MANAGEMENT OF SOIL-BORNE BACTERIAL WILT, *Ralstonia solanacearum*, THROUGH ESSENTIAL OIL PLANT EXTRACTS AND CHOICE OF ROTATION CROPS

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DECLARATION AND RECOMMENDATION

DECLARATION

This thesis is my original work and has not been submitted or presented for examination in any other university either in part or as whole.

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Signature…… Date…12/02/2015

RECOMMENDATION

This thesis has been submitted for examination with our recommendation and approval as university supervisors.

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ABSTRACT

Bacterial wilt disease is a major constraint to production of crops in solanaceae family that include potatoes (*Solanum tuberosum)*, which is a major food crop in Kenya. Control of bacterial wilt in areas conducive to disease development is generally very difficult as there are no effective curative chemicals. It is for this reason that this study was undertaken to investigate the potential of essential oils for use in control of bacterial wilt. The study also searched for suitable non-host crops for use in rotation with host crops in managing the *R. solanacearum* pathogen *solanacearum* under various temperature conditions in greenhouse conditions. The effects of each extract on the growth of *R. solanacearum* were determined using a modified Kirby-Bauer disc diffusion method that included incubation of pathogen inoculated plates at different temperatures and measurement of the size of inhibition zones. Investigations on the effect of host and non-host plants on survival and rhizosphere infestation by *R. solanacearum* were also conducted. Analysis of variance test was used to compare differences between variables and Fisher's protected least significant difference test used to separate the means. Essential oils from *Ocimum suave, Tarchonanthus camphorates* and *Lippie javanica* were found to possess strong and significant antibacterial activity against *R. solanacearum* at $p < 0.05$. *T. camphorates* reduced the wilting incidences by 93% confirming to have strong antimicrobial property. There was resistance observed when using synthesized antibiotics like chloramphenicol while the essential oils like *O. suave, T. camphorates* and *L. javanica* had no such resistance. The best temperature to apply the essential oils was confirmed to be between 24°C to 28°C . *T. camphorates* reduced wilt incidence on potato plants by more than 90%. Of all the host and non-host plants used in this study, cabbage was the only crop considered safe for crop rotation. Maize harboured asymptomatic survival of the *R. solanacearum* in the vascular bundles and other tissues while beans and sorghum encouraged the survival of *R. solanacearum* in the rhizosphere and roots. It can further be concluded that essential oils extracted from *L. javanica* and *T. camphorates* possess antibacterial activity that is effective in control of *R. solanacearum*. The study recommendes further study on the mode of action and appropriate method of aplication of these essential oils particulary those of *T. comphrates* in the control of *R. solanacearum.*

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LIST OF ABBREVIATIONS AND ACRONYMS

ASARECA	Association for Strengthening Agricultural Research in Eastern and Central Africa
Bv	Biovar
BW	Bacterial wilt
CABI	Commonwealth Agricultural Bureau /CAB International
CFUs	Colony-forming units
DAP	Diammonium phosphate
ELISA	Enzyme-linked immuno-sorbent assay
EPS 1	Exopolysaccharide one
FLSD	Fisher's least significant difference
IDM	Integrated disease management
IPM	Integrated pest management
LT	*Lantana trifolia*
LPJ	*Lippie javanica*
LPU	*Lippie ukambensis*
NARL	National Agricultural Research Laboratories
NCM	Nitrocellulose membrane
OC	*Ocimum suave*
PCR	Polymerase chain reaction
R3bv2	Race 3 Biovar 2
RM	*Rosmarinus officinalis*
SMSA	Semi-selective medium
TAC	*Tarchonanthus camphoratus*
TZC	Tetrazolium chloride

CHAPTER ONE
INTRODUCTION

1.1 Background information

One of the major challenging diseases of crops in the Solanaceae family is bacterial wilt (BW) caused by *Ralstonia solanacearum* (Yabuuchi *et al.*, 1995) formerly called *Pseudomonas solanacearum.* The bacterium is pathogenic on several other plant species in over 50 plant families and has long been a scourge of tropical agriculture since it is widely distributed with broad host range. Bacterial wilt is very difficult to control making it a major threat to production of solanaceous crops because *R. solanacearum* is a soil and seed-borne pathogen in potatoes with limited host resistance (Schaad *et al.*, 2001).

The main virulence factor of this bacterial pathogen is exopolysaccharide one (EPS 1) that cause wilt by vascular occlusion or rupture of vessel elements due to excess hydrostatic pressure (Schell, 2000). After invading a susceptible host, *R. solanacearum* multiplies and moves systematically within the plant before bacterial wilt symptoms occur (Elphinstone, 2005). When the pathogen gets into the xylem through natural openings or wounds, tyloses may form to block the axial migration of bacteria within the plant. In susceptible plants, this sometimes happens slowly and infrequently to prevent pathogen migration, and may instead lead to vascular dysfunction by unspecifically obstructing uncolonised vessels. Wilting is due to vascular dysfunction that prevents water from reaching the leaves. *Ralstonia's* systemic toxin also causes loss of stomatal control but there is no evidence for excessive transpiration as its consequence. The primary factor contributing to wilting is probably blocking of pit membranes in the petioles and leaves by the high molecular mass of EPS1 (Elphinstone, 2005).

Three races are more common, with race 1 having a broad host range, race 2 infecting bananas, and race 3 infecting primarily solanaceous crops. The disease is generally called 'bacterial wilt', but in potato it is also called 'brown rot' (Elphinstone, 2005). Bacterial wilt causes economic problems for about three million farming families in 80 countries leading to a devastating annual loss exceeding US $ 950 million (Elphinstone, 2005).

In Kenya, the pathogen has been reported at both low and higher elevations (Nyangeri *et al.*, 1984). Yield losses caused by bacterial wilt are estimated at 50 - 100 % in traditional potato production areas in Kenya (Ajanga, 1993). Control of bacterial wilt in areas conducive to the disease is generally very difficult as there are no curative chemicals with breeding for resistance

being only successful against race 1 of the pathogen in tropical crops like eggplant, tomato, peanut, pepper and to a very small extent potato in South America (van Elsas *et al.*, 2005). Cultural measures of diseases control such as the use of healthy seeds, a wide crop rotation, and use of certain rotation crops such as corn or rice, rouging and burning of diseased plants, and careful water management also have had only limited success (Lopez and Biosca, 2004). Suppression of soil towards bacterial wilt may be related to other factors than soil type *per se*, such as pH, organic matter content and microbial communities (van Elsas *et al.*, 2005).

Another approach in disease management is to explore the potentials of botanical extracts for their anti-microbial effects on pathogens. Essential oils contain more than 200 compounds, consisting of volatile compounds that include terpenoids, aliphatic aldehydes, alcohols and esters (Dewick, 1997). Essential oils are usually obtained by *steam distillation* or hydrodistillation (Whish, 1996). Some volatile compounds derived from plants, such as thymol (*Thymus* sp.) and palmarosa (*Cymbopogon martinii*) or other medicinal plants are antibacterial and have been widely used as general antiseptic, additive in cosmetics, food industry, or in the medical practice. Preliminary *in-vitro* as well as greenhouse experiments conducted with several plant essential oils with their components showed that some essential oils have significant efficacy against *R. solanacearum* and several soil-borne fungi of tomato (Pradhanang *et al.*, 2003).

In agricultural studies, these compounds have broad-spectrum activities against nematodes, and insects but the potential of using plant essential oils for managing bacterial plant diseases has not been adequately addressed despite the above finding that plant essential oils can manage bacterial wilt of tomato (Momol *et al.*, 2005).

1.2 Statement of the problem

Despite the increasing importance of solanaceous crops as source of food, employment and income in developing countries, bacterial wilt of these crops still remains a serious threat to their production. The main problem is in the control and management of bacterial wilt since there is no effective chemical for control as well as management of this disease. Furthermore, cultural measures have had limited success. This study was designed to explore additional strategies to complement the existing ones by using plant essential oils and enrichment of existing knowledge on the use of rotational crops for management of bacterial wilt disease.

1.3 Objectives

1.3.1General objective

To develop a sustainable strategy of managing bacterial wilt

1.3.2 Specific objectives

1) To determine the effect of some selected essential oils on growth of *R. solanacearum* under various temperature regimes.
2) To determine the effect of selected essential oil plants on bacterial wilt disease development in potato plants under greenhouse conditions.
3) To find out the effect of host and non-host plants on survival and rhizosphere infestation by *R. solanacearum*.

1.4 Justification

The most commonly used method for control of bacterial wilt is integrated disease management (IDM). This includes use of cultural measures such as the use of healthy seeds, a wide crop rotation, and use of certain rotation crops such as maize and beans, rouging, burning of diseased plants, as well as careful water management, with environmental conditions influencing the success and incidence of the disease. From the existing and available control measures, those that are feasible, suitable and effective are selected for a given location. Lack of appropriate control and economically friendly ways of managing the disease increases the cost of production in potatoes thus discouraging farmers.

Essential oils and related products from medicinal plants have been recognized as having antimicrobial effects on various plant pathogens but their efficacy as a biofumigant on *R. solanacearum* has not been thoroughly studied. They are considered to play a role in host plant defense mechanisms against diseases and can therefore be effective in efficient management of bacterial wilt disease. Crop rotation as a component of IDM has failed in most cases probably because of the length of survival of the pathogen in the soil or contribution of the most often chosen non-host plants in rhizosphere infestation. Cheaper and less time consuming ways of managing the disease is therefore necessary for increased production.Correct choice of rotational crops and those that cannot encourage survival of *R. solanacearum,* will reduce survival of this pathogen in the soil when host plants are not available.

CHAPTER TWO
LITERATURE REVIEW

2.1 Bacterial wilt pathogen

R. solanacearum is the causal agent of potato brown rot or bacterial wilt disease. The bacterium affects many plant species with some of the most susceptible being potato, tomato, eggplant, pepper, banana and weeds in solanaceae family. The bacterium has a world-wide distribution with infection spread through latently infected plant materials (Mwangi *et al.*, 2008). The pathogen can persist for a long time in soil, host plant debris, potato volunteer plants, alternative hosts or even non-host plants. It can survive for periods between 12 months to 3 years in the absence of a potato crop (Graham *et al.*, 1979).

2.1.1 Taxonomic position

In a taxonomic study of certain non-fluorescent species of the genus *Pseudomonas* (Yabuuchi *et al.*, 1992), the genus *Burkholderia* was proposed to encompass the variation found in this group and the name *Burkholderia solanacearum* was proposed. Subsequent study of this genus revealed that *R. solanacearum* was sufficiently distinct from other members of the genus to warrant assignment to the newly proposed genus *Ralstonia* (Yabuuchi *et al.*, 1995). Nucleic-acid based identification using *R. solanacearum* specific primers and pathogenicity assessment using susceptible hosts (e.g. tomato seedlings) and several rapid screening tests, such as immunostrips are available commercially for rapid field detection of *R. solanacearum* (Schaad *et al.*, 2001). According to the current classification system, the causative agent of bacterial wilt belongs to the Kingdom Procaryotae, Division Gracilicutes, Class Neisseriae, Order Burkholderiales, Family Burkholderiaceae, Genus *Ralstonia* and Species *solanacearum* (Agrios, 2005).

Two classification criteria are used for identification and differentiation of *R. solanacearum* at intraspecific level: the race and the biovar systems. The biovar system consists of biochemical tests based on the ability of the bacterium to utilize three disaccharides or oxidize three hexose alcohols (Table 1) (Schaad *et al.*, 2001). The standard biovar test uses bromothymol blue as a pH indicator in 15 ml culture tubes containing 3 to 5 ml of test media, and takes weeks to complete at 24 or 28 °C. The race system is based on host range in which under field conditions four races can be distinguished as shown in Table 2 (Schaad *et al.*, 2001).

Table 1: Classification of *R. solanacearum* into biovars

Physiological tests	Biovars 1	2	3	4	5
Utilization of disaccharides					
Cellobiose	-	+	+	-	+
Lactose	-	+	+	-	+
Maltose	-	+	+	-	+
Oxidation of alcohols					
Dulcitol	-	-	+	+	-
Mannitol	-	-	+	+	+
Sorbitol	-	-	+	+	-

+ = positive, - = negative
(Source: Schaad *et al.*, 2001)

Table 2: Differentiation of *R. solanacearum* races based on host range

Race	Natural host	Biovar
1	Many solanaceae, some diploid bananas, Numerous other crops and weeds in many families	1, 3 or 4
2	Triploid bananas, certain heliconians	1 or 3
3	Potato, tomato, and rarely, a few other hosts	2
4	Mulberry	5

(Source: Schaad *et al.*, 2001)

At the sub-species level, identification of strains of *R. solanacearum* can be assessed with several nucleic acid-based methods such as DNA probe hybridization which uses the ability of two complementary single-stranded nucleic acids to combine into a single molecule. Nucleotide probe of known sequence are used to bind complementary strands of undetermined organism for identification. DNA probe hybridization and especially the polymerase chain reaction is a technique that consists of amplifying a DNA molecule exponentially using polymerase enzyme with specific probes and primers (Schaad *et al.*, 2001).

2.1.2 Hosts of the pathogen

Ralstonia solanacearum is a bacterial pathogen that causes several diseases on a wide range of plants. The primary hosts of *R. solanacearum* include: *Solanum tuberosum* (potato) and *Solanum lycopersicon* (tomato), eggplants, pepper, solanaceous weeds and bananas. Some of the host plants are shown in the Table 3.

Table 3: Common hosts of *R. solanacearum*

Common Name (Cultivated Hosts)	Scientific Name
Geranium	*Pelargonium* spp.
Tomato	*Solanum lycopersicon*
Peppers	*Capsicum spp.*
Eggplant	*Solanum melongena*
Potato	*Solanum tuberosum*
Beet	*Beta vulgaris*
Tobacco	*Nicotiana* spp
Black nightshade	*Solanum nigrum*
Groundnut	*Arachis hypogaea*
Banana	*Musa paradisiaca*
Plantain	*Heliconia* spp

(Source : USDA, 2003; Nyangeri, 2011)

2.1.3 Biology of *R. solanacearum*

Rastonia solanacearum is believed to have originated in the temperate highland regions of Peru and Bolivia. *R. solanacearum* race 3 biovar 2 is adapted to lower temperatures than what is found for other *R. solanacearum* races (van der Wolf and Perombelon, 1997). It is a strictly aerobic, gram negative, non-spore forming, and non capsulated, nitrate-reducing, ammonia-forming, rod-shaped bacterium. On tetrazolium chloride (TZC) medium it forms irregular shaped, fluidal, white with pink centered colonies. The pathogen normally invades hosts through their roots and then systemically colonizes aerial tissues (Schaad *et al.*, 2001). The main virulence factor of *R. solanacearum* is exopolysaccharide one (EPS 1), a long polymer with a trimeric repeat unit of N-acetyl galactosamine, 2-N-acetyl-2-deoxy-L-alacturonic acid, and 2-N-acetyl-4-N-(3-hydroxybutanoyl)- 2-4-6-trideoxy-D-glucose. On various laboratory media and in plants, *R. solanacearum* produces massive amounts of EPS 1 accounting for 90% of its total exopolysaccharide.

Studies using EPS 1 specific monoclonal antibodies show that many diverse *R. solanacearum* isolates produce EPS 1 (or a very similar polymer) and that 85% of the EPS 1 exists as a released, cell-free slime; however, 15% remains in the cell surface. Mutants of *R. solanacearum* specifically blocked in synthesis of only EPS 1 rarely wilt or kill plants, even when large numbers of cells are directly injected into the stem (Schell, 2000). Studies with plants inoculated by soil infestation showed that EPS 1 also promotes stem colonization, because EPS 1 deficient mutants colonized stems much more slowly and to much lesser extent than wild types (Schell, 2000).

2.1.4 Disease symptoms and diagnosis

Leaves of infected plants become wilted during the day but recover (regain turgidity) during the night time (Smith *et al.*, 1997). Leaves may develop a bronze cast and petioles may develop epinasty. Plants may become stunted and chlorotic. In the advanced stages of the disease, the lower stem has a streaked brown appearance and the vascular ring is stained brown. If bacterial populations are high enough, a white mass of bacteria ooze from a cut made in a symptomatic stem and bacterial ooze may collect in the tuber eyes of potato and soil may stick to secretions (Smith *et al.*, 1997). Eventually, plants fail to recover and die. In potatoes, when diagnosis is done on cross sections of the stem, they reveal brown discoloration of the vascular system (Stevenson *et al.*, 2001).

The bacterial wilt agent can be isolated from a diseased tuber or stem by plating the bacterial tuber exudates or two drops of the suspension obtained in the vascular flow test on modified Kelman's medium (tetrazolium chloride containing only 2.5 g dextrose) (Stevenson *et al.*, 2001). After 48 hours incubation at 30°C, the typically fluid, slightly red-tinted colonies of virulent *R. solanacearum* are easily distinguished from other saprophytic bacteria which occur as round-shaped, uniformly dark red colonies (Stevenson *et al.*, 2001). Tubers can be screened by slicing them and looking for ooze, or if not immediately evident, incubate tubers for 3-4 weeks at 30°C and observe for ooze from eyes. This method is time consuming and may not show low infection rates. The above procedures only confirm the presence of bacteria, but do not provide information on the exact genus/species nor biovar present. Other techniques that do provide genus/species and depending on the assay, race and biovar information include: semi-selective media; immuno-fluoresence staining (IF); enzyme- linked immuno-sorbent assay (ELISA) (Ozakman and Schaad, 2002).

2.1.5 Disease cycle

A complex life cycle of *R. solanacearum* is described by Janse (1996). The source of inoculum is infected potatoes (seed tubers, harvest leftover and infected plants), infested soil, or both (Figure 1).

