ISOLATION AND CHARACTERIZATION OF ACTINOBACTERIA WITH POTENTIAL FOR CONTROL OF *FUSARIUM SPP* AND *COLLETOTRICHUM KAHAWAE*

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Declaration

This thesis is my original work and has not been presented for a degree in any other university.

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Dedication

This work is dedicated to my late parents, Mr. and Mrs. Nonoh. Thank you for all the support you gave me. You laid in me a good foundation that has seen me through to this level of education.

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Abbreviations

PDA	Potato Dextrose Agar
PDB	Potato Dextrose Broth
HCl	Hydrochloric Acid
NaCl	Sodium Chloride
Bp	Base pairs
DNA	Deoxyribonucleic Acid
rDNA	ribosomal Deoxyribonucleic Acid
PCR	Polymerase Chain Reaction
LB	Luria Bertani
G+C	Guanine and Cytosine
EDTA	Ethylene diamine tetra-acetic acid
ICIPE	International Center of Insect Physiology and Ecology
JKUAT	Jomo Kenyatta University of Agriculture and Technology
NCTC	National Culture Type Collection
CBD	Coffee Berry Disease
EPA	Environmental Protection Agency

Abstract

Smallholder subsistence farming is important in the production of food crops for basic livelihoods and income generation in rural areas. However, yields have remained low due to high disease, weed and pest incidences. Currently, crop pests and diseases are largely controlled by use of chemicals, which have adverse effects on the environment and non-target organisms. There is need for new methods that are environmentally safe to supplement existing control strategies in order to enhance pest and disease control. Microorganisms and their natural products are potentially important alternatives. Identification and characterization of such microorganisms is critical in creating biological control alternative strategies.

In the present study, actinobacteria isolated from soil collected from selected protected areas in Kenya (National Parks and Reserves) were screened for antagonistic activity on three prevalent plant fungal pathogens. A total of four hundred and twenty eight (428) isolates were screened for antifungal activity on the three test fungi, *Fusarium oxysporum*, *Fusarium spp* and *Colletotrichum kahawae*. Three hundred and seventy nine (379) isolates did not show any observable antagonistic effects on the three test fungi *in vitro*. Twenty (20) isolates showed very minor antagonism in most of the fungi tested with the activity disappearing within a week. Eighteen (18) isolates showed *in vitro* activity on only one or two of the test fungi. Eleven isolates showed broad-spectrum activity on the three test fungi and were therefore studied further. The isolates that were not antagonists (379), those that showed inhibition on only one or two of the test fungi (18) and those isolates with minor inhibition whose activity disappeared with time (20),

were not investigated further. The eleven isolates that were studied further and characterized were selected based on *in vitro* broad-spectrum antifungal activity on all the three test fungi. They were isolated from different National Parks with Chyulu having the majority (6 isolates) while Ruma, Kakamega, Imenti, Aberdares and Shimba Hills National Parks produced one each. The isolates were characterized using morphological, biochemical and molecular methods. Phylogenetic analysis of amplified 16S rDNA sequence revealed that the eleven isolates belonged to the genus *Streptomyces*. Majority clustered with known *Streptomyces* species. This was supported with physiological, cultural and morphological studies that demonstrated that the isolates displayed characteristics typical of streptomycetes. Three isolates out of the eleven showed very strong *in vitro* antagonistic effects than the rest indicating that the three are likely to produce novel antifungal secondary metabolites which can find useful application in biological control of crop diseases caused by fungi.

1.0 CHAPTER 1: INTRODUCTION

1.1 Background

Crop production is an important source of food and is expected to play a key role in meeting the needs of the world population, which was reported in 1999 to be growing by 160 persons per minute (Hoisington *et al*, 1999). Losses to pests and diseases are a serious limitation to the productivity of farming with 50% of all crops in some cases being lost to pre- and post harvest pests (Jonathan, 2002). Small-scale farmers sometimes lack access to the technologies they need to control plant disease problems. In some cases, reliance on heavy overuse of broad-spectrum chemical pesticides is a major cause of ill health due to toxicity, environmental damage as well as high residues on food crops (Elizabeth and Handelsman, 1998). The challenge is to seek alternatives to supplement existing control strategies, and to improve crop management systems so that chemical pesticides are used only where necessary and in an effective and safe manner (Benbrook et al., 1996). In order to achieve an increase in agricultural productivity in a sustainable manner, there will be an increased reliance on manipulation of microorganisms that are beneficial to soil and plant health (Conn and Franco, 2004).

1.2 Control of crop pathogens

Plant diseases have been controlled using various strategies. Cultural methods of control include crop rotation, intercropping and use of clean planting materials. Though many of the cultural practices are labor intensive, they have less adverse effects on the environment and are readily available without extra investment on equipment (Elizabeth and Handelsman, 1998).

Biological control, a component of integrated pest management (IPM) strategy, involves control of pests and diseases by natural enemies and antagonists respectively. Antagonists of plant diseases, also known as biological control agents, include inhibitors, competitors and pathogens (Fatope *et al*, 2000). Biotechnology has also been used in the control of pests through introduction of genetically modified crops. The evolution of genetically modified crops took a major step in the mid 1990s with the approval and commercial release of insect resistant maize hybrids with trans-genes derived from *Bacillus thuringiensis* (Bt maize) (Betz *et al*, 2000) in USA and South America.

Natural products derived from plants, animals and microorganisms are now used widely in medicine and agriculture (Fatope *et al*., 2000). Of the 877 small molecule New Chemical Entities (NCEs) introduced between 1981 and 2002, roughly half (49%) were natural products, semi-synthetic natural product analogues or synthetic compounds based on natural product pharmacophores (Newman *et al*., 2003). This is because natural products are readily biodegradable, specific and generally have low toxicity. There is a growing interest in the use of natural products from microorganisms, such as toxins, proteins, hormones, vitamins, and amino acids for control of diseases (Fatope *et al*., 2000). Research on the mechanisms of biological control employed by effective bacterial strains has revealed a variety of natural products that can be exploited for the development of chemical control measures (McSpadden and Fravel, 2002). One well-known example is pyrrolnitrin, a natural product produced by some *Pseudomonas* spp. Pyrrolnitrin provided the chemical model basis for development of fludioxonil, a broad-spectrum fungicide used as seed treatment, foliar spray, or soil drench (Ligon *et al*.,

2000). Research into the mechanism by which plants resist bacterial pathogens (Hutchinson, 1998) led to the discovery of harpin, a protein that is now being used to activate crop defenses prior to pathogen attack. Indeed, a variety of pathogenic and non-pathogenic microorganisms can induce plant defenses and may be useful as biological control agents (Van Loon *et al*., 1998). Focus has been directed to natural products to avoid the problem of resistance and to reduce over-reliance on chemical control of crop pests and diseases (Fatope *et al*., 2000). Because of the potential benefits of natural products, microorganisms producing fungicidal natural products are investigated in this work.

1.3 Actinobacteria

Actinobacteria have been characterized well in literature due to their economic importance as producers of two-thirds of the microbially derived antibiotics known today (Kieser *et al*., 2000). Actinobacteria are characterized by their gram-positive nature and high guanine-plus cytocine (G+C) content in their genomes (Mayfield *et al*., 1972; Coombs and Franco, 2003). Among the actinomycetes, *Streptomyces spp*. has been the major source of all types of antibiotics (Shiburaj, 2003). According to Williams *et al*., (1983), the genus *Streptomyces* of the family Streptomycetaceae contains the largest number of species among the order of Actinomycetales and are involved in a number of processes in the soil. They are found worldwide in soil and are important in soil ecology (Goodfellow *et al*, 1987). Streptomycetes are metabolically diverse and can "feed on" almost anything, including sugars, alcohols, amino acids, organic acids, and aromatic compounds (Goodfellow *et al*., 1987). This is achieved by producing extracellular

hydrolytic enzymes (Goodfellow *et al*., 1987). Streptomycetes grow as vegetative, long, branching hyphae that rarely contain septae and are thus multinucleoid. The formation of aerial hyphae is initiated under the control of several *bld* (bald) genes. Subsequent action of different *whi* (white) genes induces a curling of the aerial hyphae, their septation, and finally spore formation (Kieser *et al*., 2000). There is considerable interest in these organisms as agents for bioremediation. Streptomycetes are also of medical and industrial importance because they synthesize antibiotics (Williams *et al*., 1983).

