg/ml respectively. The recommended rate (6.25 ml/l) of copper hydroxide was used. Copper hydroxide and *Artemisia annua* had antibacterial inhibitory zone of 12.8mm. This zone was significantly different from that of *Zingiber officinale* and mixture of *Zingiber officinale* and *Artemisia annua* which was 10.6 and 10.2 mm respectively ($P \leq 0.05$). The research therefore revealed that *Artemisia annua* and copper hydroxide inhibited bacterial growth better than *Zingiber officinale* and mixture of *Zingiber officinale* and *Artemisia annua*. Results of treatments done on inoculated rose plants in the greenhouse in terms number of plants with galls, gall weight and stem length showed similar tread to *in vitro* bacterial growth inhibition. The ultimate stem length of *Artemisia annua* and copper hydroxide treatment were 69.4cm and 65.8cm respectively. These lengths were better and significantly different from that of *Zingiber officinale* and mixture of *Artemisia annua* and *Zingiber officinale* which were 48.8cm and 54.4cm respectively ($P \leq 0.05$). From the results of this research, *Artemisia annua* and *Zingiber officinale* extracts are promising biocontrols for crown gall in roses. Farmers are recommended to continue with integrated crown gall control methods and pursue plant extracts as an alternative. The performance of *Artemisia annua* extracts compared well with that of conventional copper hydroxide and is therefore the better option.

CHAPTER ONE

INTRODUCTION

1.1 Background Information

Roses are the main cut flowers in Kenya. Other cut flowers include carnations, statice, carthumus, solidago, arabicum, chrysanthemums, rudbekia, lilies, gypsophila, molucela, eryngium, and tuberoses. The main market for Kenya flowers is European market with Netherland auction being the largest in the world (FloraHolland, 2017). Kenya is world's fourth largest cut flower exporter to the European market behind Netherlands, Columbia and Ecuador (Macharia, 2018). The Kenya cut flowers export has been on upward trajectory from 10,946 tonnes in 1988; 80,480 tonnes in 2006; 120,220 in 2010;136,601 tonnes 2014 and 159,00 in 2017 (Benoit, 2019). The value of Kenya cut flowers sales in 2016 was 65 billion, 71 billion in 2017 and 113 billion Kenya shillings in 2018 representing a 7% of global cut flower sales (Jacob, 2018). The only other Africa country with significant global sales was Ethiopia with a share of 2% (Macharia, 2018).

Owing to favorable weather conditions, well drained sandy- loam soils, availability of technical and financial assistance and cheap labor, Kenyan flower sector has attracted a lot of investors. As a result, the sector employs one hundred thousand people, has an estimated five hundred thousand dependants and impacts over two million livelihoods (Ethical Trading Initiative, 2018).

In terms of roses, Kenya roses form a third of the roses sold in European market (FloraHolland, 2017). Kenya has 2,900 hectares of roses and in terms of production area, is ranked third globally behind china and India. Ethiopia, with 1,200 hectares,

is the only Africa's closely ranked country at number seven (Rikken, 2018). Other Africa countries with developed floriculture industries include Zimbabwe, Uganda, Tanzania and South Africa (Rikken, 2018).

Diseases and pests are the main challenges of rose production in the Kenya. Crown gall disease, caused by *Agrobacterium tumefaciens* which is found worldwide, is the most challenging disease to control (Furuya *et al*., 2004). The bacteria affects species belonging to over ninety three plant families (Kado, 2002). Such plants include apples, pears, cherries, apricots, grapes, tomatoes, sweet pepper, and chrysanthemums (Rhouma *et al*., 2006). Crown gall was first noted in Kenya in the year 1998 and information indicates that the disease was introduced through infected roses from Israel (Arim, 2011). Currently, the disease is spread across the country in rose nurseries and in the main production regions in Naivasha, Thika, and Nanyuki. Crown gall infected flowers show galls, slow growth, stunting, leaves chlorosis and general decreased production (Agrios, 2005). Depending on age and variety of roses, a 60% production loss is experienced (Arim, 2011).

Currently, there is no effective conventional bactericide for crown gall (Arim, 2011). However, dipping the roots and the crown of rose seedlings in a solution containing non-pathogenic *Agrobacterium radiobacter* bacteria strain 84 before planting, offers protection (Lopez *et al*., 1989; Kado, 2002). Many farmers in Kenya and worldwide have reported re-infection after *A. radiobacter* use, implying its protection is short-lived (Arim, 2011). There is no data showing existence of any rose variety which is resistant to crown gall among the popular varieties in the world market (Arim, 2011). Many bactericides have been used against crown gall and only copper

compounds produce good results. The result of copper compounds is seldom satisfactory owing to the pathogen resistance and the phytotoxicity reported in many plant species (Agrios, 2005).

1.2 Statement of the problem

Survey from rose flower major production areas in Kenya indicated crown gall disease as a major problem. The bacterium that causes crown gall is common in the soil and it is able to swim towards photo assimilates that accumulate around the roots of plants with a help of a flagellum. A study done on management of crown gall disease of roses in Kenya revealed that farms planted seedlings from certified propagation houses and practiced integrated disease management to avoid infections and manage crown gall (Kariuki, 2015). Trials of biocontrol using *Agrobacterium radiobacter* K84 that produces agrocin 84 which has antibacterial activity against *A. tumefaciens*, the causative agent of crown gall has been done. However, farms in Kenya have reported the recurrence of crown gall in roses that had been treated with K84 (Arim, 2011).In addition, the control of pathogen is effected by regulation of the nutrition of the roses by raising the pH therefore enhancing uptake of copper thus increasing the resistance of the host to infection by crown gall disease (Teitel, 2007). Despite the above interventions, crown gall still remained a menace.

Disinfection of tools and equipments, especially cutting tools using oxidizing agents like sodium hydroxide was common in all farms. The wounds on the plants, mainly caused by harvesting were painted with copper based fungicides. Antibiotics and copper based compounds have been suggested to control bacterial pathogens in agriculture but their effect has never been satisfactory (Gitonga *et al*., 2014). Many

of these chemicals have been forbidden in many countries owing to their toxicity to human being and negative impact on yields and environment (Kumari *et al*., 2014; Damalas and Koutroubas, 2015). Export of horticultural products has been negatively affected by use of synthetic pesticides (Nashwa and Abo-Elyousr, 2012). Some pesticides like alphadime, dimethoate demeton and cypermethrin have been banned and detection of traces above regulatory residue levels lead to market loss (Business Daily, 2013; Business Daily, 2014). Apart from use of copper based chemicals, many other synthetic chemicals have been used to control crown gall with little success. Synthetic chemicals are expensive, lead to disease resistance; adversely affects non- target organism, cause crop phytotoxicity and have unacceptable chemical residues (Sande *et al*., 2011; Wimalawansa and Wimalawansa, 2014).

Plant antimicrobials are biodegradable and do not accumulate in the environment hence do not affect ozone layer or cause soil and water pollution. Most importantly, plant antimicrobials do not cause disease resistance because they have different modes of actions; a run away from conventional chemicals hence crucial for disease resistance management (Joseph and Sujatha, 2012; Hernandez *et al*., 2013). Plants extracts have been used extensively in Turkey and India for control of bacterial and fungal diseases in vegetables and flowers (Nas, 2004; Pavela, 2014; Pavela, 2016). In Kenya, extracts from *Azadirachta indica*, *Allium sativa* and *Zingiber officinale* has been used to control a wide range of pests; nematodes, white flies, mites and mealy bugs (Arim, 2011). Survey in the farms indicated that management of crown gall using plant extracts had not been fully explored. This research therefore

explores the efficacy of *Artemisia annua* and *Zingiber officinale* extracts in crown gall control.

1.3 Justification

Kenya earned 113 billion Kenya shillings in the year 2018 from flower industry (Jacob, 2018). Apart from earning revenue to the country, the sector employs one hundred thousand people, has an estimated five hundred thousand dependants and impacts over two million livelihoods (Ethical Trading Initiative, 2018). Among the pests and diseases affecting roses, crown gall disease has remained one of the biggest challenges in greenhouse roses growing for the last two decades, causing a production loss of up to 60% (Arim, 2011). The disease causing bacteria is spread across the world, not only causing losses in roses flowers but also to over sixty different plant families (Rhouma *et al*., 2006; Arim, 2011).

Plant bacteria have remained a challenge not only because the chemicals used are not effective but due to the bacteria ability to genetically transmit and acquire resistance to antibiotics (Brent and Holloman, 1998). Flavonoids, which are found in *Artemisia annua* leaves and *Zingiber officinale* rhizome, have been reported to act on *Agrobacterium tumefaciens* directly or synergistically with other antibiotics (Cushnie and Lamb, 2011). Their mode of action is different from those of existing antimicrobial agents hence lack cross resistance to currently used chemicals (Joseph and Sujatha, 2012).

Owing to the limitations of conventional agrochemicals and the strengths of plant phytochemicals, plant extracts has become an option in management of crop diseases. Extracts are non-toxic to beneficial organisms and are environmentally friendly. However, botanical pesticides have not been fully adopted due to challenges in formulation and commercialization which are attributed to lack of chemical data and positive controls (Joseph and Sujatha, 2012). Another challenge is the need for large scale production of extracts for commercial scale of flowers industry.

1.4 Research Questions

(i) Is crown gall disease prevalent in different varieties of roses in selected flower farms in Kenya?

(ii) Which methods do the selected flower farms in Kenya use to control crown gall disease?

(iii) Do *Artemisia annua* leaves and *Zingiber officinale* rhizome extracts contain secondary metabolites responsible for control of crown gall?

(iv) Do *Artemisia annua* leaves and *Zingiber officinale* rhizome extracts inhibit growth of *Agrobacterium tumefaciens in vitro* and *in vivo?*

1.5 Hypotheses

(i) Crown gall disease is prevalent in different varieties of roses grown in selected flower farms in Kenya.

(ii) Farmers use various methods to control crown gall disease in selected flower farms in Kenya.

(iii) *Artemisia annua* leaves and *Zingiber officinale* rhizome extracts contain secondary metabolites responsible for control of crown gall.

(iv) *Artemisia annua* leaves and *Zingiber officinale* rhizome extracts inhibit the growth of *Agrobacterium tumefaciens in vitro* and *in vivo*.

1.6 Objectives

1.6.1 General objective

To contribute to increased rose flower productivity through management of crown gall disease using *Artemisia annua* leaves and *Zingiber officinale* rhizome extracts.

1.6.2 Specific objectives

(i) To determine the prevalence of crown gall disease in different varieties of roses in selected flower farms in Kenya through field survey.

(ii) To determine various methods used to control crown gall disease in selected flowers farms in Kenya.

(iii) To determine the secondary metabolites present in *Artemisia annua* leaves and *Zingiber officinale* rhizome extracts.

(iv) To determine *in vitro* and *in vivo* growth inhibition of *Agrobacterium tumefaciens* after use of *Artemisia annua* leaves and *Zingiber officinale* rhizome extracts.

CHAPTER TWO

LITERATURE REVIEW

2.1 Agronomy of *Rosa hybrida*

Rosa hybrida (rose plant) belongs to family Rosaceae. It is a woody perennial herb whose stems that can be erect, climbing or trailing. The genus *Rosa* has between 100 to 300 species and numerous cultivars (Ertter, 2001). Basically, a shoot comprises of a succession of 8-15 repeating units each one consisting of a leaf, prickles, an auxiliary bud, a node and internodes (Bloom and Tsujita, 2003). The origin of most rose varieties; wild and cultivated dates back 34 million years ago from Asia and others from Europe, North America and North West Africa (Rits *et al*., 2005).

Taxonomic classification of different cultivars of roses is challenging because the morphological characteristics which include stem height, size of the bud, fragrance, stem shape and color of the petals of the cultivars differs a lot (Ertter, 2001). Further, different rose species hybridize naturally making cultivar identification difficult. The scientists have as a result adopted chemotaxonomy (for example fragrance) and molecular taxonomy such as DNA fingerprinting to classify cultivars of roses, albeit little success (Fiasson *et al*., 2003). Rose species range from wild to thousands of cultivated species (Ertter, 2001). Hybrid tea and floribundas, which are the modern rose cultivars, have resulted from many years of complex crosses of different species. The interspecific hybridization intended to improve one characteristic resulted to changes in other characteristics, causing a lot of variations in cultivars (Zlesak, 2006).

Growing of commercial cut roses dates back 500 BC. Over the years roses have been produced for landscaping and ornamentals where stems are cut and preserved indoors in vases for their physical attractiveness and fragrance. Rose petals have volatile essential oil used in manufacture of perfumes (Collin, 2003). Roses can also be used in food and drinks industry in manufacture of jams, jelly or soup which is rich in vitamin A and C (Cinar, 2005). Rose petals are as well treated to obtain rose water which is used as medicine (Cuttler, 2003).

Rose propagation involves budding or grafting, where a root stock is joined with a scion for the two to unite and form one plant. All agronomical practices like propagation, bending, pruning and harvesting causes wounds to the plants; predisposing it to crown gall disease. The processes therefore, require continuous disinfection of propagation houses, materials and equipments.

2.2 Crown gall disease

Crown gall disease is caused by a bacterium called *Agrobacterium tumefaciens*. The crown galls appear after the bacterium transfers genes that cause uncontrolled cell proliferation to the plant cell (Schell *et al*., 2009). The galls grow and increase in size independent of *A. tumefaciens* and finally decay, split and fall releasing the *Agrobacterium tumefaciens* back into the soil (Shams *et al*., 2012). Once released to the soil, *A. tumefaciens* remains active for a minimum of two years within which it is able to incite crown gall again once a plasmid free *Agrobacterium tumefaciens* successfully re-gains a new Ti-plasmid from its neighboring pathogenic *Agrobacterium tumefaciens* (Shams *et al*., 2012).

Crown gall is widely spread and affects dicotyledonous plants in more than sixty different plant families for example apple, pear, peach, cherry almond, raspberry and several ornamentals (Rhouma *et al*., 2006). The disease was discovered in 1900s and in Kenya the first case was reported in roses in 2003 (Aysan and Sahin, 2003). The disease symptoms begins as a small swelling that looks like callus tissue; usually found on the roots, crown and occasionally on aerial parts of stem or leaves (Plate 2.1). Crown gall is usually not fatal unless if the infection occurs in young susceptible crop; however production and crop vigor is highly affected (Schroth, 2008). The bacterium allocates water and nutrients to the rapidly dividing cells in the gall at the expense of other plant tissues leading to poor production (Agrios, 2005). Galls also serve as entry route for secondary infections which further weakens the plants (Agrios, 2005; Schroth, 2008).

Fresh galls in rose foliage Corky old gall at the plant crown

Plate 2. 1: Crown gall symptoms

Source: Author (2017).

2.3 *Agrobacterium tumefaciens*

Agrobacterium tumefaciens belongs to the kingdom Bacteria, phylum Proteobacteria, class Alphaproteobacteria, order Rhizobiales, family Rhizobiaceae and genus *Agrobacterium*. *Agrobacterium tumefaciens* is aerobic, gram negative, motile, non-sporing, rod shaped bacterium measuring 1 × 3µm (Collins, 2001). It has one, up to five laterally inserted flexuous flagella on its cell body.