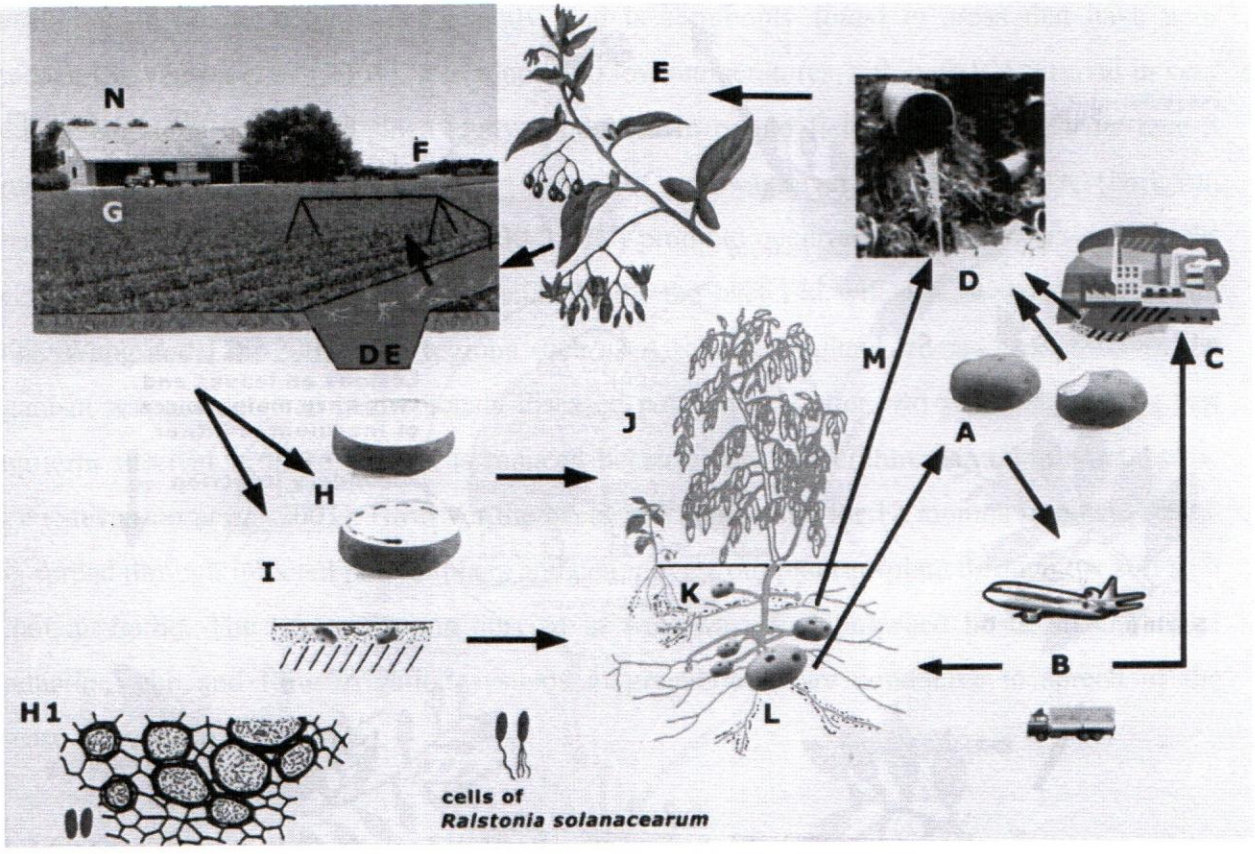


Figure 1: Life cycle of *R. solanacearum* race 3, biovar 2 in association with potato (Janse, 1996). (A) Seed potato as a primary source, (B, C, D) transport via airplane, truck or processing industry, and water to the plants in the field, (DE) contaminated surface water, (E) latently infected bittersweet plants (*Solanum dulcamara*), (F) irrigation water, (G) contaminated machinery, (H, I) infected potato tubers, (H1) vascular tissue infected, (J) wilted potato plant, (K) latently infected daughter tubers which contaminate the soil, (L) infected rhizosphere, (M) transmission to surface water and eventually to bittersweet, (N) seed storage sheds, grading belts, packing materials and machines contaminated by infected tubers.

2.1.6 Epidemiology

R. solanacearum race 3 biovar 2 is a soil-borne pathogen that persists in wet soils, deep soil layers (more than 75 cm), and reservoir plants (van der Wolf and Perombelon, 1997). Its distribution in potato fields can be spotty, and is commonly found in areas that have poor drainage (Stevenson *et al.*, 2001). It is adapted to low temperatures, although it's survival in very cold temperatures is reduced. In a study conducted in potato fields, *R. solanacearum* race 3 biovar 2 population densities declined at 15 and 20°C and was severely reduced at 4°C (Dirk van Elsas *et al.*, 2000). High temperatures (30-35 °C) promote occurrence of this disease, whereas soil temperatures below 20 °C are not suitable for the bacterial wilt disease (Gadewar *et al.*, 1999; Wang and Lin, 2005a). In regions such as Australia, England, Kenya, and Sweden the organism was not detected in previously diseased potato fields after two years, suggesting that long-term survival in these regions is reduced because soil temperature sometime goes below 20°c (Stevenson *et al.*, 2001). However the bacterium can persist for 12 months in potato fields. It is spread through infected potato tubers and can move from plant-to-plant through the soil as it is not air-borne. The bacterium can survive in waterways and on weed hosts. In Geranium production, ebb and flow irrigation systems in greenhouses are conducive to spread of the disease (Stevenson *et al.*, 2001).

2.1.7 Geographical distribution of the pathogen

Biovars 3, 4, and 5 are widely distributed in Asia and Australasia and unknown in most parts of the America while biovar 3 also occurs in northern Brazil, parts of Central America, the West Indies and southern Africa (Akiew *et al.*, 1990). *R. solanacearum* race 3 biovar 2 causing brown rot of potatoes is thought to have evolved on potatoes in the Andes of Bolivia as well as Peru and to have spread worldwide in latently infected potato tubers (Janse, 1996). The noticeable differences in the geographic distribution of biovars suggest separate evolutionary origin. In general, biovar 1 is predominant in the Americas and biovar 3 in Asia. By contrast, biovar 1 is absent from most parts of Asia, and until its introduction on *Heliconia* (Akiew *et al.*, 1990), biovar 1 has never been found in Australia. Brown rot, race 3 biovar 2 has been found throughout central and southern Africa, and results in serious constraints to potato production in Uganda, Ethiopia, Kenya, Madagascar, Rwanda, Burundi, Nigeria and Cameroon (Janse, 1996).

2.1.8 Survival of *R. solanacearum*

The pathogen can be introduced into an area by planting an infected host crop like potato tubers, where it can be disseminated; it can also survive in contaminated surface water and weed hosts (Akiew *et al.*, 1990). Volunteer plants or weeds can be a reservoir, and responsible for transmission of the pathogen through successive seasons. The pathogen can persist for a long time in soil, in infected host plant debris or by colonizing potato volunteer plants, alternative hosts or even non-host plants.

The *R. solanacearum* can survive in soil or infected plant debris for prolonged periods (Buddenhagen, 1986; Grey and Steck, 2001). Many asymptomatic weeds harbor the bacteria in their roots (Wang and Lin, 2005b). The disease can prevail in different soil types (Granada and Sequeira, 1983). According to some reports, potato brown rot in Europe is mainly spread through irrigation with contaminated water (Carusu *et al.*, 2005; Danial *et al.*, 2006). This bacterium may also survive by colonizing the rhizospheres of non-host plants (Wenneker *et al.*, 1999). High temperatures (30-35 °C) promote occurrence of this disease, whereas soil temperatures below 20 °C are not suitable for the bacterial wilt disease (Wang and Lin, 2005a).

2.2 Control of bacterial wilt disease

Survival of *R. solanacearum* was found to be affected by soil type (texture and organic matter), soil temperature and moisture content (Grey and Steck, 2001). Despite reports on suppression of various pathogens in organically managed soils, it was found that *R. solanacearum* survived least in loamy soil with relatively high organic matter content (4%) whilst survival was highest in soil with lower organic matter content of 2.0-2.5% (van Elsas *et al.*, 2005). Yet, it has been stated that efficient soil management would be related to composition and/or activity of the soil microorganisms by enhancing natural biological control capacity (van Elsas *et al.*, 2005). Soil amendment with organic materials or NPK fertilizers or with different combination of these organic and inorganic amendments significantly affected bacterial wilt incidence and increased potato yields (Smith *et al.*, 1997).

Altering soil pH depending on the time of year may also be effective. For potatoes, lowering the pH to 4-5 in the summer and raising it to 6 in autumn helped to eradicate the pathogen (Smith *et al.*, 1997). Apparently resistant plants are often latently infected with various strains of *R. solanacearum* which are additional means of dissemination of the pathogen and, as such, to control and eradicate bacterial wilt, integrated disease management (IDM) is employed.

The main components of IDM are the use of healthy seeds and planting in clean soils. However, many additional factors influence the incidence of the bacterium, such as environmental conditions (temperature and soil moisture), rotation with non-host plants, the use of less susceptible varieties and cultural practices (Stevenson *et al.*, 2001). In East Africa, *R. solanacearum* can be very destructive at the lower altitude in Kenya. Since the pathogen is transmitted through tuber seed into the soil, availability of "clean" seed and adequate rotations are the most effective control measures (Lutaladio *et al.*, 1995).

Potato Research Centre has initiated several breeding programs with special emphasis to resistance against latent infection (Priou and Alley, 1999; Priou and Gutarra, 2005; Priou *et al.*, 2006). Tremendous research efforts have been carried out to identify resistance sources against tomato bacterial wilt (Grimannet *et al.*, 1995). Molecular markers for identification of bacterial wilt resistance have been developed. Using tomato variety Hawaii 7996 major Quantitative trait loci (QTL) were found on chromosome number 12 (Wang and Lin, 2005).

2.3 Potential of plant extracts in the control of plant diseases

There has been constant and increasing alternative search on efficient compounds for plant disease control, aiming at partial or total replacement of antimicrobial chemicals (Simões *et al.*, 1999). Systematic investigation of biological interactions between microorganisms and plant products has been a valuable source of new and effective antimicrobial substances, which could act differently on or in the microbial cell compared to other conventional antimicrobials.

Plants synthesise secondary metabolites and some of them as well as their derivatives have antimicrobial effect. Among these secondary metabolites are found alkaloids, flavonoids, isoflavonoids, tanins, cumarins, glycosides, terpens and phenolic compounds (Simões *et al.*, 1999). In agricultural studies, these compounds have broad-spectrum activities against fungi, nematodes, and insects (Lee *et al.*, 1997; Wilson *et al.*, 1997 and Calvet, 2001). Spices offer a promising alternative for food safety and plant protection due to their antimicrobial effect. Inhibitory activity of spices and their derivatives on the growth of bacteria, yeasts, fungi and microbial toxin synthesis has been reported (Notermans and Hoogenboon-Verdegaal, 1992; Sagdiç *et al.*, 2003). Studies on plant extracts commonly used by Aborigines found out that approximately 20% of the samples tested were able to inhibit bacterial growth (Semple *et al.*, 1998; Palombo and Semple, 2001). Some plant extracts have been shown to possess growth inhibitory effects towards plant pathogenic bacteria (Pradhanang *et al.*, 2003).

2.3.1 Essential oils

Essential oils which are part of secondary metabolites are usually obtained by steam distillation or hydrodistillation (Whish, 1996). In steam distillation, the steam is passed through plant material that is placed on a mesh between steam inlet and condenser (Whish, 1996). Steam distillation makes use of the principle that two immiscible liquids, when mixed, each exert a vapour. The total vapour pressure of the boiling mixture is therefore equal to the sum of the partial pressures exerted by each of the individual components of the mixture. When the total vapour pressure reaches atmospheric pressure, the mixture starts to boil. This implies that the boiling point of the mixture is reached at a lower temperature than the boiling points of the individual components. Steam distillation is therefore able to separate volatile from nonvolatile components with a reduction in boiling point, thereby avoiding extreme temperatures. An added advantage is the displacement of atmospheric oxygen by the steam, which protects compounds from oxidation (Krell, 1982).

However, hydrolysable compounds such as esters, as well as thermally labile components, may be decomposed during the distillation process (Houghton and Raman, 1998). In addition, partial loss of more polar constituents of the oil, due to their affinity for water, may also occur (Masango, 2004). In hydrodistillation procedures, the material is immersed in water, which is heated to boiling point using an external heat source. In both hydro- and steam distillation techniques, the vapours are allowed to condense and the oil is then separated from the aqueous phase. Care must be taken to ensure efficient condensation of steam, thereby preventing the loss of the more volatile oil components (Houghton and Raman, 1998). It has been suggested that that steam distillation is more efficient than hydrodistillation in removing oil from plant material (Charles and Simon, 1990).

2.3.2 Use of essential oil in control of bacterial wilt disease

Many plant species produce volatile essential oil compounds. Essential oils are aromatic oily liquids obtained from plant materials (flowers, buds, seeds, leaves, twigs, bark, herbs, wood, fruits and roots). They can be obtained by expression, fermentation or extraction but the method of steam distillation is most commonly used for commercial production (McMurry, 1996). Essential oils have been used for thousands of years as medicines, flavourants and perfumes. In modern times, they are widely used in pharmaceutical products, cosmetics, as well as in the food and human nutrition field (Luque de Castro *et al.*, 1999).

These oils are considered to play a role in host defense mechanisms against plant pathogens. Essential oils have been shown to possess antibacterial, antifungal, antiviral, insecticidal and antioxidant properties. Thousands of terpenoid components of essential oils have been identified from natural sources (Dewick, 1997). A study conducted by Ooshiro *et al.* (2004) found out that incorporation of dried young aerial leaves of some essential oil plants like *Geranium carolinianum* in the soil effectively counteract the microbial activities of *R. solanacearum* in the soil. Preliminary *in-vitro* as well as greenhouse experiments conducted with several plant essential oils with their components showed that some essential oils have significant efficacy against *R. solanacearum* and against several soil-borne fungi of tomato (Pradhanang *et al.*, 2003).

2.4 Summary

Ralstonia solanacearum is a bacterial pathogen that causes several diseases on a wide range of plants. The primary hosts of *R. solanacearum* include: *Solanum tuberosum* (potato) and *Solanum lycopersicon* (tomato), eggplants, pepper, solanaceous weeds and bananas. Volunteer plants or weeds can be a reservoir, and responsible for transmission of the pathogen through successive seasons. The pathogen can persist for a long time in soil, in infected host plant debris or by colonizing potato volunteer plants, alternative hosts or even non-host plants. These oils are considered to play a role in host defense mechanisms against plant pathogens. Essential oils have been shown to possess antibacterial, antifungal, antiviral, insecticidal and antioxidant properties. Among these secondary metabolites are found alkaloids, flavonoids, isoflavonoids, tanins, cumarins, glycosides, terpens and phenolic compounds.

CHAPTER THREE
MATERIALS AND METHODS

3.1 Collection of essential oil plants

Essential oil plants listed in Table 4 with known antimicrobial activities were used in this study. They were collected from Egerton University Botanic Garden and identified with reference to taxonomic keys (Cowan, 1999).

Table 3: Essential oil plants tested for antimicrobial effects against *R. solanacearum*

Common name	Scientific name	Family name
Rosemary	*Rosmarinus officinalis*	Labiatae
Cambodia	*Ocimum suave*	Labiatae
Camphor brush	*Tarchonanthus camphorates*	Asteraceae
Popcorn lantana	*Lantana trifolia*	Verbenaceae
Sage brush	*Lippie javanica*	Verbenaceae
Augustus lippi	*Lippie ukambensis*	Verbenaceae

All the plants were harvested by cutting the stem above the soil level to collect stems, leaves, flowers and the side branches. Harvesting was done in the mid-morning and packed in plastic sacks (each plant type per sack) that were used to ferry them to Egerton University biotechnology laboratories for extraction. The fresh plant materials were used in the extraction by first cutting them into appropriate sizes that could fit in the still. The fresh plant materials were weighed to determine the weight of the plant used for every distillation. All the essential oil plants used are as shown in the photographs in Plate 1.

3.2 Preparation of plant extracts

Essential oil products obtained from different plant species listed in Table 4 and plate one were prepared as per the method of Awuah (1989).

(a)

(b)

(c)

(d)

(e)

(f)

Plate 1: Essential oil plants used in the study (a) *Ocimum suave*;
(b) *Tarchonanthus camphorates*; (c) *Rosmarinus officinalis*; (d) *Lantana trifolia*;
(e) *Lippie javanica*; (f) *Lippie ukambensis.*

A known weight of fresh leaves, stems and flowers was washed in sterilized water and the oils extracted by hydrodistillation (Plate 2). The plant materials were added in bits of 500g and packed tightly in the still without further chopping or cutting. A hydrodistillation procedure was used for the extraction in which the plant materials were immersed in water, which was heated to boiling point using an external heat source. The vapour was allowed to condense and the oil was then separated from the aqueous phase. Care was taken to ensure efficient condensation of steam by passing plenty of cold water in the condenser, thereby preventing the loss of the more volatile oil components as observed by Whish (1996).

Essential oil was skimmed off the surface of the hydrosol by running down the hydrosol below leaving it behind the oil in the separator that allowed the oils to be collected and dried using anhydrous calcium chloride. The oil was then packed in the storing containers that were tightly sealed and wrapped with parafilm. The essential oils were then stored at 4°C until the time of use after having been passed through 0.22µm filters (Millipore) to remove contaminants. To determine the quantity of oil produced, the volume of the essential oil skimmed was measured using a 10.0ml measuring cylinder and recorded. Quantification of essential oils for each plant was important to give an idea on the quantity of plant material required to give sufficient amount of oil for carrying out antimicrobial test.