1.3.1 Diversity of actinomycetes

Actinomycetes are gram-positive bacteria with a high G+C content of their DNA (60-70 mol%) belonging to the order Actinomycetales. A characteristic of many actinomycetes is that they form branching filaments, giving them a fungal appearance (Demain and Davies, 1999). Ecologically, actinobacteria and, particularly, *Streptomyces spp*. are generally saprophytic, soil dwelling organisms that spend the majority of their life cycles as semi-dormant spores (Mayfield *et al*., 1972). Soil is the most important habitat of streptomycetes. Most soils contain 10^4 to 10^7 colony-forming units of streptomycetes representing 1 to 20% or even more of the total viable counts (Demain and Davies, 1999). Grass vegetation or soil rich in organic matter contains the highest numbers of streptomycetes. Most *Streptomyces* species prefer a neutral to mildly alkaline pH (Goodfellow *et a*l., 1990). However, several reports prove that some strains also grow under acidic (pH 3.5) or alkalophilic (pH 8 to 11.5) conditions. As expected, streptomycetes settle in soil as microcolonies on particles of organic matter (Goodfellow

*et a*l., 1990). In terrestrial habitats, streptomycetes are the most abundant actinomycetes (90% or more) (Demain and Davies, 1999).

1.3.2 Actinobacteria as sources of antibiotics and other secondary metabolites

Metabolites from actinobacteria continue to be an important source of antibiotics, enzymes, and bioactive products (Bull *et al*, 1992). The first identified antibiotic was streptomycin, detected about 67 years ago; since then, thousands of low-molecular-weight, chemically different compounds with antibacterial, antifungal, antiparasitic, agroactive, herbicidal, cytostatic, and other activities have been found within many *Streptomyces* species and some other actinomycetes (Demain and Davies, 1999). Because of their ability to synthesize numerous compounds that exhibit extreme chemical diversity, *Streptomyces* strains are major part of industrial strain collections used in screening for new bioactive molecules (Demain and Davies, 1999). Most of these compounds are synthesized as secondary metabolites. Genes encoding these pharmacologically active substances have been found clustered within DNA stretches of 20 kb to more than 100 kb. Using various approaches, the biosynthetic genes for actinomycin, aminoglycosides, β-lactams, rifamycin, tetracycline and many other substances have been identified (Demain and Davies, 1999).

1.3.3 Activity of actinobacteria in degradation of waste

Actinomycetes, particularly streptomycetes play an important role in the turnover of chitin, the second most abundant polysaccharide in nature (Demain and Davies, 1999). Contrary to other chitinolytic bacteria, almost every *Streptomyces* species uses chitin not

only as a carbon source but also as a nitrogen source (Kurtzner, 1981). The few analyzed *Streptomyces* species produce several chitanases. Enzymes catalyzing the hydrolysis of soluble forms of cellulose have been investigated for a few *Streptomyces* strains including *S. lividans* (Demain and Davies, 1999). Extra-cellular proteases are abundant among streptomycetes, and several corresponding genes (Goodfellow *et a*l., 1990) have been characterized. They are involved in important natural processes in a wide range of habitats (Williams *et al*, 1984). In addition to enzymes degrading macromolecules, streptomycetes produce a large repertoire of enzymes, including those for the modification of pharmacologically relevant compounds and xenobiotics (Goodfellow *et a*l., 1990). They are active in the decomposition of organic materials in soil, including lignin, starch, chitin and other recalcitrant polymers, and can degrade agricultural and urban wastes (Crawford, 1988). Mycolic acid-containing actinomycetes are involved in filamentous foaming in activated sludge systems (Reyes *et al*, 1997). The capability of actinomycetes to degrade a variety of biopolymers, to modify xenobiotics, and to detoxify harmful compounds is of great biotechnological and ecological relevance (Demain and Davies, 1999).

1.3.4 Use of actinobacteria in farming

Members of the Genus *Frankia* fix nitrogen in nodules of non-leguminous plants. Actinomycetes have also been used in biological control of plant pathogens (Liu *et al*, 1996), and a few are known to be plant pathogenic (Takeuchi *et al*, 1996). Preliminary descriptive research shows that actinomycetes are a promising group of the fungus-antagonistic root colonizing microbes (Crawford *et al*., 1993). *Streptomyces* species and a

few other actinomycetes have been shown to protect several different plants to various degrees from soil-borne fungal pathogens (Reddi and Rao, 1971). Recent evaluations of streptomycetes as potential antagonists of soil-borne plant pathogens have focused on their ability to produce natural antifungal metabolites (Kieser *et al*., 2000). *Streptomyces rochei* and *Streptomyces rimosus* from the chickpea rhizosphere were strong antagonists of *Fusarium oxysporum f. sp. ciceri* (Bashar and Rai, 1994). *Streptomyces hygroscopicus* var. *geldanus*, grown in sterile soil, antagonized *Rhizoctonia solani*, the pea root-rot fungus, via geldanamycin production (Rothrock and Gottlieb, 1984). A few actinomycetes have also been shown to produce herbicidal and insecticidal compounds (DeFrank and Putnam, 1985).

1.3.5 Characterization of actinobacteria

Molecular biology techniques have become increasingly popular methods for discovering bacterial diversity. Actinobacteria have been characterized by their 16S rRNA gene sequences (Muyzer and Ramsing, 1996; Andrew, 2000). A whole array of taxonomic tools has been used to define genera and suprageneric groups of actinomycetes (Goodfellow, 1989; Embley and Stackebrandt, 1994), but partial sequence analysis of 16S rRNA is the most significant. The characterization of bacteria has a great potential to assist in further investigation and exploitation of the organisms (Teske *et al*., 1996). One of these molecular tools is the PCR amplification of variable region of genes encoding 16S rRNA (16S rDNA) by use of primers homologous to the conserved regions of the gene (Muyzer *et al.*, 1993). Subsequent electrophoretic separation of the PCR products in a polyacrylamide matrix, give comparable fingerprints of microbial communities

(Muyzer *et al*., 1993). Some members of the order Actinomycetales were already identified about a century ago (Demain and Davies, 1999). Initially, the phylogeny of actinomycetes was primarily based on the great diversity of their morphological traits (Demain and Davies, 1999). Chemotaxonomy has been employed as an efficient tool for the rapid differentiation of genera (Kurtzner, 1981). Recent comparisons of 16S rRNA sequences or corresponding rDNA have provided a basis of classification. The relationships between different lineages within the actinomycetes are still largely uncertain, and interrelationships are only known for a few taxa (Demain and Davies, 1999). The genus *Streptomyces* is defined by both chemotaxonomic and phenotypic characters (Kieser *et al*., 2000). All members of the Streptomycetaceae family contain partially saturated (H2, H4, H6) menaquinones with nine isoprene units, and their peptidoglycan has LL-diaminopimelic acid as diagnostic amino acid, but not characteristic sugar (Embley and Stackebrandt, 1994). An enormous number (>800) of *Streptomyces* species have been described, and their classification is frequently based on numerical taxonomy, applying more than 350 physiological traits (Goodfellow *et a*l., 1990). However, only about 70 species have been investigated by phylogenetic methods. More molecular studies are required to determine phylogenetic relationships (Demain and Davies, 1999).

1.4 Justification

Control of crop disease pathogens is critical for optimal agricultural production. The increasing demand for a steady food supply by the human population will require controlling diseases that reduce crop yield. Current control methods such as cultural and use of chemical fungicides among others have been faced with a myriad of limitations ranging from slow nature of action to non-specificity and pollution of the environment, hence need for improved control strategies. One possible strategy lies in the biological control using microorganisms such as actinomycetes and/or their secondary metabolites to suppress crop disease causing pathogens. It has been suggested (Shiburaj, 2003) that screening and isolation of promising strains of actinomycetes with potential antibiotics is an important area of research. The exploration of materials from new areas and habitats have a pivotal role to play in the search for new microbes and novel metabolites and is urgent to counter the threats posed by the fast emerging phenomenon of antibiotic resistance (Shiburaj, 2003). The use of microorganisms and their products for biological control does not experience rapid resistance and environmental damage noted in the conventionally used chemical control. Additionally, the genetic diversity of microorganisms with disease suppressive potential allows identification of various bioactive compounds with immense potential in biological control and bioremediation. It is therefore important to investigate microorganisms with potential inhibitory effects on crop pathogens. Molecular characterization of such microbes will help to identify closely related species that are well described. This will facilitate the identification of genes involved in production of bioactive secondary products and to construct novel biological

control strains by genetic modification. The ultimate benefit is the development of improved disease control to reduce crop losses.

1.5 Objectives

In this study, Actinobacteria were isolated using differential agar media, from soils collected in selected National Parks and Reserves in Kenya. The isolates were subcultured in differential broth media and incubated under appropriate conditions to extract secondary metabolites. Metabolites extracted from the isolates were screened for activity on selected crop pathogens *Fusarium oxysporum sp. lycopersici*, *Fusarium spp.* and *Colletotrichum kahawae*, the causative agents of tomato wilt, rose flower wilt and coffee berry disease respectively. Actinobacteria producing secondary metabolites with broad-spectrum bioactivity on the selected fungal pathogens were characterized by morphological and biochemical methods, and finally by phylogenetic analysis of the PCR-amplified 16S rDNA sequences.

1.5.1 General objectives

To bioprospect for soil actinobacteria, which produce secondary metabolites with antifungal potential in Kenya.

1.5.2 Specific objectives

- To isolate actinomycetes from soil samples from selected National Parks and Reserves in Kenya.
- To screen for isolates producing antifungal metabolites against selected fungal pathogens.
- To characterize and identify the antagonistic isolates using morphological, physiological and molecular methods.

