SCREENING OF *STREPTOMYCES* ISOLATES FROM MAU FOREST COMPLEX FOR ANTIMICROBIAL ACTIVITY AGAINST SELECTED PLANT PATHOGENS

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DECLARATION AND RECOMMENDATION

DECLARATION

I hereby certify that this thesis is my original work and has not been taken from the work of others, save and to the extent that such work has been cited and acknowledged within the context of my work.

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RECOMMENDATION

This thesis has been prepared with our guidance and assistance; it has been submitted for examination with our approval as the University supervisors.

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DEDICATION

I dedicate this thesis to my son Michael Njoroge. May you excel academically beyond me and be successful in your life.

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ABSTRACT

The search for new principles in bio-control of plant pathogens different from the classical fungicides is of worldwide interest. The genus *Streptomyces* is the producer of about 80% of all known world antibiotics. It is of a major interest in bio control of plant pathogens. The study consisted of three steps: (i) collection of soil samples from different sites in the Mau Forest Complex and isolation of *Streptomyces* from these samples, (ii) screening for antimicrobial activity of the isolates against selected plant pathogen in the laboratory i.e. *Fusarium moniliforme* and *Ascochyta rabie; Erwinia carotovora, Xanthomonas campestris* and *Pseudomonas savastanoi* pv. *phaseolicola* and other indicator reference cultures which were : *Staphylococcus aureus* ATCC 25923; *Escherichia coli* ATCC2 5922; *Pseudomonas aeruginosa* ATCC 27853 and *Bacillus subtilis* ATCC 6633. (iii) Identification of the isolates most effective in inhibiting the growth of the pathogens. A total of 270 isolates were screened for antimicrobial activity, 14 of the isolates showed antibacterial activity against the test bacteria while 39 isolates had antifungal activity. Most of the selected isolates had more effective inhibitions to fungal plant pathogens than bacterial plant pathogens. Ethyl acetate extracts of culture filtrates from the isolates produced significantly different ($P<0.05$) inhibitory effects to some of the tester microorganisms. Five of these isolates that had both antifungal and antibacterial activity were identified using molecular methods. Out of these isolates, isolate WHF2B16 was identified as *Streptomyces* Mau 1 (Accession number-KR780774), isolate BFOR3B14 as *Brachybacterium* Mau 1 (Accession number KR476396), these two isolates belong to the phylum actinobacteria. Two bacterial isolates belonged to the genus *Bacillus*; isolate WHF3A15 as *Bacillus subtilis* strain and WHF1A17 as *Bacillus* Mau 1 (Accession number KR780775), finally isolate BFOR1B22 was identified as uncultured *Acinetobacterium*. The study found that soils from Mau Forest complex harbor diverse group of bacteria including *Streptomyces* species that could be exploited for control of plant diseases. There is need to establish their effectiveness in green house and field studies.

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LIST OF ABBREVIATIONS AND ACRONYMS

ATCC	American Type Culture Collection
BLAST	Basic Local Alignment Search Tool
CFUs	Colony forming units
DNA	Deoxyribonucleic acid
GPS	Global positioning system
ISP	International Streptomyces Project
Kbp	Kilobase pair (unit of length of nucleic acids)
NCBI	National Center for Biotechnology Information
PCR	Polymerase Chain Reaction

CHAPTER ONE

INTRODUCTION

1.1 Background information

The bacterial genus *Streptomyces* is widely known for its ability to produce a variety of secondary metabolites, including valuable antibiotics, anti-tumour agents, and immuno-suppressants (Hranueli *et al.,* 2005). *Streptomyces* are one of the most attractive sources of biologically active substances such as vitamins, alkaloids, plant growth factors, enzymes and enzyme inhibitors (Shahidi *et al,* 2004). About 80% of all known antibiotics are derived from this genus (Kieser *et al.,* 2000). These include a diverse array of antibiotics including aminoglycosides, anthracyclins, glycopeptides, β-lactams, macrolides, nucleosides, peptides, polyenes, polyethers and tetracyclines. *Streptomyces* are the producers of more than 5,000 known bioactive compounds (Anderson and Wellington, 2001), and estimates of the total number of antimicrobial compounds produced by representatives of *Streptomyces* screened for new antibiotics are in the order of 100,000 (Watve *et al.,* 2001). In addition, not only has the overall versatility of these compounds been studied in great detail, but also a high proportion of them have known biological effects, which is unparalleled in the living world (Kieser *et al.,* 2000).

Plant diseases have resulted in epidemics in many countries in the world. This has in turn led to famines and loss of economic returns. Plant diseases are caused by a diverse collection of microbes including fungi, bacteria, viruses, viroids, nematodes and parasitic plants. The largest numbers of plant diseases are caused by fungi, and the most common chemical tools for plant disease control are fungicides. Synthetic protectant fungicides such as ferbam, zineb, maneb, captan, moncozeb, chlorothalonil, copper are used widely. These compounds are applied at high rates (pounds per acre or per 100 gallons). They leave residues on the crops, possibly preventing use of the crop residues for forage and are often phyto-toxic. The high application rates raise concerns about environmental effects and chronic low dose health effects on humans (Dutky, 2006).

Development of resistance to systemic fungicides can occur in a population of pathogenic fungi. This is because some individuals within a population have alternate metabolic pathways; if these alternate pathways are not destroyed by the fungicide the individuals develop resistance. The susceptible fungi are eliminated while the resistant types survive and propagate. Spontaneous mutations also play a role in the development of fungicide resistance.

If the resistant type is also fit, it can remain a major part of the population even after exposure to the fungicide is halted (Dutky, 2006).

Resistance development among the plant pathogens due to usage of chemical pesticides coupled with the environmental pollution and pesticide residues in the treated crops has been an issue of great concern necessitating the need to investigate other strategies such as use of natural products including antimicrobial metabolites. Metabolites from antagonistic microorganisms have shown promising results in the management of plant diseases and their rapid breakdown renders them environmentally friendly and safe to use (Muiru *et al.,* 2007).

The search for new principles in biocontrol of plant pathogens different from the classical used fungicides is of worldwide concern and hence the genus *Streptomyces* is of major interest in biocontrol of plant pathogens. Application of antibiotics in plant disease control has been expanding rapidly and hundreds of antibiotics are already commercially available for plant disease control (Mustafa *et al*., 2004). Natural forests, extreme environments, and other undisturbed sites are known to harbour a high diversity of organisms and a repertoire of microbes suitable for search of new microbes with industrial potential. This study aimed at exploring the Mau Forest Complex for potentially useful streptomycetes.

Mau Forest Complex is the largest closed-canopy forest ecosystem in Kenya and the largest indigenous forest in East Africa, stretching across 400,000 hectares (1,544 square miles (Kanyinke, 2005). The importance of the Mau Forest Complex lies in the ecosystem services it provides to Kenya and the East Africa region, these include; river flow regulation, flood mitigation, water storage, reduced soil erosion, biodiversity, carbon sequestration, carbon reservoir and microclimate regulation. The Mau Forest Complex is of critical importance for sustaining current and future ecological, social and economic development in Kenya. Over the last two decades, the Mau Forest Complex has lost approximately 25 % of its forest cover around 107,000 hectares (413 square miles) due to irregular and unplanned settlements, illegal resources extraction, in particular logging and charcoal burning, the change of land use from forest to unsustainable agriculture and change in ownership from public to private. Perennial rivers are becoming seasonal, storm flows and downstream flooding are increasing and wells and springs are drying up. The water stress in the Mau Forest Complex is attributed to land degradation and deforestation (Nkako *et al.*, 2005).

Microbial resources in the Mau Forest Complex remain unexploited and hence underutilized. There is no documented information on the microbial resources in the Mau Forest Complex. The study involved isolation of *Streptomyces* from the Mau Forest

Complex, identification of these bacteria using morphological, cultural, biochemical and molecular procedures and finally screening for the antimicrobial effect against selected plant pathogenic bacteria and fungi. Mau Forest Complex provided a good study area where the diversity and distribution of *Streptomyces* was observed. Different sites were used in this study; these include soil from natural areas, floriculture soil, agricultural farm soil and tea farm soil. The study obtained information to show the diversity and distribution of *Streptomyces* in the different sites, the antimicrobial potential of the isolates against plant pathogenic fungi and bacteria, and biochemical, morphological and molecular characterization of the bioactive isolates from the study.

1.2. Statement of the problem

Plant diseases have resulted in epidemics in Kenya leading to famines and loss of economic returns for example wheat rust disease and maize lethal necrosis disease. The use and misuse of agrochemicals to control plant diseases has led to resistance development in plant pathogens against these chemicals, and increased environmental pollution. Agrochemicals are also expensive to use. Developing plants that are resistant to diseases is also very costly and may take a long time. There is a need to search for new methods in plant disease management. Bio-control of plant diseases using antagonistic organisms provides such an option. *Streptomyces* produce metabolites that have the potential to be used in plant disease management. In addition, microbial resources in Kenya are underexploited. Information on useful antimicrobial producing bacteria like *Streptomyces* and their application is limited. There is no documented information on the diversity and distribution of *Streptomyces* from the Mau Forest Complex. Documented information in the use of *Streptomyces* spp. in managing plant diseases in other countries is an eye opener in exploring the same option in Kenya.

1.3. Objectives

1.3.1. General objective

To isolate *Streptomyces* from Mau Forest Complex, screen them for activity against selected plant pathogens and characterize the isolates using biochemical and molecular methods.

1.3.2. Specific objectives

1. To determine distribution and diversity of *Streptomyces* spp. in soil from the Mau Forest Complex.

2. To screen for antimicrobial activity of *Streptomyces* spp. against selected plant pathogenic bacteria and fungi.
3. To carry out molecular characterization of the *Streptomyces* spp. with antimicrobial activity.

1.4. Hypotheses

1. There was no significant difference in distribution and diversity of *Streptomyces* spp. in soil from selected sites in Mau Forest Complex.
2. The *Streptomyces* spp. isolated have no significant antimicrobial activity against plant pathogenic bacteria and fungi.
3. Isolates with antimicrobial activity did not belong to different species of genus *Streptomyces*

1.5. Justification

Plant diseases need to be controlled so as to improve the quality and abundance of food, feed and fiber produced by farmers around the world. Farmers often rely heavily on chemical pesticides. The environmental pollution caused by excessive use and misuse of agrochemicals and appearance of resistant strains to these chemicals has led to an increased concern and the search for alternative means of managing plant diseases. Presently, as is the case for all microbial and other biodiversity resources, there is little documented information on the occurrence of *Streptomyces* spp. with potential to produce antimicrobial compounds in Kenya. The Mau Forest Complex provided a good study area that helped to compare the effect of different cropping systems on microbial distribution and diversity.

The study is important because it involved the search for antibiotics producing *Streptomyces* from the Mau Forest Complex, which has the potential to be suppressive to plant pathogens and the disease they cause. There is an increase in the number of resistant and multi-resistant strains of bacteria and this is a major concern of health officials worldwide, particularly with the decline in the number of new antibiotics available for treatment. Not only can the *Streptomyces* identified be used in treating plant diseases, but also the pharmaceutical industry in the country can try them in managing animal and human diseases.

CHAPTER TWO

LITERATURE REVIEW

2.1 Mau Forest Complex

Mau forest is the largest closed-canopy forest ecosystem in Kenya and the largest indigenous forest in East Africa, stretching across 400,000 hectares (1,544 square miles). The latitude is between 0º 20′ 60″S and the longitude is between 35º 27′ 32″E. It lies between 2,000 m and 2,600 m above the sea level, on the Western slope of the Mau Escarpment. The Mau has deep, fertile, volcanic soils, and annual precipitation ranges from 1,000 mm in the east, with a seasonal regime, to 2,000 mm in the west, where it is more-or-less continuous around the year. Soil samples from natural forest, tea farm, floriculture farm and an agricultural farm was used in the proposed study.

2.2 Biology of the genus *Streptomyces*

The genus *Streptomyces* belong to the order Actinomycetales within the class Actinobacteria. This is a group of filamentous, Gram positive bacteria and has a DNA G+C content of 63-78 mol%. Most actinomycetes form spores; the manner of spore formation varies and is used in separating subgroups (Stackebrandt *et al.,* 1997). The genus *Streptomyces* contains a large number of species and varieties, over 500 species of *Streptomyces* are recognized (Kieser *et al.,* 2000).