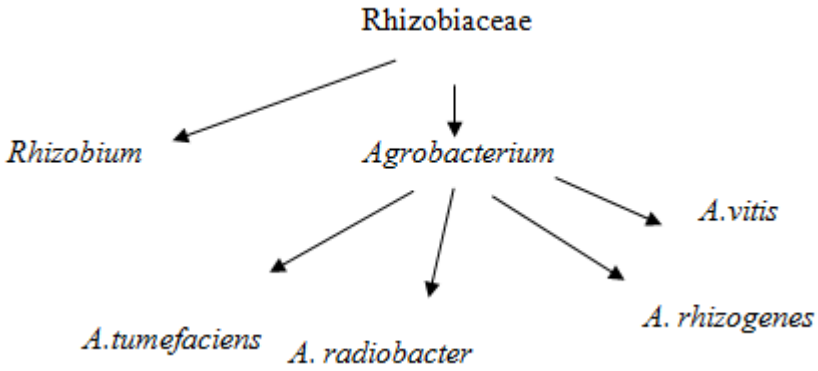


Figure 2. 1: Phylogeny within Rhizobiaceae
Source: Lindstrom and Young (2010).

Agrobacterium is a rhizospheric bacterium which lives saprophytically within the plants rhizosphere of numerous plants. The *Agrobacterium* found to have a dispensable tumor inducing plasmid are plant pathogenic and they cause crown gall disease of most dicotyledonous and few monocotyledonous plants (Rhouma *et al*., 2006). The tumor inducing plasmids are conjugative and they spread to plasmid free Agrobacteria making them to become also pathogenic (Genetello *et al*., 1977).

Before discovery of the plasmids, the first Agrobacteria which were isolated from plant tissues and reproduced the symptoms were named *Bacterium tumefaciens* and

Phytomonas rhizogenes (Smith and Townshed, 1979). A non- pathogenic Agrobacteria had earlier been isolated and named *Bacillus radiobacter* because they showed star shape in certain growth conditions. The genus name *Agrobacterium* was adopted in the year 1942 in association with another similar but different genus *Rhizobium* which does not have the tumor inducing plasmid like genus *Agrobacterium* (Genetello *et al*., 1977).

Through consideration of genomic information, modern bacterial taxonomy has been able to separate *Agrobacterium* species (Figure 2.1). In this respect, isolates of *Agrobacterium* are determined by chromosomal genes and not by plasmids therefore tumorigenic, rhizogenic and non-pathogenic strains can be found within the same biovar (Stackebrandt *et al*., 2002). Isolates of *Agrobacterium spp* are classified in three biovars (1, 2 and 3). Biovar 1, include *Agrobacterium tumefaciens* and *Agrobacterium radiobacter* (Lindstrom and Young, 2010). Biovar 3 isolates are mainly confined in grapevines and they include *Agrobacterium vitis* (crown gall disease causing bacterium) and *Agrobacterium rubi* (cane gall causing bacterium). Biovar 2 appeared more phylogenetically related to *Rhizobium* spp. therefore a proposal for biovar 2 to be classified in genus *Rhizobium* while other species remained in genus *Agrobacterium* (Costechareyre *et al*., 2010). In agreement with the previous taxonomical classification, biovar 2 was suggested to be renamed *Rhizobium rhizogenes* (Young *et al*., 2011). *Rhizobium rhizogenes* are closely related to the hairy- root forming *A. rhizogenes* (Kado, 2002).

2.4 Virulence of *Agrobacterium tumefaciens*

The virulence of *Agrobacterium tumefaciens* depends on the presence of dispensable tumor inducing plasmid (Ti-plasmid). Ti-plasmid is a large transferable extra-chromosomal DNA element which confers tumorigenic ability to *A. tumefaciens*. It is compost of two components; T-DNA part that carries oncogenes which change the normal cell division and a set of virulence (v*ir*) genes that promotes transfer of T-DNA from the *A. tumefaciens* to plant cells (Gelvin, 2003). *Agrobacterium tumefaciens* can therefore be said to be a vector for Ti-plasmid which causes crown gall (COST 873, 2011). Successful pathogenesis happens when the tumorigenic T-DNA gets integrated into the plant genome and the plant cell metabolism is altered resulting to production and accumulation of auxins and cytokinins that bring about tumorigenesis (Lee *et al*., 2009).

Wounded susceptible plant cells produce phenolic compounds (amino acids, organic acids and sugars) which attract *A. tumefaciens* to bind itself to the plant cell (Agrios, 2005). The attachment is made possible by the polysaccharides and extruded cellular fibrils found on the cell (Gelvin, 2003). A long flexuous appendage called transfer pillus aids the transfer of T-DNA from *A. tumefaciens* to the plant cell. To encode proteins that produce a pilus-like structure which bridges *A. tumefaciens* and the host plant cell, the cell requires *vir*B gene. The integration of T-DNA into the genome of recipient cell is then carried out by *vir*C (Gheysen *et al*., 2001). Once the section of DNA of host plant that will be replaced is identified, *vir*C cuts into this particular strand of DNA and initiates integration process through illegitimate recombination.

T-DNA has oncogenes and opines related genes which are responsible for tumor formation. The oncogenes are responsible for phytohormone production that leads to

production and accumulation of auxins and cytokinins that bring about tumorigenesis.

The discovery that T-DNA region and virulence genes can be split into two plasmids lead to a T- DNA binary system which occurs naturally in Ti-plasmid found in *A. tumefaciens* resulting to two artificial vectors. The oncogenes can be removed and substituted with a gene of interest (Patentlens.net, 2014). The helper plasmid has its entire T-DNA region removed while retaining the virulence genes region. These two plasmids are used together to produce genetically modified plants. *Agrobacterium tumefaciens* mediated transformation has been used over years since 1983 in genetic improvement of major economic crops like vegetable, fruits, ornaments and pasture (Islam *et al*., 2010). For instance, an herbicide tolerant soybean commonly known as soybean MON 89788 was developed through *Agrobacterium* mediated transformation of soybean meristematic tissue using the binary vector PV-GMGOX20 (Powell *et al*., 2009). *Agrobacterium* spp. strain CP4 allowed *cp4 epsps* gene to be expressed, making the plants to be resistant towards action of an herbicide called glyphosate. The transformative characteristics of interest in most commercial crops include disease and pest resistance, high yielding, tolerance to different ecological zones and early maturity (Arim, 2011).

2.5 Life cycle of *Agrobacterium tumefaciens*

Pathogenic strains of *Agrobacterium tumefaciens* live saprophytically within the rhizosphere of woody and herbaceous weeds (Agrios, 2005). The bacterium enters the plant through natural openings or a fresh wound that is less than twenty four

hours old on the crown, the roots, or the branches. The bacteria come from the galls that were broken or sloughed off from infected plants during farm operations (Alsup, 2004). *Agrobacterium tumefaciens* is carried from on one place to another through cutting and tilling equipments, irrigation water and workers hands and feet (Arim, 2011).

2.6 Management of crown gall disease

Breeding for pest and disease resistance is the best approach in pest and disease control. In roses however, breeding for resistance has not been explored because the varieties grown depends on customers' tastes and preferences. Some rose varieties remain in the market for a very short period because roses taste and preference is very dynamic, making breeding for resistant uneconomical (Arim, 2011). Propagators of roses avoid crown gall by getting pathogen free materials from reputable growers for grafting or budding. Most often, the propagators do not carry laboratory tests to confirm absence of *A. tumefaciens* but largely depend on visual examinations of galls. Absence of crown galls on plants does not guarantee crown gall free field since *Agrobacterium* resides in the rhizospere and systemically in certain host plants. The effort to get agrobacteria free planting materials has forced the farmers to adopt integrated crown gall disease management strategy.

2.6.1 Cultural methods of crown gall control

Agrobacterium tumefaciens, like other bacteria require natural opening or fresh wounds to enter the plant cell (Agrios, 2005). Careful cultural activities, control of chewing insects and nematodes prevents wounding the plants to enhance fight

against crown gall (Arim, 2011). The fact that the entry of *A. tumefaciens* into plant cell is through wounds, cultural methods to control crown gall has remained a challenge because it is difficult to avoid wounds during agronomical activities like propagation, harvesting and pruning.

Abandoning infected soils for five years and planting non- susceptible host plants like cereals within that period has been practiced amid challenges of inadequate land (Arim, 2011). Fields grown with flowers, fruits and nut crops require rotation with monocotyledonous plants. Some weeds like morning glory are natural hosts and should be avoided in the farms (Burr, 1993).

2.6.2 Chemical methods of crown gall control

Antibiotics and copper compounds have been used to control bacterial pathogens in agriculture but their effect has never been satisfactory. In fact, they have been forbidden in many countries owing to their toxicity and negative impact on yields and environment (Arim, 2011). *Agrobacterium tumefaciens* have an ability to genetically transmit and acquire resistance to antibiotics making their control using conventional antibiotics a challenge (Brent and Holloman, 1998).

It is difficult to avoid wounding flower plants during many field operations. Consequently, tools used for these operations are disinfected using copper or bleach-based bactericides to reduce *A. tumefaciens* on tools and plant surfaces. Secateurs or cutting tools are dipped into 25% sodium hypochlorite for one minute before proceeding with an operation from one plant to other (Arim, 2011). Chemicals use

remain a challenge because most them work by contact and are therefore ineffective for *A. tumefaciens* systemically infected plants (Alsup, 2004). Dipping cutting tools after every plant operation and painting all cut ends is as well very laborious.

2.6.3 Biological methods of crown gall control

Agrobacterium radiobacter, a closely related soil borne avirulent bacterium is used to control *Agrobacterium tumefaciens*. *Agrobacterium radiobacter* strain K84 is the most commonly used biocontrol agent (Burr, 1999). It produces agrocin 84 which has antibacterial activity against *A. tumefaciens* with nopaline- type Ti plasmid (Penyalver *et al*., 2000). The strain also produces agrocin 434 and AL584 which affect the *A. tumafaciens* without nopaline- type Ti plasmid hence broadening the scope of control.

Agrobacterium radiobacter K84 is a widespread, naturally occurring bacterium found in the soil near plant roots. *Agrobacterium radiobacter* K1026 and K84 are essentially identical and have similar characteristics except that K1026 strain is a genetic alteration of K84 by removing a small portion of K84 DNA to prevent transfer of resistance from strain K1026 to other strains (Penyalver *et al*., 2000). *Agrobacterium radiobacter* K1026 is registered in USA as a biological control agent and its ecotoxicology studies on non –target organisms and risks on environment are currently under research. Work on a bacteria which is able to synthesize enzymes that inhibit ethylene production hence reduce tumor growth is as well going on (Penyalver *et al*., 2000). Incidences of *Agrobacterium tumefaciens* resistant to K84 has been reported, making biological control non effective (Burr, 1993). Farmers in

Kenya have also reported recurrence of crown gall in roses which has been treated with K84 (Arim, 2011).

2.7 Medicinal plants

For a very long time plant pathogenic microorganisms have been controlled using synthetic fungicides and bactericides (Kumar and Rathinam, 2013). Farmers have mainly relied on synthetic chemicals because their effects are quick (Shabana *et al*., 2017). However, extensive reliance on these chemicals has brought about diseases and pests' resistance which has resulted to higher chemical dosages being used (Nkechi *et al*., 2018).

Antibiotics and copper compounds have been used to control bacterial pathogens in agriculture but their effect has never been satisfactory. They have been forbidden in many countries owing to their toxicity and negative impact on yields and environment (Arim, 2011). Bacteria have remained a challenge not only because the chemicals used are ineffective but due to the bacteria ability to genetically transmit and acquire resistance to antibiotics (Brent and Holloman, 1998). Over 80% of individuals in developed countries use traditional medicine whose compounds are derived from medicinal plants. As such, the need to understand these plants secondary metabolites safety and efficacy has become important (Eloff, 1998). Recent studies have shown that essential oils and secondary metabolites from plants and algae have interesting antibacterial activity (Nair *et al*., 2005; Kothari, 2011), antifungal (Khan and Wassilew, 1987), antidiabetic (Kumar *et al*., 2008), antioxidant (Wong *et al*., 2009; Kothari *et al*., 2010) and radio protective activity (Jagetia *et al*., 2005). Consequently, a research on their use in pests and diseases

control in agriculture has intensified and several metabolites have proven to be effective bio-control (Fravel, 2005).

Antimicrobials from phytochemicals have different modes of action as opposed to existing antimicrobial agents hence lack cross resistance to currently used chemicals (Mcchesney, 2003). Natural products are therefore core in diseases and pests' resistance management programs. Many pesticides are natural or in a way are derived from natural products, as such 42% of the new active ingredient registrations for pesticides between 1997 and 2010 were natural (Cantrell *et al*., 2012). Extensive research has been done on many plants genera including *Artemisia annua*, *Mellissa Allium*, *Zingiber officinale*, *Rosmarinus spp*., *Lantana camara*, *Thymus spp*., *Cymbopogon spp*., *Sesbania sesban*, *Psidium caryophyllus*, *Michelia spp*., *Passiflora spp*., *Punica spp*., *Salvia spp*., *Azadirachta spp*., *Vitex ssp*., *Ipomoea spp*., *Tegetes spp*., *capscum spp*. and *cassia spp*. (Ahmad *et al*., 2017; Ali *et al*., 2014; Isman, 2017; Isman and Grieneisen, 2014; Mkenda *et al*., 2015). Phytochemicals from these plants genera have shown varying degree of antimicrobial activities which results from a combination of secondary metabolites.

2.7.1 *Zingiber officinale* rhizome

The botanical classification of *Zingiber officinale* is as below;

Kingdom	Plantae
Phylum	Spermatophyta
Class	Monocotylendonae
Order	Zingiberales

Family Zingiberaceae

Genus *Zingiber*

Species *officinale*

Zingiber officinale, also commonly known as ginger is a perennial herb indigenous to southern China but has spread to all other continents especially within the humid tropics. The plant has a reddish stem, underground rhizome from which roots grow and has a cluster of white and pink flower buds which bloom to yellow flowers.

Most of the *Zingiber officinale* rhizome consumed in Kenya comes from Uganda. However, in Kenya it is grown in coastal regions of Kwale and around Lake Victoria. The sensory perception of *Zingiber officinale* rhizome in the mouth and nose is attributable to volatile oils and mixture of terpenoids (sesquiterpenoids and monoterpenoids) and non-volatile compounds called phenylpropanoids (Bailey *et al*., 2012). The volatile oils and terpenoids are responsible for characteristic aroma and taste while the non-volatile pungent compounds which include gingerols, shogaols, paradols and zingerone are responsible for the “hot” sensation in the mouth (McGee and Harold, 2004). The phenylpropanoids (gingerols, shagaol, paradol, gingerdione and gingereone) are lipid soluble compounds primarily isolated from the rhizome of *Zingiber officinale*; other parts of *Zingiber officinale* contain very little metabolites (Koh *et al*., 2009).

The concentration of secondary metabolites in *Zingiber officinale* rhizome increases with plants age hence the rhizome for pharmacological use is harvested nine months after planting. The concentration of *Zingiber officinale* metabolite used in this

research was compared with those used by other researchers because the proportion of individual components can vary with country of origin (Schwertner *et al*., 2006)

The pharmacological properties of bioactive compounds from *Zingiber officinale* rhizome have been extensively studied towards human and crop diseases control (Ahmad, 2017). These compounds have been used over the years to control human bacterial and viral infections such as colds, nausea, arthritis, hypertension, cancer and indigestion (Wu *et al*., 2008). Extracts from *Zingiber officinale* rhizome have shown antioxidant and antibacterial effects on *Staphylococcu*s spp., *Escherichia* spp., *Pseudomonus* spp. and *Candida albicans* (Wong *et al*., 2009). The extracts have shown effects on damping off, bacterial wilt diseases and crown gall (Monaim *et al*., 2011). A mixture of *Azadirachta indica*, *Allium sativa* and *Zingiber officinale* has been used by Kenya flower growers to control a wide range of pests; nematodes, white flies, mites and mealy bugs (Arim, 2011).

