

**MANAGEMENT OF CROWN GALL DISEASE OF ROSES USING
AGROBACTERIUM RADIOBACTER, CORN OIL AND COPPER
HYDROXIDE AND OXYCHLORIDE IN KENYA**

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DECLARATION

I, Gitari Judith Murugi, declare that this thesis is my original work and has not been presented for the award of a degree in any other University or any other award.

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DEDICATION

This work is dedicated to my daughter Jenascia Wangari.

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ABBREVIATIONS AND ACRONYMS

ALS	Antibiotic-likes
BOD	Biological Oxygen Demand
CRD	Complete Randomized Design
DNA	Deoxyribonucleic acid
EPZA	Export Processing Zone Authority
HCDA	Horticultural Crop Development Authority
ITC	International Trade Centre
KARI	Kenya Agricultural Research Institute
LSD	Least Significant Difference
µg/l	Microgram per liter
PANUPS	Pesticides Action Network Updates Services
NaOCl	Sodium Hypochlorite
T-DNA	Transfer Deoxyribonucleic Acid
Vir	Virulent
YEMA	Yeast Extract Mannitol Agar
YEP	Yeast Extract Peptone

ABSTRACT

The cut-flower industry is one of the fastest growing sub-sectors of the horticultural industry contributing significantly to the continued growth of Kenyan economy. Roses are the most popular cut-flowers world-wide. Rose flower production in Kenya faces significant challenges particularly with regard to pests and diseases. Crown gall disease caused by *A. tumefaciens* is one of the limiting factors in the production of roses. The disease is widespread in rose flower farms and nurseries in Kenya causing a substantial loss of yield and therefore there is an urgent need to manage it. The study aimed at evaluating the antagonistic activity of naturally occurring *A. radiobacter* isolates from different flower farms in four ecological regions notably Nanyuki, Naivasha, Murang'a and Timau against *A. tumefaciens*. In addition, Elianto oil, copper hydroxide (kocide 2000) and copper oxychloride were evaluated as chemical control agents. Field survey was conducted over the period between August, 2012 and October, 2012. Gall and soil samples were collected during the period between November, 2012 and December, 2012. Laboratory work was conducted at Kenyatta University, Microbiology Department Laboratory while greenhouse experiments were carried out at Branan farm (Muranga County). The gall and soil samples were isolated on Yeast Extract Mannitol Agar supplemented with Congo red and Yeast Extract Peptone media. Colony morphology and standard biochemical tests including: Gram staining, catalase test, urease test, salt tolerance test, 3-ketolactose test, motility test and H₂S production were performed for identification and confirmation of the isolates based on their Gram staining and cultural characteristics. Kirby-Bauer disc diffusion technique was used to determine antimicrobial sensitivity patterns of bacteria to antibiotics. Carrot disc assay test was conducted on *A. tumefaciens* for pathogenicity test while *in vitro* antagonistic test was done on *A. radiobacter*. In the greenhouse experiments, pathogenicity test was carried out using *A. radiobacter* and *A. tumefaciens* isolates on four varieties of rose plants. Minimum incidence (6%) of crown gall was observed on Topsun, Fuschiana and H3O rose varieties when *A. radiobacter* isolates were applied compared to 54% in the control. In Furiosa variety, there was minimal incidence of crown gall disease on treated plants and in control. There was no significant differences ($P>0.05$) in the interaction between rose flower variety and treatments on the number of plants with galls. In chemical control, there was no significant difference ($P>0.05$) in the interaction between treatment and variety with regard to number of plants with galls. The study confirmed that *A. radiobacter* had the potential in the management of crown gall disease on rose flower plants. Therefore, the study recommends the use of *A. radiobacter* (biological control agent) and chemical control methods to minimize spread of the disease.

CHAPTER ONE

INTRODUCTION

1.1 Background to the study

1.1.1 Cut flowers in the global economy

The world market for cut flowers has grown consistently since the early 1980s. The total acreage allocated to cut flower production worldwide is now over 200,000 hectares, with roses, carnations, and chrysanthemums being the dominant varieties (ITC, 2001). While the Netherlands remains the largest producer of cut flowers worldwide, developing countries such as Colombia, Kenya, Ecuador, and Zimbabwe have become strong players in global markets. Exports from developing countries by 1998 comprised 29 percent of the world total (ITC, 2001) and provided employment to approximately 190,000 people (PANUPS, 2002). The Kenyan flower industry is the third largest flower exporter by value and volume behind the Netherlands and Colombia on a global level (Rikken, 2011). It is ranked second in foreign exchange earnings, with cut flowers dominating the sub-sector (Bolo *et al.*, 2006). Kenya is the top supplier of cut flowers to the Dutch flower auction, accounting for 44.6% of total supplies in 2011 (FloraHolland, 2011).

1.1.2 Kenya flower industry

The flower industry contributes about half of fresh horticultural exports and it is estimated that by 2010, the flower industry provided direct employment to over 90,000 and over 500,000 in related industries (Arim, 2011).

The industry is dominated by large-scale production which contributes 97 percent and small scale contributing a paltry three percent. The main focus of production in the country has been large scale and export market oriented. Cut flower is one of the two commodities where large-scale production dominates in the country (Nyangito, 2008).

Kenya's cut-flower industry is widely seen as a great success and a model of the benefits of export oriented production (Dolan and Opondo, 2005). It has been an extremely profitable activity for Kenya. The production costs of growing flowers in Kenya (45 million EUR) (Wijnands, 2005) is offset by the high value of the crop as the production per hectare is extremely high (EPZA, 2005). This industry is the fastest growing agricultural sub-sector in Kenya. It is the second largest agricultural source of foreign exchange (Hale, 2005). The value of Kenya's flower exports was US \$613-640 million in 2007 (Vogt, 2008; Satter, 2008). The annual average revenue from cut-flowers is at US\$350 million. This growth accounts for the overall expansion of Kenya's economy (Council of Canadians, 2008). Kenya has now become the largest producer in Africa and the leading supplier to Europe. It produces approximately \$200 million in cut flowers and foliage annually. Nearly all of it is exported, with 94% of the exports going to the European Union. The latest figures released by the Kenya Flower Council (KFC) show that the country's flower exports now control 32% of the European Union market, consolidating the lead Kenya achieved in 2000 after edging out Israel and Columbia. This is a point above the 2006 European market share of 31%, which the country attained after climbing from 25% in 2003 (Riungu, 2007). There are over 140 flower farms in Kenya experiencing a growth rate of 15% per year (Wijnands, 2005).

The major flower-growing areas are Naivasha, Thika, Limuru, and Nairobi, Athi river plains the west, Nakuru, Nanyuki, Mount Kenya region and Eldoret in the North. Kenya has had a 25% share of the world market for cut-flowers since 2000 (Barlow, 2007; Council of Canadians, 2008) and is the world's fourth largest exporter (Wijnands, 2005). Roses make up over 70% of all trees, plants, buds, roots, flowers and foliage exported from Kenya (Muhammad, 2009). Roses alone account for 35% of the total value of horticultural exports and stand out as Kenya's single most important export product (HCDA, 2010). Carnations follow as the next most popular, especially on less romantic holidays. Kenya is considered to be the largest supplier of Fair trade flowers to Europe, accounting for 50-60% of total volumes (Patton, 2008).

1.1.3 Crown gall disease as a constraint to flower production in Kenya

There are many constraints to flower production in Kenya. Among them are pests and diseases. Among the diseases, crown gall caused by *Agrobacterium tumefaciens* causes a significant damage to roses in Kenya. *Agrobacterium tumefaciens* is found in the soil and is responsible for the tumorous growth found in infected plants (Deacon, 2002). *Agrobacterium tumefaciens* is a widespread naturally occurring soil bacterium that causes crown gall disease in many plant species and has the ability to introduce new genetic material into the plant cell (Gelvin, 2003). Agrobacteria are also widely distributed in the nursery and even in non-nursery conditions and do affect a variety of other crops. In Ethiopia, the only soil-borne pathogen of economic importance in commercial greenhouse sector in the country is the crown gall forming bacteria (Derso and Yalemtesfa, 2011).

In Kenya, the incidence of crown gall disease was noticed in 1998, when many flower farms that went into commercial production of roses were severely affected by the outbreak of the disease. Survey findings revealed by Smit (2011) indicated that the disease was introduced in Kenya through infected root stock of roses imported from Israel. Smit further reveals that *A. tumefaciens* is widely spread in Kenya in nurseries, commercial production areas and uncultivated fields. Despite losses incurred in flower exports due to effects of diseases and high residual effect of pesticides, empirical research especially focusing on management of *A. tumefaciens* is limited. Therefore there is paucity of information to farmers on the best practices to leap maximum profits.

Various remedial methods have been employed worldwide to control *A. tumefaciens* including utilization of a strain of closely related bacteria *A. radiobacter* (Riley and Wertz, 2002). The use of bacteriocins has been acclaimed as one of the safest means to control the plant disease of microbial origin. Bacteriocins have been described as extracellular macromolecular proteins or peptides antibiotics produced by certain bacteria, which exert their lethal effects on bacteria of the same or related groups (Russel, 2002). This is with particular reference to the risks in using broad- spectrum agro-chemicals and antibiotics to control the plant pathogens. Thus, bacteriocins have most of the attributes considered desirable for microbial control. They have been reported to inhibit a wide range of gram-positive and gram negative bacteria. Bacteriocins and Bacteriocins-like inhibitory substances (BLIS) are medically, industrially and agriculturally very important (Riley and Wertz, 2002).

Many phytopathogenic bacteria including members of the *Corynebacteria*, *Erwinia*, *Pseudomonas*, *Xanthomonas* and *Agrobacterium* produce proteinaceous bacteriocins (Heu *et al.*, 2001). These bacteriocins are specific, cost effective and are safe for the users and the environment and appear to be excellent candidates for agricultural use in controlling plant pathogens. Genetically modified *A. radiobacter* releases a bacteriocin (agrocin) active against *A. tumefaciens*. This agrocin is a novel nucleic acid derivative that prevents the formation of crown gall tumors in the infected plants (Kado, 2002).

A study conducted in Australia by Ryder and Jones (1991) to investigate the effectiveness of using the nopaline-producing strains of *A. radiobacter* to control the disease was found to be a highly effective biological control system. This method of control is now used world-wide though the practice is yet to be embraced in Africa and especially Kenya. In general, bacterial diseases of plants are very difficult to control owing to the lack of effective chemicals to manage them. There is need therefore to explore *A. radiobacter* isolates naturally occurring in Kenya which has potential effect on the management of *A. tumefaciens*.

1.2 Problem statement and justification

Crown gall disease is a chronic and resurgent disease problem that affects many perennial fruit, nut and ornamental crops. Growers and nursery industries suffer significant annual losses of between 5-6% worldwide due to crown gall disease in the form of low quality rose flower stock, lowered productivity from galled trees and increased susceptibility of infected plants to pathogens and environmental stress (Bliss *et al.*, 1999).

Crown gall disease of rose plants in various flower farms in Kenya has been a recurrent problem presenting a major threat to rose flower production thus severely affecting the yield hence economic losses in the flower industry. There is a paucity of data focusing on *A. tumefaciens* contributing to crown gall disease of roses in Kenya.

1.3 Research Questions

- i) What is the status of *A. tumefaciens* in selected flower farms in Kenya?
- ii) What is the antagonistic effect of *A. radiobacter* naturally occurring in the Kenyan soils against *A. tumefaciens*?
- iii) What is the effect of the use *A. radiobacter*, corn oil (Elianto oil), copper hydroxide and oxychloride in the management of crown gall disease of roses?

1.4 Research hypotheses

- i) There is a no significant difference on the status of *A. tumefaciens* in selected rose flower farms in Kenya.
- ii) There is no antagonistic effect of naturally occurring *A. radiobacter* isolates in the Kenyan soils on *A. tumefaciens*.
- iii) There is no significant effect of the use *A. rabactedor*, corn oil (Elianto oil), copper hydroxide and oxychloride in the management of crown gall disease of roses.

1.5 Objectives of the study

1.5.1 General objective

To assess the status of *A. tumefaciens* in rose flower farms in Kenya and evaluate the use naturally occurring *A. radiobacter* isolates, corn oil (Elianto) and copper hydroxide and oxychloride in management of crown gall disease.

1.5.2 Specific objectives

- i) To assess the status of *A. tumefaciens* in selected rose flower farms in Kenya.
- ii) To assess the antagonistic effect of naturally occurring *A. radiobacter* isolates in the Kenyan soils against *A. tumefaciens*.
- iii) To determine the effect of *A. radiobacter*, corn oil (Elianto oil), copper hydroxide and oxychloride in the management of crown gall disease of roses.

CHAPTER TWO

LITERATURE REVIEW

2.1 Rose plant taxonomy

A rose is a woody perennial of the genus *Rosa*, within the family *Rosaceae*, which contains 200 species and more than 18,000 cultivars (Gudin, 2000). It is closely related to apple, pear, plum, cherry, blackberry and strawberry. The genus is divided into four sub-genera of which subgenus *Rosa* (previously known as *Eurosa*) includes nearly all the species (Gudin, 2000 and Nybom *et al.*, 2005). In turn, subgenus *Rosa* is divided into sections, the actual number of which is still debatable (Gudin, 2000). Roses have complex nomenclatural history and taxonomic difficulties of this genus have resulted in reports of anywhere from 100 to 300 *Rosa* species, of which there are innumerable cultivars (Ertter, 2001). These taxonomic problems stem from the fact that the morphological characters used to distinguish species are highly variable and are compounded by the ease with which species naturally hybridize.

2.2 Morphology

2.2.1 Plant morphology

Across the *Rosa* genus, stems can be erect, trailing or climbing (Harden, 2001). Recurrent flowering roses such as the hybrid teas are perennial woody shrubs that continuously produce new shoots. Basically, a shoot comprises a successive of 8-15 repeating units, each one consisting of a leaf, an auxiliary bud a node and internodes (Bloom and Tsujita, 2003).

Stems of rose plants bear prickles, mistakenly referred to as ‘thorns’ that originate from the epidermal layer and are usually more densely distributed at a proximal (basal) end of the stem (Andre, 2003). Roses have compound leaves (a number of leaflets borne on an axis called a rachis) and the arrangement of the leaflets is odd-pinnate. The middle leaves of hybrid tea roses usually have J-T leaflets, while Asiatic species may have up to 19 leaflets (Torre, 2003). The morphology of the root system on a rose plant differs according to whether the root system develops on a seed-propagated plant, a rooted cutting (own-rooted plant) or a rootstock (grafted plant). In addition, factors such as nutrition and pruning have a strong influence on root morphology (Skyt and Fuchs, 2003).