The vegetative hyphae (0.5–2.0 μm in diameter) produce an extensively branched mycelium that rarely fragments. The aerial mycelium at maturity forms chains of three to many spores. Some species may bear short chains of spores on the substrate mycelium. Sclerotia, pycnidia, sporangia, and synnemata like structures may be formed by some species. The spores are non-motile. On complex agar media, discrete and lichenoid, leathery or butyrous colonies are formed. Colonies are initially relatively smooth surfaced, but later they develop an aerial mycelium that may appear floccose, granular, powdery or velvety (Ambarwati *et al.,* 2012). Strains belonging to the genus *Streptomyces* may produce a wide variety of pigments responsible for the color of the vegetative and aerial mycelia (Flardh and Buttner, 2009). They are catalase positive, and generally, reduce nitrates to nitrites. Most representatives can degrade polymeric substrates like casein, gelatin, hypoxanthine, starch and also cellulose (Smaoui *et al.,* 2012). Many strains produce one or more antibiotics (Mohanraj and Sekar, 2013)

They have oxidative metabolism and are chemoorganotrophs that are strictly aerobic in growth (Ikeda *et al.,* 2003). The optimum temperature for most species is 25–35°C; however, several thermophilic and psychrophilic species are known (Kampfer, 2006). The optimum pH range for growth is 6.5–8.0 (Cabello-Gonzalez and Genilloud, 2003). Growth of *Streptomyces* occurs at the tips of the filaments, often accompanied by branching. Thus, the vegetative phase consists of a complex, tightly woven matrix, resulting in a compact, convoluted mycelium and subsequent colony. As the colony ages, characteristic aerial filaments called sporophores are formed, which project above the surface of the colony and give rise to spores (Madigan and Martinko, 2007).

2.3 Ecology of the *Streptomyces*

Members of the family Streptomycetaceae are ubiquitous in nature. Streptomycetes can be isolated in high numbers in soil, which is their natural habitat, though a few can be isolated in aquatic habitats (Vetsigian and Kishony, 2011). Members of the genus *Streptomyces* are involved in the biodegradation of various polymers abundant in soil owing to their ability to produce extracellular enzymes. In most soils, 10^4 to 10^7 colony forming units (CFUs) per gram can be expected, accounting for about 1–20% of the total viable count; in some soils however streptomycetes dominate (Korn-Wendisch and Kutzner, 1992).

Streptomycetes, like other soil bacteria, may also be found in the intestinal tracts of earthworms, the gut of arthropods and the pellets produced by millipedes and woodlice (Kampfer, 2006). With respect to moisture, Streptomycetes resist desiccation because they form arthrospores. In addition they need a lower water tension for growth than other bacteria, but they are sensitive to water-logged conditions (Subbarao, 1999). Streptomycetes have also been found to occur in the rhizosphere of plants, they have been suggested to play an important role in controlling root fungal pathogens of plants (Ghadbane *et al.,* 2015). In addition, many Streptomycetes are often successful in competition with other rhizospheric bacteria such as pseudomonads and bacilli, especially in relatively dry soil. An added advantage over Gram negative bacteria is their ability to spread through relatively dry soil with hyphal growth (Kieser *et al.,* 2000).

A small proportion of the described *Streptomyces* species are known to be plant or animal pathogens. *Streptomyces scabies* is the causal agent of common scab of potato, this species is the best studied of the pathogenic *Streptomyces. S. acidoscabies* produces symptoms like those of *S. scabies* on potato and on other taproot crops. *S. ipomeae* causes soil rot of sweet potato, which is characterized by necrosis of fibrous roots and cankers on

expanded storage roots (Fatope *et al.*, 2000). *Streptomyces somaliensis* and *Streptomyces sudanensis* causes a disease in humans known as actinomycetoma (Quintana *et al.*, 2008). The diseases is a chronic subcutaneous infection which results in a granulomatous inflammatory response in the deep dermis and subcutaneous tissue, which can extend to the underlying bone and cause disfigurement to the tissues. It is characterized by the formation of grains containing aggregates of the causative organisms that may be discharged onto the skin surface through multiple sinuses (Basilio *et al.*, 2015).

2.4 Characteristics of *Streptomyces* species

The genus contains mainly mesophilic species in addition to some thermotolerant (growing up to 45°C) and a few thermophilic species. The thermophilic streptomycetes described so far grow at 28–55°C, and several grow at even higher temperatures (Kampfer, 2006). In thermophillic streptomycetes active growth takes place at sites of high temperature such as in compost, manure, and self-heating hay or grain (Kim and Goodfellow, 2002). The vegetative phase ends with the formation of a large number of spores. These are returned with the compost or manure to the fields and pastures where they infect plant material and hay directly or via soil dust. For this reason thermophilic actinomycetes are widespread and can be isolated from various sources like soils, pig feaces, sewage-sludge compost, and freshwater habitats (Kampfer, 2006).

The characteristic earthy odour of soil is caused by the production of a series of streptomycete metabolites called geosmins. These substances are sesquiterpernoid compounds – unsaturated ring compounds of carbon, oxygen and hydrogen. A common geosmin is trans-1, 10-dimethyl-trans-9-decalol. Geosmins are also produced by cyanobacteria (Madigan and Martinko, 2007). Alkaline and neutral soils are more favorable for the development of *Streptomyces* than are acidic soils. Well drained soils, like sandy loams or soils covering limestone, have higher numbers of *Streptomyces*. This group of bacteria requires lower water potential for growth than many other soil bacteria.

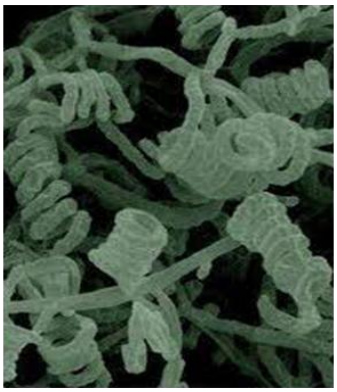
Nutritionally, the *Streptomyces* are quite versatile. They don't require growth factors and can utilize a wide variety of carbon sources, such as sugars, alcohols, organic acids, amino acids and some aromatic compounds. Most isolates produce extracellular hydrolytic enzymes that permit utilization of polysaccharides (starch, cellulose, and hemicelluloses), proteins and fats, and some strains can use hydrocarbons, lignin, tannin, or even rubber. *Streptomyces* can often be obtained by spreading a soil dilution on an alkaline agar medium containing polymers such as casein and starch (Madigan and Martinko, 2007).

2.5 Identification procedures for *Streptomyces*

2.5.1 Morphology

Colony morphology (colour of the aerial mycelium, color of the substrate mycelium, and soluble pigment) is very useful in identification. A microscopic characterization (particularly the morphologies of the aerial mycelium, arthrospores and vegetative mycelium) is of high value in identification (Korn-Wendisch and Kutzner, 1992).

According to Shirling and Gottlieb, (1966) characterization of the *Streptomyces* species requires the use of the standard culture media for morphological studies. Yeast malt extract agar, oatmeal agar, inorganic salts agar and glycerol-asparagine agar can be used. Determination of spore bearing hyphae can be done by direct microscopic examination of the culture at x100-x700 magnification. The spores bearing hyphae can either be: Straight-Rectus (R), Flexible-Flexible (F), Open loops Retinaculum-Apertum (RA), Spirals (S), Monoverticillus (MV), Monoverticillus-Spira (MV-S), Biverticillus (BV), Biverticillus- Spira (BV-S).



Biverticillus- Spira

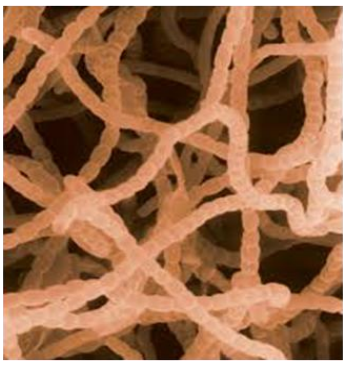


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Figure 1: Examples of spores in *Streptomyces* (Kampfer, 2006).

Spore morphology and surface is studied using an electron microscope. The spores of *Streptomyces* can be smooth, warty, spinous or hairy (Kampfer, 2006). Colonies of *Streptomyces* are discrete and they may form diffusible pigment in the media. The formation of the pigment depends on nutritional and growth conditions.

2.5.2. Biochemical identification

Streptomyces have ability to utilize various compounds as sources of carbon. Majority of *Streptomyces* have positive catalase reactions and they are able to reduce nitrates to nitrites. *Streptomyce*s have the ability to utilize different organic substances as the sole

carbon source. Casein, gelatin, starch, hypoxanthine and cellulose have been shown to be degraded by many of the members of streptomycetes (Korn-Wendisch and Kutzner, 1992). Glycolysis (Embden-Meyerhof-Parnas) and the hexose monophosphate shunt pathway is mostly used in *Streptomyces* for energy generation (Salas *et al.,* 1984)

2.5.3 Molecular Identification

The development of molecular methods to analyze bacterial genomes has provided a new basis for studying bacterial taxonomy and in some cases phylogenetic relationships of the prokaryotes at the genus, species and subspecies level. rRNA sequence comparisons are important in the taxonomy of *Streptomyces.* Genes for 16S RNAs are highly conserved within bacteria. Within the genus *Streptomyces*, three regions within the gene have enough sequence variation to be useful as genus-specific (*a* and *b* regions) and species-specific (*c* regions) probes (Anderson and Wellington, 2001). rRNA sequences cannot be used alone because of the intraspecific variation and intragenomic heterogeneity (Kieser *et al.,* 2000). DNA-DNA hybridizations of total chromosomal DNA have also been used within the genus *Streptomyces*. Randomly Amplified Polymorphic DNA (RAPD) Polymerase Chain Reaction (PCR) (RAPD-PCR) is used as a rapid screening method to detect similarity among *Streptomyces* strains. Single primers with arbitrary nucleotide sequences to amplify DNA are used in addition to a low annealing temperature so that polymorphisms can be detected (Kampfer, 2006). Stringent standardization of the reaction parameters is required. These include primer sequence, annealing temperatures, buffer components, concentration and quality of template DNA. The resulting characteristic fingerprint of PCR products enables detection of chromosomal differences between individual isolates without having any prior knowledge of the chromosomal sequence (Williams *et al.,* 1990). This method however is disadvantaged in that it cannot resolve interspecific relationships. The detection of LL-A2pm in cell wall or whole-cell hydrolysates, the lack of mycolic acids, the predominance of mainly *iso* and *anteiso*-methyl branched fatty acids, and the 16S rRNA sequence are used for genus identification.

2.6 Secondary metabolites produced by *Streptomyces*

Secondary metabolites are metabolic products produced at idiophase when cells have completed active growth (Madigan and Martinko, 2007). Actinomycetes are the most economical and biotechnologically valuable class of prokaryotes producing bioactive secondary metabolites such as antibiotics, anti-tumor agents, immunosuppressive agents and

enzymes (Procópioa *et al.,* 2012; Nagpure *et al.,* 2014; Aftab *et al.,* 2015). Antibiotics production in *Streptomyces* is generally a growth-dependent phase. In liquid cultures it begins as the culture enter stationary phase while in agar cultures it start upon the onset of the morphological differentiation (Kieser *et al.,* 2000). The onset of antibiotic biosynthesis is determined and influenced by a variety of physiological and environmental factors. These include growth rate and diffusible-γ-butyolactone signaling molecules, imbalances in metabolism and various physiological stresses. In addition to these positive effectors of antibiotic production, antibiotic synthesis may also be subject to metabolite repression and/or inhibition by readily utilized sources of nitrogen (generally NH_4^+), phosphate and/or glucose (Kieser *et al.,* 2000).

Actinomycetes are the main source of clinically important antibiotics, most of which are too complex to be synthesized by combinatorial chemistry; the *Streptomyces* are especially prolific, producing around 80% of total antibiotic products (Bull and Stach, 2005; Kim and Garson, 2005). Streptomycin was the first antibiotic which was classified from a *Streptomyces* strain by Waksman and Henrici (1943). Since then, streptomycetes have proved to be the richest sources of thousands of low molecular weight, chemically different compounds having antibacterial, antifungal, antiparasitic, agro-active, cytostatic or other biological activities. Additionally, they have provided many enzymes which are required for the natural turnover of many macromolecules in soil (Kampfer, 2006).

Streptomyces is the largest antibiotic-producing genus in the microbial world discovered so far. The number of antimicrobial compounds reported from the species of this genus per year has increased almost exponentially for about two decades (Kampfer, 2006). This group of microorganisms still remains an important source of antibiotics (Watve *et al.,* 2001). Evidence for antibiotic production is often seen on the agar plates used in the initial isolation of *Streptomyces* (Zarandi *et al*., 2009). Adjacent colonies of other microorganisms show zones of inhibition.

The same antibiotics may be formed by different species found in widely scattered parts of the world. An antibiotic producing organism is resistant to its own antibiotics but sensitive to antibiotics produced by other *Streptomyces.* More than 60 streptomycete antibiotics have found practical application in human and veterinary medicine, agriculture and industry (Madigan and Martinko, 2007) (Table 1.).