1.6 Hypothesis

Actinomycetes isolates produce secondary metabolites with antifungal activity.

2.0 CHAPTER 2: LITERATURE REVIEW

2.1 Biological factors affecting agricultural productivity

It is well known that plant damage can be caused by soilborne phytopathogenic bacteria, fungi, nematodes, insects and other organisms (Chet, 1990). Soilborne pathogens can harm or destroy the seed or the roots of growing plants. Plant pests include insects (Hill, 1993), mites, nematodes, other invertebrate animals, disease organisms, and noxious weeds that are injurious to plants and plant products (Cook, 2000). Diseases caused by viruses, bacteria, and fungi, as well as physiological disorders lead to serious losses in agricultural productivity the world over (Jonathan, 2002; Cook, 2000). Crop losses of billions of dollars annually are caused by these organisms (Bell *et al*., 1982). Some experts estimate such losses to be as high as 20 per cent and even more (Hag and Khan, 2000). Disease prevention and control, as well as actual yield loss account for large proportion of the annual monetary cost experienced by producers (Fatope *et al*., 2000). Disease management is important for producing acceptable yield and quality of crops such as cabbage, cauliflower, maize, tomatoes, cassava, potatoes and many more crops grown for the fresh market, processing, and for storage (Hag and Khan, 2000). Current control methods often rely on chemicals such as soil fumigants. The use of methyl bromide is very widespread, but other fumigants are also used as fungicides, nematicides and insecticides (Chet, 1990). These compounds tend to be highly toxic, not only to the target organism but also for humans. Fumigation methods kill soil organisms indiscriminately, including beneficial organisms as well as the soilborne pathogens (Bell *et al.,* 1982; Chet, 1990). Previously fumigated soils can be rapidly recolonized by soilborne pathogens and nematodes. There is an urgent need for alternative products that

are effective against a broad range of soilborne pathogens and nematodes with benign environmental effects (Chet, 1990). Biological control organisms such as predators, parasites, parasitoids and antagonists may be utilized to adversely impact plant pests and diseases for improved productivity (Cook, 2000; Elizabeth and Handelsman, 1998).

Several insects are pests of more than one crop species (Taneja and Nwanze, 1989; Overholt, 1998). Insect pests of corn are often involved in the disease cycles of ear rot and stalk rot diseases (Hill, 1983). The European corn borer is particularly well known for its involvement in these diseases. Other serious insect pests include the African stem borer (*Chillo partellus*) that attacks corn, and diamond back moth of cabbages (Hill, 1983). Lepidopterous stem and cob boring pests limit both the yield and stability of maize production in Sub Saharan Africa (Overholt, 1998). The problem is particularly acute in the small-scale, resource poor systems under which maize is typically grown in Sub Saharan Africa (Overholt, 1998). Yield losses in areas with chronic borer problems vary between 10-70% (Hill, 1983). In addition, grain damage by lepidopterous borers predisposes maize to pre- and post- harvest infestations by storage beetles, infection by *Aspergillus flavus* and *Fusarium verticilliodes* and subsequent contamination with mycotoxins (Taneja and Nwanze, 1989).

Plants can also suffer from bacterial infections (Cook, 2000; Smith *et al*., 1988). The soil bacterium *Agrobacterium tumefaciens* (cause of crown gall), now widely used in genetic engineering, is often classified as a pathogen because of the galls produced on the plants by strains that bear wild type Ti plasmids (Fatope *et al*., 2000). Other examples of

bacterial infections include bacterial blight (*Xanthomonas oryzae*) and bacterial leaf streak (*Zanthomonas oryzicola*) of rice (Helbig and Bochow, 2001; Smith *et al*., 1988). Diseases caused by soilborne bacteria including *Ralstonia solanacearum* (previously *Pseudomonas solanacearum*), the cause of bacterial wilt, and other members of this genus and other bacteria such as *Agrobacterium*, *Clavibacter*, and *Erwinia* have been controlled using microbial based products (Bell *et al*., 1982).

Viruses contain very simple genomes and depend on their host for most functions. Most viruses have a protein coat composed of many polypeptide molecules that protect the genome from degradation and may participate in infection processes (Cook, 2000; Smith *et al*., 1988). Examples of viral diseases of plants include prunus necrotic ringspot virus on roses, beet necrotic yellow vein virus (also known as rhizomania on sugar beet), cucumber mosaic virus on cucumber and many other plants, tomato mosaic virus causing deformed fruit on tomatoes and plum pox virus (IDMB report data on transgenic rice, 2007; http://www.idmb.tamu.edu/).

Fungi can severely damage plants, or can be essential as in the case of mycorrhizae, which is symbiotically associated with roots (Agrios, 1988). They are usually filamentous, eukaryotic, spore-producing organisms that lack chlorophyll (Agrios, 1988). Fungal diseases are common and cause significant economic loss of crops and ornamental plants (Ligon *et al*., 2000). Leaf blast of rice, caused by *Pyricularia grisea* is an example of a fungal disease of monocots and black spot of rose, caused by *Diplocarpon rosea* is a dicot example (IDMB, 2007; http://www.idmb.tamu.edu/). The

leaf, stalk and root rots caused by *Fusarium*, *Gibberella* and *Colletotrichum* (anthracnose) are serious as they not only reduce yields, but they also reduce grain quality. *Fusarium* can also produce mycotoxins such as fumonisins (Agrios, 1988).

2.2 Plant fungal pathogens

Plant diseases caused by plant pathogenic fungi continuously threaten the sustainability of global crop production (Elizabeth and Handelsman, 1998). Club-root, caused by *Plasmodiophora brassicae*, is a destructive soil-borne disease which affects nearly all cultivated, as well as many wild and weed members of the cabbage family (Smith *et al*., 1988). Downy mildew, caused by *Peronospora parasitica*, may be a serious foliar disease of all cruciferous crops. Susceptible hosts include canola, cabbage, broccoli, Brussel sprouts, kale, cauliflower, rutabaga, radish, horseradish and Chinese cabbage. Others include mustards, ornamentals such as stock, wallflower, and aubrietia, and many cruciferous weeds (Smith *et al*., 1988). However, there are several pathogenic varieties (physiologic races) of the fungus, which attack different groups of, but not all the aforementioned, cruciferous hosts (Agrios, 1988). The fungus *Sclerotonia sclerotiorum* causes Sclerotinia blight, also known as white mold or white rot (Agrios, 1988). This fungus attacks not only crucifers but also a wide variety of other crop plants in field and storage. Other fungal infections of plants are caused by *Alternaria alternata*, *Aphanomyces euteiches*, *Ascochyta spp*., *Bipolaris sorokiniana*, *Fusarium graminearum*, *Fusarium oxysporum f.sp*. *pisi*, *Fusarium solani f.sp*. *pisi*, *Fusarium spp*. *Mycosphaerella pinodes*, *Pythium spp*., *Rhizopus sp*., *Rhizoctonia solani*, and *Sclerotinia sclerotiorum* (Agrios, 1988). *Fusarium oxysporum* is an anamorphic species that includes both

pathogenic and nonpathogenic strains (Smith *et al*., 1988). Plant pathogenic forms cause a wilt disease and are grouped into '*formae speciales*' (f.sp) based on their host range; some are further subdivided into pathogenic races. Fungal diseases, which have been controlled using the microorganisms and their products include diseases caused by organisms in the genera *Alternaria*, *Colletotrichum*, *Fusarium*, *Helminthosporium*, *Macrophomina*, *Phoma*, *Phytophthora*, *Pythium*, *Rhizoctonia*, *Sclerotium*, *Thelaviopsis*, and *Verticillium* (Chet, 1990).