2.7.2 *Artemisia annua*

The botanical classification of *Artemisia annua* is as below;

Kingdom	Plantae
Phylum	Spermatophyta
Class	Monocotylendonae
Order	Asterales
Family	Asteraceae
Genus	*Artemisia*
Species	*annua*

Artemisia annua is commonly known as sweet worm wood, sweet annie, sagewort in English but also has other names in other languages. *Artemisia annua* is native to temperate Asia but has been naturalized across the world. In Kenya, *Artemisia annua* is grown in Nairutia in Nyeri County, Arthi River and Kitengela in Machakos County. It is an annual plant with a single stem, alternating leaves, branches and bright yellow flowers (Plate 2.2). It can grow to a height of two meters.

Artemisia annua belongs to the family Asteraceae which has over 30 species (Bremer and Hamphries, 1993). The genus has high contents of phenols and flavonoids which are responsible for oduor, appearance, taste and oxidative ability. These metabolites have shown anticancer, anti-aging, antioxidant; prevent brain damage caused by Parkinson's, Alzheimer's, and Huntington's diseases (Pillay *et al*., 2008).

Artemisia annua is the intensively studied species and is a source of over fifty flavonoids (Ferreira *et al*., 2010). Other species; *A. absinthium, A. tridenta, A. herba alba*, *A. asiatica* and *Artemisia aboratum* have also been found to contain low levels of flavonoids (Hajdu *et al*., 2014). Sesquiterpene artemisinin and its derivatives; Arteether, artemether, artesunate and dihydroartemisinin are the most studied phytochemicals in *A. annua*. They are considered to be the primary active constituents for antibacterial, antifungal and anti-cancer activity (Duke and Paul, 1993). They have also shown to be effective against malaria and parasitic diseases such as schistosomiasis, leishmaniasis (Utzinger *et al*., 2001; Sen *et al*., 2007). *Artemisia annua* essential oil was found to be active against some plant pathogenic bacterial strains and viruses such as *Pnuemocystis carinii*, *Toxoplasma gondii* and

cancer cell lines (Efferth and Willium, 2007). *Artemisia annua* is also a source of essential oils, crafting of aromatic wreaths as well as a natural herbicide (Utzinger *et al*., 2001; Sen *et al*., 2007). *Artemisia annua* extracts has been used in Turkey to control roses red spidermites and crown gall (Erel *et al*., 2012) while successful trials for control of potato aphids in Kenya has also been done (Dancewicz and Gabrys, 2008).

Artemisinin; a sesquiterpene lactose is the main active ingredient of *A. annua* as it contains an endoperoxide bridge which is important for its medicinal activity. It is highly concentrated at the beginning of flowering because it is produced at the glandular trichomes found in the leaves, floral buds and flowers (Tellez *et al*., 1999). Leaves and the flowers of *Artemisia annua* produce artemesinin ranging from 0.01 – 1.1% depending on the climate, cultivar and the time of harvesting (Wallaart *et al*., 2000). The age of the plant, species and ratooning as well has been noted to affect the level of artemesinin (Kumar and Rathinam, 2013). The highest percentage of artemesinin is found on upper third of the plant (within leaves) and the levels decrease downwards with none in the roots. Extraction of *Artemisia annua* phytochemicals therefore is done from the leaves at the beginning of flowering stage (Dhingra *et al*., 2000).

Zingiber officinale rhizome *Artemisia annua* plant

Plate 2. 2: Botanicals tested for antibacterial effect

Source: Author (2017).

Artemisia annua leaves and *Zingiber officinale* rhizome used for this research were collected in Kenya from Nairutia in Nyeri County and Kwale in Kwale County respectively. The plants parts were authenticated through Dr. Gatheri, a taxonomist in the Department of Plant Science in Kenyatta University in Kenya.

2.8 Secondary metabolites in botanical plants

Plant cells synthesize both primary and secondary metabolites. Primary metabolites which include carbohydrates, lipids and proteins are common to all plants and are involved in growth and development. Their deficiency therefore has negative effects to the plant. Secondary metabolites are considered to be the end products of primary metabolism and they include phenolics, flavonoids, alkaloids, tannins, terpenoids, sterol, steroids and essential oils. They are restricted to particular plant families, genus or species and are found in small quantities. Secondary metabolites have insignificant role in plant growth and development but are primarily involved in communication between the plant and other organisms during plants defense mechanism against virus, fungi, bacteria, nematodes, pests and predators as well as attracting beneficial organisms to the plant (Rosenthal *et al*., 2012; Hartmann, 2007).

They are also involved in plants defense against abiotic stress such as exposure to UV-B.

Commercially, secondary metabolites are used in food, pharmaceutical, cosmetic and pesticide industry where they make flavors and fragrances, food additives, drugs, dye, pigments, and pesticides. Recognition of the biological properties of myriad natural products has fueled current research on secondary metabolism with an aim to unearth new drugs, antibiotics, insecticides and herbicides (Hartmann, 2007).

Secondary metabolites are classified based on their structures or their biosynthetic pathways. Based on structure, secondary metabolites are classified into alkaloids, terpenoids, Phenylpropanoids, quinones, and steroids. These metabolites are synthesized through a series of enzyme catalyzed reactions using simple building blocks as follows; Mavalonic acid pathways (quinones), Amino acid pathways (alkaloids), Shikimic acid pathways (phenylpropanoids), Acetate- malonate pathways (fatty acids, phenols and quinines (phosphate pathways (quinones) and combined pathways (flavonoids).

2.8.1 Flavonoids

Flavonoids are a member of hydroxylated polyphenolic compounds alongside coumarins, fuvano coumarind, lignin, isoflavonoids and tannins. It is the largest class of phenolics with primary function of flower petals coloration to attract pollinators and plant defense (Kondo *et al*., 1992). Flavones and flavonols found in

flowers protects the cells against UV- B radiation (Savirnata *et al*., 2010) while allowing visible wavelengths to enter (Lake *et al*., 2009). Some plants that contain a lot of flavonoids include onions, banana, blue berries, all citrus, red wine and cocoa. Flavonoids can be consumed in large quantities since they have low toxicity and are rapidly metabolized. The chemical structure of flavonoid has a 15- carbon skeleton and contains 2 phenyl rings A and B and heterocyclic ring C. Depending on the chemical structure, flavonoids can be classified as Anthoxathins, flavapones, flavanonols, flavaus, isoflavonoids and flavonols.

Flavonoids have shown anti- inflammatory, antioxidant, antibacterial, anticancer, antifungal effects and have also been reported to act on bacteria directly or synergistically with other antibiotics (Friedman, 2007; Cazarolli *et al*., 2008; Cushnie and Lamb, 2011). Flavonoids have been intensively studied and found to have antimicrobial activity against many microorganisms *in vitro* (Cowan, 2011). Some flavonoids have showed inhibitory activity against plant diseases like *Fusarium oxysporum* (Galeotti *et al*., 2008). Flavonoids act on microbes through formation of antimicrobial barriers in response to infection and by forming complexes with extracellular and soluble proteins and bacterial cell walls (Orhan *et al*., 2010; Trease *et al*., 2012).

2.8.2 Saponins

Saponins are produced in roots and barks in many plants. They are generally used as medicine, adjuvant and cosmetics (Eskander *et al*., 2006). Structurally and chemically, they resemble human steroids like estrogen, progesterone and cortisol

and are therefore also known as steroidal saponins. All saponins are able to foam and have a compound called sapogenin which enable it to hemolytically dissolve red blood cells when taken intravenous (Rao and Gurfinkel, 2000). To destabilize the red blood cells, saponins bind with the cell membrane cholesterol to form complexes that create pores on the cell surface (Gauthier *et al*., 2009; Melzig *et al*., 2011). Saponins have been reported to have a wide range of pharmacological activities including antifungal, hypoglycaemia, antiparasitic, vasoprotective, immunodulatory, molluscicidal (Sahu *et al*., 2008).

2.8.3 Tannins

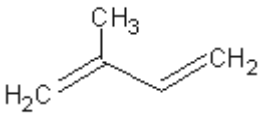
Tannins are phenylpropanoid compounds found in the roots, bark, leaves, fruits and fruit pods of many trees. Examples of plant families rich in tannins include Leguminosae (for example wattle), Anacardiaceae, Rhizophoraceae (for example magroove) and Myrtaceae (for example eucalyptus). The astringent colour and flavor in black tea, wine or unripe fruits emanates from tannins. Plant defense through tannins is achieved through their acidity, astringent taste and by their ability to bind to proline rich proteins that interfere with protein synthesis (Sanches *et al*., 2015). They are responsible for a sharp astringent sensation after binding salivary proteins making animals and pests avoid plants with tannins (Oates *et al*., 2008). The astringency brings about poor palatability and digestibility causing the pest to have depressed feed intake thus serving as feeding repellant.

Chemically, tannins are classified as either hydrolysable or condensed. While hydrolysable tannins dissociate in water to give water soluble products which are

important in leather industry, condensed tannins forms insoluble precipitates in water. Structurally, tannin is an oligomeric compound with multiple structure units with free phenolic group. Commercially, tannins are used in tanning leather, dying fabric, in manufacture of ink and in making drugs. They have well documented antimicrobial properties which are effective against bacteria, fungi, and viruses (Chung *et al*., 2007). The antimicrobial activity exhibited by tannins is achieved through enzyme inhibition, substrate deprivation and metal ion deprivation.

2.8.4 Terpenoids

Terpenoids, also called isoprenoids are the largest group of natural compounds produced in terrestrial, marine plants and fungi (Nagegowda, 2010). They give flavor and fragrance to herbal tea and essential oils. Their role in plants is to offer defense as toxins and feeding deterrents (Salminen *et al*., 2008). In fungi, terpenoids are found in actinomycetes (sesquiterpenes), streptomycetes, cyanobacteria and Myxobacteria. Traditionally, plant terpenoids have been used in human food, chemical and pharmaceutical industries as flavours, fragrances, spices, perfumes and cosmetics, drugs and food additives (Newman and Cragg, 2010). Terpenoids are derived from five carbon isoprene molecules, that is; their structure are based on various but definite number of isoprene units (Figure 2.2). Terpenoids have a chemical and structural diversity, even though their molecular skeleton is made up of two five carbon isomers: Isopentenyl diphosphate (IPP, c_5) and Dimethylallyl diphosphate (DMAPP, c_5) which condenses to give geranyl pyrophosphate (Figure 2.3).



Isoprene

Figure 2. 2: Structure of isoprene

Source: Cantrell *et al*. (2012).

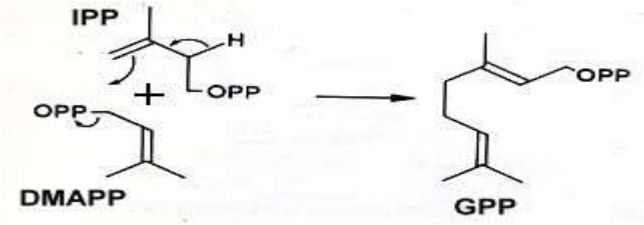


Figure 2. 3: Basic terpenoid molecular skeleton

Source: Cantrell *et al*. (2012).

The chemical and structural diversity of terpenoids depends on the numbers of isoprenes units, cyclization and the rearrangements. Consequently, terpenoids are classified based on the number of isoprene incorporated to the basic molecular skeleton (Hill, 1997). We have the following classes; Monoterpenes (C_{10}), Sesquiterpenes (C_{15}), Diterpenes (C_{20}), Sesterterpenes (C_{25}), Triterpenes (C_{30}), Tetraterpenes (carotinoids) (C_{40}) . Monoterpenes, Sesquiterpenes, diterpines and sesterterpenes have their isoprenes uniquely linked head to head and on the other hand, triterpenes and tetraterpenes contain C_{15} and C_{20} units respectively joined head to head.

Several monoterpenes have shown therapeutic and chemo-preventive activity against tumor cells. As such, limonene which is common in many trees has shown antimicrobial effect on tumor cells (Kris- Etherton *et al*., 2002). Pyrethrin from pyrethrum is a noble monoterpene with good insecticidal components that blocks the

impulse transmission in the nervous system of arthropods thereby causing paralysis and death (Jones and English, 2003). Artemisinin isolated from *Artemisia annua* is an important sesquiterpene lactone with a unique endoperoxide bridge essential for antimalaria activity, parasitic diseases, some viral and varius neoplasms (Delabays *et al*., 2001). Sponges, especially Spongiidae, Thorectidae and Dysideidae have sesquiterpenes quinines and hydroxyl quinines which have a remarkable anti-inflammatory, antibacterial, anti-HIV virus and protein kinase inhibition (Giannini *et al*., 2001).

2.8.5 Alkaloids

Alkaloids are cyclic nitrogen containing natural compounds. They contain nitrogen in amide form hence referred to us compounds containing amide nitrogens. They are synthesized from one of the few common aminoacids which include aspartic, tyrosine, tryptophan and lysine (Pearce *et al*., 2009). About 15% of plants in more than 150 families contain more than 5000 alkaloids that have been identified (Schiff, 2002). Some of the plant families which are a major source of alkaloids include; Papaveraceae (poppy), Ranuculaceae (buttercups), Papilolnacea, Rubiaceae, Rutaceae and solanaceae (night shades). Few alkaloids have been found in animals (C*astor canadensis*) and fungi (*Claviceps* spp.). Some of the well known alkaloids include morphine, quinine, coniine, strychnine and ephedrine. Most alkaloids are toxic and defend the plant by affecting the neurotransmission of the herbivores or pathogens (Konno, 2011; Hartmann, 2007). Alkaloids are not classified based on their biosynthesis origin because their building blocks come from other classes; for example terpenoids combined to phenylpropanoid. Instead, they are classified based

on their chemical structure hence we have, Pyrrolidines, Pyridines, tropanes, Pyrrolizidines, Isoquinolines, Indoles, Quinolines and terpenoid and steroids.

Most alkaloids are poisons but 50 % of plant derived pharmaceuticals are alkaloids. Nicotine and quinine have shown strong insecticidal and antimalaria agent respectively (George *et al*., 2000). Some alkaloids are poison for instance hemlock whose component is coniine from *Conium maculatum*. Alkaloids have physiological effects on central nervous system with examples of anaxiolytic (Lager *et al*., 2006), analgesic and hallucinogenic effects (Dewick *et al*., 2009). Some alkaloids are known to have antibacterial and antifungal properties allowing them to be used in treatment of skin diseases (Rao *et al*., 2009). The modes of action of alkaloids range from affecting neurotransmitter substance in the nervous system to interfering with transport at the cell membrane and with functional protein synthesis (Creelman and Mullet, 1997).

2.8.6 Glycosides

Glycocides are nitrogen containing compounds other than alkaloids. They naturally occur in plants flowers and fruits pigments. They are found mainly in graminae and leguminosae families (Seigler, 1991). They are not perse toxic but when crushed for instance when the insects and herbivores are feeding, they produce poisonous volatile substances like hydrogen sulphide and HNS to deter feeding (Taiz and Zeiger, 1995). Lima bean (*Phaseolus lunatus* L.) has been found to produce volatile compounds (HNS) believed to be defensive against herbivore (Ballhorn *et al*., 2009). Similarly, cassava has cyanogenic glycoside that makes it to be stored for long time without pest attack (Pearce *et al*., 2009).

Structurally, glycosides contain one or more sugars combined through a glycosidic bond to another functional group usually a non-sugar (aglycone). Through an enzyme (glycoside hydrolyses) reaction, glycoside can be cleavage to sugar and non-sugar component making the chemical available for use (Brito – Aris and Marco, 2007).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study area

Survey of the prevalence of crown gall disease of roses was carried from January – June 2016 in Naivasha, Thika and Nanyuki where roses are mainly grown in Kenya. Survey was done in four farms in Naivasha, three farms in Thika and one farm in Nanyuki. The number of farms surveyed from each region was a good representation since about 60% Kenya roses come from Naivasha region, 20% from Thika region and 10% from Nanyuki region (Rikken, 2018).