As a result of the increasing prevalence of antibiotic-resistant pathogens and the pharmacological limitations of antibiotics, there is a need for new antimicrobial substances.

The results of extensive screening have been the discovery of about 4,000 antibiotic substances from bacteria and fungi, many of which have found applications in medicine; 80% are produced by *Streptomyces* (Kieser *et al.*, 2000). Streptomycetes synthesize an amazing variety of chemically distinct substances, many of which act as antibiotics, fungicides, herbicides, cytostatics, or modulators of immune responses, and a huge diversity of ever increasing numbers of inhibitors for many different cellular processes. New *Streptomyces* isolates provide sources of novel natural compounds (Schrempf, 2006).

Table 1: Examples of useful antibiotics from the genus *Streptomyces* (adapted from Kieser *et al.*, 2000)

ANTIBIOTIC	PRODUCER	CHEMICAL CLASS	TARGET	APPLICATION
ActinomycinD	*S.* spp	Peptide	Transcription	Antitumour
Bialophos	*S. hygroscopicus*	Peptide	Glutamine synthetase	Herbicidal
Candicidin	*S. griseus*	Polyene macrolide (PK)	Membrane (pore former)	Antifungal
Chlortetracycline	*S. aurefaciens*	Tetracycline (PK)	R	Antibacterial
Clavulanic acid	*S. clavuligenus*	β-lactam Anthracycline (PK)	β-lactamase inhibitor	Combined with a β-lactam as antibacterial
HygromycinB	*S. hygroscopicus*	Polyene	R	Antihelminthic
Nikkomycin	*S. tendae*	macrolide (PK) Nucleoside	Chitin biosynthesis	Antifungal insecticides
Nystatin	*S. noursei*	peptide Amino glycoside	Membrane (pore former)	Antifungal
Polyoxins	*S. cacoi* var. *asoensis*	Nacrolide (PK)	Chitin biosynthesis	Antifungal (plant protection)
Streptomycin	*S. griseus*		R	Antibacterial
Tylosin	*S. fradiae*	Aminoglycoside	R	Growth promotant
Validamycin	*S. hygroscopicus*		R	Plant protectant

[1]PK= polyketide

[2]R= binds to ribosomes and thus inhibits protein synthesis

S= *Streptomyces*

2.7 Application of *Streptomyces* antibiotics in plant disease control

Plant diseases need to be controlled to maintain the quality and abundance of food, feed, and fiber produced. Farmers often rely heavily on chemicals fertilizers and pesticides.

However, the environmental hazards caused by excessive use and misuse of agrochemicals and pesticides has led to strict regulations and banning of some of these chemical substances (Pal and Gardner, 2006).

Biological control of plant diseases provides an alternative to these chemicals; this is the use of microbial antagonists to suppress diseases. Antibiotics are microbial toxins that at low concentration kill or poison other microorganisms (Pal and Gardner, 2006). *Streptomyces* antagonizes plant pathogens by producing antimicrobial secondary metabolites that inhibit the growth of the plant pathogens. In addition, *Streptomyces* also colonizes the rhizosphere of the plants displacing the pathogens and utilizes the available nutrients (Castillo *et al.,* 2002).

2.7.1. Use of *Streptomyces* in control of fungal plant pathogens

Zarandi *et al.,* (2009) isolated *Streptomyces sindeneusis* (isolate 263) from soil in Iran, and found potential antifungal metabolites against the rice blast causative agent, *Magnaporthe oryzae*. This particular bacterium showed antifungal and inhibitory effects on *M. oryzae.* Antifungal activity of *Streptomyces sindeneusis* (isolate 263) shows the importance of investigating more candidates for use in control of plant pathogenic fungi.

Wagacha *et al.,* (2007) carried out a study to control bean rust using antibiotics produced by *Bacillus* and *Streptomyces* species. Culture filtrates from *Bacillus* (CA5) and *Streptomyces* (CS35) inhibited the growth of *Fusarium oxysporum* and *Pythium* spp. in culture. Rust infection was completely inhibited on the treated leaves. Both culture filtrates from *Streptomyces* and *Bacillus* showed significant persistence by inhibiting rust infection for up to 8 days after application (Wagacha *et al.,* 2007).

A study to establish the efficacy of culture filtrates produced by antagonistic *Streptomyces* spp. against late blight of tomatoes under field conditions was done and showed that they had positive activity against late blight (Mutitu *et al.,* 2003). Antifungal activity of *Streptomyces* spp. isolated from the rhizosphere of Thai medicinal plants showed *in vitro* inhibitory activity against six plant pathogenic fungi that included *Alternaria brassicola* (rose apple anthracnose), *Alternaria porri* (shallot blotch), *Colletotrichum gloeosporioides* (potato dry rot), *Fusarium oxysporum* (Chinese cabbage leaf spot), *Penicillium digitatum* (orange green mould), and *Sclerotium rolfsii* (damping-off of balsam) (Khamma *et al.,* 2009).

Maize is a major staple food grown in the country today. In Kenya, maize is grown by small scale farmers as a source of food and also to provide income. However, fungal diseases are a limiting factor in the production of maize. *Fusarium moniliforme* J. Sheldon (*Giberella fujikuroi*) (Sawada Wollen), commonly infects a wide range of crops and is a major parasite

of the Gramineae, particularly in tropical and subtropical regions. The fungus causes seedling blight in maize *Zea mays* L. as well as root; stalk, ear and kernel rot (Gauperin *et al.,* 2003). Biological control of soil borne plant pathogens has shown to be a potential alternative disease management strategy. Actinomycetes are naturally present in soils and when tested in in-vitro condition strains of the genus *Streptomyces* have shown the potential to produce antibiotics which reduce or inhibit the growth and development of soil borne plant pathogens (Kim *et al.,* 2000; Bressan, 2003). *Fusarium* is managed by using cultural practices, fungicides or an integrated pest management approach. *Streptomyces* has been used in greenhouse management of *Fusarium* (Gopalakrishnan *et al.,* 2013). The *Streptomyces* had antagonistic activity against *Fusarium* in chickpeas in the greenhouse and also promoted agronomical traits against controls.

Ascochyta blight is one of the most frequent and damaging disease of chickpea worldwide (Jan and Wiese 1991). It is caused by *Ascochyta rabiei*, a fungus that selectively attacks chickpea. It persists in the crop's residues, seed, and volunteer plants. Infections may arise from seed borne inoculum or from windborne spores (ascospores). All parts of the plant are subject to attack by the pathogen which develops elongated, sunken, dark lesions. Within the lesions, the fungus produces fruiting bodies (pycnidia) that become visible as tiny, black, raised spots, often arranged in concentric rings. The fungus is prevalent in areas where cool, cloudy and humid weather occurs during the crop season and attacks the crop at both vegetative and podding stages. Extremely wet conditions favour disease development and spread (Ali *et al.,* 2011). Ascochyta blight is controlled by dressing seeds with fungicides, and by crop rotation. Biological control of the pathogen using members of the genus *Streptomyces* remains unexploited potential.

2.8 Use of *Streptomyces* in control of bacterial plant diseases

Liu *et al.,* (1995), used two suppressive strains of *Streptomyces (S. diastatochromogenes* strain PonSSII and *S. scabies* strain PonR) to control potato common scab which is a bacterial plant disease caused by *Streptomyces scabies*. Castillo *et al.,* (2002) described new antibiotics called munumbicins A, B, C and D obtained from *Streptomyces* NRRL 30562. The compounds had wide spectrum of activity against many human as well as plant pathogenic fungi and bacteria, and a *Plasmodium* spp. Each Gram-positive bacterium tested (*Bacillus anthracis, Erwinia carotovora, Streptococcus pneumoniae, Enterococcus faecalis* and *Staphylococcus aureus*) were sensitive to one or more of the munumbicins.

Pseudomonas savastanoi pv. *phaseolicola* formerly *Pseudomonas syringae* pv. *phaseolicola* is a rod shaped, Gram negative bacteria with polar flagella, it is the causes the halo blight bacterial disease of common bean (*Phaseolus vulgaris*) in temperate regions and above medium altitudes in the tropics (Arnold *et al.,* 2011). Gotta and Tamietti (1990), used actinomycetes isolated from Sardinian soils in Italy to inhibit in vitro growth of *Pseudomonas savastanoi* pv. *phaseolicola*. One of the actinomycetes was identified as *Streptomyces anulatus* and had an inhibited the growth of the phytopathogen by 60%.

Erwinia carotovora is a Gram negative bacterium that belongs to the family Enterobacteriacea. It causes soft rot of vegetables, fruits and post-harvest foods. Management of the pathogen is by use of cultural method and integrated pest management. Doolotkeldieva *et al.,* (2016) found that *Streptomyces diastatochromogenes* strain sk-6, and *Streptomyces graminearuss* strain sk-2, had a highly significant effect on soft rot bacteria isolates (*E. carotovora).* The pretreatment of potato tubers with the antagonistic bacteria successfully prevented the initial infection and multiplication of soft rot bacteria and reduced soft rot disease of potatoes in storage

CHAPTER THREE

MATERIALS AND METHODS

3.1. Study area

The Mau Forest Complex provided a good study area in that there are different cropping systems which can be used to compare their effect on distribution and diversity of microorganisms. Soil samples were collected from within the Mau Forest complex (Figure 2). Sites used in the study were a tea farm and a natural forest in the Tea Research Foundation in Kericho. Soil samples were collected from these sites. In addition, soil samples were also collected from a wheat farm in the Mwisho wa Lami area in Mau Narok, two samples were also collected from the Botanic Garden in Egerton University, one from the forest area and another from the grass area in the garden. GPS coordinates from the point of soil sample correction are provided in Table 2.

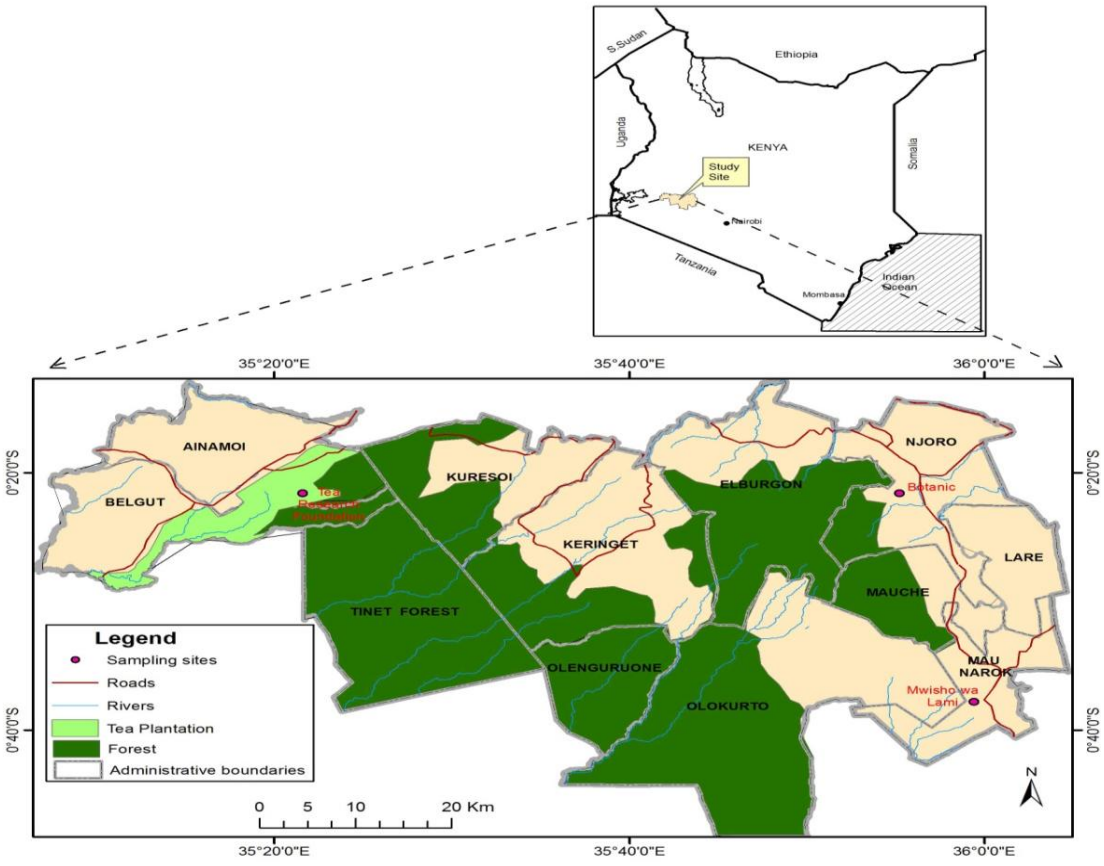


Figure 2: Mau Forest Complex from (Courtesy of GIM maps and modified by G. M. Maina, Environmental Science Department, Egerton University).