2.2.1 *Fusarium oxysporum*

Fusarium oxysporum is a filamentous soil inhabiting fungus, sometimes widely distributed on both plants and soil (Smith *et al*, 1988). *F. oxysporum* is an imperfect fungus (with no known sexual stage), belonging to the ascomycetes or sac fungi (Agrios, 1988). Hosts of *Fusarium oxysporum* include, potato, sugarcane, garden bean, cowpea, banana, and coffee among others (Smith *et al*., 1988). Like various other plant pathogens, *Fusarium oxysporum* has several specialized forms- known as *formae specialis* (f. sp.)- that infects a variety of hosts causing various diseases. These include: *Fusarium oxysporum f.sp. lycopersici* (wilt on tomato); *f.sp. cubense* (Panama disease/wilt on banana); *f.sp. niveum* (Fusarium wilt on water melon); *f.sp. pisi* (on cow pea) (Smith *et al*., 1988). Overall, the distribution of *Fusarium oxysporum* is known to be cosmopolitan. However the different special forms of *Fusarium oxysporum* often have varying degrees of distribution. *Fusarium oxysporum* and its various *formae speciales* have been characterized as causing the following symptoms: vascular wilt, yellowing on the lower side of the leaves, corn rot, root rot, and damping-off. The most important of these, is

vascular wilt. Of the vascular wilt causing Fusaria, *Fusarium oxysporum* is the most important species (Agrios, 1988; Smith *et al*., 1988). Strains that are rather poorly specialized may induce yellowing on the lower leaves, rot and damping-off, rather than the more severe vascular wilt (Smith *et al*., 1988). *Fusarium oxysporum* first appear as slight vein clearing on the outer portion of the younger leaves, followed by epinasty (downward dropping) of the older leaves. At the seedling stage, plants infected by *Fusarium oxysporum* may wilt and die soon after symptoms appear. In older plants, vein clearing and leaf epinasty are often followed by stunting, yellowing of the lower leaves, formation of adventitious roots, wilting of leaves and young stems, defoliation, marginal necrosis of remaining leaves, and finally death of the entire plant (Agrios, 1988). Browning of the vascular tissue is strong evidence of Fusarium wilt. Further, on older plants, symptoms generally become more apparent during the period between blossoming and fruit maturation (Jones *et al*., 1982; Smith *et al*., 1988). In solid media culture, such as potato dextrose agar (PDA), the different special forms of *Fusarium oxysporum* can have varying appearances. The aerial mycelium first appears white, and then may change to a variety of colors- ranging from violet to dark purple- according to the strain of *Fusarium oxysporum*. If sporodochia are abundant, the culture may appear cream or orange in color (Smith *et al*., 1988).

Fusarium oxysporum produces three types of asexual spores: microconidia, macroconidia and chlamydospores (Agrios, 1988). Microconidia are one or two celled, and are the type of spore most abundantly and frequently produced by the fungus under all conditions. It is also the type of spore most frequently produced within the vessels of infected plants.

Macroconidia are three to five celled, gradually pointed and curved towards the ends. These spores are commonly found on the surface of the plant killed by this pathogen as well as in sporodochialike groups. Chlamydospores are round, thick walled spores, produced either terminally or intercalary on older mycelium or in macroconidia. The spores are either one or two celled (Agrios, 1988).

Fusarium oxysporum is an abundant and active saprophyte in soil and organic matter, with some specific forms that are plant pathogenic (Smith *et al*., 1988). Its saprophytic abilities enable it to survive in the soil between crop cycles in infected plant debris. The fungus can survive either as mycelium, or as any of its three different spore types (Agrios, 1988). Healthy plants can become infected by *Fusarium oxysporum* if the soil in which they are growing is contaminated with the fungus. The fungus can invade a plant with its sporangial germ tube or mycelium by invading the plant's roots. The roots can be infected directly through the root tips, through wounds in the roots, or at the formation point of lateral roots (Agrios, 1988). Once inside the plant, the mycelium grows through the root cortex intercellulary. When the mycelium reaches the xylem, it invades the vessels through the xylem's pits. At this point, the mycelium remains in the vessels, where it usually advances upwards toward the stem and crown of the plant. As it grows, the mycelium branches and produces microconidia, which are carried upward within the vessel by way of the plants' sap stream. When the microconidia germinate, the mycelium can penetrate the upper wall of the xylem vessel, enabling more microconidia to be produced in the next vessel. The fungus can also advance laterally as the mycelium penetrates the adjacent xylem vessels through the xylem pits (Agrios, 1988).

Due to the growth of the fungus within the plant's vascular tissue, the plant's water supply is greatly affected. This lack of water induces the leaf's stomata to close, causing wilting, and eventual plant death. It is at this point that the fungus invades the plant's parenchymatous tissue, until it finally reaches the surface of the dead tissue, where it sporulates abundantly (Agrios, 1988). The resulting spores can then be used as new inoculum for further spread of the fungus. *Fusarium oxysporum* is primarily spread over short distances by irrigation water and contaminated farm equipment. The fungus can also be spread over long distances either in infected transplants or in soil. Although the fungus can sometimes infect the fruit and contaminate its seed, the spread of the fungus by way of the seed is very rare (Agrios, 1988). It is also possible that the spores are spread by wind. Some effective means of controlling *F. oxysporum* include: disinfestations of the soil and planting material with fungicidal chemicals, crop rotation with non-host of the fungus, or by using resistant cultivars (Jones *et al*., 1982; Agrios, 1988; Smith *et al*., 1988).

2.2.2 *Colletotrichum kahawae*

Colletotrichum kahawae also known as *Colletotrichum coffeanum* is the causative agent of Coffee Berry Disease or Green Berry Anthracnose. CBD was first reported in Kenya in 1922 (Omondi *et al*., 2000; Mitchell, 1988). It has since been recorded from most of the other coffee areas in Africa. It is not known outside of Africa, although a leaf spot and ripe berry anthracnose caused by related *Colletotrichum* species has been reported from Guatemala and Brazil (Javed, 1984; Griffiths *et al*., 1971).

Coffee Berry Disease occurs in all coffee species. Several species or strains of *Colletotrichum* occur in Coffee, but only *C. kahawae* causes Coffee Berry Disease. On the immature green berry, the disease is also known as brown blight (Hindorf, 1974).

C. kahawae is an Ascomycete, a member of the sac family. One of the features of this fungus is that it generates spores, called conidia or conidiospores, that can be easily dispersed by wind and splashing rain (Hindorf, 1970). Spread of *C. kahawae* is dependent on water, but animals can also spread it, with the spread by coffee pickers being a particular problem (Firman and Waller, 1977).

Colletotrichum invades the main body of the plant but does so without any signs of the disease (Grifiths *et al*., 1971). However, when the plant sets fruit, the fungus becomes aggressive and the disease becomes apparent. In many cases, diseases caused by *Colletotrichum* are known as anthracnose because they turn the fruit black (Hindorf, 1970). *Colletotrichum kahawae* affects green or immature berries and the coffee flower at any stage in its development (Mitchell, 1988). Infected berries often show dark sunken spits that spread and coalesce to cover the whole berry. As the fungus sporulates, a pale pink crust of conidia appears on the lesion (Griffiths *et al*., 1971). Depending on the timing of the infection, the bean can also become infected (Griffiths *et al*., 1971).

Where the virulent strains of CBD occur, serious losses have been reported. When first reported from Kenya, 75% losses were noted in some farms (Griffiths *et al*., 1971). The disease has been responsible for the abandonment of coffee growing in several districts of

Kenya and Ethiopia (Griffiths *et al*., 1971). More conservative estimates of losses occurring in Kenya are 20% (Grifiths *et al*., 1971).

Measures that ensure good airflow and prevent accumulation of water are critical for limiting the spread of the fungus. Quarantine has also been used to contain the spread of the disease since it is limited to Africa. There is also the use of resistant cultivars such as Ruiru 11. Other control measures include the use of chemicals such as benomyl, captafol, chlorothalonil, copper formulations, dithianon, thiabendazole, and thiophanate methyl (Omondi et al., 2000; Griffiths *et al*., 1971). The use of chemical strategy to control the fungus is however, discouraged because it is non-target specific and it also damages the environment. Biological control therefore, offers a good alternative, as it is biodegradable and more target-specific.

2.3 Biological control of crop pathogens

Over the past one hundred years, research has repeatedly demonstrated that phylogenetically diverse microorganisms can act as natural antagonists of various plant pathogens (Cook, 2000). Soil microorganisms have the potential for the management of crop diseases. A variety of soil microorganisms have demonstrated activity in the control of various soil borne crop pathogens, including Fusarium wilt pathogens (Bloemberg and Lugtenberg, 2001). Fusarium wilt suppressive soils are known to occur in many regions of the world. This suppression is biological in origin (Elizabeth and Handelsman, 1998). Antagonists recovered from Fusarium wilt suppressive soils have been used to reduce Fusarium wilt diseases of different crops such as tomatoes, potatoes and bananas (Faull

and Powell, 1995). Biocontrol fungi, such as *Trichoderma* and *Gliocladium sp*., have been used to control fungal pathogens, including *Rhizoctonia*, *Pythium*, *Sclerotinia*, *Sclerotium* and *Fusarium spp*. (Manwar *et al*., 2000) and may also be effective against Fusarium wilt diseases (Hag and Khan, 2000). Rhizobacterial strains of *Pseudomonas*, *Burkholderia*, and *Bacillus sp*. have been used to reduce disease caused by a variety of soil borne pathogens including *Fusarium spp*. (Goel *et al*., 2000).