2.2.2 Reproductive morphology

Flowers of the wild species in *Rosa* can be solitary, corymbose or paniced and have five petals (except *R. sericea* which often has four petals) (Wisseman and Rita, 2003). Rose pollen is elliptical in shape with a constant ratio (2:1) length: width (Jacob and Ferrero, 2003). The fruit (that develops from a fertilized ovary) of the rose plant is an achene and is often mistakenly called a seed because of its small size and hard texture.

2.3 Growth and development of rose flowers

Many roses destined for use as cut-flowers or in the garden or landscaping are commonly propagated by stem cuttings. The undifferentiated cell division (callus formation) at the basal end of a cutting is a prerequisite for optimal development of adventitious roots (Costa and Van de Pol, 2003). Recurrent flowering roses are said to produce flowers in ‘flushes’.

In commercial cut-flower roses, the stem of a shoot bearing a bloom is cut so as to leave few nodes. The auxiliary buds thus released from apical dominance then produce new shoots from which another round of bloom (a flush) is produced. In recurrent flowering plants, the secondary shoots can produce flowers. When the flowering stem is harvested the lower (proleptic) buds are released from inhibition and, in turn will develop as dependent on both genotype and environmental conditions (Roberts and Blake, 2003).

2.4 Origin and cultivation

The *Rosa* genus is wide spread in the temperate regions of the northern hemisphere, including Northern America, Europe, Asia and the Middle East, with the greatest diversity of species found in Western China (Ertter, 2001). It is also distributed in warmer areas such as New Mexico, Iraq, Ethiopia, Bengal and Southern China (Nybom *et al.*, 2005). There are no endemic *Rosa* species in the southern hemisphere. Many thousands of rose hybrids and cultivars have been bred and selected for garden use as flowering plants. Basically there are two types of systems in which cut-flower roses are grown; soil systems (Pertwee, 2000; Bloom and Tsujita, 2003) and soilless systems (hydroponics) (Carruthers, 2002; Bloom and Tsujita, 2003). The area under rose cultivation is increasing steadily due its heavy demand in domestic as well as international market (Sheela, 2008). Normally rose is cultivated on soil beds. Fertile loamy soil enriched with organic matter and mineral nutrients having good water holding capacity as well as drainage provision and a pH in between 6 and 6.5 are considered the most suitable for the cultivation of rose (Bhattacharya and Dey, 2003).

Several horticultural practices for rose cultivation which include; propagation, planting, bed-preparation, nutrient and water management, inter-culture operations have been recommended to increase the quality and quantity yield of rose flower (Sheela, 2008). However, the more scientific information on the plant growth behavior of different cultivars is still necessary to facilitate commercial cultivation of roses (Raviv and Blom, 2001). An alternative method of soilless based rose cultivation is the hydroponics system where the plants can be grown in artificial substrate like pumice or coco peat (in the nutrient solution with the help of suitable devices) instead of soil (Samartzids *et al.*, 2005). In temperate climates, cut roses are often grown in glasshouses, and in warmer countries they may also be grown under cover in order to ensure that the flowers are not damaged by weather and that pests and disease control can be carried out effectively. Significant quantities are grown in some tropical countries and these are flown by air to markets across the world (Foodnet, 2009).

2.4.1 Propagation of rose flowers

Roses are usually propagated by grafting methods (Park and Jeong, 2012) or less frequently by cutting and subsequently growing on their own roots (Welch and Manners, 2014). Propagation of roses by seed is used in breeding new cultivars or in the production of rootstock plants of some species such as *R. canina* (Gudin, 2003). Cultivars are not seed propagated because germination is often problematic and because seed derived progeny will segregate widely for traits and therefore have characteristics that differ from parents (Zlesck, 2006). However, it is generally held that own-rooted cut-flower does not perform as well as grafted plants (Safi, 2005).

Rootstock plants for grafting can be propagated from seed ('seedling rootstocks') but most often are clonally propagated using soft and hardwood cuttings and are to as 'clonal rootstocks' (Devries, 2003).

2.4.2 Commercial use of roses

Roses are the world's most traded cut-flower. Nearly 70% of this trade is within the EU. The largest production areas are in Colombia, Ecuador and Kenya (Bloom and Tsujiita, 2003). There are basically five main types of roses grown for cut-flower that is, long stemmed, medium stemmed, short stemmed, spray roses and miniature roses. The main type of cut-flower grown in Kenya is the medium and short stemmed of class Floribunda (Pertwee, 2000). Rose oil is obtained from petals of rose species such as *Rosa amascene* and *Rosa centifolia* (Collins, 2003). Rose water is also obtained through treatment of rose petals but is less much concentrated than rose oil and can be made using a variety of methods (Cutler, 2003). Rose hips (the false fruits of rose plant) particularly those from *R. canina*, *R. moschata* can be utilized to produce a range of products for human consumption such as jams, jellies, marmalades (Cinar and Colakaglu, 2005).

Roses are best known as ornamental plants grown for their flowers in the garden and sometimes indoors. Some are used as landscape plants, for hedging and for other utilitarian purposes such as game cover and slope stabilization. They also have minor medicinal uses. The majority of ornamental roses are hybrids that are bred for their flowers.

A few, mostly species of roses are grown for attractive or scented foliage (such as *R. glauca* and *R. rubiginosa*), ornamental thorns (such as *Rosa sericea*) or for their showy fruit (such as *Rosa moyesii*). Most are double-flowered with many or all of the stamens having mutated into additional petals.

2.5 Abiotic and biotic factors influencing rose flower development

In commercial rose production yield is limited by abiotic factors such as light, temperature, humidity, mineral nutrition and salinity (Lorenzo *et al.*, 2000). Roses under a commercial setting are considered to have high light requirement and optimal production is achieved with a light sum of 12-15 mol m⁻² day⁻¹ more than in plants grown under cover and this necessitates the use of supplemental lighting (Bloom and Tsujiita, 2003). In greenhouse and hydroponic systems, roses are well supplied with nutrients and are arguably oversupplied particularly with nitrogen (Cabrera, 2003). Roses growing in a garden are adaptable to a range of nutrient conditions but deficiencies may occur for example in nitrogen, manganese and magnesium availability especially under conditions of extreme pH that reduces nutrient availability (Roxburgh, 2008).

Biotic factors common in cut-flower roses include pests and diseases. Roots of roses are associated with mycorrhizal fungi (*Glomora* spp. and *Gigaspora* spp.) that enlarge the surface area for absorption of nutrients and water (Skytt and Fuchs, 2003). Fungal endophytes have been reported to occur in the vascular bundles of hybrid tea rose (*Rosa Xhybrida*) leaves (Salgado *et al.*, 2007).

Disease like stem canker is caused by a variety of fungi and result in dieback of the stem as a result of entry of spores through pruning or wounding of the crown (Botanic Gardens Trust, 2008). Other biotic factors include weed (Karlik, 2008), invertebrate pests such as thrips (*Thrip imaginis*) and nematodes (*Meloidodgyne* spp.) (Reid, 2005).

One of the major diseases affecting the industry is the crown gall disease of roses caused by *Agrobacterium tumefaciens*. *Agrobacterium tumefaciens* is a soil borne bacterium, a member of family- *Rhizobiaceae* and has worldwide distribution (Furuya *et al.*, 2004). These are gram negative, rod-shaped and motile bacteria that grow aerobically without forming endospores (Collins, 2001). Farrand *et al.* (2003) claim that members of *Agrobacterium* spp. and members of *Rhizobium* spp. differ in various aspects of chromosomal structure as reported by Goodner *et al.* (2001). However, in the studies cited, only one *Agrobacterium* spp. strain was examined. Farrand *et al.* (2003) also claim that the data of Jumas-Bilak *et al.* (1998), in which the genomic structures of *Agrobacterium* spp., *Rhizobium* spp. and *Sinorhizobium* spp. are compared, supports the differentiation of *Agrobacterium* spp. from *Rhizobium* spp. *Agrobacterium tumefaciens* can be generally found on and around root surfaces known as rhizosphere. Several studies have shown that *A. tumefaciens* can be effectively isolated from leaf, stem and crown gall of roses (Aysan and Sahin, 2003).

2.6 Economic importance of crown gall disease

Crown gall is a common disease of dicot plants, including many woody shrubs and various herbaceous plants mainly stone and pome fruit-trees, grapevines, roses and some ornamental plants (Rhouma *et al.*, 2006). It also affects some monocots and gymnosperms (Pitzscke and Hirt, 2010). It produces crown gall disease in over 600 species of trees (Wang *et al.*, 2000). It is considered to be the main bacterial disease of stone fruit trees in the nurseries of Mediterranean countries (Krimi *et al.*, 2002). The galls provide a nutrient rich environment for the growth of *A. tumefaciens* which returns to the soil as the galls decompose (Gillman, 2005).

2.6.1 Pathogenesis

In the soil with the help of flagellum, *Agrobacterium* spp. swims towards photoassimilates that accumulate in rhizosphere around roots. *Agrobacterium* spp. typically infects wounded plants where the release of plant saps, containing amino acids, sugars and organic acids, attracts the bacteria to the wound by positive chemotaxis. Once the bacterium reaches the wound, it attaches itself to the plant surface by synthesizing cellulose fibers (Tzfira and Citovsky, 2007). *Agrobacterium tumefaciens* contains an extra-chromosomal DNA designated as Ti (tumor inducing) plasmid. Ti-plasmid carries two components: vir and T-DNA regions needed for genetic transformation (Tzfira *et al.*, 2004).

The molecular machinery needed for T-DNA generation and transport into the host cell comprises of proteins that are encoded by the bacterial chromosomal virulence (*chv*) genes and encoded by the Ti-plasmid virulence (*vir*) genes (Gelvin, 2003).

In addition to these, wounded plants also produce a wide range of phenolic compounds, such as coniferyl alcohol and acetosyringone (AS) (Lee and Gelvin, 2008) which induce the bacteria to generate T-DNA, by a two component signaling system (*virA/virG*).

Detection of phenolic compounds released from the wounded plant tissues leads to adhesion and induction of the bacterial virulence (*vir*) machinery (Krispin *et al.*, 2007). Acetosyringone activates *virA*, a membrane bound receptor, which activates the *virG* (transcription factor). The activated *virG* can then interact with activator elements found in the promoters of the *virA*, *virB*, *virC*, *virD*, *virE* and *virG* operons, resulting in elevation of their expression levels and ends with the expression of its T-DNA integrated in the host genome where the host facilitates its integration into the genome, mediated by DNA repair system (Karami *et al.*, 2009).

In the case of tumorigenic strains, it consists of the transfer of a fragment of bacterial tumor-inducing plasmid (T-DNA) into the plant cell and its incorporation into the plant genome. Ti plasmids are approximately 200 kilo bases (Kado, 2002). This transfer is controlled by virulence (*vir*) genes, which are also located on the Ti plasmid but not in the T-DNA region. The T-DNA also carries genes for the biosynthetic enzymes for the production of unusual amino acids, typically octopine or nopaline. *Agrobacterium tumefaciens* also carries genes for the biosynthesis of the plant hormones (auxins and cytokinins) and for the biosynthesis of opines, providing a carbon and nitrogen source for the bacteria that most other micro-organisms cannot use, giving *Agrobacterium* spp. a selective advantage (Andrea and Heribert, 2010).

By altering the hormone balance in the plant cell, the division of those cells cannot be controlled by the plant, and tumors form. The ratio of auxin to cytokinin produced by the tumor genes determines the morphology of the tumor (root-like, disorganized or shoot-like).

Expression of T-DNA genes, which code for auxin synthesis [*tms1 (iaaM)* and *tms2 (iaaH)*] and cytokinin synthesis [*tmr (ipt)*], causes uncontrolled plant cell division and growth resulting in tumor formation (Zhu *et al.*, 2000). Tumors inhibit plant physiological functions such as transport of water and nutrients. Especially when many large tumors are formed, which may partly girdle the bigger roots or crown; plants show reduced growth and may become severely stunted. In addition, tumor breakdowns create wounds on the roots.

These wounds become entry points for other soil-borne pathogens (for example *Pseudomonas syringae* and *Armillaria mellea*) or pests such as insect borers (Escobar and Dandekar, 2003). Infected plants, especially those with tumors on the main roots and collar are unfit for marketing and must be disposed of. The highest losses occur in young plants, that is, those still in the nursery. Even though all plant material with visible crown galls is eliminated in any given nursery, healthy looking but still infected trees from the same nursery can be purchased and planted by growers. This contributes to the spread of crown gall. The disease seldom kills plants, but it can elicit lack of vigor and reduced growth.

2.6.2 Disease cycle

Infection starts from infected soil where a susceptible host is planted. Other sources of inoculums are irrigation water, infected planting materials, pruning equipment, cultivation equipment and rouged plants and detached or disintegrated galls put back in the soil. Lack of disinfection of pruning tools by field workers is a common means of bacteria dissemination (Agrios, 2005).

Prior to disease development, most pathogens undergo an epiphytic growth phase. The pathogen attaches to the plant surface and proliferates in this environment until it finds an entry site, for example wounds or stomata where the pathogen can overcome structural defense barriers provided by cuticular or cell wall (Agrios, 2005; Montesinos *et al.*, 2002). Once the bacteria enter the wound into the plant it takes about two weeks for the galls to start appearing. The gall cells are not protected by an outer epidermal layer and with time they start cracking and become brittle and start disintegrating. Old galls darken and look rugged and sometimes become infested with insects that feed on the cells. Eventually they fall off back into the soil and are released to start the infection cycle once a host is replanted and the conducive environment of wounds occur.

2.6.3 Diversity of Agrobacteria population

Agrobacteria are widely spread in soils and other natural environments. They can be isolated even from untilled soils. D'Hondt *et al.* (2004) found bacteria most closely related to *R. radiobacter* (biovar 1) in deeply buried sediments of the eastern Pacific Ocean while Süß *et al.* (2006) showed that bacteria of the same taxon is widespread in Mediterranean sediments. Some soils are found to be permissive to crown gall.

These soils allow the long term persistency of pathogenic *Agrobacteria* to continue for several decades, rendering the soils inappropriate for the culture of susceptible crops (Rhouma *et al.*, 2008). Permissive soils are those most favorable to any *Agrobacteria* with more than 10^4 cfu/g of soil during warm seasons (Krimi *et al.*, 2002). Apart from diseased foci, the great majority of soil *Agrobacteria* is, however, not pathogenic (Sobiczewski *et al.*, 2005). Other species of the genus *Agrobacterium* include; *Agrobacterium rhizogenes* which induces root tumors and carries the distinct Ri (root inducing) plasmid, *Agrobacterium vitis* causes gall in grapevines, *Agrobacterium rubi* (cane gall) and *Agrobacterium radiobacter* (non-pathogenic). There are numerous other species of the genus *Agrobacterium* either within or out the *Agrobacterium tumefaciens* complex such as unnamed species that includes strains NCPPB 1650, or the novel species that got the epithet *skierniewicense* (Pulawaska *et al.*, 2011). *Agrobacteria* usually form ecological guilds in soils consisting of several genomovars with several strains. There are also other several strains, even within a single tumor (Costechareyre *et al.*, 2010).