Table 2: GPS coordinates for the study sites

Sites	Code	GPS coordinates
Tea farm	TRF	35º21´27.87E, 0º20´49.67N
Natural forest	FOR	35º21´31.93E, 0º21´49.45N
Wheat farm	WHF	35º89´21.37E, 0º36´21.48N
Botanic Forest Area	BFOR	35º67´17.35E, 0º20´33.18N
Botanic Garden area	BGR	35º67´20.50E, 0º20´33.35N

3.2. Soil sampling

Stratified random sampling of the soil was done. Soil samples were collected from different sites in the Mau forest complex. These sites were: natural forests, agricultural areas, botanic and tea farm soil (Table 2). In each of these cases, three soil samples were obtained in sterile containers. Random soil samples were collected using a sterile Soil Auger (20 cm in depth, 2.5 cm in diameter) (Lee and Hwang, 2002). Characteristics of the site/ sample were recorded e.g. vegetation coverage, dominant plant species, pH, grain sizes, organic matter and water content of soil. The geographical location and GPS coordinates of the site was also recorded. Soil samples were taken from at two depths; 0-10 cm and 10-20 cm below the soil surface. The samples were air-dried at room temperature for 7-10 days and passed through a 0.8 mm mesh sieve and preserved in sterile polyethylene bags at room temperature before use.

3.3 Serial Dilution and isolation of *Streptomyces*

Ten grams of the samples of air-dried soil was mixed with sterile distilled water (90 ml). The mixture was shaken for 1 hour on a rotary shaker and allowed to settle for another 1 hour. Serial dilutions of the soil was done by taking 1 ml of soil suspensions (diluted 10^{-1}) and transferred to 9 ml of sterile water and subsequently diluted to 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6}. Inoculates consisted of adding aliquots of 10^{-3}-10^{-6} soil dilutions to autoclaved starch-casein agar (Kuster and William, 1964; William and Davies, 1965) supplemented with filter-sterilized (0.2 µm filters) antibiotic solution containing cycloheximide (0.005% final concentration), nystatin (0.005% final concentration), polymixin- β sulphate (0.0005% final concentration) and sodium penicillin (0.0001% final concentration) to inhibit non-actinomycete bacteria and fungi (Baltz, 2006). An aliquot of 0.1 ml of diluted samples was

spread plated on the test media. After incubation for 4 – 7 days at 28^{o}C, the colonies that had developed on the plates were enumerated and computed as colony forming units (CFU's) per gram of soil.

The isolated colonies of *Streptomyces* were transferred from the isolation media to a sterile growth media consisting of glucose (10g), yeast extract (1g), potassium nitrate (1g), potassium mono hydrogen phosphate (0.1g) and agar (15g) per liter. The plates were incubated for 3-5 days at room temperature for growth.

3.4 Characterization of Isolates

Gram staining test was done on the isolates. The isolates were tested for catalase production and the spores bearing mycelia viewed under light microscope at x250 magnification.

3.5. Test organisms

Two plant pathogenic fungi and two plant pathogenic bacteria were used for screening of antimicrobial effect of the isolates. These were: *Fusarium moniliforme* and *Ascochyta rabiei* which cause wilts in different plants. *Erwinia carotovora,* the causative agent of soft rot of Brassicas, and *Pseudomonas savastanoi* pv. *phaseolicola,* the causative agent of halo blight of beans, were the bacterial plant pathogens. Pure cultures of the fungal pathogens were made by isolation from infected plant materials and the pathogens were maintained on potato dextrose agar (Difco-39 g PDA L-1 of distilled H2O, pH 7.2). *Erwinia carotovora* was isolated from cabbages affected by the soft rot bacteria, while *Pseudomonas savastanoi* pv. *phaseolicola* was isolated from infected beans and identification done by re-infecting beans to see if they developed characteristic haloblight symptoms. The test organisms were maintained on nutrient agar. In addition to these plant pathogenic fungi and bacteria, *Staphylococcus aureus* ATCC 25923; *Escherichia coli* ATCC2 5922; *Pseudomonas aeruginosa* ATCC 27853; *Bacillus subtilis* ATCC 6633 were also used to assess for production antimicrobial compounds.

3.6 Antimicrobial bioassays

The isolates were grown in assay agar whose components were; peptone (10g), glucose (1g), agar (15g) per liter.

3.6.1 Evaluation of the antifungal activity

To test for activity of isolated *Streptomyces* against the selected plant pathogenic fungi, bioassays were performed using agar disc method. The fungal test pathogen was grown in PDA (potato dextrose agar) for three days; 8 mm disc plug of the fungi were picked using a sterile cork borer and placed in an 8 mm hole bored in the middle of a PDA plate. A disc of *Streptomyces* colony on agar was obtained using an 8 mm diameter cork borer and placed in the PDA media containing fungal test pathogens. Antifungal activity around the *Streptomyces* agar discs was evaluated as described in (Table 3.) below and the ratings used were modified from those of Lee and Hwang, (2002). Pure cultures of the test organism were grown on PDA media and used as controls.

Table 3: Rating scale for inhibition against fungal pathogens (Modified from Lee and Hwang, 2002)

Inhibition diameter	**Rating**
No inhibition	(-) mycelia growth not different from control
5-9 mm (weak inhibition)	(+) partial inhibition of mycelia growth
10-19 mm (moderate inhibition)	(++) almost complete inhibition of mycelia growth
>20 mm (strong inhibition)	(+++) complete inhibition, most mycelia will not grow

Controls included plain agar disks.

3.6.2 Antibacterial bioassay

Antibacterial activity of the isolates was done using an initial single streak method. The isolated *Streptomyces* were grown in broth of the growth media for 72 hours. A perpendicular of the broth was made on plates containing Mueller Hinton agar (Append. 2) by dipping sterile swabs in the broth and making the streaks. They were then incubated for five days at 28˚C. A single streak of the test bacteria was done at an angle of 90˚C to the *Streptomyces* streak. Incubation was done for 24-48 hours and the distance of inhibition was measured (Madigan and Martinko, 2007).

3.7 Submerged cultures and preparation of crude extract

The active isolates were cultivated on Casein Glycerol agar at 28ºC for 7 days. A 0.6 cm diameter disk of this agar culture was transferred aseptically to 250 ml Erlenmeyer flasks containing 100 ml Casein Glycerol Broth. The inoculated flasks were kept on a rotary shaker

at 130 rpm at 28-30°C for 7 days. Cells were removed by centrifugation of the broth at 5000 rpm for 20 minutes. Cell free supernatant was separated using 0.2 µm pore size membrane filter (Millipore) and the filtrate collected as the antibiotic sample (Minas *et al*., 2000).

3.8 Antimicrobial activity of the culture filtrate

Antimicrobial activity of the culture filtrate was done using the well diffusion method. The test bacteria was grown in Nutrient Broth and compared to a Macfarland standard of 0.5. The test bacteria were spread plated on Mueller Hinton agar plates. Using a sterile 8 mm cork borer, three wells were dug on the seeded agar plates and each well was filled with 100 µl of the filtrate collected as the antibiotic sample. The plates were incubated at 30°C and the diameter of inhibition was measured round the well. For estimation of the antifungal activity, a 10^6 spore suspension of the fungi was prepared and spread plated on PDA plates. The well diffusion method was used to assay for antifungal activity (Acar and Goldstein, 1996).

3.9 Isolation of antimicrobial metabolites

Antibacterial compound was recovered from the filtrate by solvent extraction method following the process described by Westley *et al.,* (1979). Ethyl acetate was added to the filtrate in the ratio of 1:1 (v/v) and shaken vigorously for 1 hour for complete extraction. The ethyl acetate phase that contains antibiotic was separated from the aqueous phase. It was evaporated to dryness in water bath at 80°- 90°C and the residue obtained was weighed. The obtained compound was used to determine antimicrobial activity. Six millimeters wet paper disks were added to the obtained residue and used to investigate the zone of inhibition around the disk.

3.10 Characterization and Identification of active isolates

3.10.1 Cell morphology and cultural characteristics

The cell morphology of the isolates was done by observing the spore morphology. Colour determination was also done by cultivating the isolates on yeast-extract malt extract agar, starch casein agar, nutrient agar and inorganic salt agar. Observation was done after 7, 14 and 21 days. The aerial mass colour, presence of soluble pigment and colour on reverse side of the plate was also observed. Microscopic observation was carried out by cover slip method as described in the ISP project. Arrangement of spores on mycelium was observed under high power objective in the light microscope (Shirling and Gottlieb, 1966).

3.10.2 Biochemical Characteristics

Gram staining test was done by spreading the broth culture on a glass slide followed by heat drying. The smear was covered with crystal violet for 30-60 seconds and rinsed off with water. The smear was covered with Gram's iodine for 30-60 seconds then decolorized with alcohol and washed with water. Finally the smear was stained with safranin counter stain for 2 minutes. It was rinsed off and left to dry. The slides were viewed at x100 under the light microscope. Citrate utilization was done by inoculating the test isolates in Simon citrate agar media. The isolates were then incubated at 30 ºC for 48 hours. Positive results showed growth and blue colour in the media. Nitrate reduction was also tested where the isolates were grown in 5 ml nitrate broth and incubated for 96 hours at 30 ºC. 0.1 ml of the test reagent (equal volumes of 0.8% sulphilinic acid and 0.5% α-naphthylamin in 5M acetic acid). Positive results showed red colour while negative results were yellow in colour. Catalase test was done by taking a drop of 10% hydrogen peroxide on a clean glass slide. A colony of each isolates was picked using a wire loop and placed on the drop of hydrogen peroxide production of bubbles showed positive catalase test, while absence of bubbles showed negative results. Carbon utilization was done by growing the isolates in a basal media of peptone water, the source of carbon was 1% of either glucose, lactose, starch, mannitol, L arabinose, fructose and other sugars. Turbidity of the broth was measured using a photometer to observe the amount of growth (Bharti and Arora, 2007).

3.11 Identification using molecular procedures

Isolates that showed antagonistic activity against the test pathogens were characterized using molecular methods. Identification using molecular procedures was carried out at the Institute of Biotechnology Research, JKUAT. Pure cultures of the isolates were grown in yeast extract broth with 34% sucrose + 0.5% glycine (Kieser *et al.,* 2000). DNA was extracted using the phenol/chloroform/isoamyl method (Kieser *et al.,* 2000).The DNA was semi quantified on a 1% agarose gel in 1 x TAE buffer and visualized under UV by staining with ethidium bromide (Sambrook *et al.,* 1989). Amplification was done using universal primers 8f (5'-AGA GTT TGA TCC TGG CTC AG -3') and 1492r (5'-TACCTTGTTACGACTT-3') under the following condition: 94˚C for 5 min, 30 cycles of 94˚C for 45 sec, 48˚C for 2 min, 72˚C for 90 sec and final extension at 72˚C for 10 min. The PCR reaction mixture (50 µl) contained PCR beads 1 µl from each primer *8f* and *1492r,* 2.5 µl of dNTPS, 5 µl of buffer, 0.2 µl of Taq polymerase, 2 µl of BSA and 1 µl of template DNA up to final volume 50µl which was reached by adding distilled water. Electrophoresis

of the PCR products was carried out on 1% agarose gel in 1xTAE buffer containing ethidium bromide (0.5 µg mL^{-1}), (Kim and Lee, 2000) and detected by visualizing under UV light. PCR products were purified using High Pure PCR Product Purification Kiton kit (Roche, Germany) and outsourced for sequencing in Macrogen, South Korea. The 16S rRNA gene sequences were compared to sequences in the public database using Basic Local Alignment Search Tool (BLAST) on the National Center for Biotechnology Information (NCBI), in order to determine similarity with sequences in the GenBank database (Altschul *et al.,* 1990; Shayne *et al.,* 2003).

3.12 Data Analysis

The data obtained was analyzed as follows. To determine the distribution and diversity of the isolates, mean values and standard deviation of the isolates was determined. ANOVA was done to test any variations within the mean values. T-test was carried out to determine the differences in the depths of the parameters tested. Correlations were tested to see if there was relationship between the parameters. Screening for antimicrobial activity was done and the data obtained was presented in plates showing the antimicrobial activity.

CHAPTER FOUR

RESULTS

4.1 Moisture content, pH of the soil sample and Densities of isolates in soils from the study area

The moisture content of the soil samples varied with the sites. The mean values from the wheat farm had the lowest moisture content at 4.75% while soil samples from the tea soil had the highest moisture content at 11.41% (Table 4). There was no significant difference in the means of the moisture content of the soil samples for the two depths used at ($P<0.05$).