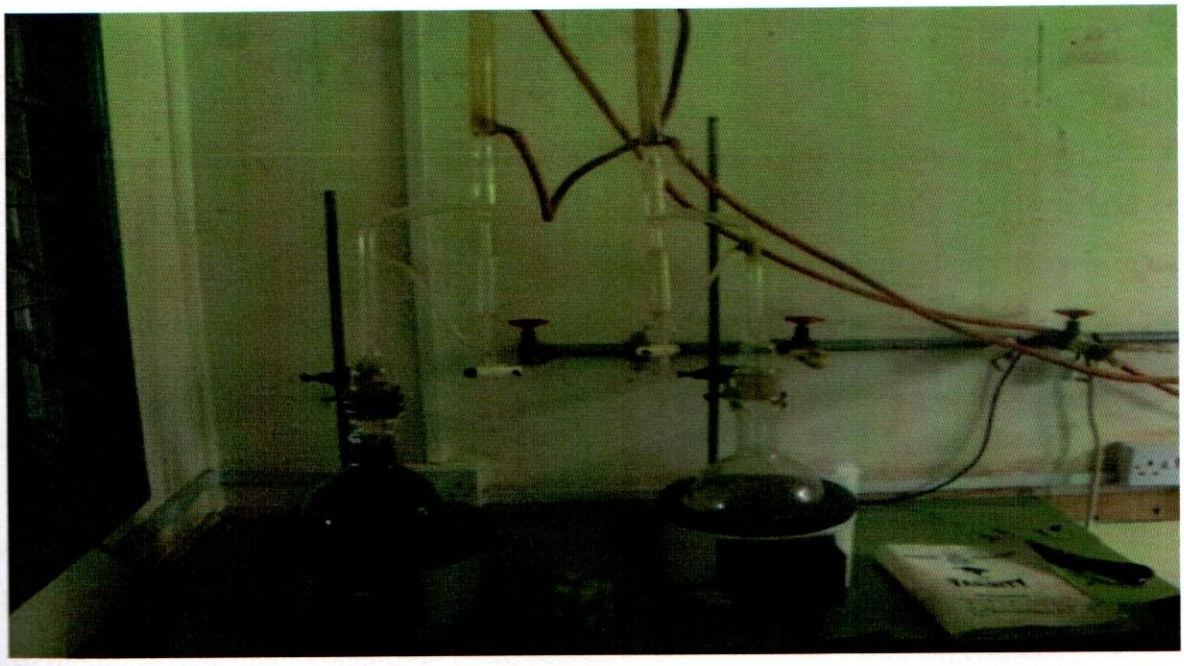


Plate 2: Process used in the extraction of essential oils

3.3 Effects of plant extracts on growth of *R. solanacearum* at different temperatures

The essential oil plant extracts collected were tested to determine their antimicrobial properties by growing *R. solanacearum* in a semi selective media modified in South Africa (SMSA) as adopted by National Agricultural Research Laboratory, Plant Pathology Department (Appendix 5).

3.3.1 Media preparation for growth of *R. solanacearum*

The SMSA test medium was prepared according to method described by Schaad *et al,* (2001). It was prepared in two parts that involved basal medium preparation and addition of antibiotics. The basal medium was prepared in portions of one liter by mixing 1g casamino acid, 10g bactopeptone, 15g bactoagar, 15ml glycerol and sterile distilled water to form a liter of the media followed by heating to boil. Dispensation was then in bits of half litre and autoclaving at 121°C for 15 minutes after which it was cooled to 40°C followed by addition of antibiotics consisting of 5ml of polymixin B, 2.5ml tetracycline salt, 0.25ml bactericin, 0.25ml penicillin, 0.25ml chloramphenicol and 0.25ml crystal violet. The medium was then gently mixed to avoid bubble formation then poured in plates at approximately 20ml per plate then left to settle for 12 hours.

3.3.2 Inoculation of plates and experimental set up

Pure cultures of *R. solanacearum* race 3 biovar 2 were obtained from Plant Pathology Department of National Agricultural Research Laboratories. Antimicrobial activity of each plant extract was determined using the modified Kirby-Bauer disc diffusion method (Schaad *et al.,* 2001). For this, 100µl of *R. solanacearum* suspension was grown in 10ml of fresh modified SMSA liquid media at 28°C up to a concentration of approximately 1.0×10^9cells/ml (Schaad *et al.,* 2001). 100µl of the pathogen suspension was then spread onto nutrient agar plates using sterile glass rod that was improvised by bending it on one end.

The effect of plant extracts on growth was tested using 12 mm diameter sterilized filter paper discs. The discs were impregnated with 20µl of the plant extract in three replicates and, allowed to dry. The discs were then placed on inoculated plates as shown in Plate 3. A standard disc of chloramphenicol containing 20µg was used concurrently in this experiment to serve as a positive control for antimicrobial activity.

Filter discs impregnated with 20µl of extraction solvent (water) were used as negative controls. The plates were allowed to stand at 4°C for 2 hours then incubation with the pathogen at 24°C, 28°C, and 32°C in a completely randomized design (CRD).

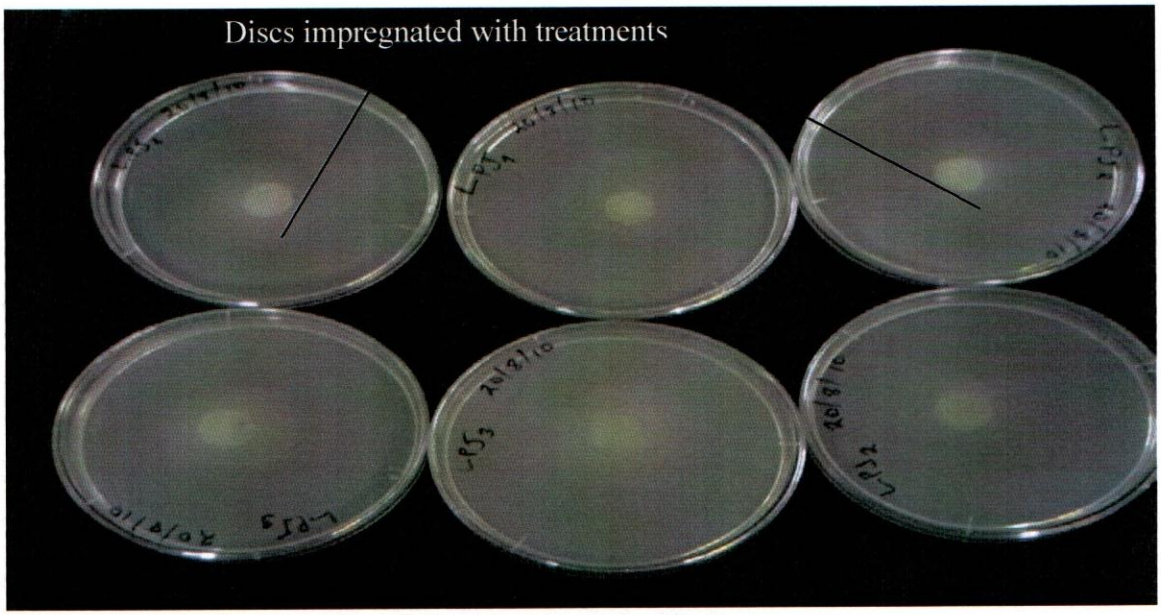


Plate 3: Inoculated plates and discs impregnated with 20µl of plant extracts before incubation

3.3.3 Investigation of antibacterial activity of essential oil plant extracts on *R. solanacearum*

Antibacterial activity was determined by recording the width (in mm) of clear zone (zone of inhibition) surrounding the diffusion discs (Reiner, 1982; Baker *et al.*, 1983; Deans and Ritchie, 1987; and Wagura *et al.*, 2011). These inhibition zones were measured at 24 hours, 48 hours and 72 hours, by measuring only the distance from the end of the disc to the end of the clear zone using ruler placed from the periphery of the disc. Each antimicrobial assay was performed in three replicates. Sensitivity of *R. solanacearum* to the essential oils was determined by enumerating the number of plates of each treatment that exhibited *R. solanacearum* growth at particular temperature interval. This data was used to calculate the percentage of plates with *R. solanacearum* growths and those without. The information obtained was used to describe the sensitivity at different temperatures. After the clear zones had developed, the plates were further observed at 48 hours and 72 hours for any change on growth, availability of other colonies on the zone, its sizes and clarity. The numbers of plates with a specific appearance that included presence of tiny colonies on the clear zone, growth of the peripheral lawn towards the clear zone

and flow of the peripheral fluidal lawn towards the clear zone were recorded and the information used to describe the appearance of the clear zone by calculation of percentages of plates with specific appearances. This was done to establish if any of the treatments could encourage development of resistance by *R. solanacearum* or any other change that may indicate unexpected effect of the essential oils on *R. solanacearum*. Distribution of *R. solanacearum* lawn in the whole plate was also carefully observed taking a special consideration on area around the clear zone. The distribution of the lawn was described as dense around the clear zone, dense in the whole plate, growth towards the zone or as no growth. The numbers of plates with a specific description were recorded and percentages calculated.

The colour of lawns were also noted at different temperatures and time intervals, and described as creamy white in order to note any deviation from the typical creamy white appearance of *R. solanacearum* colonies in SMSA media. This information was presented as percentage of presence or absence of the creamy white growth. The average width of clear zone was calculated for all the treatments at different temperature intervals. This was in order to obtain information about the possible contribution of temperature on the effect of essential oils on *R. solanacearum*. With this information available, it was know the most effective temperature for application of essential oil plants in control of *R. solanacearum*.

3.4 Effect of essential oil plants on bacterial wilt disease

This investigation was carried out in a greenhouse on raised beds measuring 1.5 meters by 2.0 meters and a depth of 30.0 cm. since the soil on the beds had previously been used for growing potatoes, disinfection was necessary to eradicate soil-borne pathogens.

3.4.1 Soil disinfestation

The soil on the beds were mixed with chicken manure at the rate of one wheelbarrow per bed followed by treatment with sodium methyl dithiocarbamate (Metham Sodium) at a dose rate of 100ml /m^2 (300ml / bed) as recommended by the manufacturer (FMC foret, Spain). This was applied as a dilute suspension after topping up to a liter. The beds were then watered slightly and small furrows made at an interval of 15.0cm along the length of each bed. The furrows were sprayed with metham sodium from a knapsack sprayer and covered with soil. Soil on the beds were leveled and then irrigated to saturation and left to stand for three weeks after which the soil in each bed was turned to vent excess volatility.

3.4.2 Application of inoculum into the beds

The beds were inoculated by using a *R. solanacearum* suspension from infected potato tubers collected from farms in Meru County. The potato tubers were cut into small pieces then soaked in 25.0 litres of tap water and the mixture allowed to stand for 1 hour then thoroughly mixed again. The content was sieved into a separate container to remove debris and mixed with 2.0 litres suspension of *R. solanacearum* containing 1.0×10^7CFU/ml. This new mixture was transferred into knapsack sprayer. Small furrows were made again at an interval of 15.0cm along the length of each bed and the inoculum sprayed at a rate of 1.0 liter/bed. The inoculated beds were watered and then left for three days (Plate 5) after which they were sampled to determine the soil *R. solanacearum* population.

3.4.3 Application of treatments

Essential oil plants that had shown strong positive results in the *in-vitro* experiment were used as the treatments in this case. 2.0 kg of freshly harvested leaves, stems and flowers from *L. javanica, O. suave* and *T. camphorates* were homogenized separately in 5.0 litres of tap water (12 hours after harvesting) as per the method used by Ooshiro *et al.* (2004) who had found out that the incorporation of dried young aerial leaves of some essential oil plants like *Geranium carolinianum* in the soil effectively counteract the microbial activities of *R. solanacearum* in the soil. The treatments were applied by turning the soil from one side of the bed followed by spreading of the plant parts to cover the soil and turning again to cover the whole bed as shown in Plate 4.

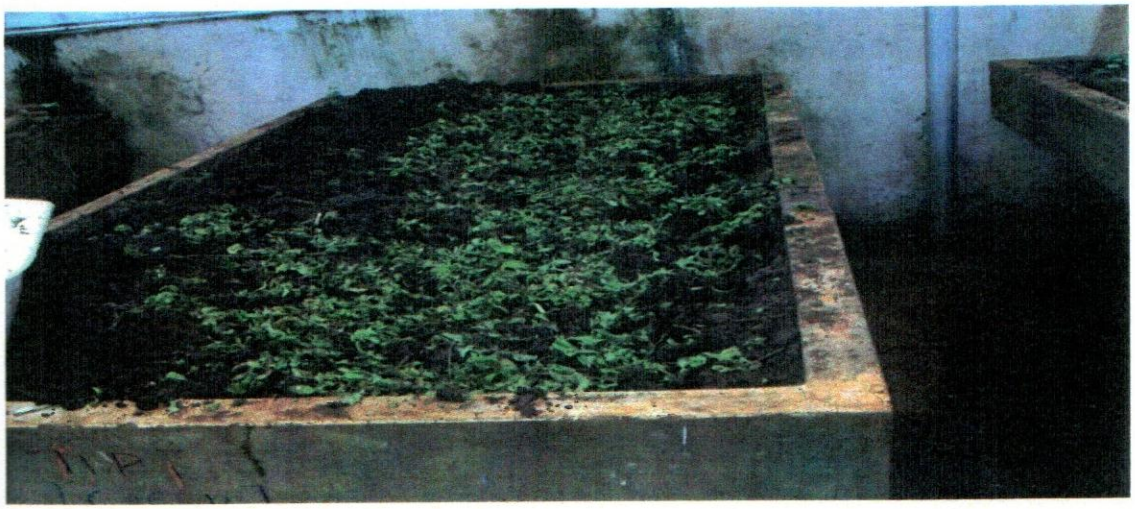


Plate 4: Application of treatments in greenhouse beds

Following treatment, the soil in the beds was mixed thoroughly each day for three days to allow fumigation, and then left to stand for four days. Plots were arranged in randomized complete block design (RCBD) with five replications (blocks) and three treatments along with two controls. One control had water used instead of extracts and this served as the positive control (T5) while the other was lacking both extracts and inocula and served as the negative control (T4).

Plate 5: Watering of treated beds

3.4.4 Planting and observation for wilt incidence

Seven days after treatment, each bed was planted with clean potato tubers (Tigoni variety) obtained from KARI Tigoni Potato Research Centre. Planting was done at a spacing of 45.0cm between the rows and 30.0cm between plants in a row giving a population of 15 plants per bed (Plate 5 and 6). The plants were watered twice a week for a duration of four months and wilt incidence recorded at 3 weeks following planting. Wilted plants were recorded for each treatment twice weekly for two months. Any abnormal symptom on leaves was observed and recorded.

Plate 6: Potato plants in the greenhouse three weeks after planting

3.5 Investigation on potential rotation crops in bacterial wilt management

Potential rotation crops in bacterial wilt management were investigated by assessing the initial (natural) soil *R. solanacearum* in the plots before planting then artificially increasing this population. After planting, rhizosphere and roots were analyzed for presence or absence of *R. solanacearum.*

3.5.1 Determination of initial *R. solanacearum* population

The natural (initial) pathogen population density in the soil was determined using a tetrazolium-based, semi-selective medium (SMSA) (Schell, 2000). This was done by randomly collecting 20g of soil from the entire plot up to a depth of 10cm three days after inoculation. From each plot, three samples were collected in a zigzag pattern throughout the plot. All the three samples per plot were thoroughly mixed to form a composite sample from which 10g of soil was picked and placed in a conical flask and then 90ml of sterile distilled water was added. The mixture was then shaken using a horizontal shaker at 200 revolutions per minute for 30 minutes. The shaken mixture was allowed to settle for 10 minutes then 1ml of the suspension was pipetted using a micro pipette and placed in an eppendorf tube and used as stock solution. 0.1ml (100ul) of the suspension was placed into 0.9ml of sterile distilled water in eppendorf tube then serially diluted up to 10^{-5} by using 0.1ml in each case. The dilutions were gently mixed after which 0.1ml was pipetted from 10^{-5}, $10^{-3}$and 10^{-1} dilutions and spread on the SMSA media using sterilized glass rod.

This was followed by incubation at 28°C for 28 hours. The *R. solanacearum* colonies were identified after 48 hours by the typical fluidal irregular shaped pink colonies with clear margins in tetrazolium-based, semi-selective medium (SMSA). The number of *R. solanacearum* colonies was determined by counting from the dilutions with 30-300 colonies. The population of *R. solanacearum* in soil was then computed from the number of colonies obtained. Since the field was naturally infested with *R. solanacearum* (race 3, biovar 2), the initial soil population density was artificially increased to a density of about 1.0×10^7 colony-forming units per gram of dried soil (CFU/g) by mixing with a known volume of a suspension of *R. solanacearum*, containing 1.0×10^9CFU/ml. The final 1.0×10^7 colony-forming units per gram of dried soil (CFU/g) was determined by sampling the soil before planting.

3.5.2 Trial set up

The crop varieties (Table 5) were bought from reputable commercial suppliers and planted in randomized complete block design (RCBD). Each unit plot was measuring 4.0m x 2.0m with a path of 1.0m between each unit in all directions. The whole experimental field had dimensions of 25.0m x 21.0m (Appendix 2).