2.7 Management of *Agrobacterium tumefaciens*

Crown gall disease development occurs when the ideal environment, the virulent pathogen and the susceptible plant host all interact at the same time to cause the disease (Agrios, 2005). Treatments designed to eliminate *Agrobacterium* spp. directly must necessarily be exercised before infection because disease development will progress independently of the causal agent following the initial transformation event. Various biological, chemical and cultural ways are used to minimize spread of the disease.

2.7.1 Chemical control

In chemical control, use of conventional pesticides has been unsuccessful and the commonly used copper hydroxide shows inadequate efficacy (Agrios, 2005). Copper compounds produce the best results against crown gall, but seldom provide satisfactory control because of the pathogen resistance and the phytotoxicity it causes in some plant species (Agrios, 2005). In situations in which causation of a wound is inevitable, grafting copper or bleached-based bactericides can be used to reduce *A. tumefaciens* populations on plant surfaces, minimizing the disease re-infection (Burr, 2004). Other farm practices reported to reduce infection of *Agrobacterium* were the use of fresh cow dung and regulating nutrients to promote uptake of copper. The abandonment of highly infested soils and intercropping with a resistant variety or soil fumigation can temporarily reduce soil populations of *Agrobacterium* (Agrios, 2005).

2.7.2 Biological control

A promising alternative approach to minimize infectious plant disease is the application of antagonizing organisms with the ability to suppress pathogen development. This concept is referred to as “biological control” and the operative organism is called bio-control agent (Wilson, 1997). Mass production of the active substance (that is fermentation of the control organism) is low-priced compared to chemical synthesis of pesticides (Shoda, 2000). Since bio-control products can be applied by conventional techniques like spraying or drenching their ability to proliferate and establish stable populations reduces application cost to a minimum (Montesinos, 2003).

The impact of a bio-control agent on the indigenous microbial communities or other organisms in the ecosystem is less severe as compared to broad-spectrum pesticides (Emmert and Handelsman, 1999). Another significant advantage of biological control results from the flexibility of the control agent as a living organism. The control organism can interact with a pathogen in many different ways and it is able to co-evolve with target and environment. This minimizes the risk of resistance formation and contributes to the potency of disease control (Emmert and Handelsman, 1999). Competition for limiting nutrient resources is a fundamental ecological principle. In bio-control systems, the pathogen and its antagonistic control agent have to compete for nutrients and space. Overlapping ecological niche enable a control agent by starvation and block possible entry sites by colonizing them (Janisiewicz and Korsten, 2002). Many bio-control agents have been shown to act by antibiosis (Raajimakers *et al.*, 2002). Production of antibiotics or inhibitory metabolites that are active against competitors is a common way of microorganisms to enhance their own ecological fitness.

Agrobacterium radiobacter is used worldwide as a commercial agent for bio-control of crown gall disease caused by tumorigenic *Agrobacterium* strains (Farrand, 2003). Another recently discovered bio-control mechanisms is quorum sensing silencing (Dong *et al.*, 2004). Many virulence determinants are regulated in a cell-density dependent manner. In gram-negative bacteria the quorum sensing signals are N-acyl homoserine lactones (AHLs). K84 produces Agrocin 434 and Antibiotic-like (ALS) range of control beyond nopaline type *A. tumefaciens* (Penyalver *et al.*, 2002).

Agrocin- 84 mimics Agrocinopine A and therefore is taken up by the same transport system used up by *A. tumefaciens* to utilize agrocinopine A. Inside *A. tumefaciens* cell, the antibiotic agrocin-84 inhibits DNA replication and cellular growth. Another improved method was by use of genetic modification by deletion of the gene responsible for exchange of DNA material among bacteria which resulted to the development of a new strain, *A. radiobacter*; strain K1026 in Australia (Manual of Bio-control agents, 2004). This strain is not capable of conferring resistance to other *Agrobacterium* that are sensitive to the agrocin antibiotic.

2.7.3 Cultural control

Agrobacterium tumefaciens can also be controlled by use of careful cultural practices that prevent unnecessary plant wound that would significantly reduce crown gall by denying the pathogen an opportunity to introduce T-DNA into plant cells (Agrios, 2005). High field hygiene is vital in every activity in minimizing incidences of *Agrobacterium*. However, these measures do not guarantee eradication of the disease. In addition, it is extremely difficult to observe hygiene practices to the expected standard in all operations.

The surest means of controlling *A. tumefaciens* is through preventing introduction of infected nursery root stocks. Since the bacteria penetrate the plant through wounds, it is important to avoid plant injury during the regular field agronomic practices such as weeding and pruning. Pruning and harvesting tools such as secateurs should be disinfected from one plant to another.

CHAPTER THREE

MATERIALS AND METHODS

3.1 The study sites

A baseline survey was conducted in rose flower farms located in various ecological regions in Kenya namely; Naivasha, Nanyuki, Timau and Murang'a. Naivasha is located in the Great Rift Valley in Kenya within Nakuru County (Kuyo, 2005). It is 1890m above sea level, longitude 36° 22' E and latitude of 0°46' S. It experiences bimodal rainfall distribution (Harper, 2004). The soils are fertile, thus attractive to floriculturalists and agriculturalists (Kuyo, 2005). The soils here have sodium and pumice content, the latter making the soils permeable with low water-holding capacity (Becht *et al.*, 2006). The temperature ranges from 15.9-18.0° C (Gaudet and Meleck, 1981), a maximum of 37°C and a minimum of 5°C (Harper, 2004). The coordinates for the study sites at Naivasha are 0° 01' N 37° 04' E. Nanyuki is located in the leeward side of Mt. Kenya. Its longitudinal extent is between 37° 04' 25" and latitudinal extent ranges between 015' S and 1°00' N. It is 1947m above sea level, climatic conditions range from humid in the alpine zone to arid conditions in the lowlands.

The coordinates for study sites are 0° 1' 31" N, 37° 04' E (Likii River Farm). Another survey was conducted at Kisima flower farm (coordinates 0° 50' 0" N, 37° 14' 0" E) which has an elevation of 2400m above sea-level. This is located in the Timau area of Meru central sub-county, on the Northern side of Mt. Kenya. Annual mean temperature ranges between 15.8-10.5°C. Rainfall annually, follows a bimodal pattern with the first rainy season, starting mid-March and a second rainy season starting mid-October.

The soil type is well-drained, moderately deep to dark reddish brown, friable to firm clay with a humic top soil. Another survey was carried out at Branan farm located at Murang'a County. Murang'a County is located between Nyeri and Thika. Its latitudinal extent is $0^{\circ} 43' 0''$ and longitude of $37^{\circ} 8' 60''$ E. The study areas were selected due to their diversity, having large number of greenhouses and being among the major commercial rose flower producers in Kenya. The study also involved a questionnaire which was filled by the production manager in various flower farms. The questionnaire addressed issues like; occurrence of crown gall on roses, flower production with respect to crown gall and management of crown gall.

3.2 Procurement of the gall and soil samples

Tumor (stem) and soil samples were collected for culturing in sterilized polythene bags. Samples were procured from the four selected flower farms located at Nanyuki, Timau, Naivasha and Murang'a.

3.3 Sample size determination

Galls were collected from each greenhouse after conducting a survey of infected rose plants in all greenhouses (Plate 3.1). The greenhouses were selected randomly per farm. Gall sample size was determined using Slovin's formula ($n = \frac{N}{1 + Ne^2}$) (Almeda *et al.*, 2010), where n = number of samples, N = total population and e = confidence level (error of tolerance). Selection was also based on susceptible rose varieties to crown gall disease and also the resistant ones. For soil samples, at each sampling point, two vertically crossing lines and two concentric circles of radius 3m were drawn. An auger of 7 cm diameter was used to take four cores of soil in the outer circle.

The 5 subsamples from 5 greenhouses in each farm were homogenously mixed to constitute a composite sample from which 500g soil was taken and placed in a plastic bag.



Young gall

(A)



(B)

Plate 3.1: Rose flower plants showing (A) rose flower plant infected and (B) rose flower plant (uninfected).

3.4. Isolation of *A. tumefaciens* and *A. radiobacter* using Yeast Extract Mannitol

Agar and Yeast peptone glucose agar

Yeast extract mannitol agar supplemented with Congo red was used to grow and culture both bacteria. It was prepared according to Beck *et al.* (1993) and it contained:- Mannitol 10.0g, MgSO₄.7H₂O 0.1g, K₂ HPO₄ 0.5g, NaCl 0.2g, Yeast extract 0.5g and agar 15.0g and 0.01g Congo red. All the ingredients were dissolved in distilled water and the volume adjusted to one liter. The pH was adjusted to 6.8 using 1.0 M sodium hydroxide. Monitoring of the pH was carried out using pH meter. The Yeast peptone glucose agar contained: - glucose 10.0 g, peptone 5.0 g, yeast extract 5.0 g, and agar 15.0 g. All ingredients were dissolved in distilled water and the volume adjusted to one liter. The pH was adjusted between 5.0 and 6.0. The combined medium mixture in both media was sterilized by autoclaving at 121°C for twenty minutes. Yeast extract mannitol broth (YEMB), was prepared in the same way as YEMA but no agar was added.

3.4.1 Yeast Extract Mannitol Agar (YEMA) plates for culturing isolates.

YEMA plates were prepared by pouring sterilized YEMA into sterile plastic plates (Petridishes). To prevent excessive water condensation in the plates, the YEMA medium was cooled down to about 45° C before dispensing it into plastic Petridishes of 100 mm at the rate of 25 milliliter of the medium per plate. Pouring of the medium into plates was carried out in a lamina flow to avoid contamination.

3.4.2 Yeast Extract Mannitol Agar (YEMA) slants for storage of pure isolates

YEMA slants were used for storage of pure isolates of *A. tumefaciens* and *A. radiobacter*. These slants were prepared by dispensing YEMA into McCartney bottles. The McCartney bottles were half filled with the YEMA medium. After autoclaving, screw caps were tightened and the bottles left to cool on a 45°C slanted surface. Before placing the sterilized YEMA bottles along the slanted surface they were allowed to cool at 45-50°C for ease in handling (Schaad *et al.*, 2001).

3.4.3 Luria-Bertani (LB) and Luria-Bertani Broth (LBB) for antagonistic test

Luria-Bertani medium was used to grow pure colonies of *A. radiobacter* for antagonistic test. The LB medium contained; 10.0g tryptone, 5.0g yeast extract, 10.0g NaCl, 15.0g agar. All the ingredients were dissolved in distilled water and the volume adjusted to one liter. The combined medium mixture was sterilized by autoclaving at 121°C for twenty minutes (Lee, 2008). Luria-Bertani broth (LBB) was prepared in the same way as LB but no agar was added. LBA was prepared using 0.6% agars at 45°C (Gerhardt *et al.*, 1994).

3.4.4 Isolation of *A. radiobacter* from the soil

The soil sample from different greenhouses of each flower farm was collected from the top 20 cm and sieved to remove particles larger than 2 millimeters. One gram (1 g) of the soil particles were suspended in 9 milliliter of sterile distilled water. The suspension was then diluted to 10^3 by serial dilution method. A loopful of suspension was streaked on YEMA supplemented with 25 ppm Congo red.

Single bacterial colony was picked up after incubation of plates at $27\pm 1^{\circ}\text{C}$ in a Biological Oxygen Demand (BOD) incubator (which stains red) for three to five days and further re-streaked on the same medium in a fresh sterilized plate. The re-streaking after picking a single bacterial colony was repeated three times to get a pure colony (Schaad *et al.*, 2001). An individual colony of each isolate was further transferred to slants of YEMA, which was then incubated at $27\pm 1^{\circ}\text{C}$ for five days.

3.4.5 Isolation of *A. tumefaciens* from the diseased plant tissues (galls)

Isolates were obtained from young tender and milky galls of infected rose plant. These materials were washed using tap water. With the help of a sharp sterilized blade, the galls were diced into small cubes (two millimeter approximately). These cubes were surface sterilized in 3% sodium hypochlorite (NaOCl) solution for 10-20 minutes according to Schaad *et al.* (2001) and were rinsed in sterilized distilled water three times to remove traces of sodium hypochlorite. Five cubes were crushed in one (1) milliliter of sterilized distilled water with the help of sterilized glass rod in a sterilized Petri plates to form the suspension which was kept undisturbed for ten minutes. A loopful suspension was then streaked on YEMA medium. A single colony was picked up after incubation of plates at $27 \pm 1^{\circ}\text{C}$ in a Biological Oxygen Demand (BOD) incubator for five days and further re-streaked in fresh medium and incubated for another twenty four hours (Schaad *et al.*, 2001). This exercise was repeated three times to get single cell bacterial colony, which was transferred to YEMA slants. The bacteria growth in each slant was checked after incubation of slants at $27\pm 1^{\circ}\text{C}$ for five days in a BOD incubator.

3.4.6 Culture maintenance and preservation

The cultures were first streaked onto YEMA plates and pure colonies selected for inoculation on YEMA slants. The slants were incubated for 4-7 days. Stock cultures of all the strains were stored in screw cap McCartney bottles at 4° C in a refrigerator.

3.4.7 Characterization of *Agrobacterium* isolates

3.4.7.1 Biochemical test

Agrobacterium tumefaciens isolates; A-Ol Jorowa (Naivasha), B-Likii River farm (Nanyuki), C-Branan farm (Muranga), D-Kisima (Timau) and *Agrobacterium radiobacter* isolates; 1-Ol Jorowa, 2-Likii River farm, 3-Branan farm and 4-Kisima farm were characterized based on the cultural, biochemical and physiological characteristics such as Congo red test (Hahn, 1996). Growth on peptone agar (Subba, 1981), Gram staining and specific tests such as growth on potato dextrose agar (PDA), 3-ketolctose test and sodium chloride tolerance test were also carried out.

Biochemical tests were performed to confirm the isolates; urease, catalase, oxidase and Tripple iron sugar (TSI) as described by Bergey's manual of Determinative Bacteriology (Moore *et al.*, 2001). For Gram staining, bacterial smears from 2-3 days colonies were prepared on clean microscope slides. The smears were air-dried, heat fixed by passing the slides over a Bunsen flame and then Gram stained as described by Beck *et al.* (1993). The slides were observed under oil immersion in a compound light microscope at magnification of $\times 400$.