The densities of the isolates indicated that population from all samples determined had a density range of an average of 15,000-930,000 CFUs g^{-1}. Samples from the TRF soil had the least numbers of isolates in colony forming units (CFUs g^{-1}) as compared to the other sites in the two depth layers showing 22,000 and 15,000 CFUs g^{-1} in the 0-10 and 10-20cm depth layers. Conversely, wheat farm recorded the highest density with a mean of 930,000 and 760,000 CFUs g^{-1} in the 0-10 and 10-20cm depth layers respectively.

Table 4: **CFUs $g^{-1,}$ pH and moisture content of the isolates**

Site	**Density (CFUs) g^{-1} dry Soil**		**pH**		**Moisture content (%)**	
Depth	**0 -10 cm**	**10 -20 cm**	**0 -10 cm**	**10 -20 cm**	**0 -10 cm**	**10 -20 cm**
FOR	$6.3\times10^{5}\pm 2.9\times10^{5a}$	$3.0\times10^{5}\pm 1.0\times10^{4a}$	4.8 ± 0.2^{a}	4.7 ± 0.3^{a}	10.27 ± 0.3^{a}	10.71 ± 0.2^{a}
TEA	$2.2\times10^{4} \pm 1.8\times10^{4\ b}$	$1.5\times10^{4} \pm 1.4\times10^{4b}$	4.3 ± 0.1^{a}	4.4 ± 0.3^{a}	11.02 ± 0.5^{a}	11.25 ± 0.2^{a}
WHF	$9.3\times10^{5} \pm 9.9\times10^{4\ c}$	$7.6\times10^{5} \pm 1.3\times10^{5c}$	5.3 ± 0.1^{a}	5.3 ± 0.1^{a}	4.81 ± 0.1^{b}	4.84 ± 0.1^{b}
BFOR	$6.1\times10^{5} \pm 1.4\times10^{5a}$	$4.5\times10^{5} \pm 1.1\times10^{5d}$	6.2 ± 0.3^{b}	6 ± 0.4^{b}	10.27 ± 0.3^{a}	10.71 ± 0.2^{a}
BGR	$3.5\times10^{5} \pm 1.9\times10^{5b}$	$2.8\times10^{5} \pm 7.8\times10^{4a}$	6.7 ± 0.1^{b}	6.2 ± 0.1^{b}	9.8 ± 0.2^{a}	9.94 ± 0.2^{b}

Values are means ±SD. The outcomes sharing a common superscript letter in the same column are not significantly different at $P<0.05$.

Soil samples with low pH value had the lowest CFUs. This was especially observed in the tea research farm soil and the natural forest soil. The pH of the soil was weakly acidic ranging from pH of 4.3 in the Tea Farm soil to 6.7 in the Botanic Garden grass area. Two depths were used in the study, the upper layer of 0-10 cm had slightly higher CFUs g^{-1} and pH, Table 4.

4.2 Distribution of the isolates in the study area

A total of 561 different actinomycetes were morphologically identified from all the samples. Higher diversities of isolates were observed in cultivated soils compared to the TRF soil and the natural forest soil.

Table 5: **Distribution of isolates in the study area (%)**

Site	**Isolates (%)**
TRF	8
Natural Forest	10.34
Wheat Farm	18.36
Botanic Garden	62.92

4.3 Antimicrobial effect of the isolates

Out of the 561 isolates, 270 of the isolates were selected based on morphological characteristics, other isolates could not grow after being sub cultured and only the 270 isolates were further investigated. Thirty nine (39) of the isolates showed antagonistic activity against the selected plant pathogenic fungi while 14 of them showed activity against one or two of the test bacteria in the primary screening (Table 6).

The isolates showed antimicrobial activity with 14.4% and 5.2 % of the total isolates showing antifungal and antibacterial activity respectively. The wheat farm soil had the highest number of isolates with antifungal activity with 17 (34%) isolates followed by the Botanic forest having 11 (42.3%) isolates with antifungal activity. Four isolates (15.4%) from the Botanic forest had

antibacterial activity; while 3 isolates from the tea farm soil (7%) and wheat farm soil (6%) showed antibacterial activity. Botanic grass area soil had the least isolates with antibacterial activity, only 2 (3.2%) showed antibacterial activity (Table 6).

Table 6: **Isolates with antimicrobial activity**

Location	**Code**	**Isolates screened**	**Isolates with antibacterial activity**	**Isolates with antifungal activity**
Tea soil	TRF	43	3 (7)	3 (7)
Forest	FOR	33	2 (6.1)	5 (15.2)
Wheat Farm	WHF	50	3 (6)	17 (34)
Botanic Grass area	BGR	62	2 (3.2)	3 (4.8)
Botanic Forest	BFOR	26	4 (15.4)	11 (42.3)
TOTAL		**270**	**14 (5.2)**	**39 (14.4)**

Values in parentheses are percentages of isolates with antimicrobial activities

4.4.1 Screening of Antibacterial Activity

Antibacterial activity of the isolates against test bacteria was in inhibition zones in millimeters as shown in Table 7. Fourteen of the isolates showed antibacterial activity to at least one of the tester pathogens. BFOR3B24 and BFOR3B14 had strong inhibition against *Staphylococcus aureus* and *Bacillus subtilis* with a mean zone of inhibition measuring above 30 mm. These two isolates did not have any effect on the growth of the other tester bacteria. BFOR1B22 inhibited the growth of *Pseudomonas savastanoi* pv. *phaseolicola, S. aureus* and *B. subtilis* with a mean zone of inhibition measuring 20 mm, 29.7 mm and 28.7 mm, respectively. BFOR1A1 inhibited the growth *Pseudomonas savastanoi* pv. *phaseolicola, S. aureus* and *B. subtilis* with a mean zone of inhibition measuring 20.7 mm, 20.7 mm and 11 mm, respectively. The isolate had no inhibitory effect on the other tester pathogens. WHF1A17 inhibited the growth of *Pseudomonas savastanoi* pv. *phaseolicola, S. aureus* and *B. subtilis* with a mean zone of inhibition measuring 26 mm, 21 mm and 27, mm, respectively. WHF2B16 also had inhibitory effect on the *Pseudomonas savastanoi* pv. *phaseolicola, S. aureus* and *B. subtilis* with mean zone of inhibition measuring 15.3 mm, 19 mm and 20.7 mm, respectively. WHF3A15 only inhibited

the growth of *S. aureus* and *B. subtilis* with mean zones of inhibition measuring 19 mm and 20.3 mm, respectively. The other isolates had minimal inhibitory effect and were not investigated further.

Table 7: **Isolates with antibacterial activity through initial screening (distance in mm values are for means and SD)**

Site	Isolates	X. c	P.s.p	E.car	P.a	E.coli	S.a	B.sp
TRF	TRF3B5	8.7±0.8	9.8±0.7	0	0	0	0	5.9±0.26
	TRF1A1	5.1±0.1	10.7±1.2	0	5±0	0	0	10.3±0.6
	TRF2B6	0	21±1	0	0	0	0	0
FOR	F3B1	0	17±0.8	5.3±0.5	10±0	16±0.9	14±0.9	9.7±0.5
	F2A1	0	6.3±0.5	0	0	0	0	0
WHF	WHF1A17	0	26.3±0.9	0	0	26±0	21±0.8	27.3±0.5
	WHF2B16	0	15.3±1.2	0	20.3±0.5	5.3±0.5	19±0.8	20.7±0.9
	WHF3A15	0	0	0	0	0	19±0.5	20.3±0.5
BGR	BGR2B3	0	0	0	0	0	15.7±0.5	18±0
	BGR1B1	0	0	0	0	0	5±0	5±0
BFR	BFOR1A1	0	20.7±0.5	0	0	0	11±0.8	20.7±1.2
	BFOR1B22	0	20±1	0	0	0	29.7±1.2	28.7±0.7
	BFOR3B14	0	0	0	0	0	31.3±0.7	31.7±1.5
	BFOR3B24	0	0	0	0	0	30.3±0.6	33.7±0.7

X.c (*Xanthomonas campestris* pv. *campestris*), P.s.p. (*Pseudomonas savastanoi* pv. *phaseolicola*), P.a (*Pseudomonas aeruginosa*), E. coli (*Escherichia coli*), S. a (*Staphylococcus aureus*), B. s (*Bacillus subtilis*) and E. car (*Erwinia carotovora* pv. *carotovora*)

BFOR3B24 and BFOR3B14 had zones of inhibition greater than 30 mm in solid media among tester bacteria. This showed they had very good inhibition against *Bacillus subtilis* and *Staphylococcus aureus*. The two isolates were the most active against Gram positive bacteria. Isolate F3B1 isolated from the forest adjacent to the tea farm had antibacterial activity to all organisms used for the study apart from *X. campestris*. Isolate WHF2B16 had antagonistic activity against five of the bacteria. Of notable interest is that *P. savastanoi*. pv. *phaseolicola* was sensitive to highest number of isolates (9) including all from Kericho tea farm and forest. *E. carotovora* was only sensitive to F3B3 isolate from the forest adjacent to the tea farm. Two gram positive bacteria *B. subtilis and S. aureus* showed sensitivity to most isolates while the Gram negative cultures were least sensitive with *P. aeruginosa* showing sensitivity to only one isolate.

4.4.2 Antifungal activity

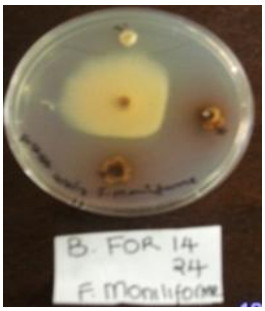
Thirty nine (39) isolates had antifungal activity. All the isolates with antibacterial activity also showed antifungal activity. Inhibition zones around the streptomycetes agar discs is described in Table 8. The zones of inhibition against the fungal pathogen ranged from moderate to strong inhibition. All the isolates tested showed strong inhibitory effects to *F. moniliforme*. Isolates WHF1A17, BFOR3B14 and BFOR3B24 demonstrated strongest inhibitory effects to the two fungal pathogens. BFOR1A1 had zones of inhibition measuring 24 mm against the two plant pathogenic fungi. BFOR1B22 had zones of inhibition measuring 20 mm for the two fungal plant pathogens. BFOR3B14 had the greatest inhibition effect against *Fusarium moniliforme* measuring at 29 mm, *Ascochyta rabiei* was inhibited at a diameter of 25 mm. BFOR3B24 had the largest zone of inhibition against *Ascochyta rabiei* at 29 mm and a diameter of 27 mm against *Fusarium moniliforme* (Table 8). WHF3A15 had a zone of inhibition of 25 mm against *Fusarium moniliforme* and 23mm against *Ascochyta rabiei*, WHF2B16 and WHF1A17 had diameters of 20 mm against the two fungal pathogens (Plate 1).

Table 8: **Antifungal activity of *Streptomyces* isolates from sites in Mau Forest Complex**

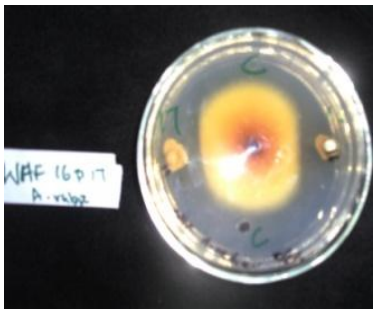
Wheat Farm			Botanic Garden			Botanic Garden Forest			Tea Farm			Kericho Forest		
Isolate	**FM**	**AR**	**Isolate**	**FM**	**AR**	**Isolate**	**FM**	**AR**	**Isolate**	**FM**	**AR**	**Isolate**	**FM**	**AR**
WHF2B3	++	+	BGR2B32	++	+	BFOR1A1	++	+++	TRF3B1	++	++	F3B1	++	++
WHF1A3	++	+	BGR1B2	++	++	BFOR1B22	++	++	TRF1A1	++	+	F3A3	++	+
WHF1A4	++	++	BGR3A4	+	++	BFOR1B18	+	++	TRF3B12	++	++	F2A1	+	+
WHF1B5	++	++				BFOR2B13	++	++						
WHF1B8	+	-				BF0R2A9	++	++						
WHF1B9	++	++				BFOR3A6	++	-						
WHF2B12	++	+				BFOR3B24	+++	+++						
WHF2A13	++	++				BFOR3B14	+++	+++						
WHF2B16	++	++				BFOR3A12	+	++						
WHF1A17	+++	+++				BFOR1A7	++	++						
WHF3A14	++	-				BFOR3A15	++	++						
WHF3A15	++	++				BFOR3A9	++	+						
WHF3B20	++	+				BFOR2B7	+	+						
WHF3B23	++	++												
WHF3B24	++	+												
WHF3B26	++	+												
WHF2B2	++	++												
WHF2B19	+++	++												

WHF, BGR, BFOR, TRF and F stand for isolates from Wheat farm, Botanic Garden grassland, Botanic Garden forest, Tea Research foundation tea farm and Kericho forest respectively. FM stands for *Fusarium moniliforme* and AR for *Ascochyta rabie*. The ratings were adapted from Lee and Hwang (2002), + is 5-9mm (weak inhibition); ++ is 10-19 mm (moderate inhibition); +++ is > 20mm (great inhibition, most mycelia will not grow)

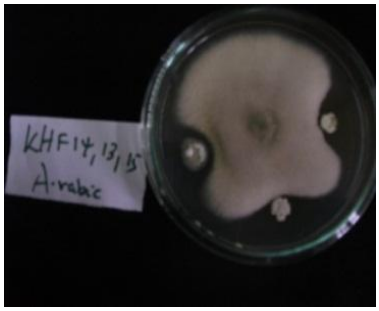
The antifungal activity of some of the isolates is shown in Plate 1. The zones of inhibition against the fungal pathogens is clearly visible.