The coordinates for the study farms in Naivasha are 0^0 43’ 1.8408’’ S 36^0 25’ 51.6936’’ E (Nini farm), 0^0 43’ 0.01’’ N 36^0 26’ 9.28” E (Orjorua), 0^0 12’ 60.00’’ N 36^0 15’ 60.00 E (Wildfire) and 0^0 46’ 4.01’’ N 36^0 21’ 1.39’’ E (Karuturi). The coordinates for the study farms in Thika are
1^0 8’ 44.53’’ S 36^0 57’ 53.472’’ E (Zena), 1^0 59.74’’ S 37^0 04’ 9.59’’ E (Penta) and 1^0 9’ 0’’ S 36^0 58’ 0’’ E (Gatoka). The coordinates of the study farm in Nanyuki are 0^0 01’0.01’’ N 37^0 04’ 22.19’’ E (Tambuzi).

Greenhouse experiment was set up in Gatoka farm in Kiambu County. The farm is located in Thika West Sub-County which is 1631 meters above sea level. The mean annual precipitation of Thika is 835mm which falls in two rainfall seasons; March to May (long rain) and from end of October to December (short rain) (KMD, 2010). The area has average temperature of 19.8^{o}C, with maximum and minimum temperature being 25.1 and 13.7 ^{o}C respectively (Ndegwa *et al*., 2009). The soils are

predominantly loamy and dominated by humic nitisols. The soil is fertile with high organic matter and has a pH ranging between 5 -7. It is well weathered with deep to shallow depth with medium grains making it to have a good drainage (FAO/UNESCO, 1990). The main agricultural products in this region include flowers, coffee, fruits, maize, beef and dairy cattle (Ntale and Litondo, 2013). The type of soil found in Thika and the prevailing temperature promote the development of crown gall diseases and the manifestation of the symptoms, notably the galls (Arim, 2011), hence the choice of Gatoka farm as the site for setting greenhouse experiments.

3.2 Determination of crown gall disease prevalence in different varieties of roses in selected flower farms in Kenya

Disease prevalence computation required establishment of the total plants population per variety and the number of plants with crown gall symptoms in that population. The numbers of plants with galls per variety were estimated through sampling and not through physical count because the plants populations per variety were high. Five population samples per variety were randomly taken, plants with crown gall symptoms counted and their mean computed. The sample size was established using the below Slovin's formula (Yamane, 1997; Hinkeimann and Kempthorne, 2008; Almeda *et al*., 2010);

$n = \frac{N}{1 + Ne^2}$ where n = sample size, N = total plant population and e = Confidence level of 95%. Computation of the disease prevalence, which is usually a percentage

of the number of plant of a particular variety with crown gall symptoms out of the total population of the same variety, was then calculated as below (Kenneth, 2012).

$$\text{Prevalence} = \frac{\text{Number of plants showing symptoms}}{\text{Total number of plants}} \times 100$$

3.2.1 Data collection

Information about varieties of roses grown in each selected flower farm and their total plant population was captured through a questionnaire presented to farm managers (Appendix I). The population sample from which plants with crown gall symptoms were counted was established using slovin's formula and recorded for each variety. From the population sample, the number of plants with symptoms were counted and recorded. This was repeated from five population samples and mean of plants with crown gall symptoms computed for each variety.

3.3 Management of crown gall disease in selected flowers farms in Kenya

The questionnaire administered (Appendix I) to farms captured information about the constraints of rose production. The information about diseases, pests and their difficulty in control was as well captured. Other information captured included crown gall management ranging from conventional, cultural, biological and integrated crown gall control methods.

3.4 Determination of secondary metabolites present in *Artemisia annua* leaves and *Zingiber officinale* rhizome extracts

3.4.1 Preparation of extracts from medicinal plants

Solvent extraction method, by use of methanol was used to get the plants extracts (Mariita *et al*., 2010). Fresh *Artemisia annua* leaves were thoroughly washed under running water and allowed to air dry to a constant dry weight for seven days at room temperature. *Zingiber officinale* rhizomes were chopped into small pieces and dried in an oven at 40^{o}C for 72 hours to facilitate quick drying. The dry materials were soaked differently in methanol at the ratio of 1:4 (Agbo *et al*., 2000). The mixture was agitated using warring blender to macerate and enhance mixing of the powder and the solvent. It was then poured in an air tight plastic and kept in refrigerator at 4^{o}C for 48 hours. The mixtures were then filtered with cheese cloth, followed by Whattman N^{o}1 filter paper (Atata *et al*., 2003; Mariita *et al*., 2010). The acquired filtrates were concentrated separately to remove methanol (boiling point 64.6^{o}c) using Rotary Evaporator in a vacuum at 40^{o}C until 10% of their original was achieved (Plate 3.1). The extracts were further concentrated to complete dryness in a water bath and transferred to vials which were kept at 4^{o}C for laboratory uses. The extraction was done in triplicate.

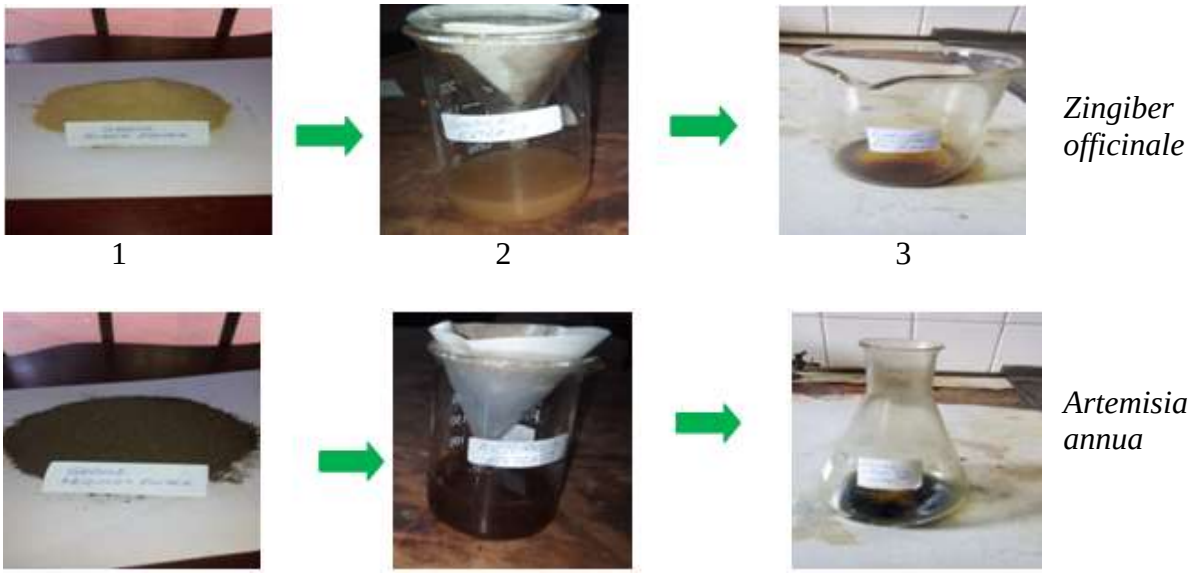


Plate 3. 1: Plant extracts preparation process

3.4.2 Phytochemical analysis of plant extracts

Chemical tests to screen and identify the bioactive chemical constituents in the *Artemisia annua* and *Zingiber officinale* extracts were carried out using the standard procedure (Trease and Evans, 2012). One milliliter of each solvent extract was used for each analysis, except for the saponin test where 3 ml of the solvent was used as recommended **(**Hasegawa *et al*., 1994**)** as indicated in Table 3.1.

Table 3. 1: Test for phytochemicals in plant extracts

TEST	PROCEDURE	INFERENCE
Alkaloids (Wagner's Test)	To 1ml of extracts in a test tube, small solution of iodide in potassium iodide was added (Kokate, 2005).	Presence of reddish brown precipitate would signify presence of alkaloids (Mueller *et al*., 2000).
Saponins (Foam Test)	To 3 mls of extracts in test tube, 10 ml of water was added and vigorously shaken **(**Hasegawa *et al*., 1994).	Formation of persistent foam would signify presence of saponins **(**Hasegawa *et al*., 1994).
Phenols and Tannins	To 1 ml of extracts in test tubes, 2 ml of 2% $FeCl_3$ was added (Ragehy *et al*., 2002).	Formation of black coloration would signify presence of phenols and tannins (Ragehy *et al*., 2002).
Terpenoids (Salkowski's Test)	To 1 ml of extracts in test tubes, 2 ml of chloroform was added. 2 ml of concentrated sulfuric acid was then carefully added (Yadav and Agarawala, 2011).	Presence of a reddish brown coloration at the interphase would signify presence of terpenoids (Yadav and Agarawala, 2011).
Flavonoids (Shinoda Test)	Into test tubes containing magnesium ribbon fragments, 1 ml of the extract was added. 2 ml of concentrated hydrochloric acid was added drop wise (Yisa, 2009).	Formation of purple coloration would signify presence of flavonoids (Yisa, 2009).
Glycoside	To 1 ml of extracts in test tubes, 2 ml glacial acetic acid containing 2 drops of 2% $FeCl_3$ was added. The mixture was poured into a test tube containing 2 ml concentrated sulfuric acid (Kokate, 2005).	Presence of a brown ring at the interphase would signify presence of glycosides (Kokate, 2005).

3.5 Determination of *in vitro* and *in vivo* growth inhibition of *Agrobacterium tumefaciens* after use of *Artemisia annua* leaves and *Zingiber officinale* rhizome extracts

3.5.1 Isolation and characterization of *Agrobacterium tumefaciens*

The method used was used and reported by Bailey and Scott (1966). Young actively growing galls from rose plant were surface sterilized with 3% sodium hypochlorite and rinsed several times with sterile water. Three grams pieces of galls were grinded

using sterile mortar and pestle and put into 100 ml sterile beaker containing 30mls of sterile distilled water (Aysan and Sahin, 2003). The mixture was shaken for 30 minutes at 70 rpm and the supernatant used to inoculate yeast extract mannitol agar and yeast peptone glucose agar (semi selective media) using an inoculating loop.

Agrobacterium tumefaciens colonies were identified using their physical colony characteristics on yeast extract mannitol agar and yeast peptone glucose agar. Biochemical tests based on Bergey's Manual of Determinative Bacteriology were further used to confirm purity of the isolated bacteria (Holt *et al*., 1994). Inoculation of rose plants with *A. tumefaciens* during the green house experiment, as well confirmed *A. tumefaciens* through presence of crown gall.

3.5.2 Preparation of media used in isolation and morphological characterization of *Agrobacterium tumefaciens*

Isolation and characterization of *Agrobacterium tumefaciens* required the following media to be prepared.

3.5.2.1 Congo red Yeast Extract Mannitol Agar (YEMA)

YEMA contains 10 g Mannitol, 0.5 g potassium hydrogen phosphate, 0.2 g magnesium sulphate, 0.1g sodium chloride, 1 g yeast extract, 1 g calcium carbonate, 20 g agar and 2.5 ml dye. The pH of the media was adjusted to 6.8 – 7.4 using sodium hydroxide.

Into 1 litre of YEM broth in conical flask, 20 g of agar was added and the mixture heated for the agar to dissolve before autoclaving at 15 psi for 15 minutes at 121^{o}C. After autoclaving, the mixture was cooled in the lamina flow hood to 45^{o}C to prevent excessive condensation of water in the plates. The cooled mixture was dispensed into Petri dishes and after overnight, the YEMA plates were inoculated using a sterile loop full of bacteria suspension. The YEMA plates were then incubated at 28^{o}C for ten days after which sub culturing by streaking on fresh media from red stained isolated colonies was done to obtain pure cultures. *Agrobacterium tumefaciens* absorbs red dye from Congo red YEMA to appear as red stained colonies. This is one of the morphological characteristic of *A. tumefaciens* (Clark, 2007).

3.5.2.2 Yeast peptone glucose agar

The media contained 10 g glucose, 5 g peptone, 5 g yeast extracts and 20 g agar in 1000 ml distilled water. The medium was autoclaved for 15 minutes at 121^{o}C at 15 psi, allowed to cool to 45^{o}C and then dispensed into Petri dishes. The medium was streaked with inoculum from single red stained colonies from Congo red YEMA using a sterile inoculating loop. The Petri dishes were incubated in dark cabinet at 28^{o}C for two days. *Agrobacterium tumefaciens* establishes as whitish cream colonies and this is one of the morphological characteristic of *A. tumefaciens* (Clark, 2007).

3.5.2.3 Mueller Hinton Agar

The media was prepared by suspending 38 g of the medium in one litre of distilled water. The mixture was heated to boil in order to completely dissolve the medium

before autoclaving for 15 minutes at 121^{o}C at 15 psi. The medium was then cooled to room temperature and poured into sterile Petri dishes. The Petri dishes were stored at 4^{o}C for later use in susceptibility tests.

3.5.2.4 YEM Broth

Yeast mannitol broth is used for multiplying/cultivation of *Agrobacterium tumefaciens* and other soil microorganisms like nitrogen fixing rhizobium. It was prepared by suspending 11.8 g of YEM broth medium into 1000mls distilled water and then adjusting the pH to 6.8 using 0.1 mls of sodium hydroxide. The mixture was heated to dissolve before being autoclaved at 15 psi for 15 minutes at 121^{o}C. The mixture was then dispensed into the tubes awaiting bacteria inoculation.

3.5.3 Preparation of media used in biochemical characterization of *Agrobacterium tumefaciens*

The biochemical tests carried out included motility, gram staining, Urease, Citrate utilization, Catalase production and 3-ketolactose. These tests ascertained the isolated bacteria as *Agrobacterium tumefaciens* or otherwise. To carry out these tests, the following media were prepared.

3.5.3.1 Motility test (soft agar stabbing – tube method)

The media was prepared by suspending 36.23 g of SIM powder into 1000 ml of distilled water and the mixture heated for the powder to dissolve. The solution was then dispensed into tubes and autoclaved at 121^{o}C for 15 minutes at 15 psi. After autoclaving, the solution was cooled to 45^{o}C and then dispensed into test tubes (third

volume). Using isolated colonies from 18-24 hour old *Agrobacteriun tumefaciens* culture, the media was centrally stabbed and aerobically incubated (caps of the tubes loose) at 35^{o}C for 18- 24 hours. The result was analyzed for motility, hydrogen sulphide and indole.

3.5.3.2 Citrate utilization test

Simmons citrate agar medium is used to test the bacteria ability to use citrate as a sole carbon source and ammonium ions as the sole nitrogen source; signified by Simmons citrate agar color change of from forest green to intense blue (Koser, 1993). The medium contained 0.2 g magnesium sulphate, 1.0 g ammonium dihydrogen phosphate, 1.0 g Dipotassium phosphate, 2.0 g sodium citrate, 5.0 g sodium chloride, 15 g agar and 0.08 g bromothymol blue. The medium was adjusted to final pH of 6.8 at 25^{o}c using 0.1 ml sodium hydroxide. Simmons medium slants were prepared by suspending 24.28 g Simmons medium into 1000 ml of distilled water. The mixture was boiled to dissolve and then dispensed into tubes. The tubes were autoclaved at 121^{o}C for 15 minutes at 15 psi and allowed to solidify at an angle to form slants. Isolates were streaked on the surface of slant agar in a zigzag manner using a sterilized inoculating loop and the inoculated tubes were aerobically incubated at 35^{o}C for 48 hours and the color change noted.