3.4.7.2 Test for antibiotic resistance on *A. tumefaciens* isolates

The antibiotic sensitivity of *Agrobacterium* spp. isolates was determined according to the method of Bauer-Kirby (Bauer *et al.*, 1966; CLSI, 2006). The test was performed on Mueller-Hinton agar using in vitro disc diffusion method. The *Agrobacterium tumefaciens* isolate was subjected to the following antibiotics; Chloramphenicol 30mcg, Penicillin 10mcg, Erythromycin 15mcg, Cotrimoxade 25mcg, Minocycline 30mcg and Ampicilin 10mcg. Whatman No.1 filter paper discs (6mm in diameter) were impregnated with 10 micro liter of antibiotic solution with particular concentration followed by air-drying and then placed on seeded Luria-Bertani (LB) agar plates. The inhibition zones were measured after 48 hours of incubation at 28⁰ c.

3.4.7.3 *In vitro* antagonistic activity using Agar well diffusion assay method

The ability of the *A. radiobacter* to produce diffusible metabolites was tested according to the agar well diffusion assay (AWDA) as reported by Rhouma *et al.*, (2008). All bacterial isolates were transferred individually to 50 milliliter of Luria-Bertani broth medium (LB broth) in a 250 ml Erlenmeyer flask and incubated by shaking at 100 rpm for 2 days at room temperature. Twenty milliliters (20 ml) LBA medium were poured into each sterile Petri dish. One ml of bacterial suspension (10^8 cfu/ml) of *A. tumefaciens* was mixed with 3 milliliter of LBA (0.6 % agar) at 45°C and quickly overlaid on plates containing LB medium, in which wells of 6 millimeter diameter were punched. The antagonist (*A. radiobacter*) cultures were centrifuged at 15,000 rpm for 30 minutes to remove cell debris.

One hundred (100) micro liter of each sample were then filtered through 0.45 micrometer filters under sterile conditions and filled into the wells. Plates were incubated at 25°C and were subsequently examined for haloes of inhibition around the wells, the size of which was recorded (Bertani, 2004).

3.4.7.4 Pathogenicity test for *A. tumefaciens* using carrot disc assay

The carrots used for the study were collected from the local market washed and sterilized with commercial bleach followed by washing with distilled water thrice. The carrots were sliced into thin disc and each disc was overlaid with 100 microliters of inocula (10^8 cfu/ ml). The Petri dishes were sealed by parafilm and incubated for three (3) weeks at 25-30°C (Soriful *et al.*, 2010). The discs were checked for development of young galls around meristematic tissue around the central vascular system.

3.5 Greenhouse experiments

The greenhouse experiments were carried out to evaluate *A. tumefaciens* and *A. radiobacter* isolates for their pathogenicity and antagonism on rose plant. Screening of isolates of *A. radiobacter* against *A. tumefaciens* was done by inoculating rose nursery stock with both organisms. The 20 centimeter diameter plastic pots were filled with sterile substrate containing soil, sand and compost in a 1:1:1 ratio. YEMA slants of three days old *A. tumefaciens* and *A. radiobacter* were suspended separately in 10 ml sterile distilled water and shaken vigorously to give suspensions of 10^8 cfu/ml. *Agrobacterium radiobacter* isolate was tested following the New and Kerr's method (New and Kerr, 1980) on rose flower cuttings, randomized split plot design (4 treatments and 4 replicates using 4 rose varieties) was used for the study.

Rose flower stocks were wounded with a blunt cylindrical sterilized steel rod of two millimeter diameter at three different portions of the stem to a depth of three millimeter. In one set, each wound was inoculated with 0.004 milliliter suspension measured using a micropipette of each *A. tumefaciens*; the second set was inoculated with 0.004 milliliter *A. radiobacter* suspension and immediately wrapped with sterilized non-absorbent cotton. The third set was inoculated with 0.004 milliliter *A. tumefaciens*, after 24 hours of inoculation, 0.004 milliliter suspension of non-pathogenic (*A. radiobacter*) containing 10^8 cfu/ml was inoculated on the same wounds after removing the cotton. The wounds were wrapped again immediately with fresh sterilized non-absorbent cotton. The fourth set was a control (rose stock treated with sterile water). Wounds were examined for the presence or absence of galls and gall size after four weeks.

3.6 Evaluation of the effect of the use of Elianto oil, copper hydroxide (Kocide) and Copper oxychloride (Curavit) on infected rose flower plants

The experiments were carried out in the greenhouse on infected rose flower plants. Galls were plucked from the infected rose plant using sterilized secateurs. Chemicals used for the study were; copper hydroxide (kocide 2000) from Drexel chemical company) and copper oxychloride (Curavit). Corn oil (Elianto oil) was also used in the study. The chemicals were prepared by dissolving 3 g and 2 g in a liter and the mode of application was in form of paste on the wounds where galls were plucked. A control of untreated rose flower stock was kept for comparison.

3.7 Experimental layout in the greenhouse

The experimental design was a randomized complete block design (Appendix I) on both biological and chemical control methods. The biological control experiment was done to study the effect of four (4) different treatments on four (4) rose flower varieties as follows; A -Rose plant + *A. tumefaciens*, B-Rose plant + *A. tumefaciens* + *A. radiobacter*, C-Rose plant + *A. radiobacter* and D-Rose plant alone. The four rose flower varieties used in the study included; V₁ (Furiosa), V₂ (Topsun), V₃ (Fuschiana) and V₄ (H₃O).

The chemical control experiment was conducted to study the effect of six (6) different treatments on one rose flower variety as follows; T₁. Rose plant + copper hydroxide (Kocide, 2000)-3 g/l, T₂. Rose plant + copper hydroxide (Kocide, 2000)-2 g/, T₃. Rose plant + copper oxychloride (Curavit)-3 g/l, T₄. Rose plant + copper oxychloride (Curavit)-2 g/, T₅- Rose plant + Elianto oil and T₆- Rose plant alone. The experiment involved one (1) rose flower variety.

In the first layout all the treatments were represented in two different greenhouses, each having two varieties of rose plants. Each plot occupied an area of 6.16 m². A total of four plots constituted the whole experimental layout. In the second layout the treatments were represented in one greenhouse having one variety of rose plant. The plot occupied an area of 18.56 m². The plant spacing between one rose flower stocks to the next rose stock was 11 cm within the row and 80 cm between rows. The rose plants were planted in pumice and nutrients supplied through irrigation.

3.8 Data collection

Data was collected using structured questionnaire (Appendix II), laboratory and greenhouse experiments. Part A of the questionnaire gathered general information of the flower farms, part B focused on the occurrence of crown gall disease and factors contributing to the crown gall disease. Part C was on rose flower production on the selected farms with respect to crown gall disease while part D dealt with management of crown gall disease in the greenhouses of the selected flower farms. The experimental studies were conducted in the laboratory and in the greenhouse. Data on Agrobacteria isolates was collected from laboratory work and included zone of inhibition of antagonist and bacterial colonies while data on rose flower plants with tumors, without tumors and gall size was collected from greenhouse experiments.

3.9 Data analysis

Data collected was analyzed and results presented using graphs, figures and tables. Data from field survey was analyzed using SPSS frequency, percentage and chi-square test. Data from laboratory and greenhouse experiments was analyzed using SAS one way and two way ANOVA using Tukey's HSD test at 5 % probability level.

CHAPTER FOUR

RESULTS

4.1 Factors contributing to the presence of crown gall disease in rose flower farms

The study sought to establish the association between several factors predisposing the rose flowers to the crown gall disease and the responses on the frequency of low production as a result of the disease. From the survey conducted, age of the plant (24%) contributed mostly to the presence of crown gall disease, followed by farm practices (20%), root stock type, poor crop management (16%) and infested soils (12%). The least was the presence of pests and climate (8% and 4% respectively) (Table 4.1). There was no significant relationship among factors contributing to crown gall disease ($P=0.05$).

Table 4.1: Factors contributing to the presence of crown gall disease

Factors	Frequency	Percentage
Age of the plant	12	24.0
Poor crop management	8	16.0
Farm practices	10	20.0
Root stock type	8	16.0
Infested soils	6	12.0
Presence of pests	4	8.0
Climate	2	4.0
Total	50	100.0
p- value		0.128

Percentage values differed significantly (chi-square test $\alpha = 0.05$)

4.1.1. Relationship between the size of the farm and *Agrobacterium tumefaciens* infection potential

The findings of the study indicated that on average 50% of the areas below 3 acres were commonly affected by crown gall disease. The areas between 4-6 acres (33.3%) were moderately affected while areas above 7 acres (16.7%) were less affected by crown gall disease in most of the greenhouses. There was no significant relationship ($P= 0.05$) between the areas affected by crown gall disease (Table 4.2).

In terms of crown gall disease distribution in greenhouses, galls had spatial distribution (45.5%) in some greenhouses while in others there was wide distribution (30.3%) and in others they showed limited distribution (24.2%). There was no significant difference ($P= 0.05$) in the distribution of crown gall disease in rose flower farms (Table 4.3).

Table 4.2: Size of the farm (in acres) in relation to crown gall disease

Areas affected	Frequency	Percentage
Below 3 acres	15	50
4-6 acres	10	33.3
7 and above	5	16.7
Total	30	100.0
p-value		0.082

Percentage values varied significantly (chi-square test $\alpha = 0.05$)

Table 4.3: Distribution of crown gall disease in rose flower farms

Distribution	Frequency	Percentage
Spatial distribution	15	45.5
Limited distribution	8	24.2
Widely distributed	10	30.3
Total	33	100.0
p-value		0.307

Percentage values varied significantly (chi-square test $\alpha = 0.05$)

4.1.2 Sources of planting materials used in the farm where survey was conducted

The planting materials used by most of the flower farms were from the local commercial propagators and a few used own-rooted or own-cuttings flower stocks. The survey results showed that on average 48.4% of the farms used locally available rose flower stocks as major planting material while 32.2% of the farms used rose flower stocks from own-rooted or own-cuttings flower stocks. Other farms however, used imported rose flower stocks (19.4%) (Table 4.4). The number of farms using rose stock from local commercial propagators was higher than those that were using own cuttings and imported ones. The study showed that the sources of planting materials were not significantly different ($P= 0.05$).

Table 4.4: Sources of planting materials used in the farm

Sources of planting materials	Frequency	Percentage
From local commercial propagators	15	48.4
Own-rooted flower stocks	10	32.2
Imported flower stock	6	19.4
Total	31	100.0
p-value		0.140

Percentage values varied significantly (chi-square test $\alpha = 0.05$)

4.1.3 Susceptible and resistant rose varieties to crown gall disease used in the farm

The common rose flower varieties that were susceptible to crown gall disease included; wild one, wild thing, top sun, impact, ace pink and H₃O. These rose flower varieties were commonly identified in most of the flower farms and they were ranked in order of priority based on the response strength.

This analysis showed that the most susceptible rose flower variety was wild thing (75%) and the least susceptible was H₃O in the varieties under the study. Generally, there was no significant difference $P=0.05$ between the susceptible rose flower varieties with regard to the occurrence of crown gall disease (Table 4.5a).

The survey results also showed that among rose flowers that were resistant to crown gall disease included; Akito, Mylo, Oops, Valentino, Shanty, Furiosa and Red calypso. Red calypso was highly resistance to crown gall (75%) followed by (Akito 50%), Furiosa (40%), Oops (35%) and the least was Valentino (20%). Therefore, there is no significant difference in the relationship between resistant rose flower variety and the occurrence of crown gall disease ($P= 0.05$) (Table 4.5b).

Table 4.5: (a) Varieties of rose flower susceptible to crown gall disease

Susceptible rose flower variety	Responses	
	Number of respondents	Percentage
Wild thing	15	25
Wild one	12	20
Impact	10	16.67
Ace pink	8	13.33
Top sun	8	13.33
H3O	7	11.67
Total	60	100
p- value	0.467	

Percentage values varied significantly (chi-square test $\alpha = 0.05$)

Table 4.5: (b) Varieties of rose flower resistant to crown gall disease

Resistant rose flower variety	Responses	
	Number of respondents	Percentage
Red calypso	15	27.27
Akito	10	18.18
Furiosa	8	14.54
Oops	7	12.73
Mylo	6	10.91
Shanty	5	9.09
Valentino	4	7.27
p-value	0.103	

Percentage values varied significantly (chi-square test $\alpha = 0.05$)

4.1.4 Cases of low production in rose flower due to crown gall disease

Galls affected quality and productivity of rose flower plants in terms of reduced harvestable stems. Cases of low production therefore, in most the farms were often (55.2%), very often (27.6%) and in a few farms the cases were not often 25%. These values varied significantly ($P= 0.05$) (Table 4.6).

Table 4.6: Cases of low production in rose flowers farms due to crown gall disease

Cases	Frequency	Percentage
Very often	8	27.6
Often	16	55.2
Not often	5	17.2
Total	29	100.0
p-value	0.035	

Percentage values varied significantly (chi-square test $\alpha = 0.05$)

4.1.5 Other major constraints affecting rose flower production in rose flower farm and measures taken

Other major constraints affecting rose flower production as perceived by the farmers on average were pests and other diseases (28%), market demand (20%) and high costs of inputs (24%). Constraints encountered during planting initiation included water scarcity (12%) and inadequate preferred planting materials in terms of varieties (16%) (Table 4.7). The survey showed that other constraints affecting rose flower production had no significant difference ($P= 0.05$).

Table 4.7: Other constraints affecting rose flower production

Constraints	Frequency	Percentage
Pests and other diseases	14	28.0
Market demand	10	20.0
Costs of input	12	24.0
water scarcity	6	12.0
Inadequate planting materials	8	16.0
Total	50	100.0
p-value		0.406

Percentage values varied significantly (chi-square test $\alpha = 0.05$)

4.1.6 Farm practices applied on the farm in the management of crown gall disease

To establish whether there existed any difference in the occurrence of the crown gall disease as a result of farm practices applied in the management of the crown gall disease, various farm practices were carried out in rose flower farms. From the survey conducted the farm practices applied included; crop rotation, plucking of galls, sanitation, removal of the whole crop, chemical and biological control. The data was collected through use of a questionnaire.

Plucking of galls was commonly practiced (28.8%) followed by removal of the crop (23.2%), crop rotation (19.2 %), sanitation (15.4%), chemical control (9.6%) and the least was biological control (3.8%) (Table 4.8). There was a significant difference ($P=0.05$) on the farm practices applied in the management of crown gall disease.

Table 4.8: Farm practices applied in the management of crown gall disease

Farm practices	Frequency	Percentage
Crop rotation	10	19.2
Plucking of galls	15	28.8
Sanitation	8	15.4
Removal of the whole crop	12	23.2
Chemical control	5	9.6
Biological control	2	3.8
Total	52	100.0
p-value		0.025

Percentage values differed significantly (chi-square test $\alpha = 0.05$)

4.1.7 Factors that hinder management of crown gall disease in rose flower farms

The study sought to gather opinions from the respondents on what makes the control and management of the crown gal disease difficult. The number of people interviewed was twenty (20) that is, the production manager and farm scouters of different flower farms. The type of farms where interviews were conducted carried out horticulture farming. Some of the farm practices carried out in these farms were plucking of galls, pruning and weeding, removal of the whole crop and crop rotation as ways of managing crown gall. Some of the choices elicited multiple responses.