Table 5: Host and non-host plants used in the study

Common name	Variety	Scientific name
Host crops		
Potato	Tigoni	*Solanum tuberosum*
Tomato	Cal J	*Solanum lycopersicum*
Egg plant	Black beauty	*Solanum melongena*
Capsicum	California wanders	*Capsicum annuum*
Non-host crops		
Maize	Hybrid 624	*Zea mays*
Sorghum	Katumani	*Sorghum bicolor*
Beans	Mwitemania	*Phaseolus vulgaris*
Cabbage	Coppenhagen	*Brassica oleracea*

Potatoes, tomatoes, egg plants and peppers served as host plants while maize, sorghum, cabbage and beans were used as the non-host plants (Table 5). The experiment was done in a randomized complete block design (RCBD) with four blocks and eight sub plots (Appendix 2).

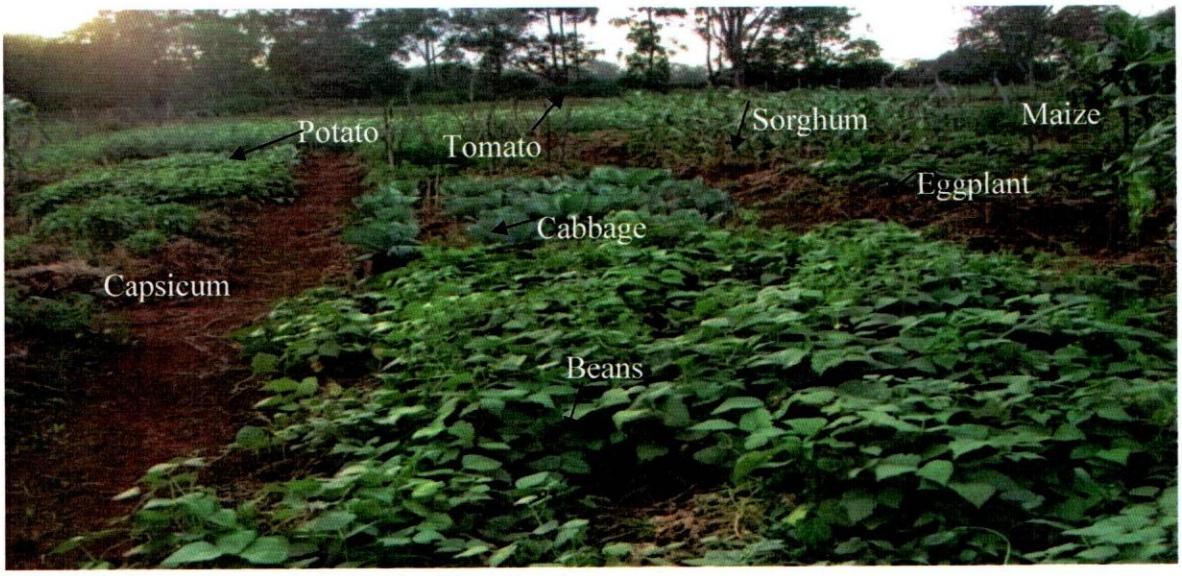


Plate 7: Two months old host and non-host crops in the field

The various crops were planted using the following spacing; Maize and sorghum were 75cm between rows and 30cm between plants with a total of 42 plants per plot. Beans and capsicum spacing were 50cm between rows and 20cm between plants with total of 56 plants per plot while potato, tomato, cabbage and eggplant were 80cm by 30cm with plant population of 37, 45cm by 45cm with population of 37, 25cm by 25cm with population of 46, and 90cm by 30cm with population of 46, respectively. After planting the plants were watered only three times since it was a rainy season. Some of the host and non-host plants are shown in Plate 7.

3.5.3 Data collection

Data on potential rotation crops in bacterial wilt management was done in three parts that involved assessment of *R. solanacearum* population, disease incidence and latent infection.

3.5.3.1 Disease incidence assessment

Wilt symptoms were observed in all the crops per plot and recorded for calculation of wilt incidence. Pathogen population in the soil was determined before planting and after harvesting. Root pathogen attachment and root invasion was also done.

Wilt incidence for all the crops (treatments) was expressed as a percentage of all the wilted plants over the total plants in the plot.

i.e.

Disease incidence (%) = (Number of wilted plants / Total number of plants) × 100

3.5.3.2 Assessment of *R. solanacearum* population

To determine survival of test bacteria in the soil and rhizosphere infestation, four sample plants were used per plot one month after planting and after every one month for three months. The number of viable bacteria in the rhizosphere was assessed by removing "bulk soil" (i.e. soil that is easily dislodged) from the roots by shaking the plants for a few seconds after dislodging them from the soil while leaving the "rhizosphere soil" still attached. The roots plus rhizosphere soil was carefully shaken for 1 minute into sterile conical flasks. The roots were removed, and the rhizosphere soil was used for population analysis (Saile *et al.*, 1997).

Each 10g of soil sample removed from the rhizosphere was suspended in 90ml of sterilized water and then the suspension was shaken in rotary shaker (120 rpm) for 30 minutes. It was diluted serially in sterile distilled water, and then aliquots (100µl) from 10-fold dilutions were spread using a glass rod on the surface of each plate of SMSA. The seeded plates were incubated at 28°C for 72 hours and then colony-forming units (CFU) counted and results expressed as total number of bacteria per gram of soil (Yue-gyukang, 2004). For evaluation of root attachments, the roots from which rhizosphere soil had been removed as described above were carefully washed for 1 minute in 100ml of sterile water in flasks. The roots were removed, and the flasks containing the suspensions were shaken on a wrist-action shaker for 1 hour. Dilutions from 1ml of this suspension were spread on SMSA to determine the number of viable cells as above.

To obtain *R. solanacearum* counts for the primary roots, entire root systems were first thoroughly washed under running tap water and the stems were cut off at 1.0cm above the former soil line. The root systems were immersed for 2 minutes in 1.0% sodium hypochlorite (20% v.v household bleach), washed under running tap water again, and the lateral roots were cut off. The remaining primary roots were rinsed with 95% ethanol, air-dried. Each segment was placed in a sterile bag with 5ml of phosphate-buffered saline (0.1M potassium phosphate buffer pH 7.3, 0.15 M NaCl, and 3.0M KCl) and crushed in stomacher lab-blender (Seward Medical, London) for 2 minutes. Dilutions of the homogenates were spread on semi selective medium, and the plates were incubated at 28°C for 48 hours. Viable cell counts were done as above. A similar experiment as above was repeated with the other host and non-host plants.

3.5.3.3 Latent infection assessment

Investigation of the presence or absence of *R. Solanacearum* on all host and non-host plant samples was done using the method developed by CIP to detect latent infection in tubers using post-enrichment NCM-ELISA (Enzyme linked immunosorbent assay on nitrocellulose membrane) as described by Priou *et al.* (1999).

3.6 Data analysis

To determine the most active essential oil plant extract compared to the array of essential oils tested, analysis of variance (ANOVA) was performed on the data. Significant mean widths were separated using Fisher's least significant difference (FLSD). Disease incidence was calculated as the percentage of plants that showed wilt symptoms. Statistical analysis was performed with GenStat 12.1 (PC/Windows XP) by which one-tailed Analysis of variance (ANOVA) was used to determine the effects of treatment on disease incidence and growth measurements while means were compared using Fisher's least significant difference (FLSD). Survival of *R. solanacearum* in the soil and the influence of different hosts on rhizosphere infestation were done in randomized complete block design (RCBD) with 4 replications (blocks) and 8 treatments. One-tailed Analysis of variance (ANOVA) was used to determine the effects of the different plants on rhizosphere infestation and survival of the pathogen. Means were compared using Fisher's least significant difference (FLSD). Survival of *R. solanacearum* in the soil and the influence of different hosts on rhizosphere infestation were analyzed by GenStat version 12.1 (PC/Windows XP). Detailed data outputs are shown in Appendix 3 and 4.

CHAPTER FOUR

RESULTS

4.1.1 Quantification of essential oils

The amount of essential oils produced per plant was quantified so that bioassay could be carried out. The essential oils obtained are as shown in plate 8. The average essential oil produced per plant species is expressed in graphical form (Figure 2).

Plate 8: Extracted essential oils before use. *Lantana trifolia* (a) *Rosmarinus officinalis* (b) *Lippie javanica* (c) *Lippie ukambensis* (d) *Tarchonanthus camphorates* (e) *Ocimum suave* (f)

There was significant difference between the amounts of essential oil produced by different essential oil plants at $p < 0.5$ (Appendix 3(i)). The values ranged from 2.6804ml/ kg of plant material in *L. trifolia* to a high of 4.040ml/kg of plant material in *R. officinalis* (Rosemary).

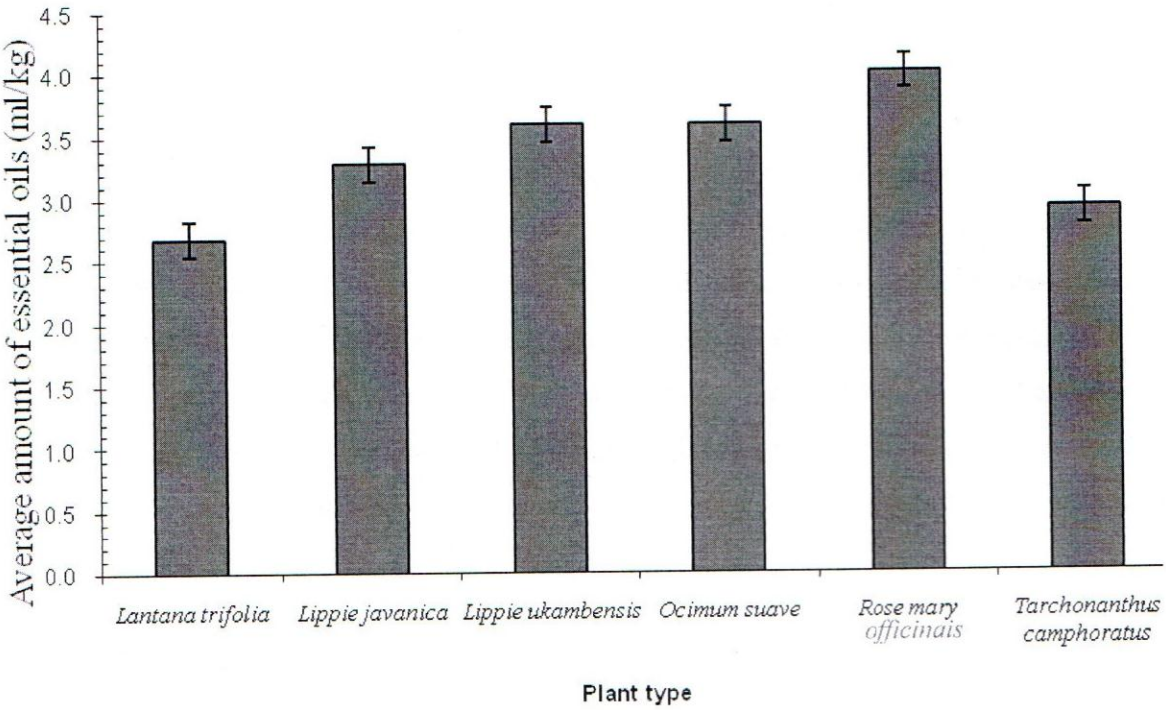


Figure 2: Average amount of essential oils extracted from the plants

Fisher's protected least significance difference test (Table 6) was done to rank the means from the least to the highest and consequently *R. officinalis, L. ukambensis and O. suave* had the highest mean with no significant difference between them. *L. javanica* also had statistically similar mean as *L. ukambensis* and *O. suave.*

Table 6: Quantity of the extracted essential oil

Essential oil plant	Extracted essential oil yield (ml/kg)
Rosmarinus officinalis (RM)	$4.04^{a} \pm 0.70$
Lippie ukambensis (LPU)	$3.60^{ab} \pm 0.60$
Ocimum suave (OC)	$3.60^{ab} \pm 0.60$
Lippie javanica (LPJ)	$3.20^{bc} \pm 0.59$
Tarchonanthus camphorates (TAC)	$2.92^{c} \pm 0.57$
L. trifolia (LT)	$2.68^{c} \pm 0.56$

Means followed by same letter are not statistically different at $P< 0.05$ by Fisher's protected least significant difference test.

4.1.2 Antimicrobial effect against *R. solanacearum*

Various aspects of the growth and development of *R. solanacearum* at different temperatures on the plates were considered. Antibacterial effects were observed as clear zones around the disc containing the extracts. The data on growth effect of essential oil extracts is described and summarised in Tables 7, 8 and 9. Apart from *R. officinalis* and *L. ukambensis,* all plant extracts that showed antimicrobial activity by development of clear zone, did so at all times and temperature intervals but *R. officinalis* only showed the development of a clear zone at 28°C and 32°C and not at 24°C . *L. ukambensis* on the other hand showed the development of clear zone at 28°C but not at 32°C and 24°C.

Table 7: Growth characteristics of *R. solanacearum* at 24°C under different treatments

Temperature (°C)	Time (hours)	Treatment	Growth on plate	Appearance of zone	Appearance of turbid ring around the zone
	24	Water (C-)	++++	no zone	-
	24	Chloramphenical (C+)	++++	very clear	*
	24	*L. javanica* (LPJ)	++++	very clear	*
24	24	*L. ukambensis* (LPU)	++++	no zone	-
	24	*O. suave* (OC)	++++	very clear	*
	24	*T. camphorates* (TAC)	++++	very clear	*
	24	*R. officinalis* (RM)	++++	no zone	-
	24	*L. trifolia* (LT)	++++	no zone	-
	48	Water (C-)	++++	no zone	-
	48	Chloramphenical (C+)	++++	tiny colonies	*
24	48	*L. javanica* (LPJ)	++++	very clear	-
	48	*L. ukambensis* (LPU)	++++	no zone	-
	48	*O. suave* (OC)	++++	very clear	*
	48	*T. camphorates* (TAC)	++++	very clear	*
	48	*R. officinalis* (RM)	++++	no zone	-
	48	*L. trifolia* (LT)	++++	no zone	-
	72	Water (C-)	++++	no zone	-
	72	Chloramphenical (C+)	++++	tiny colonies	-
	72	*L. javanica* (LPJ)	++++	very clear	*
	72	*L. ukambensis* (LPU)	++++	no zone	-
24	72	*O. suave* (OC)	++++	very clear	*
	72	*T. camphorates* (TAC)	++++	very clear	*
	72	*R. officinalis* (RM)	++++	no zone	-
	72	*L. trifolia* (LT)	++++	no zone	-

++++ = 100% colony growth. Appearance of turbid ring, Yes (*) No (-)

Table 8: Growth characteristics of *R. solanacearum* at 28°C under different treatments

Temperature (°C)	Time (hours)	Treatment	Growth on plate	Appearance of zone	Appearance of turbid ring around the zone
	24	Water (C-)	++++	no zone	-
	24	Chloramphenical (C+)	++++	very clear	*
	24	*L. javanica* (LPJ)	++++	very clear	*
28	24	*L. ukambensis* (LPU)	++++	very clear	-
	24	*O. suave* (OC)	++++	very clear	*
	24	*T. camphorates* (TAC)	++++	very clear	*
	24	*R. officinalis* (RM)	++++	very clear	-
	24	*L. trifolia* (LT)	++++	no zone	-
	48	Water (C-)	++++	no zone	-
	48	Chloramphenical (C+)	++++	tiny colonies	-
28	48	*L. javanica* (LPJ)	++++	very clear	*
	48	*L. ukambensis* (LPU)	++++	very clear	-
	48	*O. suave* (OC)	++++	very clear	*
	48	*T. camphorates* (TAC)	++++	very clear	*
	48	*R. officinalis* (RM)	++++	very clear	-
	48	*L. trifolia* (LT)	++++	no zone	-
	72	Water (C-)	++++	no zone	-
	72	Chloramphenical (C+)	++++	tiny colonies	-
	72	*L. javanica* (LPJ)	++++	very clear	*
	72	*L. ukambensis* (LPU)	++++	very clear	-
28	72	*O. suave* (OC)	++++	very clear	*
	72	*T. camphorates* (TAC)	++++	very clear	*
	72	*R. officinalis* (RM)	++++	very clear	-
	72	*L. trifolia* (LT)	++++	no zone	-