A



B



C

Plate 1: Antifungal activity of the *Streptomyces* isolates: A- antifungal activity of the agar plug of isolates BFOR3B14 and BFOR3B24 on F. *moniliforme*; B- antifungal activity of agar plug of isolate WHF2B16 and WHF1A17 on *A. rabiei*, C; WHF2A13, WHF3A14 and WHF3A15 showing antifungal activity.

4.5 Antimicrobial activity of culture filtrates

Cultures that showed antimicrobial activity were further grown in submerged cultures to monitor the antimicrobial activity of the culture filtrates. The culture filtrates had inhibitory effect on mainly Gram positive tester bacteria and the fungal pathogens (Table 9). Eight isolates showed antibacterial activity as culture filtrates. Seven of the isolates (87.5%) had inhibitory growth effect on *Bacillus subtilis* ATCC6633, five isolates (62.5%) had antagonistic effect on *Staphylococcus aureus* ATTCC 25923, and only one of the isolate (12.5%) had antagonistic effect on *Pseudomonas aeruginosa* ATCC 27853. The growth of the other bacterial plant pathogens was not affected by the culture filtrates of all the isolates. Seven isolates had antifungal activity against the two fungal tester pathogens (Table 9).

Table 9: Antimicrobial activity of culture filtrates

	B.s	S. a	P. a	E. coli	E. car	P. s. p	F. m	A. r
BFOR3B14	16±3.6	12.3±2.5	0	0	0	0	24.7±0.5	25±0
BFOR1A1	0	19.2±2.9	9.9±1	0	0	0	19.5±1	19±0.5
BFOR3B24	21±1	19±1.4	0	0	0	0	28±1	19±1.5
WHF2B2	18.7±1.8	0	0	0	0	0	0	0
WHF3A15	20.3±0.5	0	0	0	0	0	21±1	20.7±1.2
BFOR1B22	17±1.7	0	0	0	0	0	20.3±0.6	18±1
WHF2B16	10.3±0.6	5.7±1.2	0	0	0	0	25.7±1.2	22±0.5
WHF1A17	15.7±1.2	7.7±2.5	0	0	0	0	20±0.6	17±1.5

B.s (*Bacillus subtilis*), S.a (*Staphylococcus aureus*), P.a (*Pseudomonas aeruginosa*), E. coli (*Escherichia coli*), E. car (*Erwinia carotovora*), P.s.p (*Pseudomonas savastanoi* pv. *phaseolicola*), F. m (*Fusarium moniliforme*), A. r (*Ascochyta rabiei*). The values are means and SD.

4.6 Antimicrobial activity of crude extracts

Results for the effect of the crude extract on the test pathogens are represented in Plate 2 and Table 10. None of the ethyl acetate crude extracts were active on the bacterial plant pathogens *E. carotovora* and *P. savastanoi* pv. *phaseolicola* which was particularly sensitive in the initial screening test. Similar results were also seen in *E. coli* and *P. aeruginosa* which were used as reference cultures. The Gram bacteria *B. subtilis* and *S. aureus* were generally sensitive with some isolates producing inhibition zones of 20-22 mm.

A B C

Plate 2: A- antifungal activity of BFOR1B22 crude extract against *F. moniliforme*; B- BFOR3B24 crude extract inhibitory effect on the growth of *B. subtilis*; C- Inhibitory effect of BFOR3B14 on B. *subtilis.* Clear area on plates represent zones of growth inhibition.

On the other hand the fungal isolates were highly sensitive to the extracts producing zones of 20 mm and above. BFOR3B14 had the largest zones of inhibition against *F. moniliforme* of 29 mm and 25 mm against *A. rabiei*. BFOR3B24 had a zone of inhibition of 27 mm and 29 mm against *F. moniliforme* and *A. rabiei* respectively. This shows that the isolates had good inhibitory effect against the fungal plant pathogens. Significant inhibitory effects by all isolates were seen in the Gram positive tester bacteria (*B. subtilis* and *S aureus*) and in the two fungal pathogens (*F. moniliforme* and *A. rabiei*).

Table 10: Antimicrobial effect of the crude extract on the test pathogens

Isolates	**B. s**	**S. a**	**P. a**	**E. coli**	**E. car**	**P. s .p**	**F. m**	**A. r**
BFOR1A1	10 ± 0^{a}	9 ± 1^{a}	0±0	0±0	0±0	0±0	24 ± 1^{b}	24 ± 1^{b}
BFOR1B22	0±0	5±1a	0±0	0±0	0±0	0±0	20 ± 2^{b}	20 ± 2^{b}
BFOR3B14	20 ± 1^{a}	20 ± 2^{a}	10 ± 2^{b}	0±0	0±0	0±0	29 ± 1^{c}	25 ± 0^{c}
BFOR3B24	22 ± 1^{a}	21 ± 2^{a}	0±0	0±0	0±0	0±0	27 ± 1^{b}	29 ± 0^{b}
WHF3A15	20 ± 2^{a}	18 ± 0.5^{a}	0±0	0±0	0±0	0±0	25 ± 2^{b}	23 ± 1^{a}
WHF2B16	0±0	5 ± 0^{a}	0±0	0±0	0±0	0±0	20 ± 1^{b}	20 ± 1^{b}
WHF1A17	10 ± 1^{a}	8 ± 2^{a}	0±0	0±0	0±0	0±0	22 ± 1^{b}	20 ± 0^{b}

Values are means ±SD. The outcomes sharing a common superscript letter in the same column are not significantly different at $P<0.05$.

4.7 Morphological and cultural characterization of the isolates

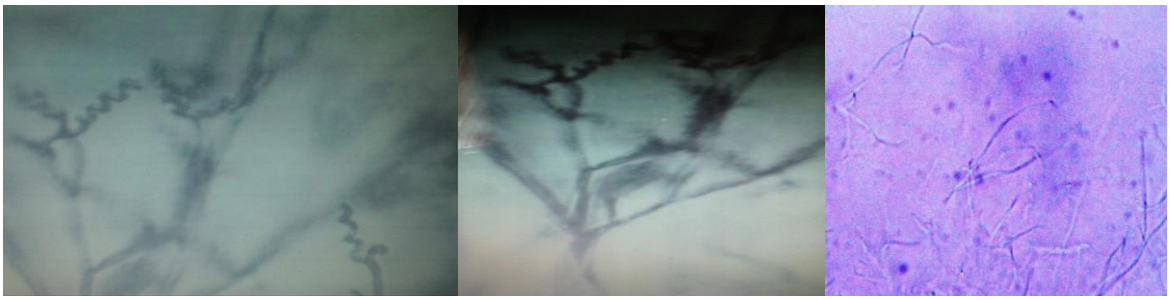
Isolates growing on different media including starch casein agar, yeast malt extract agar, inorganic salt starch agar and nutrient agar produced various colors (Table 11 and Plate 3).

The isolates were Gram positive, their colonies were discrete, butyrous, hard to pick and powdery surface in solid media which is a typical characteristic in members of actinomycetes. Microscopy was done for the aerial mycelium. The spores ranged from straight, rectus, flexous, spiral, and verticilli. Isolate BFOR3B14 and BFOR3B24 had brown diffusible pigment; the substrate mycelium for the two isolates was coffee brown. The other isolates lacked diffusible pigment. In some isolates it was possible to see spiral and straight-rectus spores which are typical *Streptomyces* spores in microscopic preparations as found in literature (Plate 4).

WHF2B16 WHF2B16 WHF1A17 WHF1A17

BFOR3B14 BFOR3B14 BFOR1A1 BFOR1B22 WHF3A15

Plate 3: Color of isolates on inorganic salt starch agar



BFOR3B14 BFOR3B14 WHF3A15

Plate 4: Spore morphology of three isolates

Table 11: Color description of the isolates after growth on different media

Media	BFOR1A1	BFOR1B22	BFOR3B14	BFOR3B24	WHF3A15	WHF2B16	WHF1A17
Starch Casein Agar							
Aerial Mycelium	Grey	White	White	Coffee brown	Ivory	Grey	Grey
Substrate mycelium	Brown	Brown	Brown	Brown	Pale yellow	Pale yellow	Yellow
Soluble pigment	None	None	Dark brown	Dark brown	None	None	Pale yellow
Yeast Malt Extract Agar							
Aerial mycelium	White	Cream	Brown	Brown	Cream	White	White
Substrate mycelium	Brown	Pale Brown	Brown	Brown	Cream	Pale yellow	Cream
Soluble pigment	None	None	Dark brown	Dark brown	None	None	None
Nutrient Agar	Grey	Cream	White	Pale brown	Cream white	Cream	Cream
Aerial Mycelium	Grey	Pale brown	Brown	Brown	Cream	Pale yellow	Cream
Substrate mycelium	None	None	Brown	Brown	None	None	None
Soluble pigment							
Inorganic salts starch agar							
Aerial Mycelium	Dull grey	White	White	Brown	White	White	White
Substrate mycelium	Brown	Brown	Dark brown	Brown	Pale yellow	Pale yellow	Cream
Soluble pigment	None	None	Brown	Dark brown	None	None	Pale yellow

4. 7.1 Biochemical tests

Results from the various biochemical tests conducted are shown in Table 12.

Table 12: Biochemical tests of the isolates with antimicrobial activity

	BFOR1B22	**BFOR3B14**	**BFOR1A1**	**WHF3A15**	**WHF2B16**	**WHF1A17**
D-Glucose	+++	+++	+++	+++	+++	+++
D-Xylose	-	-	-	-	±	++
D-Mannitol	++	±	++	±	±	++
I-Inositol	++	±	++	±	±	-
L-Arabinose	+++	±	+++	+++	+++	+++
Sucrose	+++	-	±	±	±	+++
L-Rhamnose	±	±	++	+++	++	+
Starch	+++	+++	+++	+++	+++	+++
Gelatin	-ve	-ve	-ve	-ve	+ve	-ve
Catalase test	-ve	+ve	+ve	-ve	-ve	-ve
Simon citrate	+	-	-	-	-	-
Milk Agar	±	++	++	++	++	-
Maconkey	-ve	-ve	-ve	-ve	-ve	-ve
Casein hydrolysis	+ve	+ve	+ve	+ve	+ve	+ve

(+++) = Abundant growth, (++) = moderate growth, (+) = fair growth, (±) = Doubtful, (-) =no growth; -ve = (negative), +ve = (positive)

Isolates grew well in differential media. The isolates sporulated in both solid and liquid media. On solid media the isolates were leathery, discrete and tough to pick. All the isolates had abundant growth on basal media supplemented with D- glucose, L- arabinose, starch and casein. WHF1A17 had moderate growth in basal media supplemented with D-xylose the rest of the isolates did not grow in it. The isolates had moderate to doubtful growth on D-mannitol and I-inositol. Isolates BFOR1B22 and WHF1A17 had abundant growth in basal media supplemented with sucrose while the rest had doubtful growth to no growth. Only isolate WHF2B16 liquefied gelatin. BFOR1A1 and BFOR3B14 had positive catalase test, all the isolates did not grow in Maconkey agar. BFOR1B22 had fair growth on Simon citrate agar. WHF1A17 did not grow on milk agar.

4.8 Identification using molecular methods

Five isolates that showed broad spectrum antimicrobial activity were subjected to characterization using molecular methods. These isolates are BFOR3B14, WHF3A15, WHF2B16, WHF1A17 and BFOR1B22. The extracted DNA was amplified and a photograph of gel electrophoresis of the PCR product is depicted in Figure 4.