The responses were presented in figure 4.1. From figure 4.1, most respondents felt that purchase of infected flower stock was the major hindrance with 10 responses attributing it to inadequate resistant cultivars. Lack of effective chemicals eliciting 8 and 6 responses respectively. Few respondents felt that lack of information was a major challenge with only 3 responses.

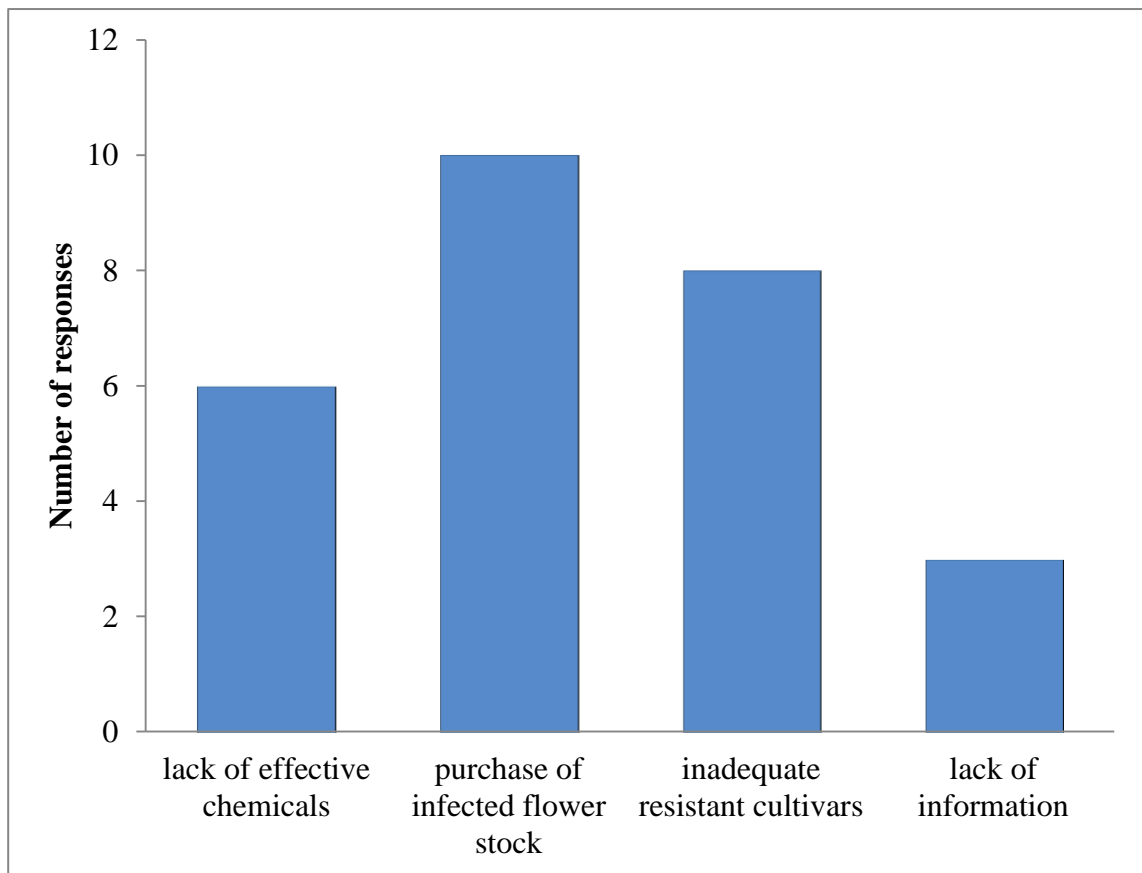


Figure 4.1 Factors affecting management of crown gall disease

4.2 Laboratory experiments

4.2.1 Isolation of *A. tumefaciens* and *A. radiobacter*

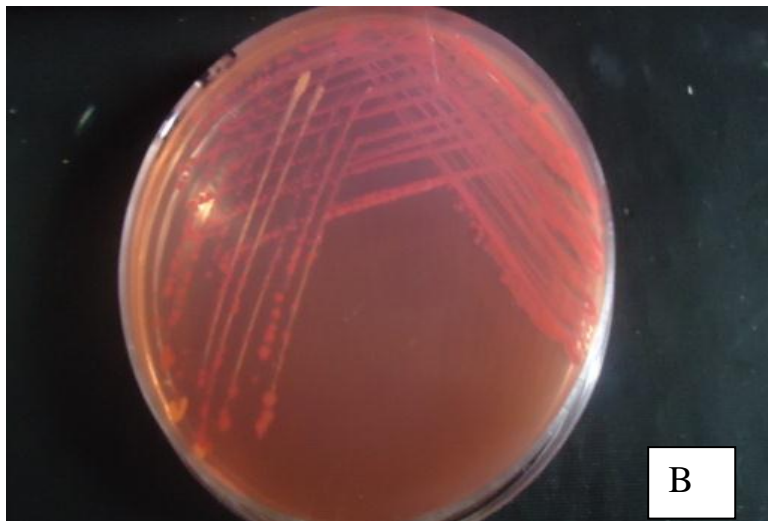
In isolation of the isolates, colonies appeared on the media within 2 days and attained full size in 4-5 days. All isolates stained pink in YEMA supplemented with Congo red, others red in color (Plate 4.1). In YEMA, *A. tumefaciens* isolates were circular in shape and had a smooth margin while *A. radiobacter* isolates in same medium was circular in shape but had a regular margin. In Yeast Extract Peptone agar (YEP), *A. tumefaciens* isolates were circular and slightly raised, cream white and translucent slime and had a smooth margin. *Agrobacterium radiobacter* isolates on YEP, had a fluorescent convex in shape, cream yellow, white shiny mucoid color and had a smooth, partially raised margin (Table 4.9 and 4.10). For the Gram stain test, *A. radiobacter* isolates the rods appeared slightly larger compared to *A. tumefaciens* isolates when observed under microscope. The micrographs were observed at magnification of $\times 400$ using an inverted microscope (Plate 4.2). Colony sizes for both isolates were ranging from 1.0 millimeter to 1.6 millimeter and generally there was a well pronounced growth within 24 hours.

Table 4.9: Morphological characteristics of *A. radiobacter*

Character	a)Yeast peptone agar	b)Yeast extract mannitol agar
Shape	Fluorescent convex	Circular
Color	Cream yellow, white shiny mucous	Red
Surface margin	Smooth, partially raised	Regular
Gram's nature	Negative	Negative

Table 4.10: Morphological characteristics of *A. tumefaciens*

Character	a)Yeast peptone agar	b)Yeast extract mannitol agar
Shape	Circular, slightly raised	Circular
Color	Cream white, translucent slime	Red/pink
Surface margin	Smooth	Smooth
Gram's nature	Negative	Negative



Plates 4.1: Gall sample inoculums on (A) YEP and (B) YEMA media. In A- colonies had a smooth margin, circular in shape and cream white in color. In B- colonies had a smooth margin, circular in shape and pink in color

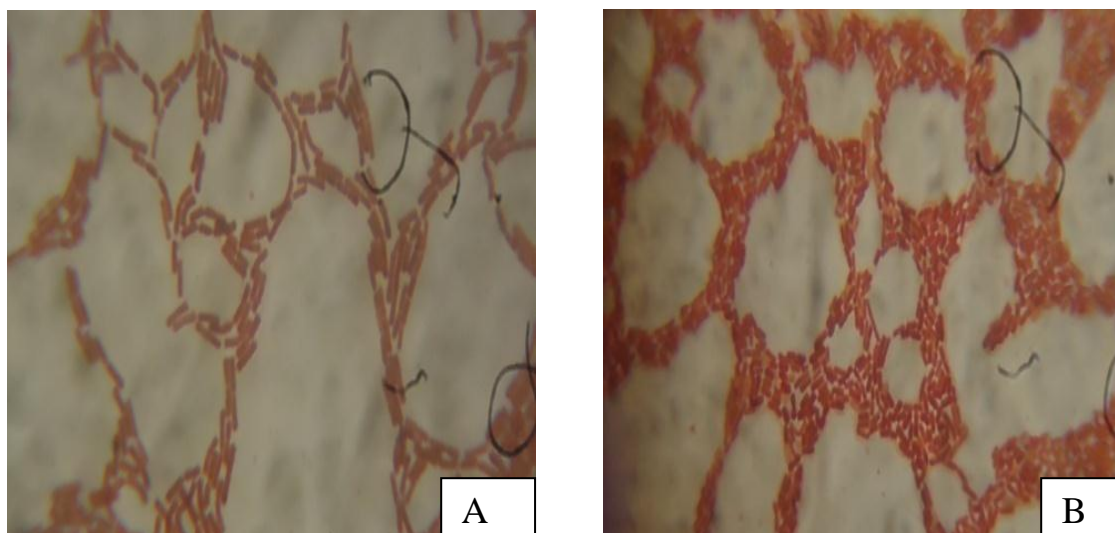


Plate 4.2: Gram stain characteristic and cell morphology of (A) *Agrobacterium radiobacter* isolate from soil sample and (B) *Agrobacterium tumefaciens* isolate from gall sample. The micrographs were observed at $\times 400$. *Agrobacterium radiobacter* had slightly larger rods compared to *A. tumefaciens* when viewed under the microscope.

4.2.2 Characterization of the isolates

4.2.2.1 Biochemical test for the isolates

The confirmation of *Agrobacterium* spp. was made by the specific tests such as growth on Potato Dextrose Agar (PDA) medium, 3-ketolactose test and sodium tolerance test. On PDA medium, all isolates that is isolates A, B, C and D showed well pronounced growth; similarly higher concentration of 3% sodium chloride did not affect the growth of isolates. The isolates showed positive oxidase reaction, positive urease reaction, positive motility test, and positive catalase test. However, *Agrobacterium tumefaciens* isolates showed negative results for 3-ketolactose test while *Agrobacterium radiobacter* isolates showed positive results that is isolates 1,2 and 3 except isolates 4 (isolates from Kisima farm) (Table 4.11).

For urease test, soil sample showed yellow coloration on the butt and pink coloration on the slant while for gall sample both slant and butt showed pink coloration (Plate 4.3) (A and B). For Triple iron sugar test (TSI) on the isolates from soil samples showed pink coloration on the slant and yellow coloration on the butt (Plate 4.4 (A and B) while isolates from the gall sample showed pink coloration on the slant, gas bubbles at the base of slant and pink coloration on the butt.

Table 4.11: Biochemical characteristics of *A. tumefaciens* and *A. radiobacter* Isolates

Biochemical tests	<i>Agrobacterium tumefaciens</i> isolates				<i>Agrobacterium radiobacter</i> isolates			
	A	B	C	D	1	2	3	4
Motility test	+	+	+	+	+	+	+	+
H ₂ S production	+	+	+	+	+	+	+	+
Urease test	+	+	+	+	+	+	+	+
Catalase test	+	+	+	+	+	+	+	+
Oxidase test	+	+	+	+	+	+	+	+
3-ketolactose test	-	-	-	-	+	+	+	-
Salt tolerance test	T	T	T	T	T	T	T	T

[Note +: positive, -: negative, T: tolerant

Agrobacterium tumefaciens isolates A-Ol Jorowa (Naivasha), B-Likii River farm (Nanyuki), C-Branan farm (Muranga) and D-Kisima (Timau).

Agrobacterium radiobacter isolates 1-Ol Jorowa, 2-Likii River farm, 3-Branan farm and 4-Kisima farm.

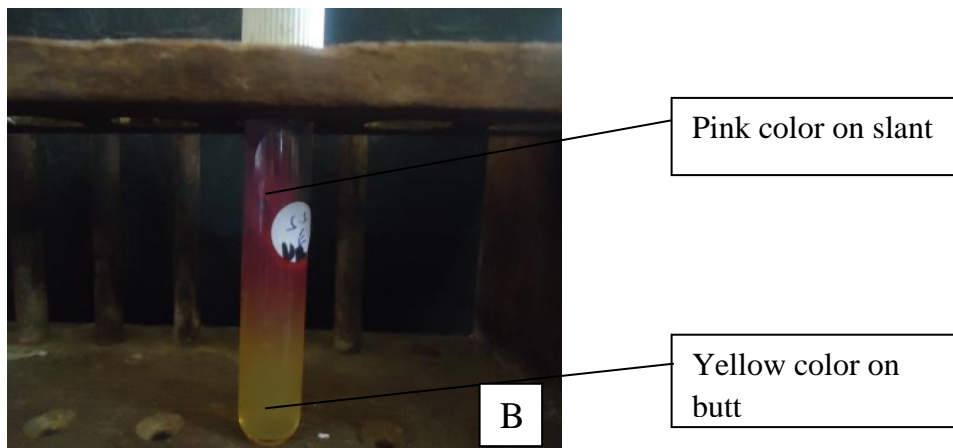
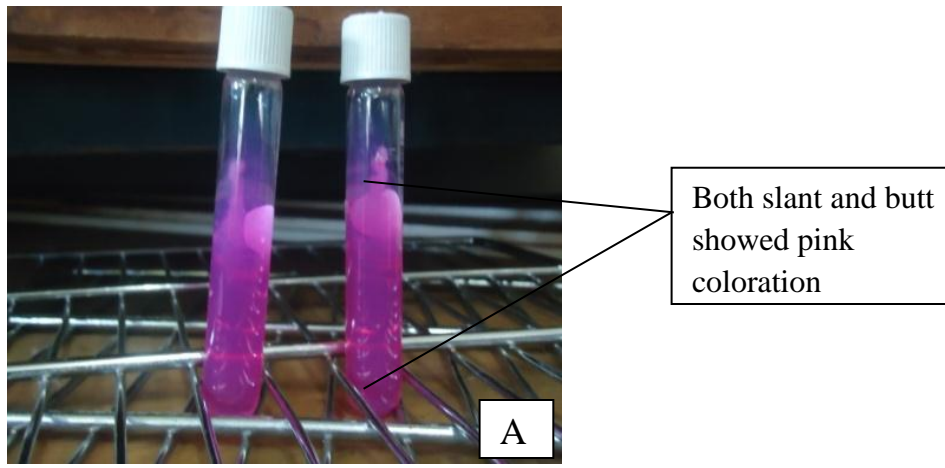


Plate 4.3: Urease test for *Agrobacterium tumefaciens* (A) and *Agrobacterium radiobacter* (B). In A-both slant and butt had pink coloration showing alkaline producing bacterium while in B- slant had pink color while butt had yellow color showing alkaline and acid producing bacterium.