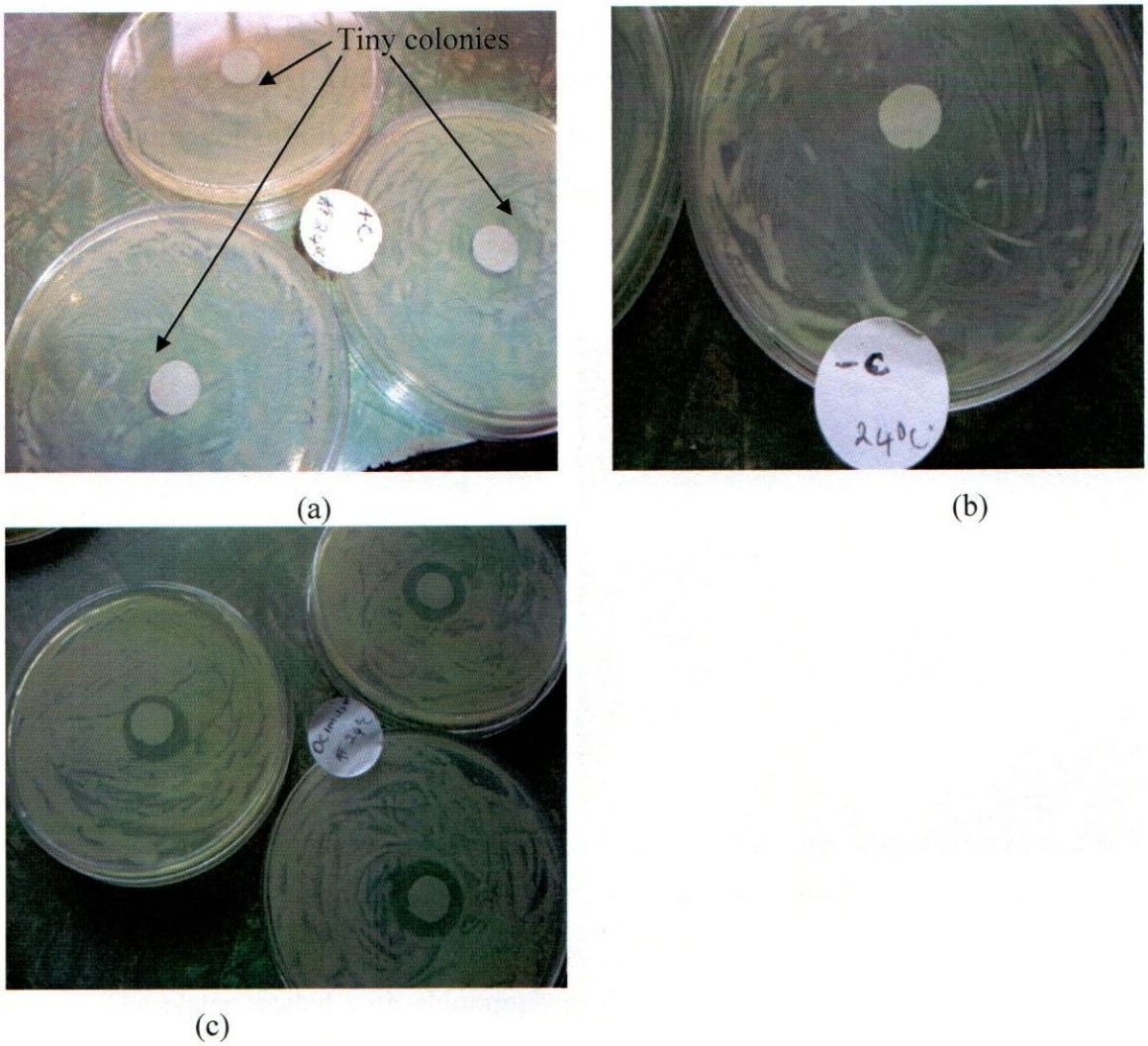
++++ = 100% colony growth. Appearance of turbid ring, Yes (*) No (-)

Table 9: Growth characteristics of *R. solanacearum* at 32°C under different treatments

Temperature (°C)	Time (hours)	Treatment	Growth	Appearance of zone	Appearance of turbid ring around the zone
	24	Water (C-)	+ + + +	no zone	-
	24	Chloramphenical (C+)	+ + + +	very clear	*
	24	*L. javanica* (LPJ)	+ +	very clear	*
32	24	*L. ukambensis* (LPU)	+ + + +	no zone	-
	24	*O. suave* (OC)	+ +	very clear	*
	24	*T. camphorates* (TAC)	+ +	very clear	*
	24	*R. officinalis* (RM)	+ + + +	very clear	-
	24	*L. trifolia* (LT)	+ + + +	no zone	-
	48	Water (C-)	+ + + +	no zone	-
	48	Chloramphenical (C+)	+ + + +	tiny colonies	-
	48	*L. javanica* (LPJ)	+ +	very clear	*
32	48	*L. ukambensis* (LPU)	+ + + +	no zone	-
	48	*O. suave* (OC)	+ +	very clear	*
	48	*T. camphorates* (TAC)	+ +	very clear	-
	48	*R. officinalis* (RM)	+ + + +	very clear	-
	48	*L. trifolia* (LT)	+ + + +	no zone	-
	72	Water (C-)	+ + + +	no zone	-
	72	Chloramphenical (C+)	+ + + +	tiny colonies	-
	72	*L. javanica* (LPJ)	+ +	very clear	*
32	72	*L. ukambensis* (LPU)	+ + + +	no zone	-
	72	*O. suave* (OC)	+ +	very clear	*
	72	*T. camphorates* (TAC)	+ +	very clear	-
	72	*R. officinalis* (RM)	+ + + +	very clear	*
	72	*L. trifolia* (LT)	+ + + +	no zone	-

+ + + + = 100% colony growth, + + below 50% colony growth. Appearance of turbid ring, Yes (*) No (-)

At 32°C it was observed that the growth in some of the plates was not as dense as in the controls probably due to increase in volatility of these essential oils. The extracts from *O. suave* (OC), *L. javanica* (LPJ) and *T. camphorates* (TAC) induced very clear zone of inhibition regardless of incubation temperature (Table 9).



(a) (b)

(c)

Plate 9: Growth characteristics of *R. solanacearum* under various treatments with inhibitory Substances (a) Chloramphenicol treatment, (b) water treatment (c) *O. suave* oil treatment

At 24 °C, there was no appearance of clear zone from all the plates that were treated with water, *L. trifolia, L. ukambensis and R. officinalis* (Table 7). However all plates treated with chloramphenicol showed development of small colonies on the clear zone by 48 hours at all incubation temperatures. Such colonies were not observed in all essential oil treatments (Tables

7, 8 and 9). The numerous tiny colonies in chloramphenicol treatment progressed in size with time. The other plates treated with *O. suave, L. javanica* and *T. camphorates* had clear zones but of varying sizes as show in Plate 9 for O. *suave.* This was the same trend at 28°C with the only difference being in the turbidity of the colonies around the disc in which *L. ukambensis and R. officinalis* had an area of turbid growth around a narrow clear zone at 28°C but no clear zone at 24°C as expressed in Table 11. At 32 °C, there was still no appearance of clear zone from all the plates that were treated with water and *L. trifolia.* Chloramphenicol plates also maintained the numerous tiny colonies on the clear zone that progressed in size with time but merged at the periphery of the clear zone at 72 hours (Plate 10). The other plates treated with *O. suave*, *L. javanica* and *T. camphorates* had clear zones.

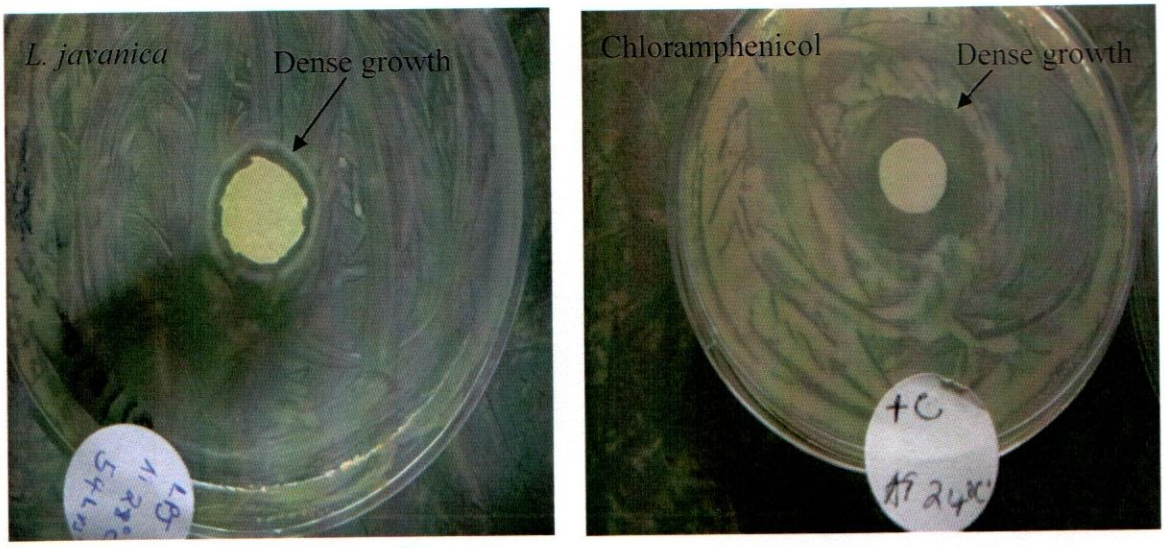


Plate 10: Dense growth around the clear zones of *L. javanica* and Chloramphenicol

The plates treated with chloramphenicol had tiny colonies that started appearing at the periphery of the zone and later developed and merged to form dense growth around the zone of inhibition and reducing its size. *L. javanica* on the other hand did not have the tiny colonies but slowly developed the dense growth around the clear zone (Plate 10). After the end of incubation period (72 hours) and 24°C, plates treated with *O. suave* had the highest width of the inhibition zone among all the treatments. This was followed by chloramphenicol and then *L. Javanica, T. camphorates, R. officinalis* in this order while plates treated with water (negative control), and *L. trifolia* had no zone of inhibition (Table 10).

Table 10: Antibacterial effects of essential oils on *R. solanacearum* after 72 hours

	Mean size of the inhibition zone (mm ± SD)							
Temp	LT	RM	OC	LPJ	LPU	TA	C+	C-
24°C	0.00^{h}	0.00^{h}	$5.78^{a} \pm 0.81$	$2.67^{cd} \pm 0.56$	0.00^{h}	$1.22^{fg} \pm 0.22$	$3.78^{b} \pm 0.60$	0.00^{h}
28°C	0.00^{h}	$0.67^{gh} \pm 0.01$	$5.22^{a} \pm 0.80$	$2.33^{de} \pm 0.22$	$0.67^{gh} \pm 0.01$	$1.22^{fg} \pm 0.22$	$4.22^{b} \pm 0.70$	0.00^{h}
32°C	0.00^{h}	$1.00^{fg} \pm 0.07$	$2.67^{cd} \pm 0.56$	$1.67^{ef} \pm 0.08$	0.00^{h}	$1.00^{fg} \pm 0.08$	$3.57^{bc} \pm 0.60$	0.00^{h}

Different letter are statistically different at $P < 0.05$ by Fisher's protected least significant difference test. C+ = Positive control (Chloramphenicol), C- = Negative control (Distilled water), LPJ = *Lippie javanica* , LPU = *Lippie ukambensis* , LT = *Lantana trifolia* , OC = *Ocimum suave* , RM = *Rosmarinus officinalis* , TA = *Tarchonanthus camphorates* .

O. suave expressed the highest width at 24°C and 28°C with a lower mean at 32°C. *L. javanica* had the second highest mean demonstrating consistency by ranking the same inhibitory effect at both temperatures (Table 10). *T. camphorates* was the next in rank demonstrating consistency as well. *R. officinalis which* had slightly higher mean at 32°C followed by 28°C and completely no clear zone at 24°C. *L. trifolia* and water demonstrated no clear zone at all temperatures. Chloramphenicol (positive control) had a consistent mean at 24°C, 28°C and at 32°C (Table 11). The highest average width of the clear zones was obtained at 28°C followed by 24°C and lastly 32°C (Figure 4).

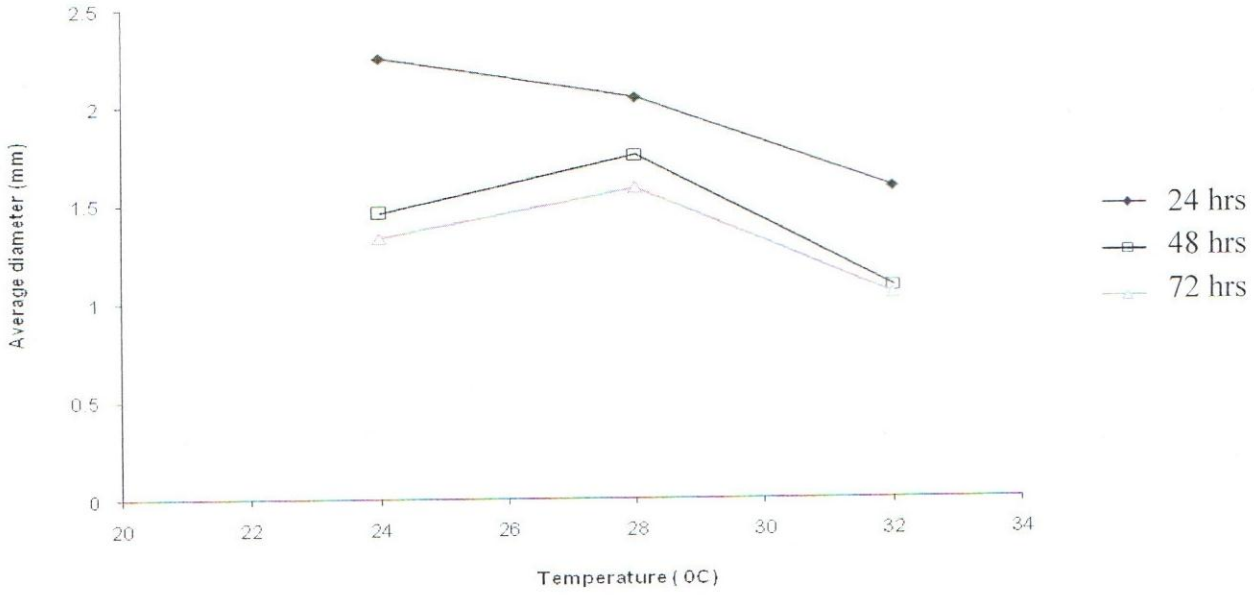


Figure 3: Mean width of clear zones at different temperatures.

The trend at 24°C demonstrated higher mean width of the clear zone when observation was made at 24 hours but slightly decreased as the temperature increased. At 28°C, there was observed general trend of increased width of the clear zone. This shows that the best temperature for realizing the highest antimicrobial activity in all the essential oils is 28°C but a range of between 24°C to 28°C is equally appropriate especially when the temperature is not constant. Mean differences are as shown in Table 11.

Table 11: Mean width of clear zone of different treatments under different temperature intervals

Treatments	Time span (hours)		
	24	48	72
OC	4.78^{b}	4.78^{b}	4.11^{b}
C+	6.69^{a}	2.67^{c}	2.22^{cd}
LPJ	2.22^{cd}	2.11^{cd}	2.33^{cd}
TA	1.67^{de}	0.89^{ef}	0.89^{ef}
RM	0.33^{f}	0.67^{f}	0.67^{f}
LPU	0.00^{f}	0.33^{f}	0.33^{f}
C-	0.00^{f}	0.00^{f}	0.00^{f}
LT	0.00^{f}	0.00^{f}	0.00^{f}

Means followed by different letter are statistically different at $P< 0.05$ by Fisher's protected least significant difference test. C+ = Positive control (Chloramphenicol), C- = Negative control (Distilled water), LPJ = *Lippie javanica*, LPU = *Lippie ukambensis*, LT = *Lantana trifolia*, OC = *Ocimum suave*, RM = *Rosmarinus officinalis*, TA = *Tarchonanthus camphorates.*

O. suave had the second highest mean ranking at all the time intervals. Growth inhibition by chloramphenicol was highest when plates were incubated for 24 hours but significantly decreased when incubated at 48 hours and 72 hours. *L. javanica* had the third highest mean at 72 hours followed by 48 hours and lastly at 24 hours with both means demonstrating consistency by ranking the same. *T. camphorates* was the next in rank with slightly higher mean at 24 hours than at 48 hours and 72 hours at which the means were the same.

R. officinalis and *L. ukambensis* had a similar trend as *L. javanica* but with very low mean value. *L. trifolia* and distilled water (negative control) had completely no clear zone thus ranking the least (Table 12). Appearance and distribution of the lawn in the whole plate and in immediate areas around the clear zone was also observed under different treatments.

There was dense growth of *R. solanacearum* in the whole plate in all plates that were treated with water, *L. trifolia* and *L. ukambensis* while plates of *R. officinalis* chloramphenicol, *O. suave*, *L. javanica* and *T. camphorates* had dense growth only around the turbid zone. At 28°C, there was dense growth of *R. solanacearum* in plates that were treated with water, *L. trifolia* and *L. ukambensis* after 24 hours and 48 hours. The trend was slightly similar in plates with *R. officinalis* in which there was dense growth after 24 hours and that changed into turbid zone after 48 hours and 72 hours. In plates with chloramphenicol, there was dense growth around the clear zone at 24 hours but the dense growth increased towards the clear zone starting as tiny colonies thereafter reducing the size of the clear zone at 48 hours and 72 hours.

This trend of chloramphenicol at 24 hours was the same with *O. suave* but there was a significant difference at 48 hours and 72 hours in which the dense growth started to move towards the clear zone thus reducing the size of the zone in some plates of *O. suave*. Plates of *L. javanica* however had dense growth around the clear zone at all times while *T. camphorates* had growth towards the clear zone at 48 hours and 72 hours thus reducing the size of the clear zone. At 32°C there was dense growth in plates that were treated with water, *L. ukambensis* and *L. trifolia*. The trend was slightly similar in plates with *R. officinalis* in which growth around the turbid zone became dense after 24 hours and was maintained at 48 hours and 72 hours. In plates with chloramphenicol, there was dense growth around the clear zone at 24 hours but the dense growth increased towards the clear zone starting as tiny colonies thereafter reducing the size of the clear zone at 48 hours and 72 hours as seen in plate 10.

Ocimum suave had dense growth around the clear zone at 24 hours. Conversely, at 48 hours and 72 hours the dense growth started to flow towards the clear zone thus reducing the size of the zone in most plates. *L. javanica* had the same trend as O. suave at 48 hours and 72 hours in all plates while *T. camphorates* had dense growth towards the clear zone at 48 hours and 72 hours that started at 24 hours as tiny colonies leading to reduction of the size of clear zone. The appearance of turbid zones are as seen in Plate 11.