3.5.3.3 Gram staining

Agrobacterium tumefaciens appears red- rod shaped when gram stained. Fresh *Agrobacterium tumefaciens* colony picked with sterile inoculating loop was thoroughly mixed with drop of sterile water on a microscope slide to form a thin

film which was air dried (Graham and Parker, 1964). The slide was held above flame for heat fixing and then cooled. Crystal violet dye was flooded on the smear and after two minute the smear was rinsed with distilled water to remove excess dye. The smear was then flooded with iodine solution and after two minute, it was discolored with 95% ethanol solution for 30 seconds. The mount was then washed in water, drained and counter stained with safranin for 1 minute. The slide was lastly rinsed with water, drained and allowed to air dry. Color and morphology of the cells were observed under x400 mg by light microscope with aid of immersion oil (Vincent, 2008).

3.5.3.4 Catalase production test

Agrobacterium tumefaciens produces catalase enzyme which decomposes hydrogen peroxide to oxygen gas (bubbling observed) and water. Fresh *Agrobacterium tumefaciens* isolates were transferred to a clean microscope slide using sterile inoculating loop and a drop of sterile distilled water added on the inoculum. After thorough mixing, a drop of 3% hydrogen peroxide was added on the smear, covered immediately with a cover slip and formation of bubbles checked (Shams *et al*., 2012).

3.5.3.5 Urease production test

Urea agar base medium contains 1 g enzymatic digest of gelatin, 1 g dextrose, 5 g sodium chloride, 2 g mono-potassium phosphate, 20 g urea, 0.012 g phenol red and 15 g agar. The medium was prepared by dissolving 24 grams of urea agar base in 950 ml of distilled water. The mixture was shaken and boiled to allow dissolving

before autoclaving at 121^{o}C for 15 minutes at 15 psi. After cooling the medium to 50^{o}C, 50 ml of 40% urea base was added to it and mixed thoroughly. About 4 to 5 ml of the mixture was aseptically poured into tubes which were allowed to cool when slanted. Heavy inoculum collected from *A. tumefaciens* culture prepared within 24 hours and streaked back and forth over the slant surface using inoculating loop (Aysan and Sahin, 2003). The inoculated tubes were aerobically incubated at 35^{o}C for two days and the color change observed. A control without urea was used.

3.5.3.6 Ketolactose test

Agrobacterium tumefaciens produces ketolactose enzyme which is responsible for precipitating cuprous oxide (yellow) from Benedict solution. Lactose broth consisted of 10 g lactose, 1 g yeast extract, 0.5 g Di potassium hydrogen phosphate, 0.2 g magnesium sulphate and 1000 ml of distilled water. The above medium was autoclaved at 121^{o}C for 15 minutes at 15 psi. After the medium was cooled to 45^{o}C, it was aseptically poured into tubes ready for inoculation after 24 hours. *Agrobacterium tumefaciens* from isolated colonies prepared within 18-24 hour was picked using an inoculating loop and stabbed at center of the medium. The inoculated medium was incubated aerobically at 28^{o}C for 18- 24 hours and then few drops of Benedict's reagent were added (Schaad, 1980). Observation was made after 2 hours.

3.5.4 Determination of Minimum Inhibitory Concentrations

This was achieved through broth micro dilution using a 96- well microtiter plate in accordance with CLSI (2011) guidelines. Extracts were initially emulsified in

Dimethylsulfoxide (50%) solution (Kelava and Cavar, 2011). Through serial two fold dilutions; *Zingiber officinale*, *Artemisia annua* and mixture of *Zingiber officinale* and *Artemisia annua* extracts of concentration 500 mg/ml, 250 mg/ml, 125 mg/ml, 62.5 mg/ml, 31.25 mg/ml, 15.6 mg/ml, 7.81 mg/ml and 3.9 mg/ml were put in different wells. In one cell, copper hydroxide (75 g/ ml copper oxide), certified by Kenya Bayer Crop Science was used as positive control. Copper hydroxide was used at manufacturer recommended rate of 6.25ml per liter of water. In one well, DMSO was used to serve as a negative control. The set up was done in triplicate. *Agrobacterium tumefaciens* inoculum prepared using Mueller – Hinton broth was adjusted to obtain turbidity comparable to that of MC, Farland 0.5 standard and then further diluted 1: 200 in Mueller Hinton broth (Kothari *et al*., 2010). About 0.5ml of this inoculum was dispensed in the wells of micro titer plate containing the extracts, copper hydroxide and DMSO and then incubated at 37^{o}C for 24 hours.

3.5.4.1 Data collection

Growth of bacteria was examined as a function of turbidity using Varioscan Flash (Simmons, 1999). Any turbidity or cloudiness in the wells signified growth of bacteria hence the concentration was unable to prevent bacterial growth. In every respective extract, the first cell that had extracts concentration that did not show any turbidity was recorded to be the minimum inhibitory rate. The minimum inhibitory concentrations of the extracts were used in greenhouse experimental treatments.

3.5.5 Preparation of sterile diffusion discs

Sterile discs were used in determination of antibacterial activity of *Zingiber officinale* and *Artemisia annua* extracts. The discs were made by punching Whatman's No.3 filter paper into 6 mm discs. The discs were then sterilized by autoclaving for 15 minutes at 121^{o}C and 15 psi.

3.5.6 Determination of antimicrobial activity of plant extracts using disc diffusion assay method

Two day old *Agrobacterium tumefaciens* suspension (0.1 ml of 10^{8}cells/ml) was swabbed on Petri dishes containing sterilized Muller Hinton Agar using sterile cotton mounted on wooden splint. Sterile Whatmman's filter dics were soaked in MIC solutions of *Zingiber officinale*, *Artemisia annua* and mixture of *Zingiber officinale* and *Artemisia annua*. Dimethylsulfoxide solvent was used as the negative control while copper hydroxide (6.25ml/l) was used as a positive control. The extracts impregnated discs were air dried and placed on the agar plates using sterile forceps. The set up was done in five replicate (CLSI, 2011). The plates were maintained for 2 hours at 4^{o}C and then incubated for 24 hours at 37^{o}C.

3.5.6.1 Data collection

Using a transparent ruler from the back of plates, the uniformly circular zones of no bacterial growth were measured (Kirby and Bauer, 1973). The zones of inhibition are usually clear areas around the disc against dense bacterial growth. The zones were measured from edge to edge crossing through the centre of the disc in

millimeter and rounded to the closest millimeter (Gooch, 2011). The measurements were taken from all the three replicates for each treatment and the average mean zone of inhibitions computed.

3.5.7 Determination of efficacy of plant extracts on roses in greenhouse

Uniform size plant seedlings of Dekora, the most susceptible rose variety were acquired from Naivasha in Kenya from a reputable propagation facility - Twiga flowers limited. Seedlings were transplanted into individual buckets of 20 cm diameter and 4.5 litre volumes. The buckets contained loamy soil that was sterilized by autoclaving for 15 minutes at 121^{o}C at 15 psi to remove soil borne pests and disease pathogens.

The experiment was laid out in a completely randomized design with six treatments with five replicates (Plate 3.2 and Figure 3.2). Treatments with *Artemisia annua* extract, *Zingiber officinale* extracts, mixture of *Zingiber officinale* and *Artemisia annua* and copper hydroxide (positive control) was done once per week for nine months from January to September, 2016. The treatments were done through stem and foliar sprays of respective extracts using a knapsack. Two negative controls, one with *A. tumefaciens* inoculated plants with no extracts application and another with uninoculated plants as well with no extracts application were added.

Daily irrigation of the treatments with 0.5 litre of water mixed with diammonium phosphate and calcium ammonium nitrate fertilizer was done every morning for nine months. Only spidermites (pest) and powdery mildew (disease) were noted.

Dynamec (active ingredient- abamectin) was used to control spidermites while score (active ingredient- difenoconazole) was used to control powdery mildew.

Plate 3.2: Arrangement of treatments in the greenhouse

	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Replicate 5
T1	Inoculated (no extracts)	Inoculated + (*Z. officinale* + *A. annua*)	Uninoculated (No extracts)	Inoculated + Copper Hydroxide	Inoculated + *Z. officinale*
T2	Inoculated + (*Z. officinale* + *A. annua*)	Uninoculated (No extracts)	Inoculated + Copper Hydroxide	Inoculated + *Z. officinale*	Inoculated + *A. annua*
T3	Uninoculated (No extracts)	Inoculated + Copper Hydroxide	Inoculated + *Z. officinale*	Inoculated + *A. annua*	Inoculated (no extracts)
T4	Inoculated + Copper Hydroxide	Inoculated + *Z. officinale*	Inoculated + *A. annua*	Inoculated (no extracts)	Inoculated + (*Z. officinale* + *A. annua*)
T5	Inoculated + *Z. officinale*	Inoculated + *A. annua*	Inoculated (no extracts)	Inoculated + (*Z. officinale* + *A. annua*)	Uninoculated (No extracts)
T6	Inoculated + *A. annua*	Inoculated (no extracts)	Inoculated + (*Z. officinale* + *A. annua*)	Uninoculated (No extracts)	Inoculated + Copper Hydroxide

Figure 3. 1: Arrangement of treatments in the greenhouse

The positive control (copper hydroxide), enabled comparison of the efficacy of plant extracts with conventional pesticide. The treatment with mixture of *Artemisia annua* and *Zingiber officinale* enabled analysis of possible antagonism or synergy of secondary metabolites from different plants. The negative controls, one with inoculation with no extracts and the other without inoculation and with no extracts enabled comparative analysis of disease progression in treatments with chemicals/extracts and the negative controls.

3.5.7.1 Data collection

The numbers of plants with crown gall symptoms (galls) in the five replicates of each treatment were counted and recorded at three months intervals and their means. The rate of increase in number of plants with crown galls at three months intervals was recorded for the five replicates of each treatment. Mature flowers stem lengths (from cut end to the tip of bud) from five replicates of each treatment were measured at three months interval using a ruler and their means computed. Analysis of galls interms of weight was done in the final month since it involved plucking of gall. Consequently, galls from all the five replicates for each treatment were plucked in the month of September, weighed and their means computed (Almeda *et al*., 2010).

3.5.8 Inoculation of the seedlings

The method used was one reported by Ark *et al*. (1960). Less than three days old YEMA slant of *Agrobacterium tumefaciens* was suspended in 10 ml of sterile distilled water. The suspension was then shaken vigorously to give a suspension of 10^8 cfu/ml. A sterile steel wire was used to make 3 mm deep wounds at three

locations on the seedling stems. In these wounds, 0.004 ml of *Agrobacterium tumefaciens* suspension was used to inoculate using sterile cotton mounted on wooden splint. The wounds were then wrapped with water soaked cotton wool for one week for successful inoculation to have taken place.

3.6 Data analysis

Crown gall prevalence data was analyzed using t- test set at 50% ($t = -8.187$, $P = 0.0001$). Data on zones of *Agrobacterium tumefaciens* growth inhibition *in vitro* was normally distributed hence did not require transformation. The data was directly subjected to one way Analysis of Variance (ANOVA) using Minitab software to test the significant difference between antibacterial activities of plants extracts at ($P \leq 0.05$). Greenhouse experiment data on stem length of mature flowers and weight of the plucked galls from different treatments was normally distributed and did not require transformation as well. It was therefore directly subjected to one way Analysis of Variance (ANOVA) using Minitab software to test the significant difference between treatments at ($P \leq 0.05$). The greenhouse data on the number of plants with galls was transformed using percentages before being subjected to one way Analysis of Variance. Significant means were separated using Tukeys' test. Crown gall progression in various treatments at three months intervals was analyzed using regression by adopting the model; $Y = \text{Constant} + \beta_1 X_1$ Where Y = Number of plants in treatment, β_1 = disease progression in the treatment as time changes. The results of data analysis were presented in figures and tables.

CHAPTER FOUR

RESULTS

4.1 Crown gall prevalence on rose varieties in the selected flowers farms

4.1.1 Crown gall prevalence in Nanyuki region

Survey from one farm in Nanyuki showed production of four rose varieties (Table 4.1). In terms of plant population, Bellarose variety had the highest production. It was closely followed by High and magic, and Mariyo. Wild calypso variety had the least plant production. Crown gall was prevalent in all four rose varieties grown in Tambuzi farm - Nanyuki region. A comparison of the level of prevalence among the varieties, done using a one sample t-test set at 50%, showed a significant difference ($t = -8.187$, $P = 0.0001$). The disease prevalence was highest in Bellarose (43.48%) and least in Wild calypso (1.43%).

Table 4. 1: Crown gall prevalence in Nanyuki region

Rose variety	No. of farms	Plant population	Sample	Plants with galls	% prevalence	Ranking (1-highest prevalence)
Bellarose	1	210000	8085	3515	43.48	1
High and magic	1	170000	6545	2463	37.63	2
Mariyo	1	140000	5390	2001	37.12	3
Wild calypso	1	116000	4466	64	1.43	4

4.1.2 Crown gall prevalence in Thika region

Survey from three farms in Thika region showed production of fourteen rose varieties (Table 4.2). In terms of plant population, Good times variety had the highest production followed by Marie Clare. Red secret variety had the least plant

production. Crown gall was prevalent in all fourteen rose varieties grown in Thika region (Table 4.2). A comparison of the level of prevalence among the varieties, done using a one sample t-test set at 50%, showed a significant difference (t = -8.187, P = 0.0001). The disease prevalence was highest in Dekora variety and least in Red secret (Table 4.2).

Table 4. 2: Crown gall prevalence in Thika region

Rose variety	No. of farms	Plant population	Sample	Plants with galls	% prevalence	Ranking (1-highest prevalence)
Dekora	3	130000	5005	2600	51.95	1
High and magic	2	320000	12320	5001	40.59	2
Labelle	1	120000	4620	1778	38.49	3
Chelsea	2	210000	8085	3105	38.40	4
Mariyo	2	125000	4812	1772	36.82	5
Bellarose	2	200000	7700	2755	35.78	6
Marie Claire	2	440000	16940	4948	29.21	7
Inka	1	350000	13475	3879	28.78	8
Good times	2	570000	21945	5267	24.00	9
Upper secret	1	90000	3465	484	13.97	10
Sonrisa	1	135000	5198	712	13.70	11
Red calypso	1	110000	4235	81	1.91	12
Madam red	1	195000	7508	18	0.23	13
Red secret	1	60000	2310	2	0.10	14

4.1.3 Crown gall prevalence in Naivasha region

Survey from four farms in Naivasha region showed production of twenty rose varieties (Table 4.3). In terms of plant population, Dekora and Red calypso variety

had the highest production followed by Sweet sher while Red alert variety had the least plant production (Table 4.3). Crown gall was prevalent in all twenty rose varieties grown in Naivasha region (Table 4.3). A comparison of the level of prevalence among the varieties, done using a one sample t-test set at 50%, showed a significant difference ($t = -8.187$, $P = 0.0001$). The disease prevalence was highest in Dekora variety and least in Moon walk (Table 4.3).