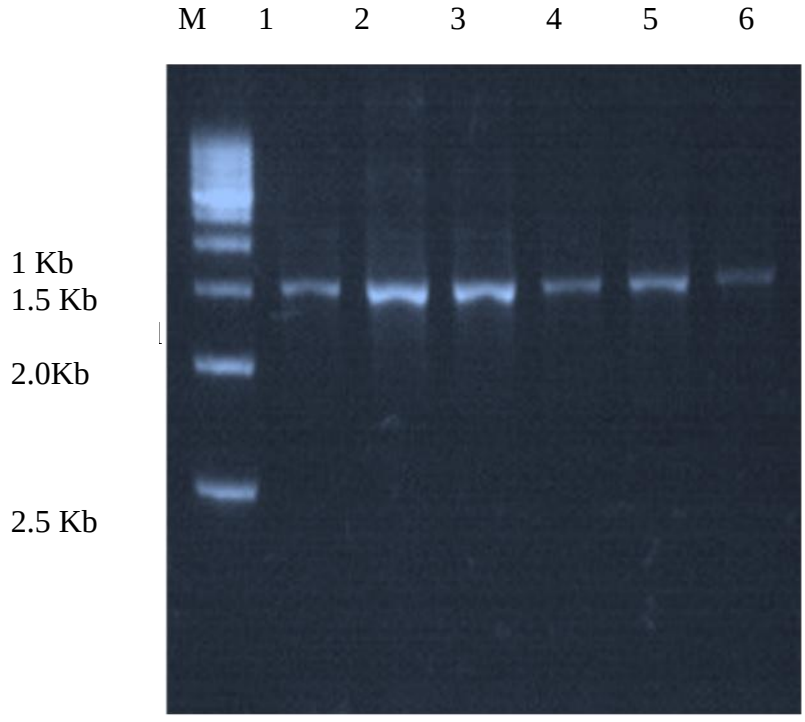


Figure 4: Photograph of gel electrophoresis for PCR products of the isolates DNA. (M represents Marker, 1- BFOR1B22, 2 - BFOR3B14, 3 -BFOR 1A1, 4 - WHF3A15, 5- WHF2B16, 6 - WHF1A17)

Sequencing of the isolates and BLAST analysis identified the isolates as follows: isolate WHF2B16 was identified as *Streptomyces* Mau 1 (Accession number-KR780774), isolate BFOR3B14 as *Brachybacterium* Mau 1 (Accession number KR476396), these two isolates belong to the phylum actinobacteria. Two bacterial isolates belonged to the genus Bacillus; isolate WHF3A15 as *Bacillus subtilis* strain and WHF1A17 as *Bacillus* Mau 1 (Accession number KR780775), finally isolate BFOR1B22 was identified as uncultured *Acinetobacterium*. (Table 13). The results from blast analysis were used to construct phylogenetic trees and these are presented in Figure 5-6.

Table 13: NCBI/BLAST database analysis results showing similarities with the sequences of the isolates

Site of soil sample collection	**Isolate code**	**Identity and Accession number from gene bank**	**Closely related strain**
Botanic Forest	BFOR3B14	*Brachybacterium* Mau 1 Accession number KR476396	*Brachybacterium* sp. MCCC1A09823 (98%)
Wheat Farm	WHF3A15	-	*Bacillus subtilis* strain BAB-1 (94%)
Wheat Farm	WHF2B16	*Streptomyces* Mau 1 Accession number-KR780774	*Streptomyces* sp. 514F (99%)
Wheat Farm	WHF1A17	*Bacillus* Mau 1 Accession number KR780775	*Bacillus* sp. FB11(99%)
Botanic Forest	BFOR1B22	-	Uncultured *Acinetobacter* sp. clone ASC732 (98%)

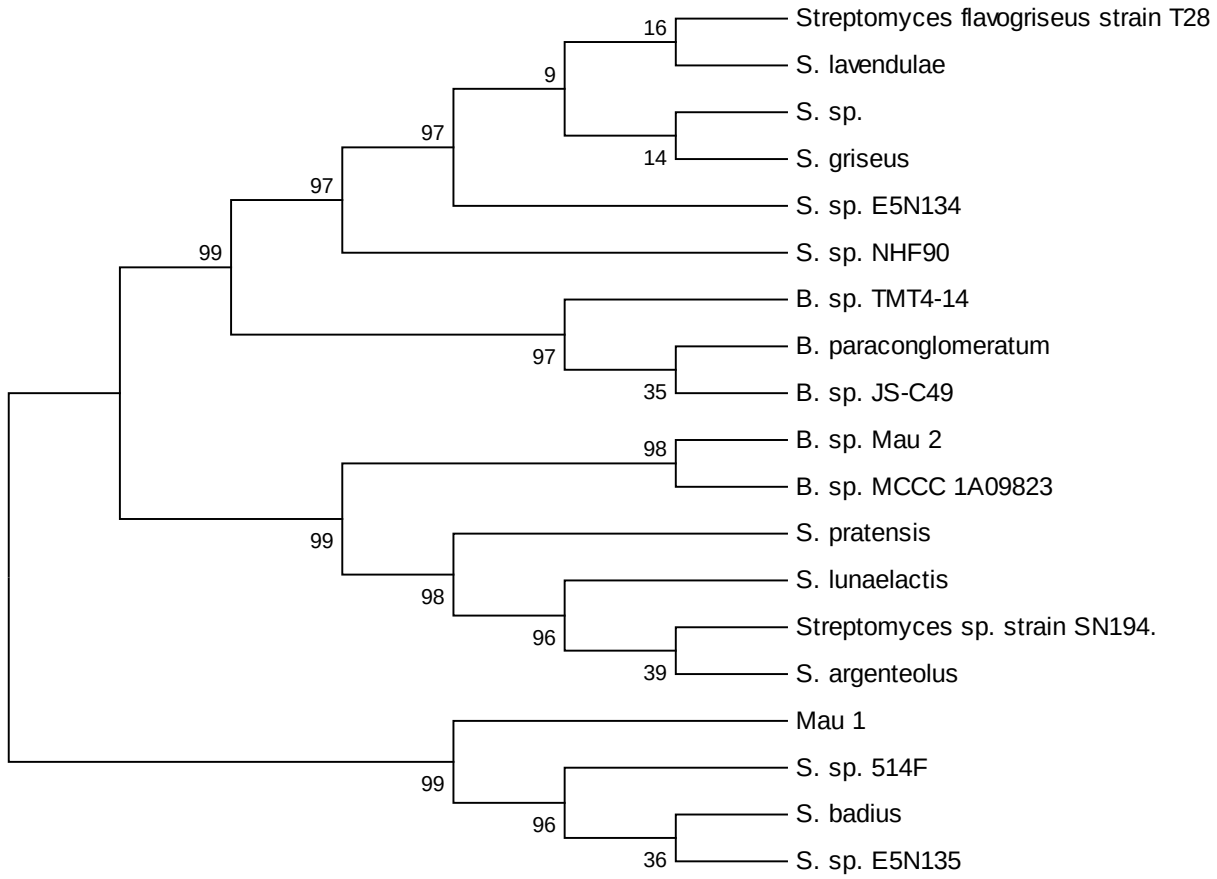


Figure 5: A maximum likelihood tree based on almost complete 16S rRNA gene sequences showing the phylogenetic relationships between the isolates with broad-spectrum antimicrobial activity against the test pathogens and known Actinomycetes from the NCBI/BLAST database.

The above phylogenetic tree shows the phylogenetic relationship of the isolate BFOR3B14 and WHF2B16 with actinomycetes. BFOR3B14 shared a sequences identity of between 98-99% with known *Brachybacterium* species. Isolates BFOR2B14 and WHF2B16 clustered together with a high bootstrap value of 100%. The two isolates had a sequence identity of 98%.

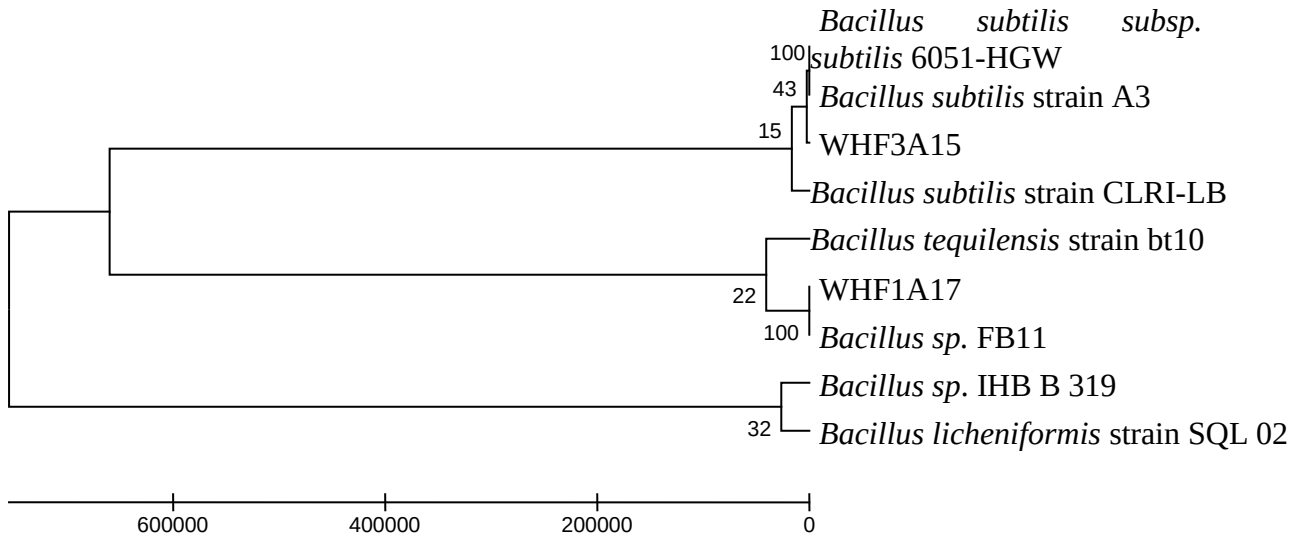


Figure 6: A maximum likelihood tree based on almost complete 16S rRNA gene sequences showing the phylogenetic relationships between the isolates with broad-spectrum antimicrobial activity against the test pathogens and *Bacillus* species from the NCBI/BLAST database.

WHF3A15 had a sequence identity to *Bacillus subtilis* strain BAB-1 of 94%. The isolate had sequence similarity of 95% -93% with known *Bacillus subtilis* species and a high bootstrap value of 100% with *Bacillus subtilis* subsp *subtilis* 6051. Isolate WHF1A17 had a sequence similarity of 99% to known *Bacillus* species and a high bootstrap value of 100% with *Bacillus* sp. FB11.

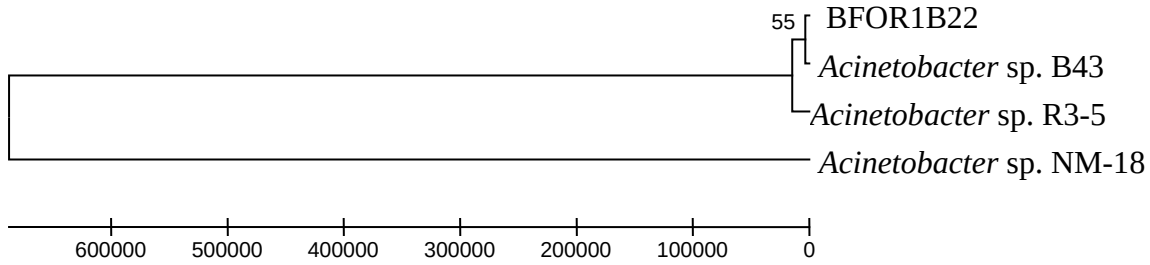


Figure 7: A maximum likelihood tree based on almost complete 16S rRNA gene sequences showing the phylogenetic relationships between the isolates with broad-spectrum antimicrobial activity against the test pathogens and related *Acinetobacter* species from NCBI/BLAST database.

BFOR1B22 had sequences similarity to known *Acinetobacter* species of 98% and a bootstrap value of 55% with *Acinetobacter* B43.