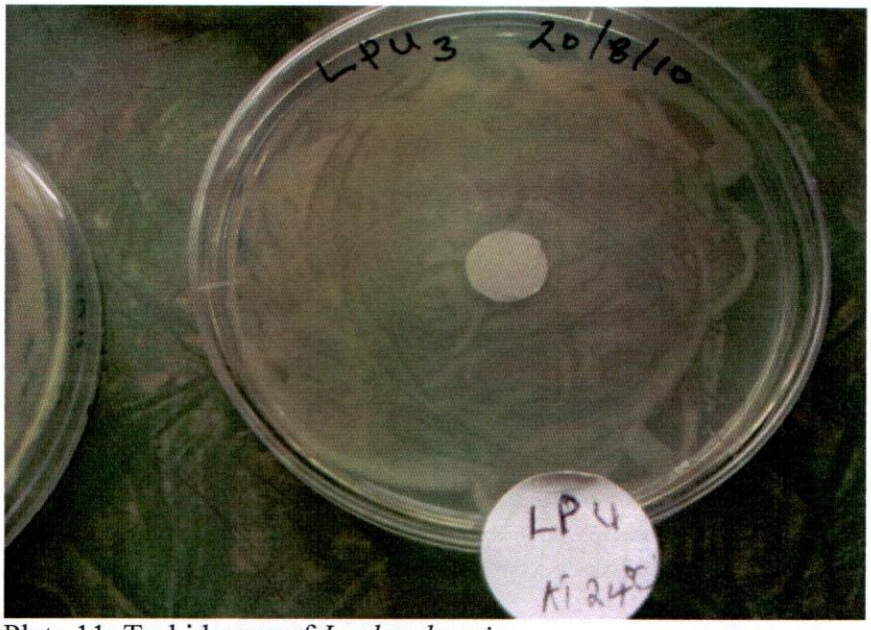


Plate 11: Turbid zone of *L. ukambensis*

At 32 °C, all water, Chloramphenicol, *R. officinalis, L. ukambensis* and *L. trifolia* plates had creamy white colonies while *O. suave*, *L. javanica* and *T. camphorates* only about 70% of the plates had colonies while the rest had completely no growth and remained clear.

4.2 Effect of essential oil plants on bacterial wilt disease

Effect of the essential oil plants on bacterial wilt in potato plants grown in greenhouse was monitored by observing the development of wilt symptoms, the progress of the disease and disease reduction.

4.2.1. Disease progress in the greenhouse foollowing treatment with oil plants

From the second month of growth, symptoms of wilts started appearing and recording was done for two months. From these records, *O. suave* (T1) had wilting increase in the earlier days of the second month (November 2011) with the highest at this time being an average of seven plants out of fifteen (46.67%) on the fifth day. This was followed by a decline for the rest of the days with an average record of three plants out of fifteen till the ninth day. The next highest wilting was observed again in the early days of the third month (December 2011). However there was a reduction in comparison of the first pick at an average of three wilted plants.

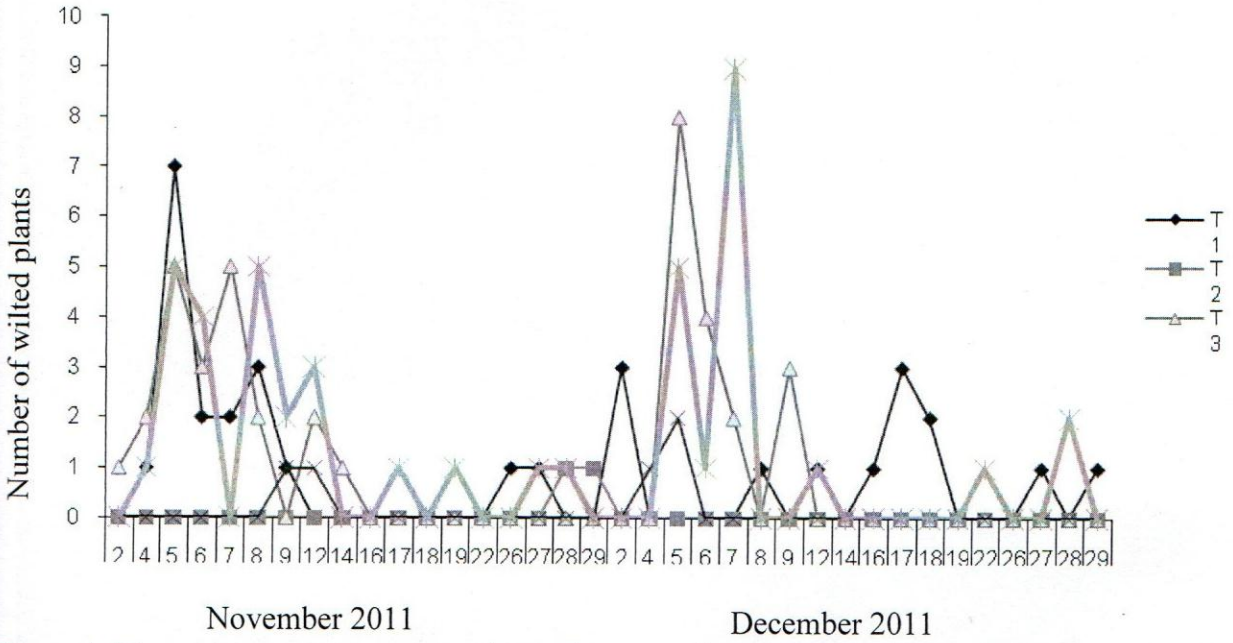
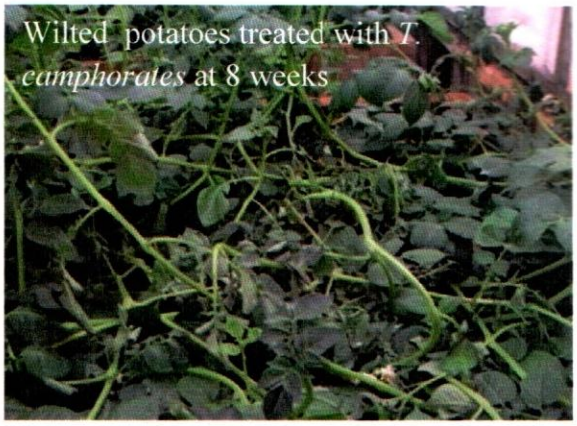


Figure 4: Disease progress in the greenhouse in comparison to the positive and negative control. T5 = Positive control (has inoculum but not essential oil plant), T4 = Negative control (lacking inoculum and any essential oil plant), T3 = *L. Javanica*, T2 = *T. camphorates* T1 = *O. suave*

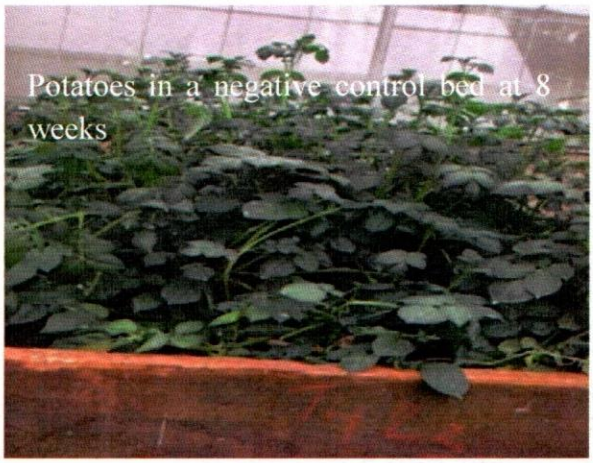
T. camphorates (T2) had almost no wilting observed for the first month with an average of one wilted plant in the last two days of the month. The negative control (T4) had wilt symptoms appearing on a few plants in comparison to the rest of the treatments towards the middle of November (second month after planting). The rest of the plants under this treatment did not wilt till early December (third month after planting). Plants treated with *L. javanica* (T3) exhibited a very high wilt in the second month with an average of eight wilted plants out of fifteen (53.33%) plants and an average of five in the first month.

In general, potatoes under the negative control exhibited slightly higher wilting than those under *T. camphorates* (T2) treatment probably due to soil-borne *R. solanacearum*. Some of the wilted and non-wilted potatoes in the greenhouse are shown in Plate 12. The trends observed above were, however, different in the beds that were treated with T5 (positive control) in which more than half the plants wilted in the plots during the second month and a maximum of five in the first month. *O. suave* (T1), *L. javanica* (T3) Positive control (T5) all had over 60% disease incidence. The most wilted beds were those that were treated with *L. javanica* (T3). By the second month, all plants treated with *L. javanica* (T3) had wilted (Figure 4).

a



b



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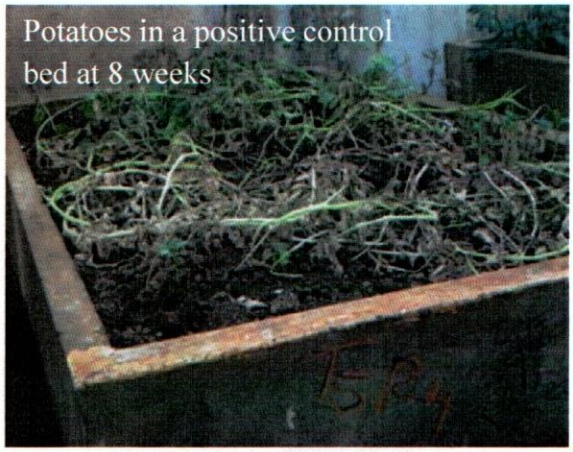

d

Plate 12: Crops in the greenhouse following treatment with plant extracts: (a) = Unwilted potato plants treated with *L. javanica* at 4 weeks (b) = wilted potato plants treated with *T. camphorates* at 4 weeks, (c) = Unwilted potato plants under negative control at 8 weeks, (d) = Wilted potato plants under positive control at 8 weeks.

4.2.2 Wilt incidence

Disease incidence expressed as percentage (%) of wilted plants in the experiments was done for five treatments. This was represented as means of wilted plants for the two months of observation and represented in Figure 5. Lowest disease incidences were recorded for treatments with *T. camphorates* and in the negative control. *O. suave*, *L. javanica* and the Positive control (inoculum only) had highest disease incidences and all had less than 40% disease reduction with *L. javanica* having only 22% disease reduction. *T. camphorates* was most effective in the control and had more than 90% disease incidence reduction.

The positive control consisted of beds inoculated with the pathogen but with no essential oil plant treatment. This bed had recorded high wilt incidence and disease reduced by only 30% compared to 69% disease reduction in negative control (Figure 5). The observed wilt incidence in the negative control could therefore have resulted from colonies of *R. solanacearum* that were not effectively eradicated by methyl sodium.

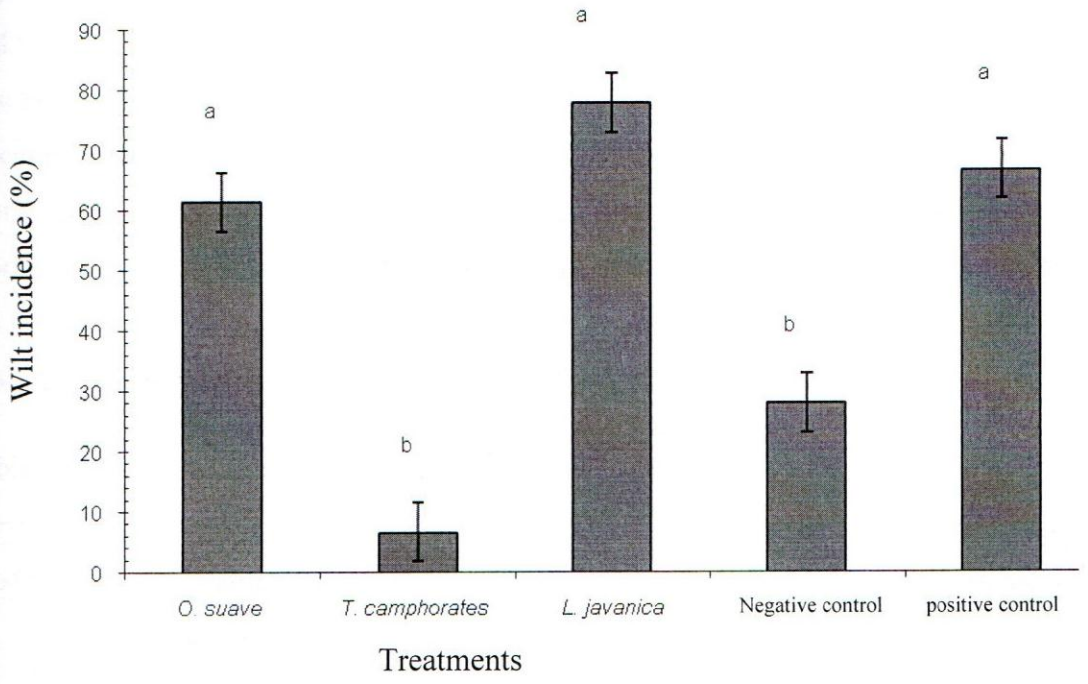

Figure 5: Disease incidence in the greenhouse experiment.

The three treatments (*O. suave*, *L. javanica* and positive control) were ranked equally with Fisher's protected least significant difference test with no significant difference between the three incidences (P>0.05) (Table 12). *T. camphorates* and negative control, had 6.67% and 28.00% disease incidence, respectively with no significant difference between the two. This indicates that *T. camphorates* reduced the wilting incidences by 93% confirming its strong antimicrobial property and success in control of bacterial wilt disease (Table 12).

L. javanica (T3) exhibited a very high wilt incidence in the second month with an average of eight wilted plants out of fifteen plants (53.33%) and average of five in the first month. The positive control (T5) all had over 60% of the disease incidence with more wilt observed in December (third month after planting). Wilting started in moderately high number in the second month with a little decline towards the end of this month only to increase again in the third month reaching the maximum of an average of eight months. Similar observation was made in the beds treated with *L. javanica* (T3) but at a slightly lower number in comparison to the positive control indicating that *L. javanica* extracts were less effective in control of bacterial wilt.

Table 12: Mean wilt incidence in the greenhouse

Code	Treatment	Mean incidence
T3	*Lippie javanica*	$77.90^{a} \pm 2.43$
T5	Positive control	$66.67^{a} \pm 2.56$
T1	*Ocimum suave*	$61.52^{a} \pm 1.81$
T4	Negative control	$28.00^{b} \pm 1.80$
T2	*Tarchonanthus. camphorates*	6.67b ± 0.72

Means followed by different letters are statistically different at P< 0.05 by Fisher's protected least significant difference test. T5 = Positive control (has inoculum but not essential oil plant), T4 = Negative control (lacking inoculum and any essential oil plant), T3 = *Lippie javanica*, T2 = *Tarchonanthus. camphorates* , T1 = *Ocimum suave*

4.3 Effect of host and non-host plants on survival of *R. solanacearum.*

The result obtained when investigating effect of host and non-host plants on survival of *R. solanacearum* are as follows:

4.3.1 Population of *R. solanacearum* in the soil.

The mean population per plot for each crop was calculated and the results are shown in Table 13. There was significant ($P< 0.05$) difference between the type of treatment and the mean of *R. solanacearum* population in the soil two months after planting as shown in appendix 4.

Table 13: Mean *R. solanacearum* population in the soil

Treatment/Crops	Mean (± SD) of soil *R. solanacearum* population (cfu × 10^3) Before planting/Treatment	After two months
Beans	155 ± 9.13	300 ± 8.05
Cabbage	161 ± 8.60	311 ± 9.56
Capsicum	192 ± 6.68	381 ± 2.58
Egg plant	166 ± 4.24	306 ± 2.16
Maize	160 ± 5.94	312 ± 1.63
Potato	131 ± 9.97	246 ± 5.16
Sorghum	142 ± 3.37	268 ± 5.09
Tomato	227 ± 9.95	439 ± 7.39

4.3.2 Rhizosphere population

Fisher's protected least significant difference test was used to compare mean rhizosphere *R. solanacearum* population three months after planting. Egg plant and potato plots had the highest *R. solanacearum* population but with no significant ($P< 0.05$) mean difference between them. This was followed by sorghum, tomato, beans, cabbage and maize that also had no significant difference between them. Capsicum had the lowest means but with no significance difference in comparison to maize and cabbage (Table 14).

Table 14: Mean values recorded for *R. solanacearum* population in the rhizosphere

Treatment	Mean population (cfu × 10^3)
Egg plant	$25.25^{a} \pm 2.00$
Potato	$16.50^{ab} \pm 1.07$
Sorghum	$16.00^{b} \pm 2.08$
Tomato	$15.75^{b} \pm 2.24$
Beans	$13.00^{b} \pm 2.01$
Cabbage	$10.75^{bc} \pm 0.95$
Maize	$7.50^{bc} \pm 1.02$
Capsicum	$3.50^{c} \pm 0.64$

Means followed by different letters are statistically different at $P< 0.05$ by Fisher's protected least significant difference test.

4.3.3 Root invasion

Potato had the highest population of *R. solanacearum* in the roots followed by egg plant, capsicum, tomato and maize without any significant difference between them (Table 15). Sorghum and beans also had comparably high populations of *R. solanacearum* in the roots in comparison to cabbage that had very low population.

Table 15: Mean observed values for *R. solanacearum* population inside the root

Treatment	Mean population (cfu× 10^3)
Potato	$82.50^{a} \pm 0.96$
Egg plant	$72.50^{ab} \pm 1.51$
Capsicum	$42.50^{abc} \pm 4.01$
Tomato	$40.00^{abc} \pm 3.00$
Maize	$37.50^{abc} \pm 1.52$
Sorghum	$32.50^{bc} \pm 2.60$
Beans	$27.50^{bc} \pm 3.06$
Cabbage	$5.00^{c} \pm 2.34$

Means followed by different letters are statistically different ($P< 0.05$), Fisher's protected least significant difference test.