Table 4. 3: Crown gall prevalence in Naivasha region

Rose variety	**No. of farms**	**Plant population**	**Sample**	**Plants with galls**	**% prevalence**	**Ranking (1-highest prevalence)**
Dekora	4	580000	22330	14550	65.16	1
Crazy sher	1	280000	10780	5416	50.24	2
Sweet akito	1	180000	6930	3152	45.48	3
Mariyo	4	240000	9240	4100	44.37	4
Bellarose	3	420000	16170	6850	42.36	5
High and magic	3	380000	14630	6050	41.35	6
Marie Claire	3	120000	4620	1778	38.48	7
Pistache	1	175000	6738	2475	36.73	8
Athena	1	120000	4620	1692	36.63	9
Sweet sher	1	490000	18865	5438	28.82	10
Bright white	1	90000	3465	456	13.16	11
Red Naomi	1	108000	4158	489	11.75	12
Proud	2	150000	5775	384	6.65	13
Red alert	1	30000	1155	76	6.54	14
Upper class	2	285000	10973	187	1.70	15
Red ribbon	3	390000	15015	177	1.18	16
Wild calypso	2	330000	12705	145	1.14	17
Red calypso	3	580000	22330	250	1.12	18
Fuschia	2	185000	7123	60	0.84	19
Moon walk	2	150000	5775	30	0.52	20

Comparison of crown gall prevalence from different rose growing regions showed that same variety had different crown gall prevalence. For instance Dekora from Naivasha had crown gall prevalence of 65.16% while the one from Thika had

51.95%. Three rose varieties; Bellarose, High and magic and Mariyo grown in the three regions had crown gall prevalence varying from one region to another.

4.2 Management of crown gall disease in selected flower farms

4.2.1 Constraints of rose production in selected flowers farms

The common challenges in the farms include; finances, land acquisition, scarcity of planting materials, pests and diseases, labor availability, market and market prices, agricultural agrochemicals, transportation and weather (Table 4.4). Only four constraints were experienced in more than 50% of the farms, with pest and diseases being the highest with 75%. Three constraints; finance, scarcity of planting materials and transportation were experienced in 25%, 37.5% and 12.5% respectively. Agrochemicals availability and weather constraints were not experienced in any farm.

Table 4. 4: Constraints of rose production

	Frequency of farms responses to constraints								**Frequency**	**percentage**
Growing constraints	**Wildfire**	**Oljorua**	**Nini**	**Zena**	**Tambuzi**	**Karuturi**	**Gatoka**	**Penta**		
Finance/ Capital	0	1	0	0	0	0	1	0	2	25.0
Land acquisition difficulties	1	1	1	0	0	1	0	0	4	50.0
Scarcity of planting materials	1	0	0	0	1	0	1	0	3	37.5
Pests and diseases	1	1	0	1	1	1	1	0	6	75.0
Labor availability	0	0	0	1	1	0	1	1	4	50.0
Agrochemicals availability	0	0	0	0	0	0	0	0	0	0.0
Market and market prices	1	0	0	1	1	1	1	0	5	62.5
Transportation	0	0	0	0	0	0	1	0	1	12.5
Weather	0	0	0	0	0	0	0	0	0	0.0

Key: 0 – non constraint, 1- a constraint

4.2.2 Roses pests and diseases control

There are several common pests and diseases in rose production that are difficult to control (Table 4.5). Amongst them, there were no challenges in aphids, mealy bugs and botrytis control in the surveyed farms. Control of crown gall and spidermites was reported to be problematic in all surveyed farms while difficulty in powdery mildew and root knot nematodes control was experienced in 75% of the farms. Challenge in thrips and downy mildew control was reported in 37.5% of the surveyed farms.

Table 4. 5: Difficulty in pest and diseases control

	Control of the pest and disease in the farms									
Pest and disease	**Wildfire**	**Oljorua**	**Nini**	**Zena**	**Tambuzi**	**Karuturi**	**Gatoka**	**Penta**	**No. of farms**	**percentage**
Red spider mites	1	1	1	1	1	1	1	1	8	**100**
Thrips	1	0	1	0	0	1	0	0	3	**37.5**
Aphids	0	0	0	0	0	0	0	0	0	**0.0**
Nematodes	1	1	0	1	1	1	1	0	6	**75.0**
Powdery mildew	1	1	1	1	1	0	1	0	6	**75.0**
Downy mildew	1	1	1	0	0	0	0	0	3	**37.5**
Botrytis	0	0	0	0	0	0	0	0	0	**0.0**
Crown gall	1	1	1	1	1	1	1	1	8	**100**
Mealy bugs	0	0	0	0	0	0	0	0	0	**0.0**

Key: 0-unproblematic to control, 1- problematic to control

4.2.3 Crown gall disease control using conventional chemicals

Results showed that five different conventional chemicals were used to control crown gall disease in the surveyed farms (Table 4.6). Sporekill and copper hydroxide were used in all farms to control crown gall. Only 50% of the farms used

sodium hypochlorite while physan and green copper was used in 25% and 12.5% of the farms respectively.

Table 4. 6: Chemicals used in controlling crown gall

	Chemical used in control of crown gall				
Farm	**Green copper**	**Copper hydroxide**	**Sporekill**	**Sodium hypochlorite**	**Physan**
Wildfire	0	1	1	0	0
Oljorua	0	1	1	1	0
Nini	0	1	1	0	0
Penta	1	1	1	1	0
Gatoka	0	1	1	1	1
Tambuzi	0	1	1	0	0
Karuturi	0	1	1	1	1
Zena	0	1	1	0	0
Frequency	1	8	8	4	2
percentage	**12.5**	**100**	**100**	**50**	**25.0**

Key: 0-Not used, 1-Used

4.2.4 Integrated management of crown gall disease

The finding showed that management of crown gall was only possible through use of different methods - integrated disease management strategy (Table 4.7). Management of crown gall through quarantine and by synthetic chemicals was reported in all farms. Wildfire, Oljorua and Zena farms, also managed crown gall by plucking galls and painting the resultant wounds. Zena and Tambuzi farm also used *Trichoderma* and *Agrobacterium radiobacter* respectively. Tambuzi and Wildfire farms also uprooted and burnt affected plants. Trials of plant extracts were only reported in Penta and wildfire farms (Table 4.7).

Table 4. 7: Crown gall management methods

Crown gall control method	Farms								Frequency	percentage
	Wildfire	Oljorua	Nini	Penta	Gatoka	Tambuzi	Karuturi	Zena		
Chemicals	1	1	1	1	1	1	1	1	**8**	**100**
Uprooting and burning	1	0	0	0	0	1	0	0	**2**	**25**
Plucking galls and painting	1	1	0	0	0	0	0	1	**3**	**37.5**
Quarantine	1	1	1	1	1	1	1	1	**8**	**100**
Agrobacterium radiobacter	0	0	0	0	0	1	0	0	**1**	**13**
Trichorderma fungi	0	0	0	0	0	0	0	1	**1**	**13**
Plant extracts	1	0	0	0	0	0	0	1	**2**	**25**

Key:1- method used: 0-method not used

4.3 Phytochemical analysis of *Artemisia annua* leaves and *Zingiber officinale* rhizome extracts

The color/ characteristic changes (Plate 4.1 and Plate 4.2) confirmed presence or absence of the secondary metabolite under investigation. The resultant colors showed that *Zingiber officinale* rhizome and *Artemisia annua* leaves extracts contained several phytochemicals known for antimicrobial activities. *Zingiber officinale* rhizome contained only three secondary metabolites; namely flavonids, alkaloids and terpenoids. On the other hand, *Artemisia annua* leaves contained six secondary metabolites; namely saponins, tannins and phenols, glycosides, flavonoids, alkaloids and terpenoids (Table 4.8).

Saponin (-) | Tannins & Phenols (-) | Glycosides (-) | Flavonoids (+) | Alkaloids (+) | Terpenoids (+)

Plate 4. 1: *Zingiber officinale* rhizome extract phytochemicals tests color changes

Plate 4. 2: *Artemisia annua* leaves extract phytochemicals tests color changes

Table 4. 8: Phytochemical constituents of *Z. officinale* and *A. annua* extracts

Plant	Saponin Test	Tannin & phenols test	Glycoside test	Flavonoids Test	Alkaloids test	Terpenoids test
Zingiber officinale	-	-	-	+	+	+
Artemisia annua	+	+	+	+	+	+

Key: + Present; - Absence

4.4 Efficacy of *Artemisia annua* leaves and *Zingiber officinale* rhizome extracts in management of crown gall disease

4.4.1 Morphological characterization of isolated *Agrobacterium tumefaciens*

The results showed that inoculum from the crown galls absorbed Congo red dye from yeast extract mannitol agar (YEMA) to form red stained, circular, raised-convex colonies (Plate 4.3). The red stained isolates from YEMA established as

whitish- cream, raised-convex with smooth margins colonies on yeast peptone glucose agar which is a selective media for *Agrobacterium tumefaciens* (Plate 4.4).

Plate 4. 3: Red stained *A. tumefaciens* colonies on YEMA

Plate 4. 4: *Agrobacterium tumefaciens* colonies on yeast peptone glucose agar

4.4.2 Biochemical characterization of isolated *Agrobacterium tumefaciens*

4.4.2.1 Gram staining

Rod shaped red stained *Agrobacterium tumefaciens* cells were seen under x400 mg light microscope oil immersion lens (Plate 4.5). The result confirmed that *Agrobacterium tumefaciens* is Gram negative.

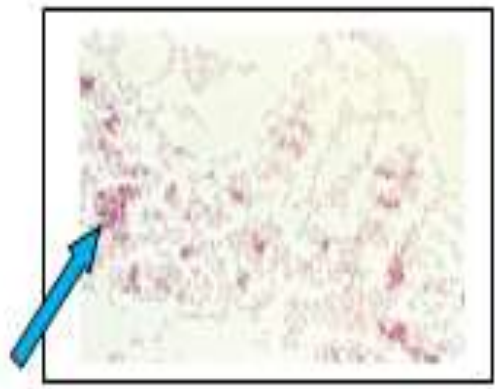
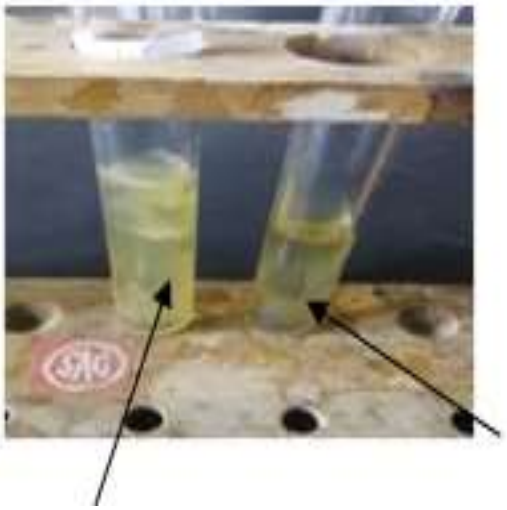


Plate 4. 5: Red stained *A. tumefaciens* colonies

4.4.2.2 Motility test (soft agar stabbing – tube method)

Agrobacterium tumefaciens caused cloudiness as they migrated along the stab line within the medium (Plate 4.6). There was no such cloudiness on unstabbed medium. Along the stab line, there was no insoluble black ferrous sulfide precipitate normally seen as black line, confirming that *Agrobacteriun tumefaciens* is negative for hydrogen sulfide reaction. A red layer on top of the medium was as well not formed when Kovac's reagent was added; confirming that *Agrobacteriun tumefaciens* is indole negative.



Unstabbed media – clear (control)

Cloudiness within the media

Plate 4. 6: Cloudiness caused by *A. tumefaciens* movements

4.4.2.3 Citrate utilization

Ammonium salts in the Simmons citrate medium was utilized by *Agrobacterium tumefaciens* as the carbon source. After citrate metabolism, the by-products (carbonates and bicarbonates) raised the pH of the medium causing the color of bromothymol blue to change from forest green to intense blue (Plate 4.7).

Uninoculated (control) Inoculated slant

Plate 4. 7: Citrate metabolism

4.4.2.4 Catalase production

Agrobacterium tumefaciens produced bubbles to confirm that it is catalase-positive (Plate 4.8). There were no bubbles liberated when bacteria was not added. Catalase-negative bacteria would not produce bubbles.

(a) (b)

(a) Bubbles and froth seen under the cover slip- pointed by an arrow

(b) No froth or bubbles- bacteria not added

Plate 4. 8: Decomposition of hydrogen peroxide

4.4.2.5 Urease production

Agrobacterium tumefaciens produced urease enzyme that hydrolyzed the urea in the medium to produce ammonia which raised the pH from 6.8 – 8.2. High pH led to medium color change from yellow to fuchsia (Plate 4.9).

Uninoculated agar Inoculated hydrolyzed agar

Plate 4. 9: Hydrolysis of urea by *A. tumefaciens*

4.4.2.6 Ketolactose test

Ketolactose enzyme produced by *Agrobacterium tumefaciens* caused the precipitation of cuprous oxide in the lactose broth leading to the yellow precipitate (Plate 4.10).

(a) (b)

(a) Intense yellow precipitate, (b) Absence of yellow precipitate (uninoculated)
Plate 4. 10: Formation of cuprous oxide

4.4.3 *In vitro* growth inhibitory of *Agrobacterium tumefaciens* by *Artemisia annua* leaves and *Zingiber officinale* rhizome extracts

Inhibitory zones of *Agrobacterium tumefaciens* around the filter disc were seen as light areas surrounding the filter discs, where *A. tumefaciens* did not establish due to the effect of products into which filter discs were soaked (Plates 4.11 – 4.14). Mean of inhibitory zone within the discs soaked in *Artemisia annua* extract and those soaked in copper hydroxide were similar and significantly higher than mean inhibitory zones of *Zingiber officinale* and the mixture of *Zingiber officinale* + *Artemisia annua* ($F = 43.93$, $P = 0.0001$). Dimethylsulfoxide, which served as negative control did not inhibit *Agrobacterium tumefaciens* growth hence did not have inhibitory zone (Table 4.9).