2.4 Metabolites from microbial sources

Bacteria and fungi cause several plants, human and animal diseases. They are also common sources of novel bioactive metabolites that have found useful applications as anti-bacterial, insecticidal, nematicidal and anti-fungal agents (Fatope *et al*., 2000). These metabolites vary enormously in structural complexity and biological activity (Demain and Davies, 1999). Actinomycetes, for example, have been and remain a major source of novel microbial metabolites (El-Tarabily *et al*., 2000). The secondary metabolites from actinomycetes may have a cidal (killing) effect or static (inhibitory) effect on the target pest or pathogen (El-Tarabily *et al*., 2000). To discover therapeutically useful metabolites, it is critical not only to design suitable and sensitive assays for screening microbial extracts but also to test extracts that contain most or all of the metabolites from culture broths with a minimum of interference (Demain and Davies, 1999). In addition to sensitive assays, novel and diverse producing microorganisms are critical to the success of any natural product program, hence biological diversity may lead to chemical diversity (Porter and Fox, 1993). The discovery of bioactive compounds usually begins with isolation of the microbes from environmental samples and growing them in appropriate

media (Fatope 1995). The chemicals they produce are scaled-up by fermentation. The fermentation broth is extracted, fractionated, tested and purified.

2.5 Biological control agents

When testing bacterial and fungal isolates from the environment for biological control activities, between 1 and 10% show at least some capacity to inhibit the growth of pathogens *in vitro* (McSpadden and Fravel, 2002). However, fewer isolates can suppress plant diseases under diverse growing conditions and fewer still have broad-spectrum activity against multiple pathogenic taxa (McSpadden and Fravel, 2002). Nonetheless, intensive screens have yielded numerous candidate organisms for commercial development (Cook, 2000). Screening is a critical step in the development of biological control agents (McSpadden and Fravel, 2002). A major factor influencing plant growth and health is the microbial population living both in the rhizosphere and as endophytes within healthy plant tissues (Conn and Franco, 2004). A portion of these microorganisms' posses the ability to suppress disease and/or promote growth and are termed plant growth promoting rhizobacteria (PGPR) (Coombs and Franco, 2003). Some success has been achieved in controlling crop pathogens and promoting plant growth by supplementing the crop soil with these PGPRs and other biocontrol microbial innocula (Bloemberg and Lugtenberg, 2001). Microbial biocontrol agents have been shown to inhibit soil-borne pathogens such as *Fusarium oxysporum*, *Gaeumannomyces graminis*, *Phytophthora spp*., *Pythium spp*., *Rhizoctonia solani*, and *Verticilium spp*. (Coombs and Franco, 2003). However, a large number of biocontrol agents fail to be effective due to the difficulty of manipulating the highly complex rhizosphere environment (Conn and Franco, 2004).

Some bacteria are able to protect plants from soil-borne fungal pathogens (DPR, 2003). The mechanisms responsible for this biocontrol activity include the production of anti-fungal metabolites (Elizabeth and Handelsman, 1998). The secondary metabolites they produce are antagonist of pests and inhibit the activities of hydrolytic enzymes, antibiotics and siderophores produced by infective fungus (McQuilken *et al*., 2001). Bacteria from the genera *Pseudomonas* and *Burkholderia* are particularly active as biocontrol agents against some crop pathogens such as *Fusarium* wilt (Manwar *et al*., 2000). Other genera, which have shown biocontrol activity against crop pathogens, include *Streptomyces* and *Bacillus* respectively (Fatope *et al*., 2000). Some of the microbial taxa that have been successfully commercialized and are currently marketed as Environmental Protection Agency-registered biopesticides in the United States include bacteria belonging to the genera *Agrobacterium, Bacillus*, *Pseudomonas*, and *Streptomyces* and fungi belonging to the genera *Ampelomyces, Candida, Coniothyrium,* and Trichoderma (USA-Environmental Protection Agency, 2007; http://www.epa.gov/pesticides/biopesticides/). Microbes that have demonstrated potential as biological control agents, can also act as a source of biologically active products (El-Tarabily *et al*., 2000).

2.6 Screening for bioactive natural products

From 1940 to 1980, the primary emphasis of natural products screening was to search for novel antibiotics to treat infectious diseases. Many novel antibiotics, such as the penicillins, cephalosporins, and tetracyclines, were discovered during this period (Demain and Davies, 1999). After 1980, the emphasis of natural products screening

shifted from antibacterial to other therapeutic areas, including antiparasitic, anticancer, antifungal and antiviral targets (Demain and Davies, 1999). There has been growing interest in bioactive natural products and the molecular basis of their activity (Fatope *et al*., 2000). The common practice to isolate, characterize and publish phytocompounds without regard to bioactivities is not adequately informative (Ligon *et al*., 1999). Three technologies are required for sourcing bioactive compounds. They are separation methods, structural elucidation and bioassay methods (Ligon *et al*., 1999; Fatope 1995). Bioassay technology for finding bioactive compounds has undergone major changes during the past decade. These methods can be grouped into two: the *in vivo* and *in vitro* methods. The *in vivo* method involves testing the efficacy of the metabolite or product on target organisms while the *in vitro* method is a mode of action or mechanism-based approach that involves testing in the laboratory set up, of the compounds on the target pest or pathogen (Fatope *et al*., 2000). Antimicrobial assays using whole organism can tolerate whole broth, while other assays such as enzyme and receptor targets or mammalian cell-based assays, are susceptible to contaminating metabolites that may be present in the broth (Demain and Davies, 1999). One way to circumvent some of these problems is to use either single-phase or two-phase extraction procedures that utilize methanol or ethyl acetate. These types of extracts are usually devoid of proteases and are suitable for testing in enzyme and receptor assays. In some cases, solid-phase extraction protocols that make use of Sep pack C-18 cartridges (Waters, Division of Millipore Corp., Milford Mass.) are employed (Demain and Davies, 1999). The adoption of bioassay methods, which correlate with prevention, inhibition and reversal of diseases or elimination of pests in screening natural products for bioactive compounds, is critical to

the discovery of new biopesticides (Fatope *et al*., 2000). Genomes now provide ever-increasing tools and platforms for designing novel *in vitro* assay models, with new targets emerging for insecticides, herbicides and anti-fungal compounds (Stover *et al*., 2000).

2.7 Advantages of biological control

One of the goals for the use of biocontrol in agriculture is to avoid the pitfalls associated with chemical control such as development of resistance in the target pathogen and environmental degradation (Benbrook *et al*., 1996). The advantage of biocontrol strategies is the slow development of resistance by the pathogens and pests to the antagonistic metabolites produced by biocontrol agents (Handelsman and Stabb, 1996). This is because most biocontrol agents produce more than one antagonistic compound and resistance to such multiple compounds should occur only at a very low frequency (Fiddman *et al*., 2000). The use of biological control strategy also minimizes the negative consequences for human health and the environment (Elizabeth and Handelsman, 1998). Also, biological control strategy does not pose serious environmental concerns.

Microbial biological control agents have been commercialized and marketed as biological control products (Helbig and Bochow, 2001). Targets of those antagonists already being commercialized are mainly soil-borne and post harvest plant pathogens (Helbig and Bochow, 2001). Example, the product 'Contans WG$^{\beta'}$ is based on the parasitic fungus *Coniothyrium minitans*, which attacks and destroys the sclerotia of *Sclerotinia sclerotiorum*. The application in soil has the advantage that environmental conditions are most stable and factors having an influence on biological control agents are limited

(Andrews, 1992). Most microbial biological control agents are very specific in suppressing other living organisms, such as plant pathogens, in their habitats (Cook, 2000). Additionally microbial antagonists compete successfully against all those inhabitants such as pathogens and are resistant to light, dryness and extreme temperatures (Helbig and Bochow, 2001). Some of the common microorganisms antagonistic against plant pathogens of fruits, vegetables and ornamental plants include fungi, yeasts and bacteria (Elizabeth and Handelsman, 1998). Fungi are very common inhabitants of a wide range of natural habitats. They are adapted to the life cycle of crops and to varying environmental conditions, such as humidity and availability of space (Helbig and Bochow, 2001). They form specific structures for survival under unfavorable conditions, for growth and reproduction. Additionally, fungi have a range of antagonistic modes of action, depending on the species. This gives the possibility to choose the mode of action according to the biology of the pathogen and other factors having an influence on effectiveness (Cook, 2000). Yeasts have some of the ecological characteristics of hyphae-forming fungi, for example the potential to grow under varying environmental conditions. Many researchers favor the use of yeasts for fruit application since yeasts normally do not produce antibiotics and, therefore, are supposed to be of minor risk to humans (Williamson and Fokkema, 1995). Bacteria able to form spores are potentially suitable for application in the phyllosphere since they can survive adverse conditions. They can successfully compete for nutrients in all habitats that they are able to colonize (Cook, 2000). Microbial control agents also produce bioactive metabolites such as antibiotics, bacteriocins and other proteins, which act in very low concentrations to suppress the growth of the microbial pathogens and the effect may cause death or otherwise interfere

in the biological processes (Helbig and Bochow, 2001). Most microbial biological control agents are easy to grow under laboratory conditions and in batch culture.