4.3.4 Wilt incidence

Wilt incidence for each crop (treatment) was done by calculating the average of the replicate blocks for all crops. The results obtained are shown in Table 16. Some of the wilted plants are shown in plate 13.

a

b

c

d

Plate 13: Wilted crops due to *R. solanacearum* in the field: (a) wilted tomato, (b) wilted tomato plant, (c) wilted egg plant (d) wilted capsicum.

From these results, potato and tomato had the highest wilt incidence that led to total death in all the plots that contained the two crops. Capsicum and egg plant had 40% and 30%, respectively while beans, cabbage, maize and sorghum exhibited completely no observable wilt symptoms. Fisher's protected least significant difference test on mean of wilt incidence of crops per plot was done to separate the means and the result shows that there was no statistical difference ($P< 0.05$) between capsicum and egg plant on the wilt incidence though wilt was observed in these plants.

Table 16: Wilt incidence on crops planted in the field infested with *R. solanacearum*

Treatment/Crops	Incidence (%)
Potato	100[a]
Tomato	100[a]
Capsicums	37.9[b]
Egg plant	29.3[b]
Sorghum	0.00[c]
Cabbage	0.00[c]
Beans	0.00[c]
Maize	0.00[c]

Means followed by different letters are statistically different at $P< 0.05$ by Fisher's protected least significant difference test.

4.3.5 Disease progress in the field

Observable wilt symptoms were first noticed one month after planting or transplanting plots with potato, egg plant and tomato showing the first symptoms. An increase in number of wilted crops was observed ten days after the first symptom appearance (Figure 6). When the soil moisture level increased drastically following intense rains, a general increase in the number of wilted plants was observed in the plots that were treated and planted with potato, tomato and capsicum while egg plants wilted five days later.

By this time over half of potato plants in all the plots had shown wilt symptoms. There was a slight decrease in the number of new wilted crops for two weeks after which a further increase in moisture level following a heavy rain caused a slight increase in this number of wilted crops in the third month. A futher increase in number of wilted plant species was observed after five weeks of reduced wilting. This trend decreased with a decline in moisture content of the soil and increased again till the end of the second month at which all the potatoes and tomatoes had died. At this time, eggplant and capsicum were showing an increase in the number of plants wilting but not all died.

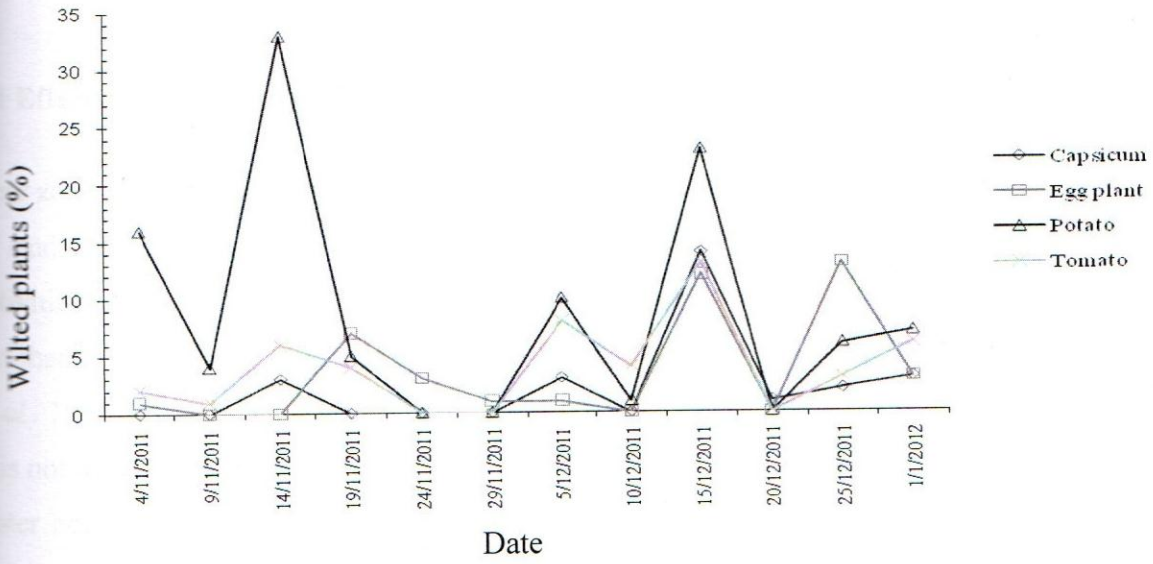

Figure 6: Disease progress in the field

4.3.6 Latent infection

All samples taken from potato, tomato, capsicum and egg plant comprising 50% of host plants tested had positive results showing dark purple colour similar to the dark purple expressed by *R. solanacearum* citrate buffer positive control. Sorghum, cabbage and beans comprising 38% of the host plants tested all showed green colours therefore giving negative results. 50% of maize sample replicates had negative results indicated by green colours while the other half had positive results with light purple colour in comparison to the dark purple colour expressed by *R. solanacearum* buffer positive control (Table 17).

Table 17: Latent infection

Response of different host and non-host plants in ELISA test	
Crop	Result (+ positive or -negative)
Beans	–
Cabbage	–
Capsicum	+
Egg plant	+
Maize	- and +
Potato	+
Sorghum	–
Tomato	+

CHAPTER FIVE
DISCUSSION

5.1 Effects of essential oil plant extracts in in-vitro assay

This study found out that there was significant difference between the mean widths of clear zones of different treatments under various temperatures. This is in agreement with findings of studies on medicinal plants that have been scientifically investigated for their antimicrobial activities (Wagura *et al.,* 2011). Studies on plant extracts commonly used by aborigines found out that approximately 20% of the samples tested were able to inhibit bacterial growth (Semple *et al.,* 1998; Palombo and Semple, 2001). Length of exposure should be at least 24 hours; though it is not directly linked to the effect of the essential oils on *R. solanacearum* could be due to the faster action of the essential oils, high rate of growth by the bacteria or the volatility of the essential oils. An important characteristic of essential oils and their components is their hydrophobicity, which enable them to partition the lipids of the bacterial cell membrane and mitochondria, disturbing the cell structures and rendering them more permeable (Seenivasan *et al.,* 2006). Extensive leakage from bacterial cells or the exit of critical molecules leads to death.

Other studies have also found out that gram-positive bacteria are more resistant to the essential oils than gram-negative bacteria (Seenivasan *et al.,* 2006). *R. solanacearum,* being a gram-negative bacterium could have easily been inhibited in its growth in this manner. At 28°C there was observed general trend of increased width of the clear zone at all time intervals. This shows that the best temperature for realizing the highest antimicrobial activity in all the essential oils is 28°C but a range of 24°C to 28°C is equally appropriate especially when the temperature is constant. *O. suave* had significant effect on growth of *R. solanacearum*; this antimicrobial property is also revealed in the finding of Birhanetensay *et al.* (2012) that proved that *O. suave* has antibacterial properties that are related to its action in reduction of inflammatory effects. *O. suave* had the highest *in-vitro* antimicrobial activity in comparison to the other essential oil plant extracts used in this investigation. *O. suave* was relatively more effective than chloramphenicol (positive control) that seemed to have degraded with time. Previous studies have shown that various species of the genus *Ocimum* produce oil of diverse characteristics and most of them with antimicrobial properties (Ntezurubanza et *al.,* 1988). However, few studies have been done to prove their effects on control of plant diseases other than their role in the individual plant defenses.

Studies by various researchers reported that essential oil derived from leaves of *O.gratissimum* collected from Meru region contained eugenol, methyl eugenol, cis-ocimene and trans-ocimene which are antimicrobial (Reuveni *et al.*, 1984; Nakamura *et al.*,1999; Lemos *et al.*, 2005; Matasyoh *et al.*, 2008; Louis *et al.*, 2011). Another study carried out to analyze essential oil of *O. gratissimum* by gas chromatography showed that they contain compounds such as thymol, eugenol and d-limoneme, among others (Masada, 1976). The oil has been reported to be effective against species of bacteria and fungi (Iwalokun *et al.*, 2003; Mbata and saikia, 2005; Matasyoh *et al.*, 2008; Malik and Singh, 2010). Though the study did not ascertain the chemical components present in the oil that has the antibacterial properties, eugenol was suspected as the most likely candidate. This has been demonstrated to have antibacterial (Nakamura *et al.*, 1999) and antihelmintic activities (Pessoa *et al.*, 2002).

From this study, the best application temperature when using essential oil extracts from *O. suave* were 24°C and 28°C because the oil was found to be less effective at high temperatures probably due to volatility. This finding is in conformity with the finding of Awuah (1989). At 24°C and 28°C the antimicrobial activity (width of clear zone of *O. suave*) was constantly indicating that temperature must be considered to realize highest level of control.

Essential oil extracted from *L. javanica* also had significant antimicrobial activity and consistently gave large inhibition zones. This is in synchrony with the findings of studies of antimicrobial activity of *L. javanica* in human pathogens since there has been very little study on its antimicrobial activities in phytopathogens. These related studies indicate that traditional uses of *Lippie* spp. include the treatment of a variety of ailments (Van Wyk, *et al.*, 1997). However, majority of species are used as remedies for gastrointestinal and respiratory complaints whose medications are usually prepared from the leaves or flowers and in some cases, all the aerial parts are generally used as either infusions or decoctions which are administered orally (Morton 1980. loc.cit. Pascual *et al.*, 2001). *Infusions* are prepared by steeping or crushing the plant material in hot or cold water for a short time (Van Wyk, *et al.*, 1997). The oils of *L. javanica* had significantly less effect on the bacteria in comparison to the positive control and *O. suave* at all the time intervals. At higher temperature, 32°C it's antimicrobial activity reduced significantly in comparison to its effects at 24°C and 28°C where there was no significant difference between mean widths of the clear zone.

Utilisation of the essential oil or other plant parts of indigenous *L. javanica* as an asset would be valuable to farmers who own land where *Lippie* spp. have invaded farm lands because they can be used to reduce bacterial wilt caused by *R. solanacearum*. Other studies have also reported that annual cropping of aerial parts could replace expensive spraying of herbicides and pesticides as well as some fungicides (Sandra, 2006). However, when using essential oil extracted from *L. javanica* the best temperature should be 24°C and 28°C because higher temperature than this can probably increase volatility of the essential oil (Awuah, 1989).

Essential oil extracted from *T. camphorates* also exhibited in-vitro antibacterial activity that was not influenced by time or temperature of incubation. Some studies found rosemary (*Rosmarinus officinalis*) to be equally effective against both gram-positive and gram-negative organisms (Seenivasan *et al.*, 2006). However this study found out that rosemary only has antimicrobial effect on *R. solanacearum* 28°C and 32°C. Extract from *L. ukambensis* had no significant effect on growth of *R. solanacearum* at all levels of temperature and time while *Lantana trifolia* only had effect at 28°C. This is probably because the concentrations of the active ingredients were too low or were unstable. Oil from a particular plant species can also vary considerably in its chemical profile, as a result of genetic, environmental, geographic and other differences (Burt, 2004). In addition, the maturity of the plant at harvest, the method of oil extraction, as well as the method of distillation, can influence oil yield and composition (Weiss, 1997). Or may be due to difference in the mechanism of action since it has been found out in other studies that, although the antimicrobial properties of essential oils and their components have been reviewed in the past (Nychas, 1995), the mechanism of action has not been studied in great detail.

Considering the large number of different groups of chemical compounds present in essential oils, it is most likely that their antibacterial activity is not attributable to one specific mechanism but that there may be several targets in the cell (Burt, 2004). At 32°C the growth of *R. solanacearum* was not 100% in all Petri dishes as was at 24°C and 28°C. However water, chloramphenicol, *R. officinalis, L. ukambensis* and *L. trifolia* had 100% growth while the rest had 70% growth. High temperature has been reported to affect the growth of *R. solanacearum* (Stevenson *et al.*, 2001). This could have been compounded by the possibility of increased volatility of some essential oils as observed in the experiments.

All plates treated with the positive control (chloramphenicol) had clear zones of different widths and all the zones were very clear at 24 hours. This clarity, however, changed at 48 hours and 72 hours as tiny numerous colonies appeared on the zone giving an indication that chloramphenicol could have induce resistance as was indicated by the re appearance of the tiny colonies on the clear zone with time, a fact that is also supported by Ajalie *et al.* (2005). Such resistance was not observed with essential oil treatments. This was a proof of their near-perfect antimicrobial effect on the growth of *R. solanacearum* (Sandra, 2006; Ozcan *et al.*, 2006; Birhanetensay *et al.*, 2012). The fluidal nature of *R. solanacearum* could affect size of clear zones as observed in some treatments following increased time of incubation (Janse, 1996). Some *Lantana* spp. have been found to contain antimicrobial activities against some pathogenic bacteria (Seenivasan *et al.*, 2006). However in this study, *L. trifolia* was not found to have any antimicrobial activity against *R. solanacearum*.

5.2 Effect of essential oil plants on bacterial wilt in potato

The effect of essential oil plant that had inhibitory effects on *R solanacearum* in the in-vitro experiment were tested in greenhouse experiment from which *T. camphorates* led to reduced disease incidence of 6.067% indicating that *T. camphorates* can be applied directly in the soil before planting to control *R. solanacearum*. This method of application has also been found effective when using *Cajanus cajans* that was used in greenhouse to reduce bacterial wilt incidences in tomatoes and potatoes (Cardoso *et al.*, 2006). Biological control measures are becoming common in integrated pest management (IPM) and integrated disease management (IDM). Research has been developed in trying to come up with plants that can be grown with potato to reduce bacterial wilt incidences, coming up with positive beneficial microbes to counter soil pathogens, and incorporation of some parts of the plant to inhibit survival of pathogens (Ooshiro *et al.*, 2004). It has been shown in certain studies that bacterial wilt in the greenhouse can be controlled almost 100% by incorporation of about 20% of fresh aerial parts of plants like *Cajanus cajan* (Cardoso *et al.*, 2006). This is an encouraging finding especially for farmers if vegetative plant parts which are easy to apply can effectively control bacterial wilt in the field to curb bacterial wilt in both tomatoes and potatoes.

Furthermore plants such as *T. camphorates* are easily available in most forests in potato and tomato growing areas in Kenya. Ooshiro *et al.* (2004) also found out that the incorporation of young aerial leaves of some essential oil plants like *Geranium carolinianum* in

the soil together with solarisation effectively counteract the microbial activities of *R. solanacearum* in the soil. The findings of this study showed that even though the extracts might have had an effect on the pathogen in *in-vitro* test, there was no significant difference between the wilt incidences of the potatoes planted in beds treated with *O. suave*, *L. javanica* and positive control (pathogen plus Chlorophenicol).

These three essential oil plants seemingly had very little effect on reduction of wilt incidence contrary to the expectation based on the result from *in-vitro* experiments that exhibited significant effect on the growth of *R. solanacearum* by appearance of large clear zones. This could be as a result of other factors that could have affected the interaction of the pathogen and the essential oils in the soil. This could range from release of essential oils from the plant materials, method of application of the essential oil plants to volatility of the essential oil in relation to the temperature of the greenhouse as was found out by Weiss (1997). When using *O. suave* and *L. javanica,* one should consider the method of application and the other possible factors that may interfere with its antimicrobial activities in the soil. This is because of the observed difference in the antimicrobial activity exhibited by the extracts from these two essential oil plants in the *in-vitro* experiment and the disease wilt incidence. Some studies suggest that cropping of some of the essential oils and subsequent incorporation in the soil can reduce soil-borne diseases (Sandra, 2006). Periodic cropping and burying of aerial parts of these essential oil plants can significantly reduce bacterial wilt disease caused by *R. solanacearum.*

5.3 Potential rotation crops in bacterial wilt management.

From this study, potato and tomato crops that are well known hosts of *R. solanacearum* (USDA, 2003) had highest number of wilting with no significant wilt difference between them. These two crops had 100% loss to *R. solanacearum* by the end of the second month *showing* that they are highly susceptible to infection as indicated in study by Schaad *et al.*, (2001). Other known host plants such as capsicum and egg plant (USDA, 2003) which had less than 50% wilt incidence. This is in agreement with the recommendation that rotating potato and tomato crop should be avoided since they are both highly susceptible to bacterial wilt caused by either race 1 or 3 strains of *R. solanacearum* (Janse, 1996). This study confirmed that non-host plants such as beans, maize and sorghum had no wilt incidences but may not be effective rotational crops in control of bacterial wilt of potato. Other studies have also suggested that rotation with non-host

plants is an effective means of decreasing the level of *R. solanacearum* populations in soil, provided volunteer potato plants are continually removed by uprooting the entire plants from the field as they emerge (Janse, 1996).

Egg plant and potato had the highest *R. solanacearum* population in their rhizosphere with no significant difference between them. French (1994) also recommended that eggplant, pepper and tobacco must never be planted after a bacterial wilt infected potato crop. This observation could be as result of their susceptibility to *R. solanacearum* that could have resulted in more pathogen oozing out of the infected plant parts into the soil or because they secreted substances that attract the bacteria to their roots or are used to support the survival of this pathogen by providing nutrients (Priou *et al.*, 1999). Tomato had significantly lower rhizosphere population compared to potato and egg plant despite the fact that it is a known host. This could be an indication that most populations of the pathogen are within the vascular bundle or that the pathogen takes slightly longer time to establish itself in tomato as was observed by Nyangeri (2011). Sorghum and beans had lower population in comparison to the first three, an observation that could have been due to their non-host nature. This finding is in agreement with the finding that legumes, e. g. fabaceous (pea, bean), can be used as rotation crops for potatoes. Moreover, they have the additional benefit of increasing soil fertility by fixing atmospheric nitrogen. A bean and maize rotation has reportedly reduced soil inoculum potential (Priou *et al.*, 1999). This study showed that maize, beans and sorghum have the ability to reduce the population of *R. solanacearum* but not to completely eradicate the pathogen from the soil since some *R. solanacearum* can still be detected in their rhizosphere.