Plate 4. 11: Inhibitory zone of Dimethylsulfoxide

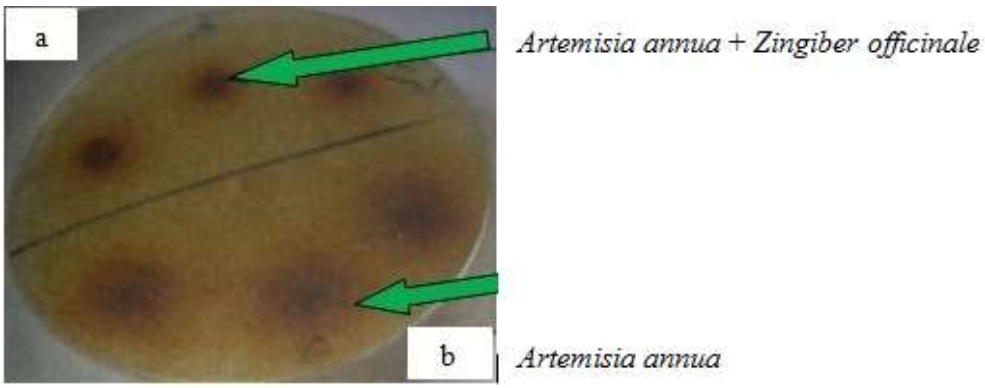


Plate 4. 12: Inhibitory zone of *Artemisia annua* and mixture *Artemisia annua* and *Zingiber officilale*

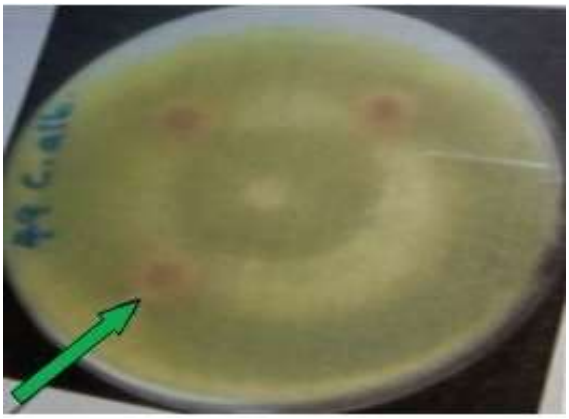


Plate 4. 13: Inhibitory zone of *Zingiber officinale*

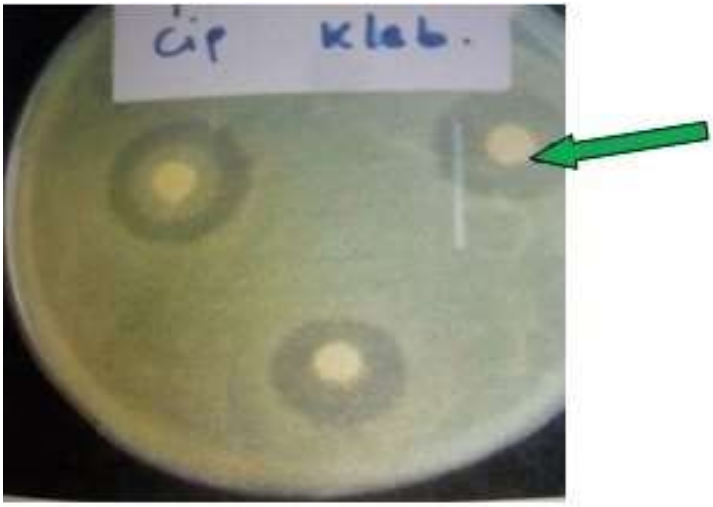


Plate 4. 14: Inhibitory zone of copper hydroxide

Table 4. 9: Inhibitory zones of *Agrobacterium tumefaciens in vitro*

Treatment	Mean zone of inhibition (mm) ± SE	F -value	P- value
Zingiber officinale	10.6 ± 0.678b		
Copper hydroxide (+ control)	12.8 ± 0.374a		
Dimethyl sulfoxide (- control)	6.0 ± 0.00c		
Zingiber officinale +Artemisia annua	10.2 ± 0.374b		
Artemisia annua	12.8 ± 0.374a	43.932	0.0001

Mean values in the same column denoted by similar letters are not significantly different at $P \leq 0.05$. Mean separated using Tukeys' HSD

4.4.4 Minimum inhibitory concentration of *Zingiber officinale* and *Artemisia annua* crude extracts

The findings showed a significant *Agrobacterium tumefaciens* growth inhibition in lactose broth by certain concentrations of *Zingiber officinale* rhizome, *Artemisia annua* leaves extracts and copper hydroxide. Presence of cloudiness in the broth signified *A. tumefaciens* growth while absence of the cloudiness signified inhibition of *A. tumefaciens* growth (Table 4.10). The minimum inhibitory concentration of *A. annua* extract was 125mg/ml; that of *Z. officinale* extract was 62.5mg/ml while

mixture of *A. annua* and *Z. officinale* was 31.25mg/ml. These were the most economical (lowest) concentrations of the extracts that were able to inhibit growth of *A. tumefaciens*. Copper hydroxide inhibited growth at manufacturers recommended rate of 6.25ml/l.

Table 4. 10: Minimum inhibitory concentration of Artemisia and Ginger extracts

Test	**Concentration of extracts in 1ml Dimethyl sulfoxide**							
	1000	500	250	125	62.5	31.25	15.6	7.81
Artemisia annua	-	-	-	+	+	+	+	+
Zingiber officinale	-	-	-	-	+	+	+	+
Zingiber officinale + *Artemisia annua*	-	-	-	-	-	+	+	+
Copper hydroxide, 6.25ml/l (+ control)	-	-	-	-	+	+	+	+
50% DMSO (Negative control)	+	+	+	+	+	+	+	+

Key: (+) cloudiness; (-) no cloudiness

4.4.5 Efficacy of *Artemisia annua* leaves and *Zingiber officinale* rhizome extracts in management of crown gall disease in the greenhouse

4.4.5.1 Number of plants with crown gall symptoms during greenhouse experiment

Results showed that all treatments had plant showing galls in the month of March and May except the uninoculated treatment. There was therefore a significant difference in the uninoculated treatment and other treatments in the month of March ($F = 1.58$, $P = 0.238$) and May ($F = 5.93$, $P = 0.005$) respectively (Table 4.11). Significant difference between uninoculated and other treatments was also noted in

the month of July (F = 5.62, P = 0.007). Similarly, there was a significant difference in the uninoculated treatment and other treatments in the month of September (F = 7.28, P = 0.002).

The results showed steady increase in number of plants with galls from the month of March to September in all treatments except uninoculated without extracts (uninoculated – no extracts). In September, the treatment with the highest number of plants with galls was the inoculated plants with no extracts application with eleven plants followed by inoculated with *Zingiber officinale* application (nine plants). Notably, crown gall symptoms appeared on uninoculated without extracts after six months and the number of plants with galls remained constant (one plant).

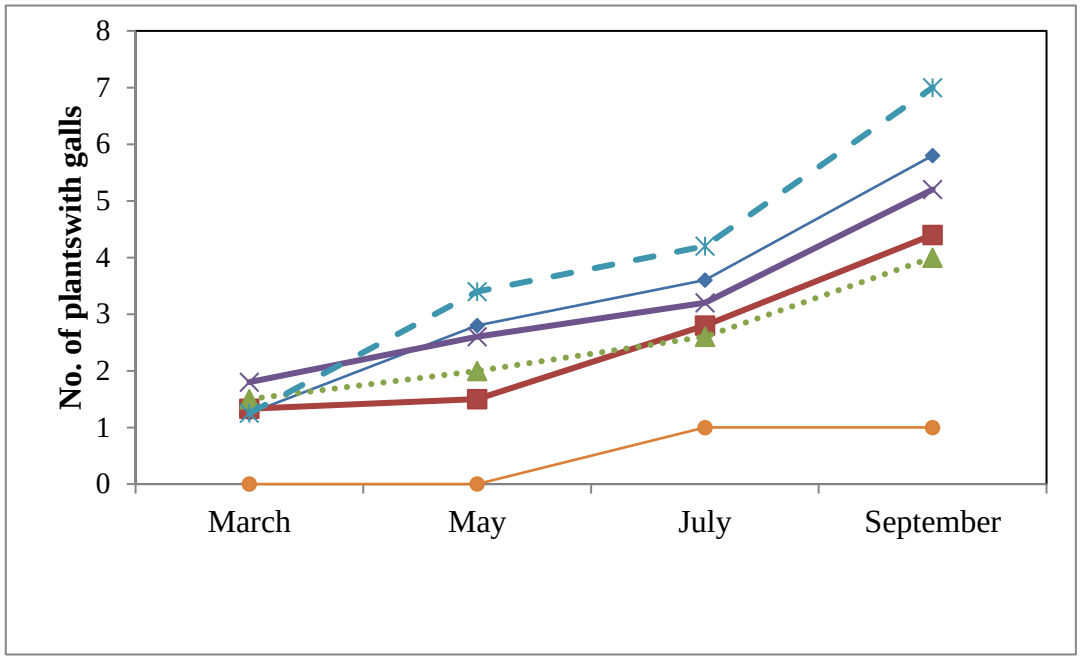
Table 4. 11: Number of plants with galls during treatments

Treatment	March	May	July	September
Inoculated + *Zingiber officinale*	1.00a	4.00b	6.00b	9.00b
Inoculated + *Artemisia annua*	1.00a	3.00b	4.00b	7.00b
Inoculated + Copper hydroxide	1.00a	3.00b	4.00b	6.00b
Inoculated + *Zingiber officinale* +*Artemisia annua*	2.00a	4.00b	5.00b	8.00b
Inoculated – no extracts	2.00a	5.00b	7.00b	11.00b
Uninoculated – no extracts	0.00a	0.00a	1.00a	1.00a
F-value	1.58	5.93	5.62	7.28
P-value	0.238	0.005	0.007	0.002

Mean values in the same column denoted by same letters are not significantly different at $P \leq 0.05$. Means separated using Tukeys' HSD

Crown gall disease progressed steadily from March to September in all treatment as shown in figure 4.1.

The progression was rapid where plants were inoculated and antibactericide was not used and lowest where plants were not inoculated and antibactericide was not used.



Key: Regression equations

Inoculated plants + Zingiber officinale = 0.250 +1.44 Time

Inoculated plants + Artemisia annua = 0.120 + 1.05 Time

Inoculated plants + Copper hydroxide = 0.500 + 1.08 Time

Inoculated plants + Zingiber officinale + Artemisia annua = 0.500 + 1.08 Time

Inoculated plants - no extracts = 0.550 + 1.80 Time

Uninoculated plants/ - no extractss = 0.500 + 0.400 Time

Figure 4. 1: Comparative crown gall progression

4.4.5.2 Length of the harvested stems

The findings showed no significant difference in stem length of harvested flowers from all treatments in the month of March (F = 0.96, P = 0.046) (Table 4.12). The stem length of harvested flowers from *Zingiber officinale* were significantly shorter than from other treatments in the month of May (F = 10.32, P = 0.0001). Stems from *Artemisia annua*, Copper hydroxide, mixture of *Zingiber officinale* + *Artemisia annua* and uninoculated were significantly taller than those from *Zingiber officinale* and inoculated plants without antibactericide treatment in the month of July (F =

18.07, P = 0.0001). In the month of September, the stem length from all treatments were significantly shorter than uninoculated plant (F = 22.04, P = 0.0001).

There was a significant stem length improvement from the month of March to May in all treatments except *Zingiber officinale* + *Artemisia annua*. A significant decline in stem length was observed from the month of May/July to September in all treatments except the uninoculated without antibactericide treatment. The results showed differences between the shortest and tallest stem amongst the treatments at every analysis becoming bigger from March to September. As such, the difference between the shortest stem 47.8 cm and the tallest stem 59.8cm in the month of March was 12 cm compared to 30cm in the month of September where shortest stem was 47.4cm and tallest 77.4cm.

Table 4. 12: Length of flower stems harvested

Treatment	**March**	**May**	**July**	**September**
Inoculated + *Zingiber officinale*	47.8a	65.8b	55.0a	48.8a
Inoculated + *Artemisia annua*	57.6a	73.2b	70.6b	61.4a
Inoculated + Copper hydroxide	48.4a	75.0b	77.8b	62.8a
Inoculated + *Zingiber officinale* +*Artemisia annua*	50.6a	62.4a	74.2b	54.4a
Inoculated + No extracts	47.2a	69.4b	54.2a	47.4a
Uninoculated + No extracts	59.8a	78.4b	78.2b	77.4b
F-value	0.96	10.32	18.07	22.04
P-value	0.046	0.0001	0.0001	0.0001

Mean values in the same column denoted by similar letters are not significantly different at P≤ 0.05. Means separated using Tukeys' HSD

4.4.5.3 Gall weights

The ultimate gall weights of copper hydroxide, *Artemisia annua* and uninoculated treatments were significantly different for the other treatments (F =4.733, P = 0.0001).

Table 4. 13: Gall weights in different treatments

Treatment	Mean gall weight ± SE	F -value	P-value
Inoculated + Copper hydroxide	30.29 ± 7.35a		
Inoculated + *Zingiber officinale* + *Artemisia annua*	33.19 ± 6.82b		
Inoculated + *Zingiber officinale*	32.47 ± 6.39b		
Inoculated + *Artemisia annua*	30.57 ± 6.55a		
Inoculated + No extracts	52.94 ± 8.06b		
Uninoculated + No extracts	0.06 ± 0.06a	4.733	0.0001

Mean values in the same column denoted by similar letters are not significantly different at $P \leq 0.05$. Means separated using Tukeys' HSD

CHAPTER FIVE

DISCUSSION

5.1 Prevalence of crown gall in different rose varieties in selected flower farms

The study established that there was a wide range of rose varieties grown depending on the market demand. Adverse effects of crown gall disease in terms of loss in production and poor flower quality were reported in all farms under the study. Breeding for resistant varieties was untenable owing to dynamic tastes and preference in the market. Rose varieties remained in the farms so far as their demand in the market was high. FloraHolland (2017) report on diversity of rose varieties confirmed that choice of rose varieties mainly depended on market trends. Availability of capital, market diversity and land availability were the other factors considered in choosing rose varieties to be grown.

Diseases and pests ranked highest among the challenges of roses production. Crown gall and red spider mites were reported to be the most challenging to control. There were no challenges of getting agrochemicals due to the availability of manufacturers and suppliers like Amiran Kenya, Bayer crop science, Elgon chemicals and Osho chemicals. The findings of this study compares well with report by Kariuki (2015), on challenges of Kenya flower industry where crown gall and red spidermites were underlined as the main problems in rose production.

Growing of roses from the studied farms was done mainly under greenhouse; only one farm had outdoor roses. Greenhouses modified temperature, humidity and

precipitation to allow growing of quality flowers. Greenhouses also enabled better management of diseases and pests which was critical to flowers quality. This result compares to the report by Teitel (2017) on the importance of greenhouses in temperature, humidity and precipitation control in flowers production. The study found that flowers affected by crown gall had galls, short stem length and poor plant growth. *Agrobacterium tumefaciens* allocates water and nutrients to rapidly dividing cells (galls) at the expense of the plant thereby affecting the plant growth as reported by Schroth (2008). Crown galls serve as route for secondary infection which weakens plant and compromise flowers quality (Agrios, 2005).

Crown gall prevalence among rose varieties showed that all varieties were susceptible but the number of affected plants differed from one variety to another for instance Dekora variety had the highest prevalence of 65.16% and Red secret had the lowest with 0.1%. The study also revealed that the same variety from different regions/farms had different crown gall prevalence. For instance Bellarose variety from Naivasha region had crown gall prevalence of 42.36% compared to that from Thika with a prevalence of 35.78%. Macharia (2018) reported on red calypso, Akito and shanty being crown gall resistant rose varieties. This was however, inconsistent with the findings of this research because the same varieties were infected with crown gall. It therefore means that, other factors of age and cultural operation influences susceptibility as reported by Arim (2011).

5.2 Management of crown gall disease

The study findings indicated that there was little investment in breeding for resistance because the changing customers taste could not guarantee a variety to remain in production. This compares well with Kariuki (2015), report on the challenges of breeding for resistance in roses.

The spread of crown gall disease in all rose varieties was attributable to age of the plant and agronomical activities such as weeding, bending, pruning and harvesting. These activities caused wounds that facilitated entry of the *Agrobacterium tumefaciens* into the plant cell. This was evident during the research when a treatment with uninoculated plants showed crown gall symptoms after six months, a sign of transmission from the other inoculated treatments during the cultural practices. This result is supported by Agrios (2005), on the requirement of a fresh wound for *Agrobacterium tumefaciens* to gain entry to the plant cell.

Farms reported the need for crop rotation as a cultural method to manage crown gall. However, many farms lacked large pieces of land to allow crop rotation because it required abandoning a field for five years with non- susceptible plants. Burr (1993) reported that soils in fields with a history of growing flowers, fruits and nuts hold *Agrobacterium tumefaciens* saprophytically within the rhizosphere of woody and herbaceous weeds for up to five years awaiting susceptible host. Rhouma *et al*. (2006) reported on the need to plant *Ailanthus*, *Berberis*, *betula*, *catalpa* and *cedrus* as rotation plants to break crown gall cycle in affected fields.

The study findings indicated that getting crown gall free planting materials was a challenge to the farms. They attributed the problem to the fact that most propagation

farms in Kenya did not ensure purity of crown gall through DNA testing but depended on visible symptoms (galls) in selecting their breeding stock. They did this largely due to limitations of capital. Rose breeders did not import certified materials from European countries but depended on local materials because they are cheap. Farms reported cases where crown gall remained systemic in the planting materials, only to manifest after seedlings establishment. Arim (2011) reported a case of crown gall free planting materials that developed crown gall after plants establishment.