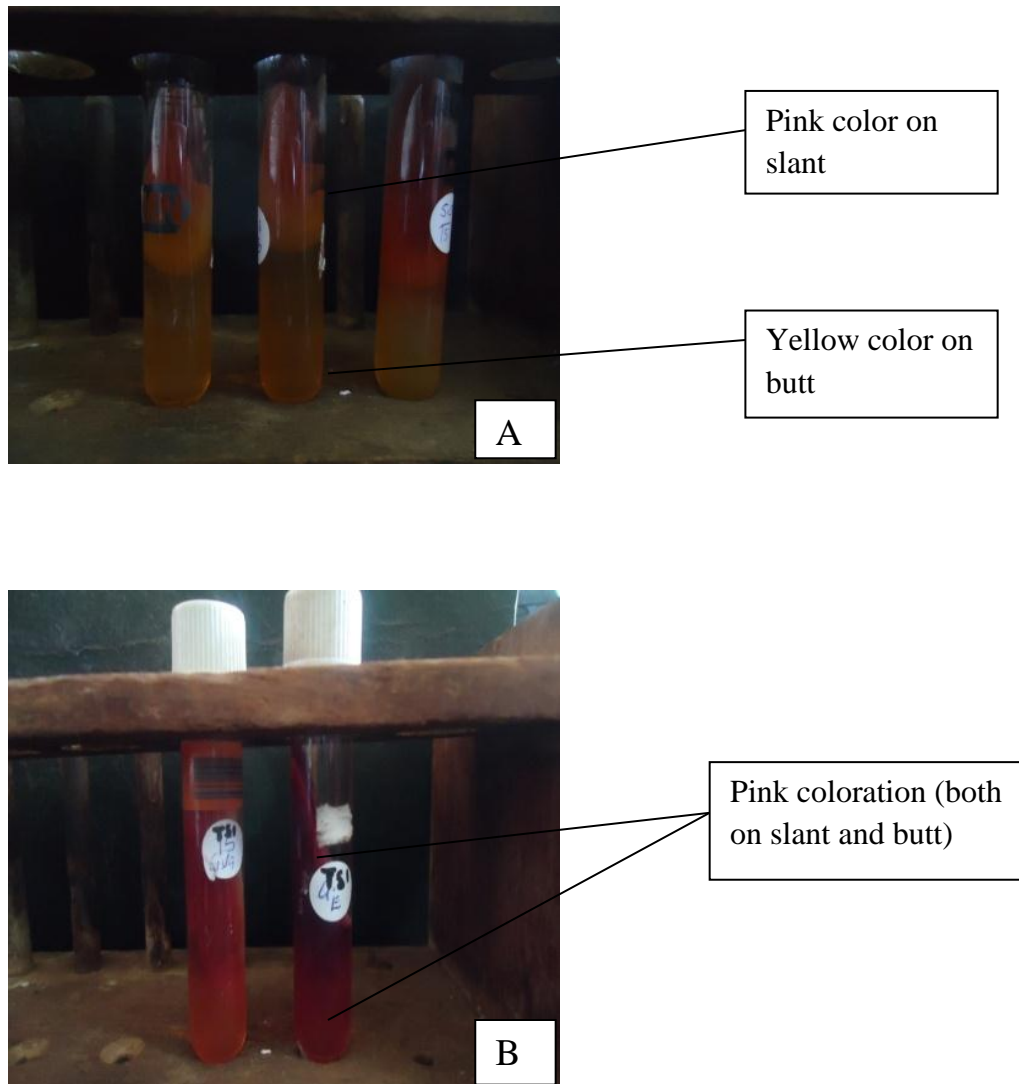
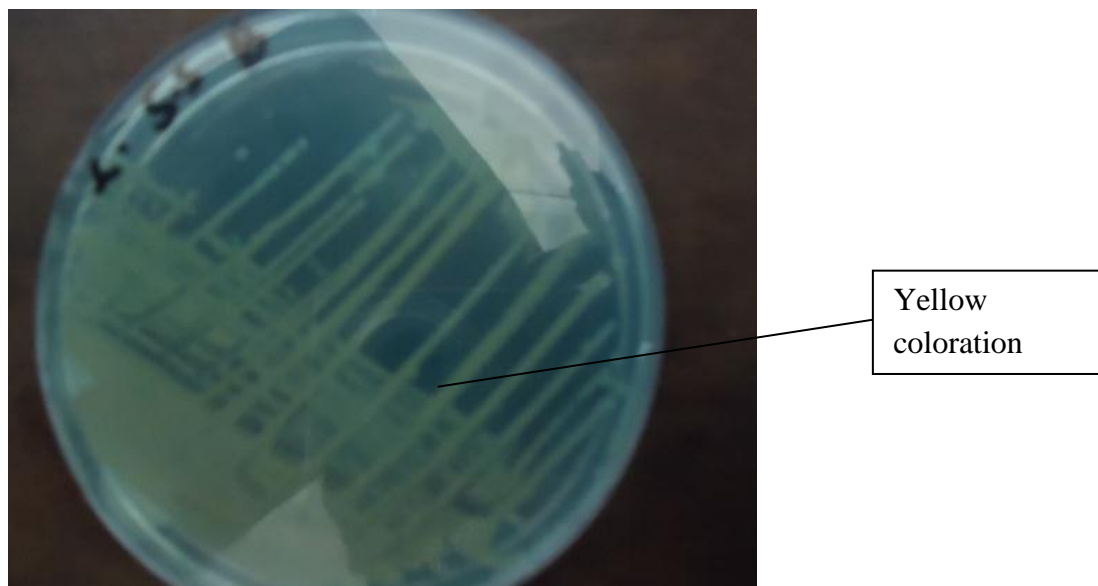


Plate 4.4: Triple sugar iron (TSI) test for (A) *Agrobacterium radiobacter* isolates and (B) *Agrobacterium tumefaciens* isolates. In A- slant had pink color while the butt had yellow color an indication of alkaline and acid producing bacterium. In B-both butt and slant had pink color showing it was an alkaline producing bacterium.

A further biochemical test was carried out to distinguish *Agrobacterium radiobacter* from *Agrobacterium tumefaciens* using 3-ketolactose test. *Agrobacterium* spp. isolated from the soil that is *Agrobacterium radiobacter* showed a yellow coloration on 3-ketolactose test (Plate 4.5).



Plates 4.5: 3-ketolactose test for *Agrobacterium radiobacter*. Formations of yellow ring around the colonies of bacterium isolate indicating positive results on the test.

4.2.2.2 Antibiotic resistance test for *Agrobacterium tumefaciens* isolates.

For antibiotic resistance test the following antibiotics were used as indicated by initials on the plate; penicillin (P), Chloramphenicol (C), Ampicillin (A), Co-trimoxazole (Co), Minocycline (Mi), Erythromycin(E), Lincomycin (L) and Methicillin (M). Antibiotic resistance test showed that the isolate showed no inhibition zones on Penicillin, Methicillin, Lincomycin and Ampicillin. However, inhibition zones were observed in Minocycline, Erythromycin, Co-timoxazole and Chloramphenicol (Plate 4.4).

The diameter of zones sizes were less than 10mm (Table 4.12) therefore classified as resistant. There was a significant difference $P= 0.05$ between the antibiotics with regard to resistance on the isolate (Table 4.12).

Table 4.12: Mean of zones of inhibition in antibiotic resistance test

Antibiotic	Zone of inhibition (diameter in mm)
Ampicillin	$6.00 \pm 0.00d^*$
Chloramphenicol	$6.70 \pm 0.06b$
Co-trimoxazole	$7.67 \pm 0.03a$
Erythromycin	$6.40 \pm 0.09c$
Lincomycin	$6.00 \pm 0.00d$
Minocycline	$6.60 \pm 0.07bc$
Penicillin	$6.00 \pm 0.00d$
Methicillin	$6.00 \pm 0.00d$
p-value	<0.0001

Means \pm standard error (SE) separated using Tukey's Honest significant difference (HSD) test, *Means within the column followed by the same letters are not significantly different at $p= 0.05$.

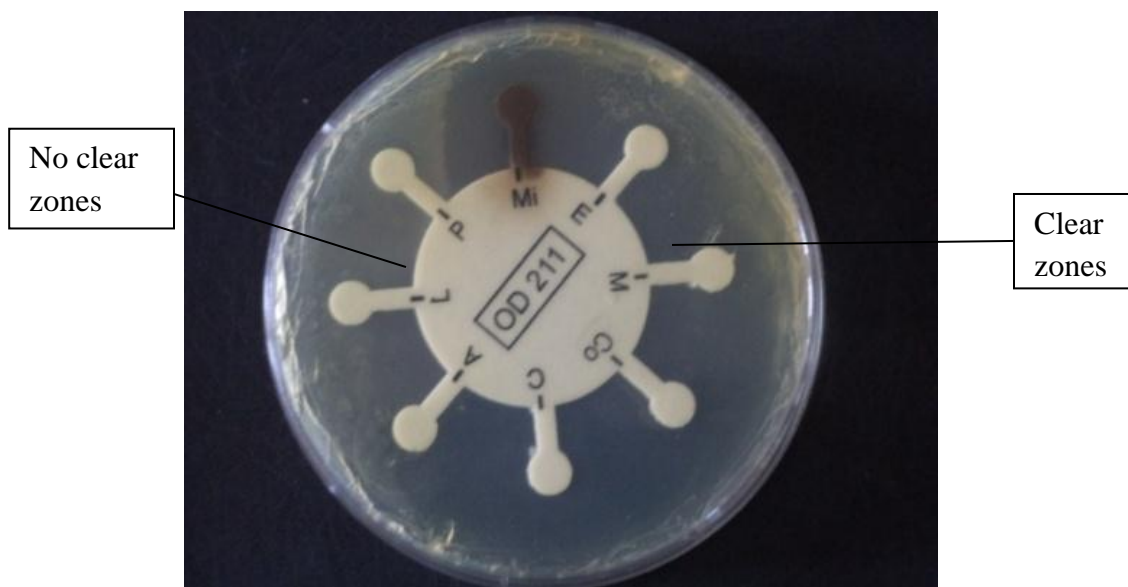


Plate 4.6: Zones of inhibition for antibiotic resistance test on *A. tumefaciens*. Clear zones an indication bacterium had not grown.

Table 4.13: Interpretation of antibiotic resistance test for zone diameter

Antibiotic	Resistant(\leq mm)	Intermediate (mm)	Susceptible(= or > mm)
Ampicil 10 μ g	11	14-16	17
Chloramphenicol 30 μ g	12	13-17	18
Erythromycin 10 μ g	13	14-22	23
Methicilin 5 μ g	9	10-13	14
Penicillin 10 μ g	14	-	15
Minocycline 30 μ g	14	15-18	19
Kanamycin 30 μ g	12	12-17	17

Source: (CLSI, 2006)

4.2.2.3 Pathogenicity test for *A. tumefaciens* isolates

The *Agrobacterium tumefaciens* isolates from Ol Jorowa farm, Likii River farm, Kisima farm and Branan farm were tested for their pathogenicity. Isolates from Ol Jorowa farm induced tumor formation when inoculated in carrot discs (Plate 4.7). Other isolates from other farms showed no gall or few tumors on the carrot disc. Young galls (tumors) developed at meristematic tissue at the central part of the carrot disc after four weeks of inoculation. No symptoms were noted on un-inoculated control indicating that these strains isolated from *Rosa* spp. were pathogenic (Plate 4.7).

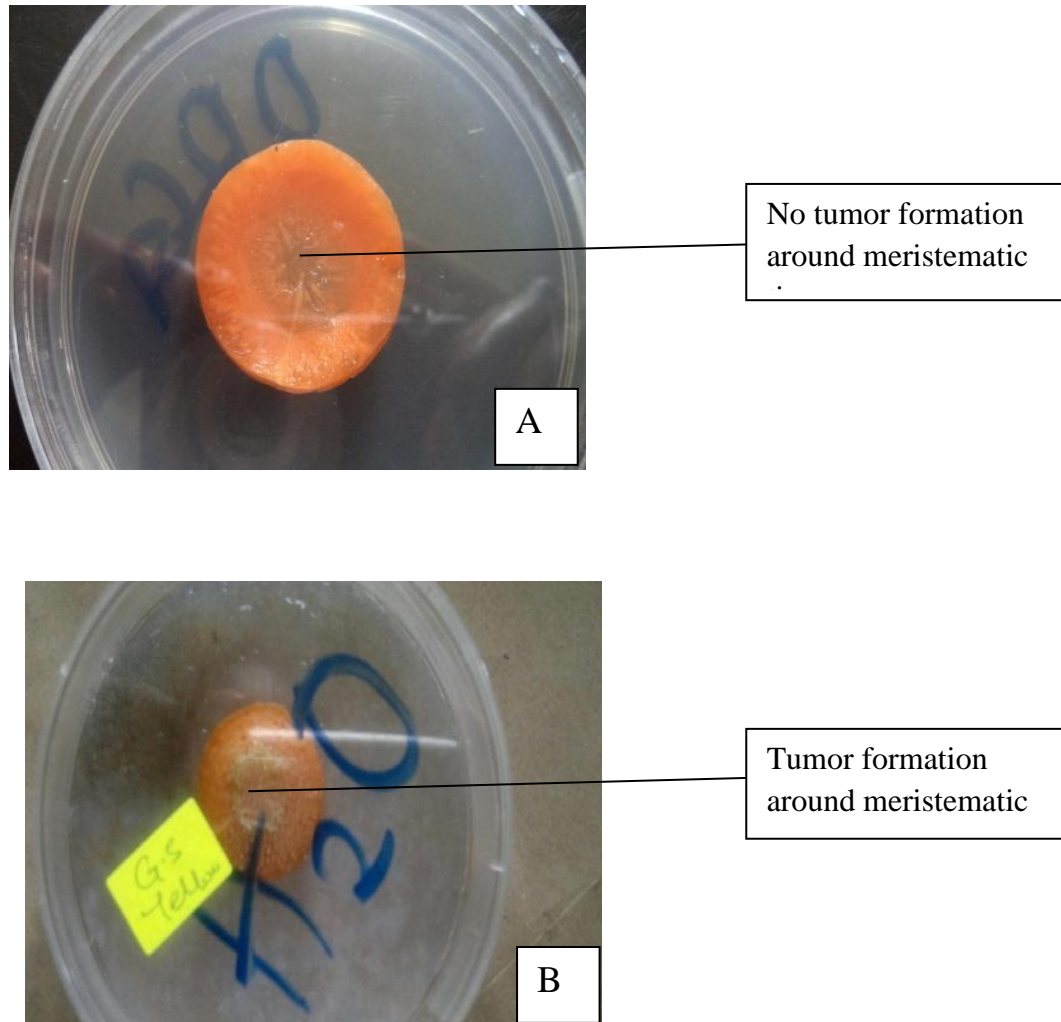


Plate 4.7: Carrot disc assay test for *Agrobacterium tumefaciens* isolate. A- Negative control test showing no galls at the central part of carrot disc. B- Positive test, showing developed young galls at meristematic tissue of the central part of carrot disc.