2.8 Genetic diversity among biological control agents

The extent of bacterial diversity is unknown and without a rationally based extrapolation. This is not the case for fungi (Allsopp *et al*., 1995). The number of known species is about 72,000 (Heywood, 1995). The case for bacteria is far more primitive. Only about 4,200 species have been described (Allsopp *et al*., 1995). It is widely recognized that this represents, at best, only 0.1 to 1% of the bacteria in nature (Demain and Davies, 1999). No rational attempt has been made to extrapolate the global extent of bacterial species as has been done for fungi, because too many coefficients for such an extrapolation are unknown (Allsopp *et al*., 1995). Several lines of evidence, however, suggest that the number of bacterial species is much higher than the currently known number of species (Demain and Davies, 1999). Microbial diversity represents the largest untapped reservoir of biodiversity for potential discovery of new biotechnology products, including new pharmaceuticals, new enzymes, new specialty chemicals, or new organisms that carry out novel processes (Demain and Davies, (1999). The bio-diversity of microbes is immense (Fatope *et al*., 2000). Microbes are exceptionally rich, diverse and easily accessible sources of novel metabolites that can inhibit enzyme pathways related to disease targets (Omura, 1992). The complexity of the interactions involved in biological control and the wide range of environmental conditions found globally in agriculture make it unlikely that any one strain will suppress even a single disease in all settings (Elizabeth and Handelsman, 1998). The genetic diversity of microorganisms with pest and disease-

suppressive potential remains an important yet largely untapped resource for biological control of crop pest and diseases (Fiddman *et al*., 2000). The revolutionary technological developments in high throughput DNA sequencing have resulted in the publication of many whole-genome sequences (Bloemberg and Lugtenberg, 2001). The sequencing of approximately 499 microbial genomes has been completed and those of another 1,299 are in progress (Liolios *et al*., 2006). The sequence data will, by comparative and functional genomics, facilitate the identification of genes present in bacteria that are involved in the regulation and production of secondary metabolites (anti-fungal metabolites) or whose expression is influenced by other rhizosphere organisms, such as fungi (Bloemberg and Lugtenberg, 2001).

3.0 CHAPTER 3: MATERIALS AND METHODS

3.1 Plant pathogens

The plant pathogens were obtained from ICIPE and the University of Nairobi Plant Science and Crop Protection departments. The pathogens used were *Fusarium oxysporum f.sp. lycopersici* -the causative agent of tomato wilt, obtained from University of Nairobi, Plant Science and Crop Protection Department, Kabete (KB 01); *Fusarium spp*., a rose flower pathogen, from ICIPE (ICIPE 06) and *Colletotrichum kahawae*, a coffee pathogen, obtained from University of Nairobi, Plant Science and Crop Protection Department, Kabete (KB 02). The pathogens were subcultured after every three months and maintained in Potato Dextrose Agar (PDA) media (Difco) prepared according to the manufacturer's instructions. For bioassays, the plates were incubated at 27^{o}C for 3 –10 days until abundant sporulation was observed. Spores were harvested from the plate with a sterile glass spreader and suspended in sterile Potato Dextrose broth (PDB) medium (Difco) prepared according to manufacturer's instructions. The suspended spores were used for bioassays (section 3.5).

3.2 Soil sampling sites

The actinobacterial cultures were isolated from soil samples randomly collected from three regions in Kenya; coastal region, western region and Mt. Kenya region. In each region, samples were randomly collected from three locations, which are representative of the region. Ten samples were collected randomly from each location, which brought the total number of samples used for the study to 90. Soil samples were collected from Kakamega Forest National Reserve, Ruma National Park and L. Bogoria National

Reserve from western region of the country; Shimba Hills, Arabuko Sokoke and Chyulu Hills National Parks from Coastal region and Mt. Kenya National Park, Aberdares National park and Imenti forest from Mt. Kenya region. These national parks represent the high altitude zones in Mt. Kenya region, Middle altitude zones in western Kenya and low altitude zones in the coastal region. Ten soil samples were collected randomly from each of the selected National Parks (Figure 1).

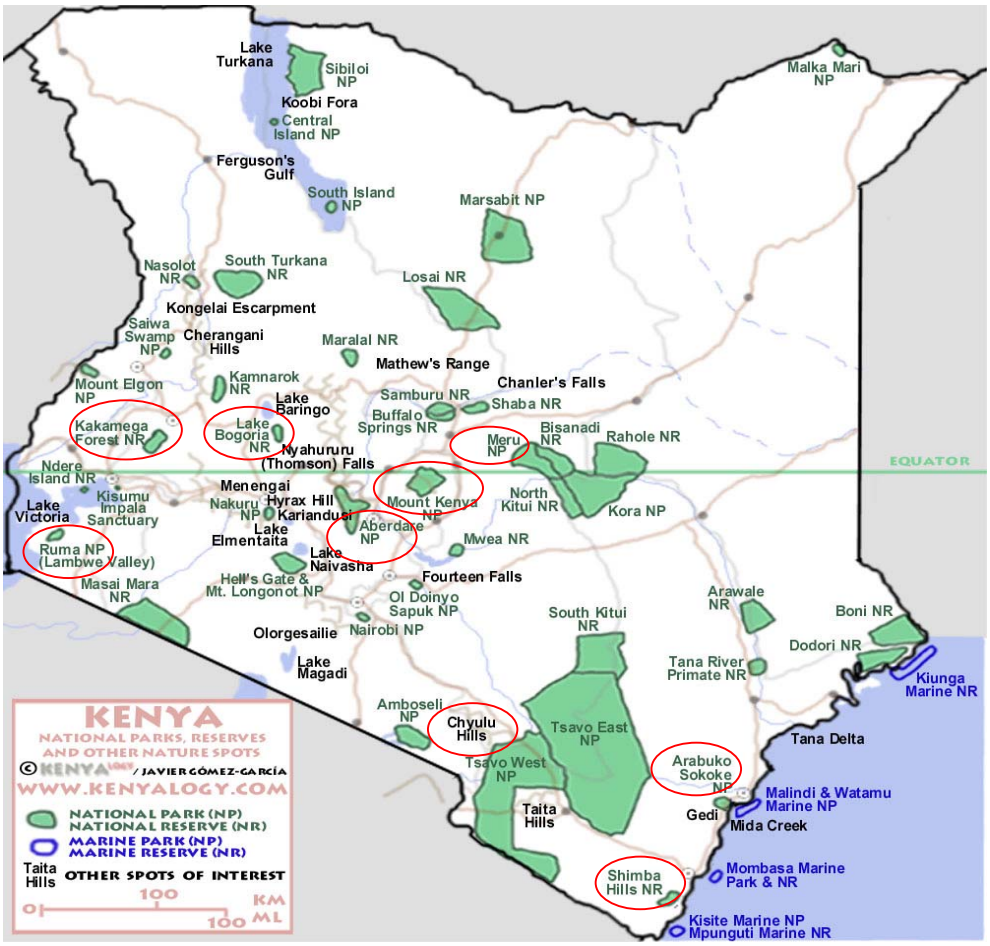


Figure.1: A map of Kenya showing the National Parks where soil samples were collection. The selected Parks are circled in red.

3.3 Isolation and maintenance of actinobacterial cultures

About 2 g of soil sample per site were collected using a sterile sample collection spoon, put in sterile soil sample bags and kept in a cool box at 4^{o}C for transportation to the laboratory. The samples were stored at 4 ^{o}C in the cold laboratory. About half a gram (0.5) of the sample was air dried for six hours at room temperature and subsequently suspended in 49.5 ml of sterile distilled water and shaken for one hour in a shaker incubator (200 rpm, 30 ^{o}C). Twenty microlitres of the supernatant was plated on differential agar media (Appendix 1). This media is poor in organic carbon, which effectively controlled eubacterial and fungal growth and aided in isolating the more slowly growing actinomycetes (Reddi and Rao, 1971). The plates were incubated at 28 ^{o}C for six days. Individual colonies of the cultures were isolated and subcultured into freshly prepared agar plates in differential media (Appendix 1) until pure cultures were isolated. The isolated pure actinomycete strains were streaked on differential agar slants. After incubation, the cultures were maintained at 4 ^{o}C and subcultured after every three months (Demain and Davies, 1999).

3.4 Growth in broth cultures and extraction of secondary metabolites

Isolates were inoculated into differential broth medium and incubated for six days in a shaker incubator at 28 ^{o}C and 200 rpm. The fermentation broth was centrifuged for 20 minutes at 12000 rpm to remove the microbial cells. The supernatant was passed through Waters Oasis HLB extraction cartridges in a solid-phase extraction chromatography protocol according to manufacturer's specifications (Waters Corporation, Milford U.S.A.). The bound sample was eluted using a solution containing 10% Water, 20%

Methanol, and 70% Acetonitrile. The eluate was freeze dried to remove the solvents and then stored at –20 ^{o}C (Demain and Davies, 1999).