Synthetic chemicals were the main component of crown gall control methods, where copper based chemicals were considered the most effective in all farms. Ammonia based chemicals, which acted through oxidizing plant and working tools surfaces were as well used. The dependency on synthetic agrochemicals was driven by the fact that they offered quick results. Shabana *et al*. (2017) reported a similar study where use of agrochemicals was found to be the main strategy by most farmers in disease and pest control.

The study findings indicated that farms did not use biological methods of crown gall disease control except one farm which used *Agrobacterium radiobacter* K84. *Agrobacterium radiobacter* K84 is a widespread, naturally occurring avirulent bacterium found in the soil near plant roots. *Agrobacterium radiobacter* strain K84 is the commonly used biocontrol agent. It produces agrocin 84 which has antibacterial activity against *Agrobacterium tumefaciens*. Farms reported inefficiency of *Agrobacterium radiobacter* in controlling crown gall. This compares to the study by Burr (1999) and Arim (2011) on use of *Agrobacterium radiobacter* in crown gall control where the disease was found to recur.

Farms reported that crown gall disease developed resistance to copper based fungicides. There were also concerns of reliance on synthetic chemicals because it faced resistance from European nations flower markets. As such, use of some chemicals was restricted due to their effects on yields and environment. Chemicals like alphadime, dimethoate and cypermethrin were banned and their traces above regulatory levels led to rejection of exported flowers. This compares to the finding by FloraHolland (2017) on acceptable maximum residue levels of chemicals to European nations market. Kumari *et al*. (2014) and Dalamas and koutroubas (2015) reported on the development of synthetic agrochemicals resistance to various diseases and pests, hence supporting the findings of this study. Owing to the challenges faced by farms in management of crown gall, they emphasized on quarantine and adopted integrated management strategy to control crown gall disease.

5.3 Determination of secondary metabolites present in *Artemisia annua* leaves and *Zingiber officinale* rhizome extracts

Methanolic extracts of *Artemisia annua* leaves revealed presence of flavonoids, tannins and phenols, alkaloids, terpernoids, saponins and glycosides. *Zingiber officinale* rhizome extracts revealed presence of Alkaloids, flavonoids and terpenoids. A study by Eloff *et al*. (1998) reported methanol's better results than ethanol, water and hexane in getting medicinal plants extracts. Ethanol was found to have poor selectivity hence required efficient purification that led to loss of metabolites. Another study by Lin *et al*. (2007 reported that water was unable to dissolve essential oils making it inefficient. Quantitative study to detect amount of

flavonoids and phenols from *Zingiber officinale* using different solvents; butanol, methanol, ethyl acetate and chlorofoam revealed that methanol extracts had maximum antioxidant and antimicrobial activity (Alabri *et al*., 2014).

Phytochemical analysis results on *Artemisia annua* leaves in this research compares well with results from India and Turkey which shown majority of the metabolites found in this study (Kumar and Rathinam, 2013). .*Artemisia annua* leaves from Nigeria revealed alkaloids and flavonoids and lucked tannins and saponins (Ajah *et al*., 2013). Phytochemical analysis results of *Zingiber officinale* rhizome in this research compares well with results by Jiang *et al*. (2005) which revealed presence of alkaloids, flavonoids, phenylpropanoid and terpenoids. Similarly, report by Siddiqui *et al*. (2009) also found the same secondary metabolites in analysis of *Zingiber officinale* rhizome extracts.

5.4 Determination of *in vitro* and *in vivo* growth inhibition of *Agrobacterium tumefaciens* after use of *Artemisia annua* leaves and *Zingiber officinale* rhizome extracts

5.4.1 Isolation and characterization of *Agrobacterium tumefaciens*

Agrobacterium tumefaciens isolated from crown galls absorbed Congo red dye in Congo red YEMA medium and appeared convex, raised red stained and circular colonies. Mannitol in the YEMA served as carbon source while the yeast extract served as nitrogen source and growth factors for *Agrobacterium tumefaciens*. Magnesium in the medium provided cations required for bacterial growth. The red

stained isolate showed well pronounced growth in glucose peptone agar which is a selective media for *Agrobacterium tumefaciens*. These results were in agreement with those reported by Aysan and sahin (2003) and Islam *et al*. (2010) on physical characteristics of *Agrobacterium tumefaciens*.

Gram stained colonies were seen as rod shaped and red stained under x400 mg light microscope oil immersion lens. *Agrobacterium tumefaciens* caused cloudiness as they migrated along the stub line of soft agar (SIM) signifying motility. The semisolid nature and light amber color of the medium enabled the bacteria motility to be noted. The ferrous ammonium sulfate component of the medium served as hydrogen sulfide indicator. Holt *et al*. (1994), in Bergey's manual of determinative bacteriology reported that gram negative bacteria generally grow red in YEMA supplemented with Congo red dye.

Agrobacterium tumefaciens did not produce hydrogen sulfide which combines with ferrous ammonium sulfate to form insoluble black ferrous sulfide precipitate seen along the stab line, confirming *Agrobacterium tumefaciens* to be negative for hydrogen sulfide reaction. The tryptophan in the medium was not hydrolyzed to produce indole, pyruvic acid and ammonium which are responsible for formation of a red layer on top of the medium, also confirming that *Agrobacterium tumefaciens* was indole negative. These results are similar to those reported in Bergy's manual of determinative bacteriology (Holt *et al*., 1994).

Agrobacterium tumefaciens isolates utilized ammonia salts in Simmons citrate medium, leading to metabolites that increased the pH of the medium hence the color

change from forest green to intense blue. The isolates also produced catalase enzyme which decomposed hydrogen peroxide to oxygen gas and water. The isolates as well produced urease enzyme that hydrolyzed urea in the urease medium causing release of ammonia which changed the pH of the media from acidic to alkaline leading to medium color change from yellow to fuchsia. Unlike most bacteria, *Agrobacterium tumefaciens* produced ketolactose enzyme which precipitated cuprous oxide in Benedict's reagent found in lactose broth. These results were completely in agreement with biochemical results carried out by Koivunen *et al*. (2004) and Aysan and Sahin (2003) when distinguishing *Agrobacterium tumafaciens* with *Agrobacterium radiobactor*.

During the greenhouse experiments, all inoculated plants developed crown galls after two months except the uninoculated treatment which developed crown gall after six months. It was presumed that cultural activities among the treatments caused transmission of crown gall from inoculated treatments to uninoculated treatment. Galls developed when *Agrobacterium tumefaciens* entered the plant cell and transferred a gene that caused uncontrolled cell proliferation to the plant cell. This result compares well with report by Schell *et al*. (2009) and Aysan and Sahin (2003) on *Agrobacterium tumafaciens* pathogenesis. The study findings indicated steady increase in galling from the month of March to September while the stem length steadily declined in all treatments within the same period. Increased galling and decline in stem length resulted from compromised plant growth due to build up of *Agrobacterium timefaciens* within the plants. These results were in agreement with report by Arim (2011) on the effects crown gall disease on rose plant growth.

5.4.2 Determination of antimicrobial activity of plant extracts using disc diffusion assay method

Both *Artemisia annua* and *Zingiber officinale* extracts had antibacterial activity against *Agrobacterium tumefaciens*. The zones of inhibition for all extracts in this research were greater than 9mm and were therefore considered active. Zones of inhibition greater than 9mm are considered to have significant antimicrobial activity (Celikel and Kavas, 2008). *Artemisia annua* and Copper hydroxide had similar antibacterial effect with an inhibition zone of 12.8mm. *Zingiber officinale* and mixture of *Zingiber officinale* and *Artemisia annua* also had remarkable activity against *Agrobacterium tumefaciens* with an inhibition zone of 10.6mm and 10.2mm respectively. This result compares with study on *Artemisia annua* in USA which had inhibition zones of 13mm (Mikicinski *et al*., 2012). De-Souza *et al*. (2015) reported tests of *Artemisa anuua* with inhibition ranging from 9.15mm to 13.6mm which also compares well with the result of this study. *Zingiber officinale* rhizome extracts antimicrobial activity done by Chandarana *et al*. (2005) and Onyeagba *et al*. (2014) revealed zones of inhibition comparable to the results of this study.

5.4.3 Determination of efficacy of plant extracts on roses in greenhouse

The number of plants with crown galls, ultimate weight of plucked galls and mature flowers stem length from greenhouse treatments were directly related to the size of zone of inhibition achieved by the extracts *in vitro*. As such, the treatment with the biggest zone of inhibition had the highest number of plants with crown galls, shorter stem length and higher plucked gall weight. These results were in agreement with report by Arim (2011) on the effects crown gall disease on rose plant growth.

The antibacterial effects noted *in vitro* and *in vivo* were attributable to the secondary metabolites in *Artemisia annua* and *Zingiber officinale* extracts. Flavonoids, one of the phenolic compounds found in both *Artemisia annua* and *Zingiber officinale* acted on *Agrobacterium tumefaciens* by forming antibacterial complexes with extracellular soluble proteins of bacterial cell wall. In a study of antimicrobial effect of phytochemicals, Orhan *et al*. (2010) and Trease *et al*. (2012) reported on the activity of flavonoids on a bacterium.

Artemisia annua extracts had better performance than *Zingiber officinale* and mixture of *Artemisia annua* and *Zingiber officinale* in terms of the number of affected plants, stem length, and galls weight. This was attributable to the combination of flavonoids and tannins (phenolic compounds) which worked synergistically to achieve better results. The poor performance of the mixture of *Artemisia annua* and *Zingiber officinale* can only be explained in terms of antagonistic functioning of secondary metabolites. Yadav and Agarawala (2011), in a study of comparative performance of different medicinal plants metabolites reported on the synergy of phenolic compounds.

The presence of alkaloids in both *Artemisia annua* and *Zingiber officinale* contributed to antibacterial activity. The alkaloids acted by interfering with the impulses transmission, altering the cell membrane and interfering with synthesis of functional protein. Saponins contributed to antibacterial activity by binding with the cell membrane cholesterol and forming complexes that made pores on the cell surface of *Agrobacterium tumefaciens*. Schiff (2002) and Gauthier *et al*. (2009) reported on the activity of alkaloids and saponins on plants bacteria.

CHAPTER SIX

CONCLUSION AND RECOMMEDATIONS

6.1 Conclusion

Through the research, the following were revealed;

1. Crown gall is prevalent in rose flower industry, with Dekora variety being the most susceptible and Red secret being the most resistant.
2. Farmers relied on quarantine and integrated crown gall disease management approach to control the disease since no one specific control method is efficient.
3. *Zingiber officinale* and *Artemisia annua* extracts demonstrated antibacterial effects *in vitro* and *in vivo*; hence applicable in managing crown gall disease of roses.
4. The *in vivo* and *in vitro* performance of *Artemisia annua* extract was better than *Zingiber officinale*, the mixture of *Zingiber officinale* and *Artemisia annua* and only compared well with that of copper hydroxide.
5. *Artemisia annua* extracts can be used to replace conventional copper hydroxide in control of crown gall disease.

6.2 Recommendations

The research recommends the following;-

1. Farmers to continue with integrated crown gall control methods.
2. Farmers to include *Artemisia annua* and *Zingiber officinale* extracts in crown gall cross resistance management program in roses.

3. Other extractants apart from methanol to be used and their results compared with those of methanol.
4. A protocol to be designed for use of plant extracts in management of crown gall as replacement to synthetic chemicals.
5. More greenhouse experiments, with different varieties in different environments to be done and their results compared to this study.

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APPENDICES

Appendix I: Questionnaire

I am a Masters student investigating prevalence of crown gall disease in roses. I intend to set up experimental plots for controlling the disease using *Artemisia annua*, *Zingiber officinale* rhizome extracts, and copper hydroxide. Faithfully respond to the questions below by ticking the appropriate place or by giving brief responses on the spaces provided. The information collected will remain confidential and will only be used for the purposes of the research.

FARM IDENTIFICATION

Name of the farm _____

County _____

Section A: Varieties of roses grown and their susceptibility to crown gall

1. (a) List the varieties of roses grown in your farm

b) List the plant population per each rose variety

2. (i) Do we have rose varieties in your farm which are not affected by crown gall disease?

a) = Yes b) = No

(ii) If yes, list the varieties

Section B: Constraints of rose production

5. Tick in the list below the rose production constraints experienced in your farm.

Finance/ Capital	
Land acquisition difficulties	
Scarcity of planting materials	
Pests and diseases	
Labor availability	
Agrochemicals availability	
Market and market prices	
Transportation	
Weather	

6. (i) Is diseases and pests a problem in your farm?

a) = Yes b) = No

(i) Tick in the below list the disease or pest which is a problematic to control in your farm.

Thrips	
Nematodes	
Aphids	
Red spider mites	
Mealy bugs	
Crown gall	
Downy mildew	
Powdery mildew	
Botrytis	

Section C: Crown gall control strategies

7. Is it easy to control crown gall disease?

a) = Yes b) = No

8. List the methods used in your farm to control crown gall under the following approaches.

a) = Chemically, which chemicals do you use?

b) = Culturally

c) = Biologically

d) = Use of resistant varieties

e) = Others (specify)

9. (i) Have you tried plant extracts in managing crown gall in your farm?

a) = Yes b) = No

(ii) If the answer above is yes, give the name of the extracts.

Appendix II: Approval of Research Proposal

KENYATTA UNIVERSITY
GRADUATE SCHOOL

E-mail: dean-graduate@ku.ac.ke
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P.O. Box 43844, 00100
NAIROBI, KENYA
Tel. 810901 Ext. 57530

Internal Memo

FROM: Dean, Graduate School

DATE: 13th November, 2015

TO: Njagi Alfred Kariuki
C/o Plant Sciences
Department.

REF: 156/CE/24566/12

SUBJECT: APPROVAL OF RESEARCH PROPOSAL

This is to inform you that Graduate School Board, at its meeting of 4th November 2015, approved your Research Proposal for the M.Sc. Degree Entitled, "Use of Artemisia and Ginger Extracts in Management of Crown Gall Disease of Roses in Selected Flower Farms in Kenya".

You may now proceed with data collection, subject to clearance with the Director General, National Commission for Science, Technology and Innovation.

As you embark on your data collection, please note that you will be required to submit to Graduate School completed Supervision Tracking forms per semester. The form has been developed to replace the progress report forms. The supervision Tracking Forms are available at the University's website under Graduate School webpage downloads.

Thank you.

ANNBELL MWANIKI
FOR: DEAN, GRADUATE SCHOOL

c.c. Chairman, Department of Plant Sciences Department

Supervisors:

1. Dr. Jonah Birgen
   C/o Department of Plant Sciences
   Kenyatta University

2. Dr. Ezekiel Mugendi Njeru
   C/o Department of Microbiology
   Kenyatta University

AM/nn

Appendix III: Research License

REPUBLIC OF KENYA

NATIONAL COMMISSION FOR SCIENCE,TECHNOLOGY & INNOVATION

Ref No: **546421**

Date of Issue: **18/November/2020**

RESEARCH LICENSE

This is to Certify that Mr.. Njagi Kariuki alfred of Kenyatta University, has been licensed to conduct research in Kiambu, Nakuru on the topic: USE OF ARTEMISIA AND GINGER EXTRACTS IN MANAGEMENT OF CROWN GALL DISEASE S OF ROSES IN SELECTED FLOWER FARMS IN KENYA for the period ending : 18/November/2021.

License No: **NACOSTI/P/20/7108**

546421

Applicant Identification Number

Director General
NATIONAL COMMISSION FOR SCIENCE,TECHNOLOGY & INNOVATION

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