4.2.2.4 *In vitro* antagonistic activity of *A. radiobacter* against *A. tumefaciens*

In *In vitro* antagonistic test, isolates from three farms were used for the study. *Agrobacterium radiobacter* isolates inoculums were poured in wells labeled (X) on the plate while *Agrobacterium tumefaciens* isolates inoculums were spread on the plates as labeled (Y) on the plate (Plate 4.6).

Agrobacterium radiobacter isolates isolated from soil samples from Ol Jorowa farm (Naivasha), Likii River Farm (Nanyuki) and Branan Farm (Murang'a) coded as; A, B and C respectively. The inhibition zones from these isolates showed 0.6 mm, 0.53 mm and 0.37 mm in diameter and 0.00 mm in their controls respectively in Agar Well Diffusion Assay (Table 4.14). Therefore, there was a significant difference ($P= 0.05$) in the zones of inhibition detected in antagonistic activity of *A. radiobacter* against *A. tumefaciens*. Diameters were determined by the zones of inhibition (Plate 4.6). Isolates in farm A had larger inhibition zones compared to isolates in farm B and C.

Table 4.14: *In vitro* antagonistic test of *A. radiobacter* isolates against *A. tumefaciens* in Agar Well Diffusion Assay

Isolates (<i>A. radiobacter</i>)	Zone of inhibition (diameter in mm)
A	$0.600 \pm 0.12^{a*}$
B	0.530 ± 0.12^a
C	0.367 ± 0.09^{ab}
D (Control)	0.0 ± 0.0^b
p-value	0.0082

Means \pm standard error (SE) separated using Tukey's Honest significant difference (HSD) test, *Means within the column followed by the same letters are not significantly different at $p < 0.05$.

Agrobacterium radiobacter isolates A- Ol Jorowa farm, B- Likii River farm and C- Branan farm

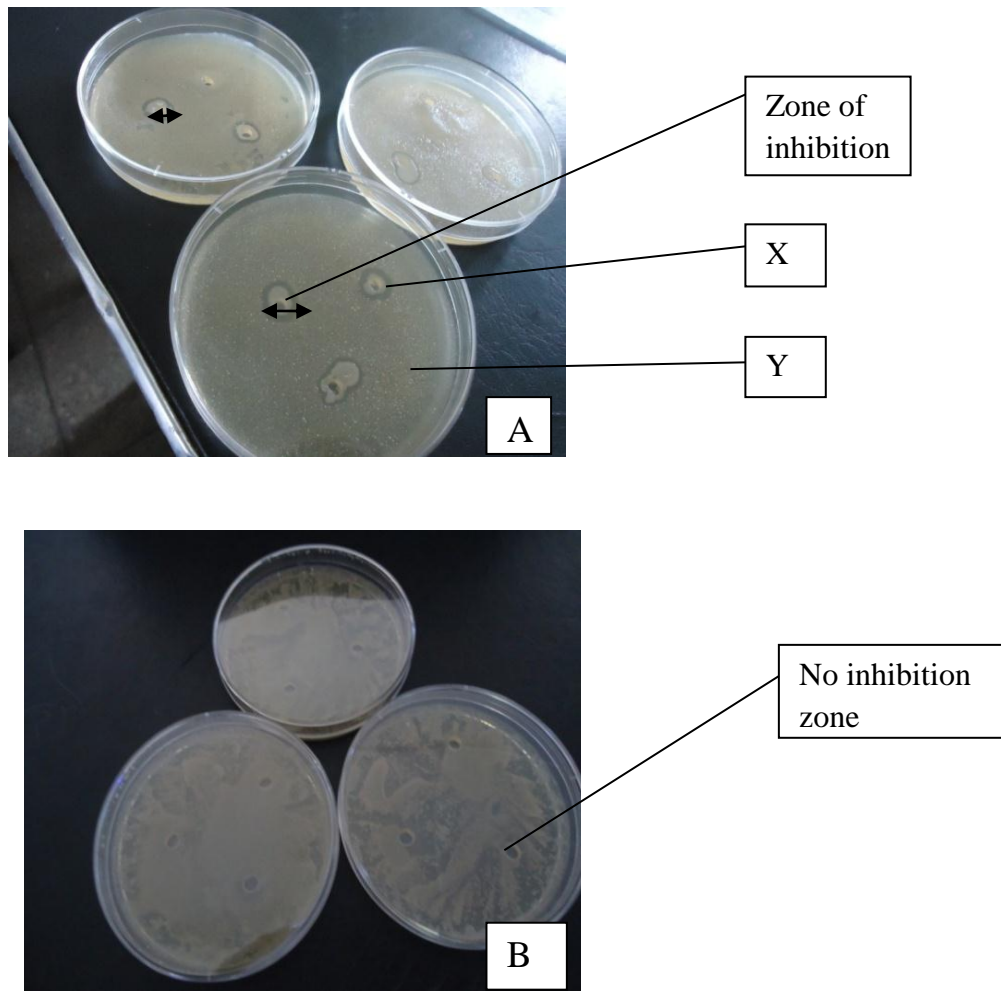


Plate 4.8: Zones of inhibition which antagonists shows (A) positive results for *A. radiobacter* isolates against *A. tumefaciens* isolates and (B) negative control results in agar-well diffusion assay (no zones indicated around the wells).

4.3 Greenhouse experiments

4.3.1 Evaluation of *A. tumefaciens* and *A. radiobacter* isolates for their pathogenicity and antagonism on rose plants

Agrobacterium radiobacter isolates and *A. tumefaciens* isolates from Ol Jorowa farm (Naivasha) were used for antagonism and pathogenicity test. The *A. radiobacter* isolates from the Ol Jorowa farm were used for the study because of their efficacy compared to isolates in other farms. *Agrobacterium tumefaciens* isolate screened for their pathogenicity on rose plants showed tumor forming ability on those rose flower stocks where it was inoculated. *Agrobacterium radiobacter* isolates when tested for antagonism against *A. tumefaciens* on rose plants showed that it was able to control gall development. Rose flower stocks treated with *A. tumefaciens* alone had gall sizes ranging from 1.0 cm, 0.57 cm and 1.0 cm in Top sun, Fuschiana and H₃O respectively. The gall sizes in their controls were 1.0 cm, 1.0 cm, 1.0 cm and 1.7 cm in Furiosa, Top sun, Fuschiana and H₃O respectively. There was a significant difference (P= 0.05) (Table 4.15a) and (P= 0.05) (Table 4.14b) on the treatment applied on rose flower plants in trial one and trial two respectively. Similarly, there was a significant difference (P= 0.05) (Table 4.15a) and (P= 0.05) (Table 4.15b) in terms of gall size with regard to the treatment applied on rose plants in trial one and two respectively.

The varieties of rose plants used for the study were Topsun, Fuschiana, Furiosa and H₃O. There was no significant difference (P= 0.05) (Table 4.15a) and (P= 0.05) (Table 4.15b) on the plants with galls after inoculation in trial one and trial two respectively.

In determination of gall sizes on varieties of rose plants used in the study there was no significant difference ($P= 0.05$) (Table 4.14a) in trial one but there was a significant difference ($P=0.05$) in trial two. There was no significant difference ($P= 0.05$) (Table 4.15a) and ($P= 0.05$) (Table 4.15b) in the interaction between the four varieties rose plants used in the study and the treatment applied in trial one and two respectively.

Table 4.15 a) Effectiveness of the antagonist (*A. radiobacter*) on tumor formation

TRIAL ONE

Treatment	Number of plants with galls	Gall size (diameter in cm)
<i>Agrobacterium radiobacter</i>	0.1875 ± 0.1001 ^{ab}	0.06875 ± 0.0435 ^b
<i>A. tumefaciens</i> + <i>A. radiobacter</i>	0.0625 ± 0.0625 ^b	0.00625 ± 0.0063 ^b
<i>Agrobacterium tumefaciens</i>	0.4375 ± 0.1281 ^{ab}	0.36875 ± 0.1306 ^{ab}
Control	0.5625 ± 0.1281 ^a	0.79375 ± 0.2242 ^a
Variety		
Furiosa	0.2500 ± 0.1118 ^a	0.1563 ± 0.0584 ^a
Fushiana	0.3125 ± 0.1197 ^a	0.0723 ± 0.1500 ^a
H3O	0.3125 ± 0.1197 ^a	0.5063 ± 0.2020 ^a
Topsun	0.3750 ± 0.1250 ^a	0.4250 ± 0.1974 ^a
P- values		
Treatment	0.0127	0.0001
Variety	0.8959	0.0697
Variety* Treatment	0.7906	0.0224

Means ± standard error (SE) separated using Tukey's Honest significant difference (HSD) test, Mean values followed by the same lowercase within the same column are not significantly different (two way ANOVA, $\alpha= 0.05$).

Table 4.15 b) Effectiveness of the antagonist (*A. radiobacter*) on tumor formation

TRIAL TWO

Treatment	Number of plants with galls	Gall size (diameter in cm)
<i>Agrobacterium radiobacter</i>	0.1250 ± 0.0853 ^b	0.1125 ± 0.0774 ^b
<i>A. tumefaciens</i> + <i>A. radiobacter</i>	0.1118 ± 0.1188 ^{ab}	0.11875 ± 0.0564 ^b
<i>Agrobacterium tumefaciens</i>	0.6250 ± 0.1250 ^a	0.3563 ± 0.3563 ^{ab}
Control	0.5625 ± 0.1281 ^a	0.7625 ± 0.7625 ^a
Variety		
Furiosa	0.2500 ± 0.1118 ^a	0.0563 ± 0.02700 ^b
Fushiana	0.3750 ± 0.1250 ^a	0.3976 ± 0.1518 ^{ab}
H3O	0.5000 ± 0.1291 ^a	0.7500 ± 0.2405 ^a
Topsun	0.4375 ± 0.1281 ^a	0.1500 ± 0.0619 ^b
P- values		
Treatment	0.0131	0.011
Variety	0.5122	0.0010
Variety* Treatment	0.9700	0.0451

Means ± standard error (SE) separated using Tukey's Honest significant difference (HSD) test, Mean values followed by the same lowercase within the same column are not significantly different (two way ANOVA, $\alpha= 0.05$).

4.3.2 Effects of chemical methods in the management of crown gall disease

There was no significant difference ($P= 0.05$) (Table 4.16a) and ($P= 0.05$) (Table 4.16b) in number of plants with regards to chemical control methods applied on infected rose plants in trial one and two respectively.

In terms of gall sizes with regard to chemicals applied on the study there was a significant difference ($P= 0.05$) (Table 4.16a) in trial one while there was no significant difference ($P= 0.05$) in trial two (Table 4.16b)

Table 4.16 a) Evaluation of the effect of chemical methods in management of crown gall disease

TRIAL ONE

Treatment	Plants with galls	Gall size (diameter in cm)
Copper oxychloride (Curavit) 3 g/l	0.2500 ± 0.1118^a	0.3438 ± 0.1317^{ab}
Copper oxychloride (Curavit) 2 g/l	0.3750 ± 0.1250^a	0.7500 ± 0.2500^{ab}
Copper hydroxide (Kocide) 3g/l	0.3125 ± 0.0475^a	0.7344 ± 0.2812^{ab}
Copper hydroxide (Kocide) 2 g/l	0.3750 ± 0.1250^a	1.1200 ± 0.3750^{ab}
Corn oil (Elianto oil)	0.1250 ± 0.0854^a	0.1125 ± 0.0769^b
Control	0.4375 ± 0.0475^a	1.2250 ± 0.3580^a
P-value	0.4737	0.0319

Means \pm standard error (SE) separated using Tukey's Honest significant difference (HSD) test, Mean values followed by the same lowercase within the same column are not significantly different (one way ANOVA, $\alpha= 0.05$).

Table 4.16 b) Evaluation of the effect of chemical methods in management of crown gall disease

TRIAL TWO

Treatment	Plants with galls	Gall size (diameter in cm)
Copper oxychloride (Curavit) 3 g/l	0.2500 ± 0.1118 ^a	0.2000 ± 0.0874 ^a
Copper oxychloride (Curavit) 2 g/l	0.4375 ± 0.1281 ^a	0.5608 ± 0.1665 ^a
Copper hydroxide (Kocide) 3g/l	0.3125 ± 0.1197 ^a	0.4688 ± 0.1795 ^a
Copper hydroxide (Kocide) 2 g/l	0.3750 ± 0.1250 ^a	0.5250 ± 0.1537 ^a
Corn oil (Elianto oil)	0.1875 ± 0.0101 ^a	0.1500 ± 0.0806 ^a
Control	0.4375 ± 0.1281 ^a	0.7500 ± 0.2500 ^a
P-value	0.6015	0.0922

Means ± standard error (SE) separated using Tukey's Honest significant difference (HSD) test, Mean values followed by the same lowercase within the same column are not significantly different (one way ANOVA, $\alpha=0.05$).

CHAPTER FIVE

DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 DISCUSSION

The study findings indicated that the age of the plant contributed highly to the occurrence of crown gall followed by infested soils and variety of rose flower stock. These factors on average contributed 50% (below 3 hectares) to the occurrence of crown gall disease in most farms where the survey was conducted. Rose flower stock were planted on either soil or soilless culture therefore, rose flower stocks planted on soil culture had spatial distribution of crown gall compared to those planted on substrate. This showed that soils were more permissive to the pathogen compared to substrate like pumice. Krimi *et al.* (2002) reported a similar study where some soils were found to be permissive to crown gall which contributed highly to the spread of pathogen. Another factor highlighted was use of contaminated water for irrigation which also contributed to the spread of pathogen.

On average 48.4% of planting materials used on flower farms were purchased from local commercial propagators. Few disease incidences 19.4% were reported on the use of imported planting materials despite some of the imported varieties competing well in the market. Cases of low production due to crown gall disease were noted in most of the farms. The disease lowered the quality of the stems, reduced length of harvestable stems and production. These cases were often and common in all farms.

Crown gall disease made growers suffer significant annual losses in form of unsalable nursery stock and lowered quality of galled rose plants which compares with study conducted by Bliss, (1999). The study findings indicated that some rose flower varieties were highly susceptible to crown gall disease such as Wild thing and Wild one. Due to susceptibility of these varieties, farmers had to be selective on the varieties to grow and those to avoid. The study also showed that the levels of susceptibility varied with rose flower varieties. Reynders (1998) reported varying levels of susceptible agricultural plant species like *Rosa* spp. The study also confirmed that some varieties survived attack by the pathogen for example Furiosa and red calypso. Apart from the crown gall disease, other major constraints affecting the rose flower were the costs of inputs, use of chemicals and fertilizers and presence of pests for example plant parasitic nematodes. Pests like root chewing insects and plant parasitic nematodes create wounds on rose plants which become entry point of pathogens.