CHAPTER FIVE

DISCUSSION

5.1 Distribution of *Streptomyces* isolates in soils from various vegetation types

The Mau Forest complex provided a good study area that has a wide range of soil types with different distribution of the actinomycetes. The wheat farm soils had higher densities of the actinomycetes as compared to the Tea farm soil and natural forest soils. This result agrees with the work of Dharumadurai *et al.,* (2009), who also observed that there were more numbers of isolated actinomycetes in agricultural soils as compared to non-agricultural soils. The actinomycetes were isolated at mesophillic temperatures of between 25-37ºC; this is in agreement with Kutzner, (1986). The pH of the soil samples was between an average of 4.6 in Tea farm soil, 5.3 in the wheat farm and 6.0 in the Botanic garden soil. Isolation of actinomycetes in acidic soils has been noted by other workers. Gerrettson and Kelly, (1981) isolated actinomycetes from forest soil at a pH of between 4.6 and 4.9. According to Kampfer, (2006) most *Streptomyces* occur in neutral to alkaline soils, however acid tolerant *Streptomyces* have been isolated by many workers such as Williams *et al.* (1977) who showed acidic forest soils contained numerous Streptomycetes which had amylase and chitinase activity. Kontro *et al.* (2005) performed studies on *Streptomyces* species and found that most of them had the optimum pH ranges of growth and sporulation at between 5.5 and 11.5. Soil samples with low pH value had the lowest populations. This was especially observed in the tea research foundation soil and the natural forest soil.

5.1.1 Influence of land use type on soil bacterial populations

The results showed that wheat farm soil and the grass area soil had higher densities of the bacteria. Garcia-Orenes *et al.,* (2013) carried out a study that showed that intensively managed soils have higher abundance of bacteria and actinobacteria. According to Shange *et al.,* 2012 actinomycetes have highest abundance in heavily disturbed cultivated system with low soil organic carbon values. This study agrees with the research findings since high densities were found in the wheat farm and the grass area soil which is heavily disturbed as compared to the tea farm soil and the forest soil. Ederson *et al.* (2009) observed that pasture and crop soil communities had the most diversity of bacteria in the study conducted in Western Amazon soils. In a study conducted by Ding *et al.* (2013) actinomycetes had lower relative abundance in natural scrublands compared to alfalfa soils. Lauber *et al. (*2009) compared four different land uses (cultivated, pasture, pine plantation and mixed wood

forest) in George, USA and found out that actinobacteria were higher in number in pasture and cultivated soils. Also Burck *et al.* (1989) found that actinomycetes were abundant in agricultural land compared to forest soils. Related literature agrees with findings obtained in this study.

5.1.2 Influence of pH and Moisture on Bacterial Populations in the soils

All the soil samples were slightly acidic. However the tea farm soil and the natural forest soil in Kericho was more acidic as compared to the rest of the soil samples. These two sites had the lowest densities of the isolated bacteria. Bacterial populations in the soil are influenced by factors like soil pH, water content and temperature (Grandy *et al.*, 2009). Shange *et al.* (2012) showed that acidic soils have low diversity and numbers of bacteria. Their study observed forest soils had low soil pH which had the lowest richness in bacteria populations. This study agrees with their findings, the natural forest and the tea farm had least number of isolated bacteria. Nasrabadi *et al.* (2013) noted that agricultural soils with a pH range above 7 to 7.7 had the highest numbers of actinomycetes. In their study the forest soil had a pH of 6 and the numbers of actinomycetes were lower as compared to the agricultural soils with a higher pH. In this study the agricultural soil had higher population of isolates, this agrees with their findings. Davis and Williams, (1970) observed that actinomycetes were greatly influenced by moisture content and pH. They also reported that the lowest numbers of actinomycetes were obtained in soils with high pH and low moisture content. Although the wheat farm had a low moisture content, the soil still had a higher density of isolated bacteria. This can be attributed to the fact that actinomycetes are able to sporulate, the spores can survive in low moisture content and propagated when conditions are favourable. Nasrabadi *et al.* (2013) reported that semi-arid soils had high numbers of actinomycetes. In the current study, the wheat farm soil had the lowest moisture content and the highest CFUs/g as compared to the other sampling sites.

5.1.3 Isolation and characterization of *Streptomyces*

The media for the isolation used was starch casein agar, which from other studies it has been shown to yield the highest numbers of actinomycetes. Kuster and Williams, (1964) suggested that the best media for isolation of streptomycetes should contain starch as the source of carbon pand casein, arginine or nitrate as source of nitrogen. At 0-10cm the number of isolates were more compared to 10-20cm. The results are in agreement with what other researchers have found out. Waksman, (1967) noted that the numbers of actinomycetes

decreased with decrease in organic matter in the soil profile. Gerrettson and Kelly (1981) reported that the CFUs/ml of streptomycetes decreased with increase in depth and low pH.

5.2 Screening of isolates for antimicrobial activity

5.2.1 Antibacterial activity

Isolates grown in broth media showed reduced antibacterial activity as compared to their activity in solid media. This has been observed by other researchers, (Kumar and Kokati, 2012) noted that antibacterial activity of actinobacteria was more in solid media than in culture filtrates. This is probably due to breaking of the mycelium when agitated in liquid media which may affect the strains producing the antibiotics; the pH of the medium can change due to fast metabolism as compared to solid media. The concentration of active ingredient in solid media is probably higher than in the culture filtrates (Atta, 2015).

These results show that there was production of extracellular bioactive compounds. The literature reviewed agreed that actinomycetes produce antibiotics through extracellular means (Kampfer, 2006, Silambarasan *et al.,* 2012, Shirling and Gottlieb, 1966).

The results from both primary and secondary screening showed that the active isolates had more inhibitory activity against Gram positive bacteria than Gram negative bacteria. These results agree with the findings of Panwar and Saini (2012) who observed that 10.76% of their isolates had antibacterial activity against *B. subtilis* as compared to 6.45% isolates which showed antibacterial activity against *E. coli.* Silambarasan *et al.* (2012) obtained the same findings where actinomycetes isolates showed more inhibitory activity against Gram positive bacteria than in Gram negative bacteria. Qin *et al.* (2009) found that actinobacteria isolated from medicinal plants had more inhibitory activity against *Bacillus subtilis* and *Staphylococcus aureus* as compared to *Pseudomonas aeruginosa*. This can be attributed to the fact that Gram positive bacteria only have an outer peptidoglycan layer which is not an effective permeability barrier as compared to the Gram negative bacteria which has an outer polysaccharide membrane that have structural lipopolysaccharide components which make the cell wall impermeable to lipophilic solutes (Scherrer and Gerhadt, 1971). It may also mean that the antibiotics have effect on the peptidoglycan development in Gram positive bacteria.

The results show that the isolates are potential sources of bioactive compounds which can be further investigated for pharmaceutical and crop protection in agriculture. New information

has been generated by this study that has shown presence of antibiotics producing bacteria from the Mau Forest Complex.

5.2.2 Antifungal activity

When the active actinomycetes isolates were grown in submerged culture and the culture filtrate used, the number of bioactive isolates decreased. Studies have shown that the production of antibiotic compounds is more efficient in solid culture media, compared with submerged media, where activity may decrease or even cease completely. Research done by Thakur *et al.* (2007) showed that out of 65 isolates that showed antibacterial activity in solid medium, 15 failed to do so in liquid medium. Similar results were described by other authors (Salamoni *et al.* 2010, Anibou *et al*., 2008). Oliveira *et al.* (2010) suggested this is because the production of antibiotic compounds in liquid media is generally low, and the detection of bioactive compounds requires high concentrations of the compounds.

The results obtained in this study show that the presumptive *Streptomyces* isolates were more effective in inhibition of fungal plant pathogens (*Fusarium moniliforme* and *Ascochyta rabiei*) than the bacterial plant pathogens. Only the halo blight pathogen, *P. savastanoi phaseolicola* showed remarkable sensitivity in the screening stage but other bacterial pathogens were not affected by any of the isolates.

5.3 Molecular identification of the isolates

Phylogenetic analysis showed that only isolate WHF2B16 belonged to the genus *Streptomyces*. The isolate was obtained from the wheat farm in Mau. The results also agree with other researchers who obtained antagonistic *Streptomyces* from agricultural soils. Dharumadurai *et al.*, (2009) obtained antagonistic *Streptomyces* from agricultural farms.

Blast analysis showed that isolate BFOR3B14 had 99% sequence similarity to *Brachybacterium* sp. L58. Orsod *et al*., (2012) isolated *Brachybacterium* sp. with antifungal activity against Gram positive and Gram negative bacteria. Gontia *et al*., (2011) also isolated a *Brachbacterium saurashtrense* sp. nov., which had a 99% sequence similarity with *Brachybacterium conglomeratum*, the isolate had antagonistic activity against phytopathogenic bacteria and growth promoting activity on plants.

Isolates WHF 3A15 and WHF1A17 had sequence similarity to the genus *Bacillus*, with the later having 94% sequence similarity to *Bacillus subtilis* strain BAB-1 and WHF1A17 with 99% sequence similarity with *Bacillus* sp. FB 11. Moshafi *et al.* (2011) isolated a *Bacillus* sp from soil that had antimicrobial activity against bacteria and fungal plant pathogens. The

isolate was identified using 16S RNA analysis as *Bacillus* sp. strain FAS1. Finally isolate BFOR1B22 had 98% sequence similarity with an uncultured *Acinetobacter* sp. Clone ASC 732.

CHAPTER SIX

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

The wheat farm soil had high populations of isolated putative streptomycetes isolates while the natural forest and the tea farm soil had the least densities of isolates.

Bacteria isolates with antimicrobial activity were isolated from different soils in the Mau Forest Complex, one of the isolate was a *Streptomyces*. The isolates had more antimicrobial activity against fungal plant pathogens than in bacterial plant pathogens. The isolates also showed greater antimicrobial activity against Gram positive bacteria than Gram negative bacteria.

Five bacteria isolates had broad spectrum antimicrobial activity. Characterization using morphological, biochemical and microscopy showed that the isolates were streptomycetes. Further characterization using molecular characterization the isolates were as identified as follows: WHF2B16 was identified as *Streptomyces* Mau 1 (Accession number-KR780774), isolate BFOR3B14 as *Brachybacterium* Mau 1 (Accession number KR476396), these two isolates belong to the phylum actinobacteria. Two bacterial isolates belonged to the genus Bacillus; isolate WHF3A15 as *Bacillus subtilis* strain and WHF1A17 as *Bacillus* Mau 1 (Accession number KR780775), finally isolate BFOR1B22 was identified as uncultured *Acinetobacterium*. The sequences were deposited to the gene bank.

In conclusion, soils from the Mau Forest Complex have shown that they contain antibiotics producing bacteria. This can be exploited by pharmaceutical industries and agrochemical industries in developing new antimicrobial agents.

6.2 Recommendations

1. Further studies are recommended to see if farms have higher numbers of bacteria with antimicrobial activity as compared to natural forest and undisturbed soils.
2. Antimicrobial activity of the isolates can be tested further in the green house or in the field to see if they will reduce disease incidences in crops. The isolates can be further investigated for other useful secondary metabolites such as enzymes and antibiotics.
3. Use specific primers for *Streptomyces*. This is to ensure that only 16S RNA from streptomycetes are amplified unlike when using universal primers which can amplify even contaminants 16S RNA.

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APPENDICES

APPENDIX 1
Culture media

YEAST EXTRACT MALT EXTRACT AGAR

Yeast extract	4g
Malt extract	10g
Dextrose	4g
Distilled water	1liter
Agar	20g

Final pH 7.3 at 25ºC

APPENDIX 2

MUELLER HINTON AGAR

Beef extract	2g
Acid Hydrolysate of Casein	17.5g
Starch	1.5g
Agar	17g
Distilled water	1liter

Final pH 7.3 at 25ºC

PAGE 2

THIS IS TO CERTIFY THAT:

Prof./Dr./Mr./Mrs./Miss ANASTACIA WAIRIMU MUIA NGURE

of (Address) EGERTON UNIVERSITY P.O. BOX 536, EGERTON

has been permitted to conduct research in Nakuru, Kakamega, Nyeri, Nioro, Kisumu, Baringo, Nyeri, Molo, Kericho, Bomet, Koibatek Districts Western, Riftvalley, Central Provinces

on the topic A SEARCH FOR NEW BACTERIAL ANTIBIOTICS FROM THE GENUS STREPTOMYCES OCCURING IN NATURAL ENVIRONMENTS.

for a period ending 30TH JUNE 20 11

PAGE 3

Research Permit No. NCST/RRI/12/1/MAS/71

Date of issue 11/05/2010

Fee received SHS 5,000

Applicant's Signature

Secretary
National Council for
Science and Technology

CONDITIONS

1. You must report to the District Commissioner and the District Education Officer of the area before embarking on your research. Failure to do that may lead to the cancellation of your permit
2. Government Officers will not be interviewed with-out prior appointment.
3. No questionnaire will be used unless it has been approved.
4. Excavation, filming and collection of biological specimens are subject to further permission from the relevant Government Ministries.
5. You are required to submit at least two(2)/four(4) bound copies of your final report for Kenyans and non-Kenyans respectively.
6. The Government of Kenya reserves the right to modify the conditions of this permit including its cancellation without notice

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