In other studies, rotation with cereals and gramineaceae family has also been observed to decrease soil inoculum potential even though the time necessary for eradication has to be determined in each location (French, 1994). This study also found out that cabbage had significantly low *R. solanacearum* population that may be related to their non-host nature. When roots of the crops in study were analysed for the presence of *R. solanacearum* cells, there were significantly high populations of the bacteria in potato tubers which must have been as a result of its susceptibility to the pathogen. It has been documented that, the bacterium affects more than 30 plant species but the most susceptible crops being potato, tomato, eggplant, pepper, banana and groundnut (Priou *et al.*, 1999). This same reason could be true for egg plant capsicum and tomato. *R. solanacearum* usually enters the plant via natural wounds (created by excision of

flowers, genesis of lateral roots) as well as unnatural ones (by agricultural practices or nematodes and xylem-feeding bug attack) this wounds would become entry sites for *R. solanacearum*. The bacteria get access to the wounds partially by flagella-mediated swimming motility and chemotaxic attraction towards root exudates (Elphinstone, 2005). After invading a susceptible host, *R. solanacearum* multiplies and moves systematically within the plant before bacterial wilt symptoms occur (Wilting should be considered as the most visible symptoms that usually occurs after extensive colonization of the pathogen) (Elphinstone, 2005). When the pathogen gets into the xylem through natural openings or wounds, tyloses may form to block the axial migration of bacteria within the plant (Smith *et al.*, 1997).

Wilting is due to vascular dysfunction that prevents water from reaching the leaves (Stevenson *et al.*, 2001). Ralstonia's systemic toxin also causes loss of stomata control but there is no evidence for excessive transpiration as its consequence while the primary factor contributing to wilting is probably blocking of pit membranes in the petioles and leaves by the high molecular mass EPS1 (Elphinstone, 2005). From this study it is clear that some non-host plants like maize and beans did not exhibit wilt symptoms but had the pathogen surviving within their vascular bundle. This possibility was observed in maize that exhibited significantly high root infestation despite the fact that it is a non-host a trend that was similar in sorghum and beans indicating that they also can harbour the pathogen in their roots.

Cabbage exhibited negligible amount of the pathogen in its roots and can therefore provide a very safe choice of crop for rotation with host plants in order to reduce the pathogen population. This is despite the finding that, *R. solanacearum* has a poor survival ability in soil, but it can survive on roots of alternate hosts, undecayed infected plant tissues, volunteer tubers from precedent crops or in deeper layer of soil where they do not confront the antagonism from others soil microorganisms (Wenneker *et al.*, 1999). When *R. solanacearum* population from roots was analysed, eggplant, tomato, potato and sorghum had the highest number of *R. solanacearum* attached on its roots in comparison to the others with no significant difference between them. Pradhanang *et al.* (2000) compared artificial inoculation to natural infestation in some common weeds in UK and Nepal. In that study, inoculation of summer weeds *Drymaria cordata* and *Polygonum capitata* did cause systemic infection, this gives a possibility of the pathogen surviving on roots of some non-host plants. Sorghum which is considered a non-host exhibited significant attachment making it not a suitable choice of rotational crop.

Beans and maize had very low attachment which could be attributed to their non-host nature but this still means that they cannot be used for eradication of the pathogen in the soil since the pathogen was detected in their roots. Maize and sorghum are commonly known not to suffer from bacterial wilt (Denny, 2006) can encourage survival of the pathogen as attachments on their root system. From this study, cabbage remained the safest crop for rotation with the host plants in order to eliminate the pathogen from the soil. This is because of the insignificant amount of the pathogen detected in its root system and on the rhizosphere soil. Other studies have found out that many more dicots suffer from the disease than do monocots and that the reason why some families are more susceptible to bacterial wilt is still unknown (Denny, 2006).

Potato, tomato, eggplant, and capsicum gave a clear positive result for the presence of *R. solanacearum* from the NCM-ELISA test perfomed on them, a result which is consitent with the one obtained from root invetion experiment above. This could be because they are hosts of *R. solanacearum* and are therefore highly susceptible to the pathogen (USDA, 2003).

At this time, extracellular polysaccharide (EPS1) content is about 10 µg/g tissue in the taproot, hypocotyl and midstem; EPS1 concentration is higher later on at more than 100 µg/g tissue in fully wilted plant (Elphinstone, 2005). This makes detection of the pathogen easy as the cells are already abundant in the xylem. Some samples of maize showed positive result for the presence of *R. solanacearum* while some gave negative results indicating that maize has the ability to habour *R. solanacearum* within its roots and stems. This observation is similar to the one obtained when the roots were analysed for systemic infections and from other studies that have proved that maize can be used as rotation crop for potatoes, and that bean and maize rotation has reportedly reduced soil inoculum potential but has failed to eradicate the pathogen completely (Priou *et al.*, 1999). Beans has been reported as host of *R. solanacearum* (USDA, 2003) even though Priou *et al.* (1999) found out that beans can be used together with maize in rotation program to reduce *R. solanacearum* population in the soil. This study however did not give similar result probably due to the type of ELISA test done which is suitable for potato tubers (CIP, 2001). Cabbage and sorghum on the other hand gave negative result and can for this reason be used as the best choice in rotation as a control mechanism for *R. solanacearum*. In line with the recommendation by Priou *et al.* (1999) that the best rotation crops when the aim is to reduce the soil population of *R. solanacearum* are crops in gramineceae and brassicaceae families.

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

6.1 CONCLUSIONS

The study concluded that essential oils extracted from possess antibacterial activity that is effective in control of *R. solanacearum*. And further demonstrates that there are active compounds against *R. solanacearum* contained in the essential oils of *O. suave, L. javanica* and *T. camphorates*. Biologically active essential oils represent a rich potential source of alternative and perhaps environmentally more acceptable disease management compounds that are active against the pathogen. They also pose no challenge of resistance as was observed when using synthesized antibiotics like chloramphenicol and clear contradiction of this state when using essential oils that showed no resistance to *R. solanacearum*.

The application of these essential oils should however be done at a temperature that is below 32°C to reduce the effect of their volatility. The application can be done by incoporating chopped essential oil plant parts in the soil during the time of land preparation before planting when the temperature is moderately high (between 24°C to 28°C). It is also worth noting that the amount of essential oils produced by different essential oil plants significantly differ and that one should consider the quantity of essential oil to be used depending on the plant.

On the other hand, if one decides to control *R. solanacearum* by crop rotation, of all the host and non-host plants used in this study, cabbage is the most effective non-host crop to used for eradication of *R. solanacearum* from the soil. Maize that has been considered as a safe non-host for crop rotation was found to encourage asymptomatic survival of the pathogen in it's vascular bundles and assesory tissues. Beans and sorghum were also found to encourage the survival of *R. solanacearum* in the rhizosphere and as attachment on their roots.The rest of the plants used were known host crops of *R. solanacearum* and were found not to be effective in control of the pathogen because of their proved susceptibility.

6.2 RECOMMENDATIONS

Following these findings, the following are recommended for advancement of the undertanding in this sphere of study:

1. Further study on the mode of action of these essential oils particulary those of *T. comphrates* in the control of *R. solanacearum.*
2. Appropriate method of application of the essential oil when using them to control *R. solanacearum* in the field since there was strong evidence of atimicrobial activity in the in-vitro experiment that was not directly reflected in the disease incidence.
3. Need to search for more non-host crops that can be used safely in crop rotation programs without encouraging the survival of *R. solanacearum.*

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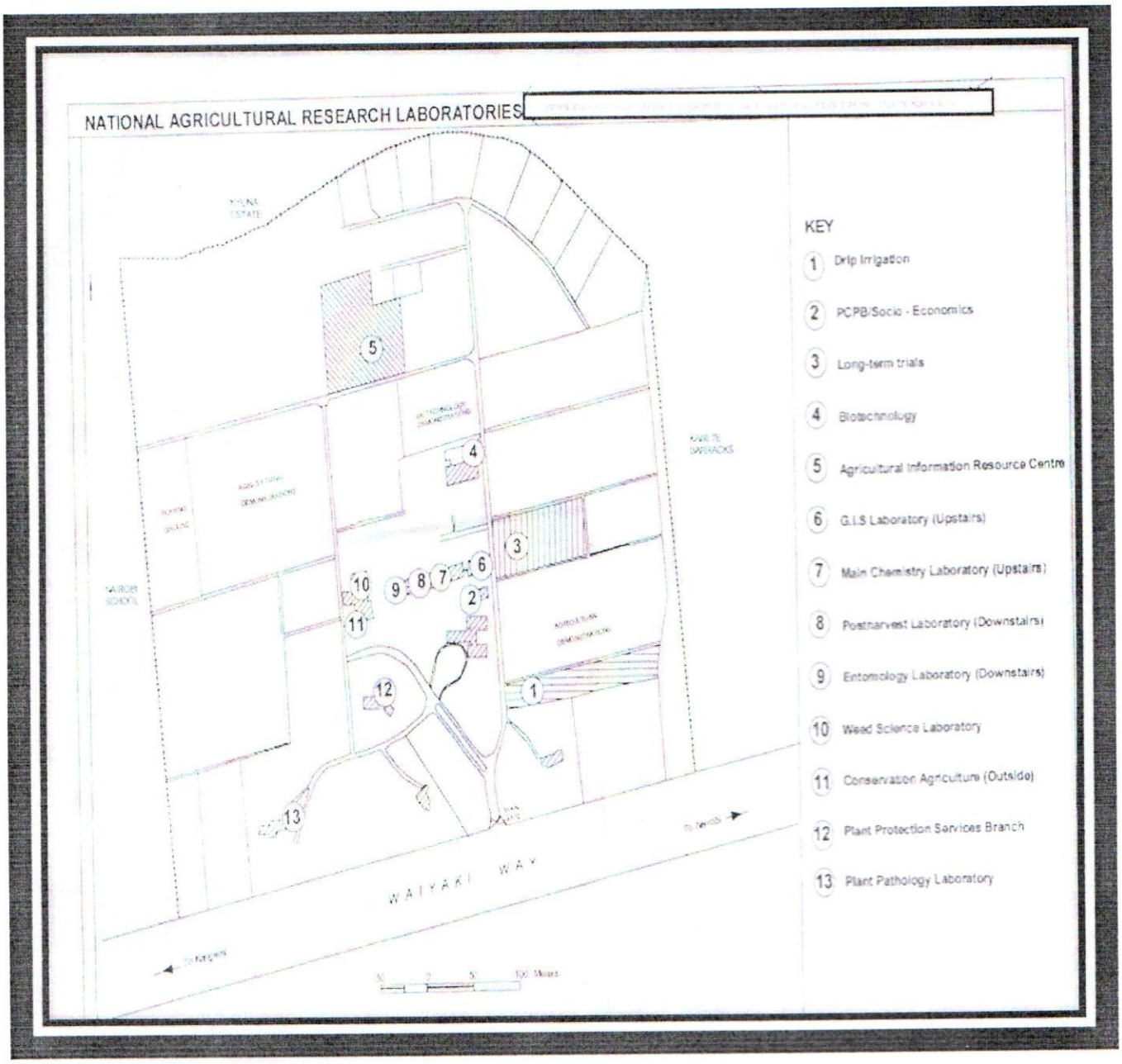
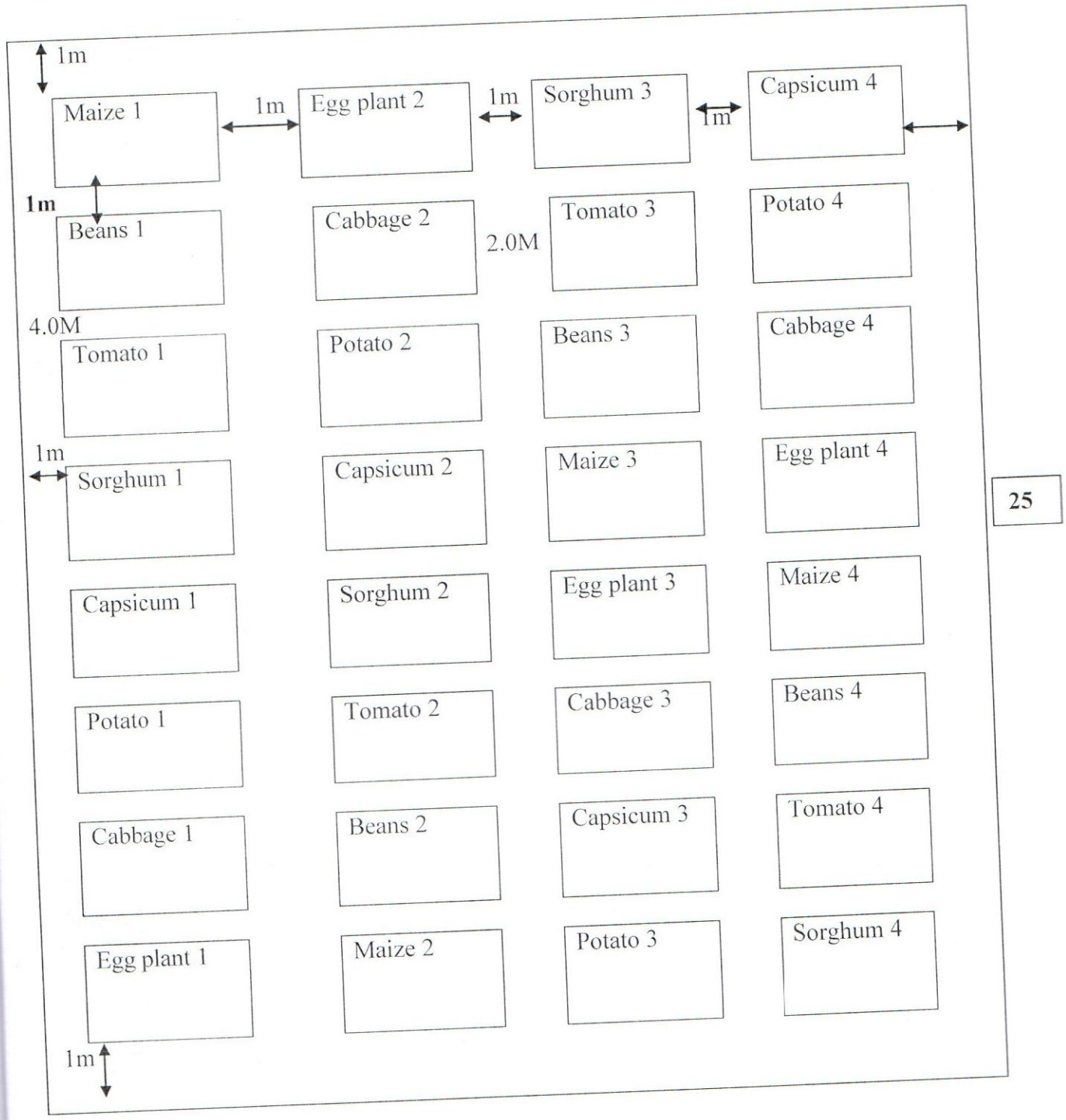
APPENDICES

Appendix 1: Map of the study site.



Appendix 2: RCBD of effect of host and non-host crops on *R. solanacearum*



Appendix 3 (i): **ANOVA of mean quantity of essential oil extracted**

Source of variation	df	ss	ms	vr	F pr
Rep	4	0.53533	0.13383	2.77	
Plant type	5	1.55867	0.31173	6.46	<.001
Residual	20	0.96467	0.04823		
Total	29	3.05867			

Appendix 3(ii): **ANOVA of mean root invasion of host and non-host plants by *R. solanacearum***

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Time	2	0.0000	0.0000	0.00	1.000
Treatment	7	3.4100	0.4871	2.40	0.035
Time. Treatment	7	0.0000	0.0000	0.00	1.000
Residual	48	9.7500	0.2031		
Total	63	13.1600			

Appendix 3(iii): **ANOVA of mean root attachment of host and non-host plants by *R. solanacearum***

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Time	2	0.0000	0.0000	0.00	1.000
Treatment	7	5.0134	0.7162	4.33	<.001
Time. Treatment	7	0.0000	0.0000	0.00	1.000
Residual	48	7.9477	0.1656		
Total	63	12.9610			

Appendix 3(iv): **ANOVA of mean *R. solanacearum* population in the soil**

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Time	1	0.0000	0.0000	0.00	1.000
Treatment	7	3.1094	0.4442	2.35	0.038
Time. Treatment	7	0.0000	0.0000	0.00	1.000
Residual	48	9.0874	0.1893		
Total	63	12.1968			

Appendix 4: Paired t-test of population of *R. solanacearum* in the soil before and two months after

		Paired Differences					t	df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference				
					Lower	Upper			
Pair 1	PREVIOUS - AFTER	-153.63	32.049	11.331	-180.42	-126.83	-13.558	7	.000

Appendix 5: Modified SMSA medium preparation

Basal medium

Add	Per litter
Casamino acid (casein hydrolysate)	1 g
Peptone	10 g
Glycerol	15 ml
Agar	15 g

Autoclaves at 121°C for 15 minutes then cool the medium to 40°C and add the antibiotics

Add	Per liter
Crystal violet	0.25ml
Polymyxin ß sulfate	2.5ml
Bacitracin	0.25ml
Chloromycetin	0.25ml
Penicillin	0.5 mg
Tetracycline	0.25ml