Flower farms employed different measures in the management of crown gall disease for example, plucking of galls from infected rose plants, crop rotation, sanitation and removal of whole affected crop. Removal of the whole crop (23.2%) and plucking of galls (28%) were the commonly practised methods. It therefore follows that crop rotation and plucking of the galls played an important role in managing the crown gall disease. It was also noted that timely removal of the infected plant material prevented the continued 'seeding' of the soil with a large population of pathogenic *A. tumefaciens* from crown gall tissues.

Another cultural method which most farms practiced was use of clean secateurs when harvesting flowers and plucking galls. This prevented the spread of pathogen from one rose flower stock to another. This compares to a study conducted by Sige (1993) and Ryder and Jones (1991) on the use of clean tools when pruning and plucking galls as a matter of routine practice to prevent spread of the pathogen. Farmers also employed use of resistant rose plant to crown gall disease.

This was another measure that helped reduce the spread of pathogen and management of crown gall disease. Otten *et al.* (2004) and Escobar and Dandekar (2003) reported on the use of crown gall resistant cultivars in *Rosa* spp. The study findings also showed that in most of the farms, the gall removal with the concomitant application of disinfecting paste was used to prevent gall from regenerating where galls were plucked as one of the chemical control method. This compares with the study done by Burr (2004), on the use of copper bactericides that are able to kill the bacterium on contact but did not penetrate the plants therefore offering temporal management of crown gall disease. Various farms however, faced a challenge in the management of crown gall disease due lack of effective chemicals to manage the disease.

Another hindrance was inadequate resistant cultivars and purchase of already infected rose flower stocks from commercial propagators. This made the pathogen spread very fast making its management difficult. *Agrobacterium tumefaciens* was effectively isolated for identification from gall tissue of *Rosa* spp. A similar report was given by Collins (2001) when pathogenic strains were isolated from gall tissues, soil and water.

Crown gall disease is a common disease of dicot plants, including many woody shrubs and various herbaceous plants like grapevines, roses and some ornamental plants (Rhouma *et al.*, 2005). Crown galls are often found at or just below the soil surface on the roots or crown region of plants (Ogawa *et al.*, 1999). Holt *et al.* (1994) in Bergey's manual of determinative bacteriology reported that gram negative bacteria generally grow pink to red in YEMA supplemented with Congo red. In this study both isolates grew pink to red colonies on YEMA supplemented with Congo red, showing that they absorbed the dye strongly compared to *Rhizobium* which absorbs the dye weakly. During Gram staining test, both isolates stained pink showing that they were gram negative bacteria.

This helped in further identification of bacteria. On catalase test both isolates were positive as indicated by production of gas bubbles on 3% hydrogen peroxide. On potato Dextrose Agar (PDA) all isolates indicated a well pronounced growth, similarly higher concentration of sodium chloride did not affect growth of isolates and they utilized citrate. The triple sugar iron test and urease test for both isolates were positive showing that both isolates were acid and alkaline producing bacteria.

On 3-ketolactose test, there was formation of yellow ring of precipitate of copper (I) oxide (CuO_2) around the colonies of bacterium when the plates were flooded with Benedict's reagent, especially on the isolates from the soil sample (*A. radiobacter*). This test helped distinguish *A. radiobacter* from *A. tumefaciens*. Gauer *et al.* (1973) also distinguished *Agrobacterium* spp. from *Rhizobium* spp. by conducting 3-Ketolactose test.

In antibiotic resistance test the diameter for zones of inhibition on antibiotics used for the study were less than 10 mm as indicated on the interpretation data. This was an indication that all the antibiotics used were resistant. The observation made was important particularly considering that this bacterium had the ability to withstand effects of an antibiotic. Karthy *et al.* (2009) reported on the ability of micro-organisms to withstand effect of antibiotic. However, the pathogen was susceptible to Minocycline, Erythromycin and Cotrimoxazole. The clear zones around each disc were an indication of the extent of test organism's inability to survive in the presence of the antibiotic.

This parameter was of importance in confirming the presence of the pathogen in the gall sample. Another study was conducted by Marja *et al.* (2004) on the sensitivity of *A. tumefaciens* strain to many antibiotics including tetracycline and kanamycin. Carrot disc assay test was also used in the study to confirm the presence of *A. tumefaciens*. Young galls (tumors) developed from meristematic tissue around the central vascular system. The tumor forming ability of isolates from the gall sample was an indication that the isolates were virulence. This compares to the report given by Aysan and Sahin (2003) on tumor forming ability on isolates extracted from *Rosa* spp.

There was a significant difference in antagonistic test for *A. radiobacter* with *A. tumefaciens* ($P=0.05$). It was noted that *A. radiobacter* created zones of inhibition against *A. tumefaciens* an indication that it can be used in the management of galls through antibiosis process.

The non-pathogenic strain competes for food and space in mixed inoculations preventing the pathogenic bacterium from becoming established as reported by Farrand (1990).

Various microorganisms with antagonistic activity against phytopathogens have been isolated from suppressive soils. In these soils pathogens are either unable to persist or cause low damage to plants and antagonistic microorganism account for a large part in elimination of plant disease. A similar study has been conducted by Mazzol, (2002).

The non-pathogenic isolates used on the study were isolated from the soils and substrates where rose varieties were planted on greenhouses. *Agrobacterium radiobacter* constitutes important agents for bio-control of soil-borne disease and for plant growth promotion as reported by Rajkumar *et al.* (2005). The pathogenicity and antagonism test using *A. tumefaciens* and *A. radiobacter* respectively showed there was positive interaction between the treatment and plant varieties on the number plants with galls. In terms of gall sizes there was a negative interaction between the treatment and varieties of rose plants used in the study.

Copper bactericide (Copper oxychloride (curavit) and Copper hydroxide (Kocide) were used in the evaluation of the effect of chemicals in the management of crown gall disease. There was temporal management since the gall regenerated after one month. The results showed that the chemicals cleared the galls on areas where the paste was applied since galls developed on the same plant where another wound was created. Copper bactericides do not penetrate plants and therefore fail to come into contact with the pathogen residing systemically as reported by Burr, (2004).

In gram negative bacteria, plasmid-mediated resistance genes produce proteins that can bind to DNA gyrase or Topoisomerase IV and can decrease their binding affinity to quinolones, decreasing the drug's effectiveness (Robicsek *et al.*, 2006).

The use of corn oil (Elianto oil) was able to reduce number of galls significantly. Areas where galls were plucked and oil applied appeared dry and galls did not regenerate. This was an indication that oil worked generally on suffocation.

5.2 CONCLUSIONS

- i. The study showed that crown gall is still one of the important diseases often limiting nursery and greenhouse production of rose flowers in Kenya. Agrobacteria causing this disease are soil-borne pathogens commonly occurring in the soils and other natural environments. The study established that crown gall is influenced by susceptible host, cultural practices, virulence pathogen and an environment stress. Increased moisture enhances the rise of pathogen infection and also some pests like nematodes, caterpillar and mites that initiate wounds in plants acting as entry point of the pathogens.
- ii. The study confirmed that use of naturally occurring *A. radiobacter* isolates had the potential in the management of crown gall diseases of rose flower stocks in Kenya.
- iii. Use of Copper oxychloride (Curavit), Copper hydroxide (Kocide) and Elianto oil showed that there was a temporal management of galls since there was regeneration of galls afterwards.

5.3 RECOMMENDATIONS

- i. More elaborate studies should be carried out to formulate *A. radiobacter* as a commercial biopesticide and apply it on a larger scale and also establish its ability for root colonization and survival in the rhizosphere.
- ii. A study should be conducted on different strains of *A. radiobacter* occurring in the soil and characterize them.
- iii. The study recommends the use of *A. radiobacter*, corn oil and copper based bactericides using different concentrations in flower farms.

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APPENDICES

APPENDIX I: Experimental layout for greenhouse experiment

Randomized complete block design

Four treatments (Factor I)

A- Rose plant + *Agrobacterium tumefaciens*

B- Rose plant + *Agrobacterium radiobacter*

C- Rose plant + *Agrobacterium radiobacter* + *Agrobacterium tumefaciens*

D- Rose plant alone

Four varieties of rose plants- V₁, V₂, V₃ and V₄ (Factor II)

Replicates 4

V1	V2	V3	V4		V1	V2	V3	V4		V1	V	V3	V4		V1	V2	V3	V4
										2								
A	B	C	D		D	A	B	C		D	A	C	B		B	C	A	D
B	A	D	C		C	B	A	D		C	B	D	A		D	B	C	A
C	D	A	B		B	D	C	A		B	D	A	C		A	D	B	C
D	C	B	A		A	C	D	B		A	C	B	D		C	A	D	B
BLOCK 1					BLOCK 2					BLOCK 3					BLOCK 4			

Green houses where experiments were conducted.

Variety	Greenhouse
V ₁	9
V ₂	10
V ₃	17
V ₄	1

Treatments (Randomized complete block design)

T₁. Rose plant + copper hydroxide (Kocide 2000)-3 g/l

T₂. Rose plant + copper hydroxide (Kocide 2000)-2 g/l

T₃. Rose plant + copper oxychloride (Curavit)-3 g/l

T₄. Rose plant + copper oxychloride (Curavit)-2 g/l

T₅- Rose plant + Elianto oil

T₆- Rose plant alone

Replicates 4

Variety 1

Layout of the design

T1	T2	T3	T4	T5	T6		T4	T4	T2	T5	T3	T1
T2	T4	T1	T3	T6	T5		T3	T5	T6	T2	T1	T4
T3	T5	T6	T2	T1	T4		T2	T4	T1	T3	T6	T5
T4	T6	T2	T5	T3	T1		T1	T2	T3	T4	T5	T6

BLOCK 1**BLOCK 2**

T2	T1	T1	T6	T4	T5		T6	T5	T4	T3	T2	T1
T1	T2	T3	T4	T5	T6		T5	T4	T6	T1	T3	T2
T3	T5	T6	T2	T1	T4		T3	T1	T2	T4	T1	T3
T4	T6	T2	T5	T3	T1		T1	T3	T5	T2	T6	T4

BLOCK 3**BLOCK 4****APPENDIX II: Questionnaire for production managers**

I am post graduate student at Kenyatta University carrying out a study on management of crown gall disease of roses caused by *Agrobacterium tumefaciens* using locally available isolates of *Agrobacterium radiobacter*, corn oil and copper hydroxide and copper oxychloride in selected flower farms in Kenya.

You are kindly requested to respond to all the questions with honesty. All information given will be treated with confidentiality. Your cooperation will be highly appreciated.

LOCATION OF THE FARM

COUNTY

DISTRICT

LOCATION

SUB-LOCATION

PART A: General information

1. Please mark the box that describes you.

Male

Female

2. Please mark the time you have served as production manager.

Less than 1 year

1-3 years

4-6 years

7 or more years

PART B: Occurrence of crown gall on rose

3. Are you aware of crown gall disease?

1 = Yes 2 = No

If yes, how do you identify the disease from the rest of the disease?

4. Factors that contribute to the presence of crown gall disease. (Tick where applicable)

Age of the plant	
Poor crop management	
Farm practices	
Root stock type	
Infested soil	
Presence of pests	
Climate	

5. Size of the farm (Acreage) affected by crown gall disease. (Tick where applicable)

Below 3 acres	
4-6 acres	
7 and above	

6. How is the distribution of the crown gall disease on the rose farm?

1= Widely distributed

2= Spatial distribution

3= Limited distribution

7. What are the sources of types of planting materials used on the farm? (Tick where applicable)

1= Imported

2= From local commercial propagators

3= Own cutting.

8. List some of the rose varieties used on the farm that susceptible and resistant to crown gall disease. Name the varieties in terms of priority: 1.....
2..... 3.....

PART C: Flower production

9. How frequent are the cases of low production in roses due to the presence of crown gall disease. (Tick where applicable)

Very often	
Often	
Don't know	
Not often	
Very rare	

10. Other major constraints in flower production apart from crown gall disease. (Tick where applicable)

Market demand	
Pests and other diseases	
High cost of inputs	
Scarcity of preferred rose varieties	
Soil infertility	
Water scarcity	

PART D: Management of crown gall disease

11. How do you manage the crown gall disease on your farm? (Tick where applicable)

Chemical control	
Sanitation	
Crop rotation	
Plucking of galls	
Biological control	
Removal of whole crop	
Crop rotation	

12. Factors that hinder in the management of crown gall disease (tick where applicable)

Lack of information on proper control methods	
Lack of effective chemicals	
Inadequate resistant varieties	
Purchase of infected rose stock from the propagators	

THANK YOU FOR YOUR COOPERATION.

APPENDIX III: Media for isolating, culturing and maintenance *Agrobacterium* isolates.**YEMA supplemented with Congo red.**

Mannitol 10.0g

MgSO₄.7H₂O 0.1g

K₂HPO₄ 0.5g

NaOCl 0.2g

Yeast extracts 0.5g

Agar 15.0g

Congo red 0.01g

Adjust to 1L, PH 6.8

YEP (Yeast extract peptone agar)

Glucose 10.0g

Peptone 5.0g

Yeast extracts 5.0g

Agar 15.0g

Adjust to 1L, PH 5.0-6.0

***In vitro* antagonistic media**

Luria-Bertani (LB)

Tryptone 10.0g

Yeast extracts 5.0g

NaCl 10.0g

Agar 10.0g

Adjust to 1L, PH of 7.0 and autoclave at 121⁰c for 15min.

CHARACTERIZATION MEDIA

Biochemical test

Potato Dextrose Agar

Potato infusion 200.0g

Glucose 200.0g

Agar 150.0g

Distilled H₂O 1L

3-ketolactose test

Yeast extracts 10.0g

Lactose 10.0g

Agar 15.0g

Adjust to 1l with H₂O

Lactose Agar

Yeast extracts 1.0g

Lactose 10.0g

Agar 15.0g

Distilled water 1L

Benedict's solution

Solution I

Mix, 17.3g sodium citrate, 10.0g sodium carbonate, 70ml hot distilled water.

Solution II

1.73g/10ml water, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$. Cool solution I, add 10ml solution II bring up to 100ml with deionized water.

Catalase test

Use of 3% Hydrogen peroxide.

Oxidase test

Tetramethyl-para-phenylenediamine hydrochloride 0.5g

Distilled water 50ml.

Urease test

L-tryptophan 3g

Urea 20g

KH_2PO_4 1g

K_2HPO_4 1g

NaCl 5g

Ethanol 95% 10ml

Phenol red 25mg

Adjust to pH 11 with H_2O

Antibiotic resistant test

Minocycline 30 mcg

Penicillin 1.0 mcg

Erythromycin 15 mcg

Methicillin 5 mcg

Cotrimoxazole 25 mcg

Chloramphenicol 30 mcg

Amplicin 10 mcg

Lincomycin 2 mcg