3.5 Anti-fungal screening assays

To test the ability of individual actinomycete isolates to inhibit the growth of *Colletotrichum kahawae*, *Fusarium spp*. and *Fusarium oxysporum*, an *in vitro* plate assay was adopted. The screening assay procedure of DeFrank and Putnam (1985) was used to assay each isolate for its effect on germination of test fungi spores. Freeze-dried extracts was dissolved in 1 ml of sterile distilled water and mixed thoroughly. The fungal spores were collected by scrapping with a sterile inoculating loop across the surface of sporulating plate. Concentrated suspensions were made in half strength Potato Dextrose Broth. Counting of the spores for assay was done using a Neauber chamber. Fungal spore suspensions were diluted to give a concentration of 250 spores per 90 µl of half strength PDB. Ten microlitres of the test sample in sterile distilled water was applied to the 96 well plates (Demain and Davies, 1999). Ninety microlitres (90 µl) of fungal spore suspension was added to each well and thoroughly mixed. The plates were covered and incubated for 48 hours at 28 ^{o}C after which the bioassay results were read. The control well had sterile distilled water added instead of the sample extract. Antagonistic activity was evaluated visually under a dissecting microscope by scoring for inhibition of test fungal growth in the wells. Samples in wells where the growth of the test fungi was observed were recorded as negative and were not investigated further. The samples that inhibited fungal growth for all the three test fungi after 48 hours were scored as positive and were investigated further (Fatope *et al*., 2000).

3.6 Characterization of isolates

Actinobacterial cultures that showed antagonistic activity against the test pathogen were selected for characterization. The isolates were selected based on broad-spectrum activity on all the three test fungi tested. A polyphasic approach of characterization was adopted in this work. Preliminary characterization was performed using morphological and cultural characteristics. The cultural features were observed on a number of standard media after 7 days incubation at 28 °C. Morphological identification of the isolates was done under the dissecting and compound microscope to observe colony and growth characteristics. Further characterization was done through biochemical and molecular studies to support the findings of the morphological characterization.

3.6.1 Morphological characterization

3.6.1.1 Sporophore studies

The morphological characteristics of antagonistic strains were examined by dissecting and compound microscopy of 14-day cultures grown on differential agar and inorganic salts-glycerol agar. The coverslip technique (Zhou *et al*., 1998; Kawato & Shinobu, 1959) was used to observe the hyphae and spore-chain characters by light microscope. Spore-chain morphology and spore-surface ornamentation were studied by examining gluteraldehyde-fixed ethanol dehydrated specimens with a compound microscope. Mycelial and spore arrangement were determined by plating the organism on freshly prepared agar block aseptically placed at the center of the glass slide in a sterile glass petri dish. Cover slips were gently used to cover the inoculated agar block on the glass slides to allow the spread of mycelia on both the glass-slides and cover slips. The petri

dishes were covered. The plates were then inoculated for six to eight days to allow growth of the isolates after which both the cover slips and the glass slides were gently pulled out of the agar block. The cover slips and the glass slides were fixed overnight in 2% gluteraldehyde at 4^{o}C then gently washed with several changes of distilled water to remove excess gluteraldehyde. It was further dehydrated with successive passages through increasing concentrations of ethanol in the ranges of 10, 30, 50, 70, 80, and 95% allowing the cover slips and the slides to sit for 15 minutes at each ethanol concentration (Hopwood, 1960). The cover slips were then air-dried, gram stained and observed under an inverted microscope at x100 (Keast *et al*., 1984).

3.6.2 Biochemical characterization

The test strains were examined for a range of biochemical and physiological properties as described by Williams *et al*. (1983) and Kämpfer *et al*. (1991).

3.6.2.1 Carbohydrate fermentation

Carbohydrate fermentation was carried out in glucose manitol broth media containing Durham tubes according to the method of Harold (2002 and Williams *et al*. (1983). The fermentation media were phenol red, glucose, lactose, and sucrose broths (Cappuccino and Sherman, 2002). Following incubation, carbohydrates that were fermented produced acid that caused phenol red to turn yellow, there by indicating positive reaction.

3.6.2.2 Glucose oxidation fermentation (O-F) test

Glucose oxidation test was done using Hugh and Leifon's O-F media (pH 7.1) containing bromothymol blue indicator based on Harold (2002) protocol. One set of the tubes was covered with hot liquid Vaseline, which solidified to provide an anaerobic environment and the second set left open. Following incubation acid production from glucose was indicated by color change from blue to yellow. Oxidation was characterized by acid production in open tubes and by acid production in open and covered tubes (Cappuccino and Sherman, 2002).

3.6.2.3 Methyl Red-Voges- Proskauer (MR-VP) test

MR-VP test was used to determine the ability of the isolates to oxidize glucose with production and stabilization of high concentrations of acid end products according to Harold's (2002) protocol. MR-VP broth was inoculated with each of the isolates and incubated at 27 °C for 48 hours. Methyl red indicator or Barrit's reagent was added to aliquots of each culture. For positive culture, methyl red appeared red and in VP positive culture gave a rose coloration (Cappuccino and Sherman, 2002).

3.6.2.4 Starch hydrolysis

Starch agar (1.5%) containing 0.2% soluble starch served as the polysaccharide substrate. The detection of the hydrolytic activity following bacterial growth was determined by addition of iodine, a clear zone around the colonies indicated positive test (Cappuccino and Sherman, 2002; Harold, 2002).

3.6.2.5 Gelatin liquefaction

Nutrient Broth supplemented with 12% gelatin was used to demonstrate the hydrolytic activity of gelatinase (Harold, 2002). After incubation at 28 ^{o}C the cultures were placed in the refrigerator at 4°C for 30 minutes. Cultures that remained liquefied were considered positive for gelatin hydrolysis (Cappuccino and Sherman, 2002).

3.6.2.6 Indole production and Hydrogen sulfide production

Sulfur-Indole Mortility (SIM) agar media was used to test the production of tryptophanase enzyme and the ability to produce hydrogen sulfide from substrates such as sulfur containing amino acids and organic sulfur. Presence of indole was detected by addition of Kovac's reagent to 48-hour cultures of each isolates (Harold, 2002). Positive results were indicated by production of a cherry red layer. Absence of black coloration in the media following incubation indicated absence of hydrogen sulfide (Cappuccino and Sherman, 2002).

3.6.2.7 Catalase test

Catalase production was determined by addition of 3% hydrogen peroxide to Tryptic Soy Agar (TSA) cultures of each isolate based on the methods outlined by Cappuccino and Sherman (2002). A positive reaction was indicated by formation of bubbles, which indicated production of catalase activity.

3.6.2.8 Oxidase test

Cytochrome oxidase production was determined using oxidase strips containing tetramethy-p-phenylenediamine dichloride. Blue purple coloration indicated a positive test (Cappuccino and Sherman, 2002; Harold, 2002).

3.6.2.9 Citrate utilization

Simmons' Citrate agar slants were used to determine the capability of the isolates to use citrate as a carbon source for their energy (Harold, 2002). Bromothymol blue indicator incorporated in the media turned from green to prussian blue indicating positive tests (Cappuccino and Sherman, 2002).

3.6.2.10 Nitrate reduction test

The ability of the isolates to reduce nitrates to nitrites or beyond was carried out using nitrate broth medium containing 1% potassium nitrate according to the methods of Harold (2002) and that of Cappuccino and Sherman (2002). Following inoculation and incubation, addition of sulfanilic acid and alpha-naphthylamine produced a cherry red coloration, which was indicative of positive results (Cappuccino and Sherman, 2002).

3.6.2.11 Urease test

The ability of the isolates to attack nitrogen and carbon bonds in amide compounds was determined using urea broth media containing phenol red indicator according to the methods of Harold (2002). A positive reaction was indicated by development of deep pink color (Cappuccino and Sherman, 2002).

3.6.3 Molecular characterization of the isolates

3.6.3.1 DNA extraction from actinobacterial cells

Pure subcultures of the antagonistic isolates were inoculated in freshly prepared LB broth (Appendix 2) and incubated for six days in a shaker incubator at 28 ^{o}C and 200 rpm. Total genomic DNA was extracted using Ultra- Clean Microbial DNA Isolation kit (Mo Bio Laboratories, Calif. USA) according to the manufacturer's specifications based on the method of Stach *et al*, 2003. One point eight milliliters (1.8 ml) of bacterial cultures was added into the centrifuge tube and centrifuged for 30 seconds at 10000 x g. The supernatant was discarded and the tubes spinned one more time for 30 seconds at 10000 x g. The media supernatant was removed completely with a pipette tip. The cell pellet was resuspended in 300 µl of MicoBead solution, vortexed gently to mix and then the resuspended cells transferred to a Micro Bead tube. Fifty microlitres (50 µl) of solution MD1 (Mo Bio Laboratories, Calif. USA) was added to the Micro Bead tube and heated in an incubator at 60 ^{o}C for 10 minutes. The tubes were secured horizontally on a flatbed vortex pad with a tape and vortexed at maximum speed for 10 minutes. The tubes were centrifuged for 30 seconds at 10000 x g and the supernatant transferred to clean microcentrifuge tubes. One hundred microlitres (100 µl) of solution MD2 (Mo Bio Laboratories, Calif. USA) was added to the supernatant, vortexed for 5 seconds and incubated at 4^{o} C for 5 minutes. The tubes were centrifuged for 1 minute at 10000 x g and the entire volume of the supernatant transferred to clean 2 ml tubes. Nine hundred microlitres (900 µl) of solution MD3 (Mo Bio Laboratories, Calif. USA) was added to the supernatant and vortexed for 5 seconds. Seven hundred microlitres (700 µl) was loaded into the spin filter, centrifuged at 10000 x g for 30 seconds and the flow-through

discarded. The remaining supernatant was added to the spin filter, centrifuged at the same conditions and all the flow-through discarded. Three hundred microlitres (300 µl) of solution MD4 (Mo Bio Laboratories, Calif. USA) was added and centrifuged at 10000 x g for 30 seconds and the flow-through discarded. The tubes were centrifuged again for 1 minute and the flow-through discarded. The spin filter was carefully placed in 2 ml tubes, 50 µl of solution MD5 (Mo Bio Laboratories, Calif. USA) added to the center of the white filter membrane and centrifuged for 30 seconds. The spin filter was discarded and the DNA in the tube was ready for application (Stach *et al*., 2003). The DNA was semi quantified on a 1% agarose gel in 1xTAE buffer (Appendix 3) and visualized under UV by staining with ethidium bromide (Sambrook *et al*., 1989).

3.6.3.2 PCR amplification of actinobacterial 16S rDNA

Purified total DNA from each isolate was used as a template for amplification of the 16S rDNA genes. This was done using the HotStar Taq Master Mix Kit (Qiagen, USA) according to the manufacturer's instructions. Nearly full-length 16S rRNA gene sequences were PCR-amplified using bacterial primer pair 27F forward 5'-TAG AGT TTG ATC CTG GCT CAG-3') and 1392R reverse, 5'-GAC GGG CGG TGT GTA CA-3' (Sigma) according to the position in relation to *Escherichia coli* gene sequence (Embley and Stackebrandt, 1994; Lane, 1991). Amplification was performed using a model PTC-100 thermal cycler (MJ research inc., USA). Amplification was carried out in a 50 µl mixture containing 25 µl of HotStar Taq Master Mix, 0.2 µl (pmol) of 27F forward primer, 0.2 µl (pmol) of 1392R reverse primer, 1 µl of template DNA and 23.6 µl of water. The control contained all the above except the DNA template. Reaction

mixtures were subjected to the following temperature cycling profiles repeated for 35 cycles: Initial activation of the enzyme at 95^{o}C for 15 minutes, denaturation at 94^{o}C for 30 seconds, primer annealing at 55^{o}C for 45 seconds, chain extension at 72^{o}C for 2 minutes and a final extension at 72^{o}C for 10 minutes (Roux, 1995). Amplification products (20 µl) were separated on a 1% agarose gel in 1X TAE buffer and visualized by ethidium bromide staining (Sambrook *et al*., 1989).

3.6.3.3 Purification of PCR products

The PCR products were purified using the QIAquick PCR purification Kit protocol (Qiagen, Germany) according to manufacturer's instructions. Five volumes of buffer PB (Qiagen, Germany) was added to 1 volume of the PCR sample and thoroughly mixed. The QIAquick spin column was placed in a 2 ml collection tube, the sample applied to the QIAquick column to bind the DNA, and then centrifuged for 30-60 seconds at 13000 rpm. The flow-through was discarded, and the QIAquick column placed back into the same tubes. To wash the DNA, 0.75 ml buffer PE was added to the QIAquick column and centrifuged for 1 minute. The flow-through was discarded and the column centrifuged again for an additional 1 minute at 13000 rpm to remove residual ethanol from buffer PE. The QIAquick column was placed in a 1.5 ml microcentrifuge and 30 µl of buffer EB (10mM Tris-Cl, pH 8.5) added to elute DNA. The tubes were then centrifuged for 1 minute, the spin column removed and DNA stored at –20^{o}C for application (Sambrook *et al*., 1989).

3.6.3.4 Phylogenetic data analysis

Sequencing of purified PCR products was done without cloning, using a commercial service provider. The CHECK-CHIMERA program (http://rdp.cme.msu.edu/html/) of the Ribosomal Database Project (Maidak *et al*., 2001) was used to check for the presence of possible chimeric artifacts (Janssen *et al*., 2002). Sequence data was analyzed with ARB software package [version 2.5b; O.Strunk and Ludwig, Technishe Universitat Munchem (http://www.arb-home.de)]. The new sequences were added to the ARB database and aligned with the Fast Aligner Tool (version 1.03). Alignments were checked and corrected manually where necessary, based on conserved regions. 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) website (http://www.ncbi.nih.gov) in order to determine similarity to sequences in the Genebank database (Altschul *et al*., 1990; Shayne *et al*., 2003). The 16S rRNA gene sequences with high similarities to those determined in the study were retrieved and added to the alignment based on BLAST results. Phylogenetic trees were constructed by Maximum likelihood method. Bootstrap analysis-using PHYLIP for 100 replicates was performed to attach confidence estimates for the tree topologies (Felsentein, 1989; Saitou and Nei, 1987).

4.0 CHAPTER 4: RESULTS

4.1 Isolation

A total of five hundred and thirty six (536) pure isolates were obtained from the 90 soil samples. Five hundred and three (503) isolates were grown on broth media for antifungal assays, the rest were lost due to contamination or inability to grow after subculture. A total of four hundred and twenty eight (428) isolates (Table 1) were screened for antifungal activity, 75 were lost due to contamination. Of the 428 screened, forty-nine (49) isolates showed antagonistic activity to all the three test fungi. Eleven (11) isolates, out of the 49 antagonists were investigated further, based on their broad-spectrum *in vitro* activity on all the three test fungi (Table 1). They were found to suppress the growth of fungal phytopathogens *in vitro*.

Table 1. A summary of soil collection sites, number of isolates screened and the number showing broad-spectrum activity on test fungi

Location	Code	No. of soils	No. of isolates	No. of isolates brothed	No. of isolates screened	No. active on 3 fungi
Chyulu	Chy	10	80	73	67	6
Arabuko	Arj	10	75	69	63	0
Aberdares	Ab	10	70	65	49	1
Imenti	Imt	10	77	75	70	1
Mwaluganje	Mws	10	43	40	35	1
Ruma	Ruj	10	67	66	50	1
Bogoria	Boj	10	60	58	53	0
Kakamega	Kkj	10	42	38	24	1
Mt. Kenya	Mkj	10	22	19	17	0
TOTAL	9	90	536	503	428	11

Most of the isolated actinobacteria grew well on the differential agar media used for isolation. There was different numbers of actinobacterial isolates between different geographical areas, as well as between different sites that were sampled. There was a high number of isolates in some soils as compared to others. For example, Chyulu National Park soils had a high diversity of isolates (Figure 2 a), while Mount Kenya soils had very few colonies observed (Figure 2 b).

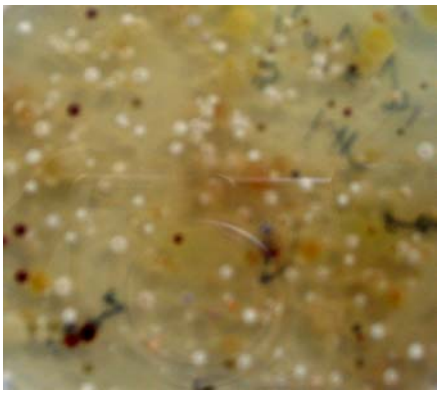


Figure 2a. A photograph showing soil culture plate of Chy 18 above from Chulu Park with different colonies of actinobacteria before isolation of individual colonies. There is high diversity with colorful looking colonies

Figure 2b. A photograph showing soil culture plate of Mkj 13 above from Arabuko Sokoke Park with different colonies before isolation. There is low diversity with a few colonies

Pure isolates grew well on the differential agar media forming well-isolated colonies. The isolates formed colored tough, leathery and filamentous colonies that were hard to pick from the culture media (Figure 3a and 3b). The majority of the isolates also produced colored pigments, which were secreted into the culture media within a few days of incubation (Figure 3a and 3b). However, not every isolate that produced colored pigment into the media was an antagonist. Upon screening *in vitro*, isolate Arj 12-8 (Figure 3b) unlike isolate Chy 4-10 (Figure 3a) was not antagonist.

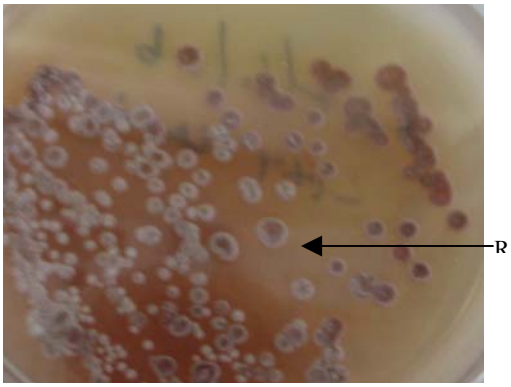


Figure 3a. A pure plate culture of antagonistic Chyulu 4-10. The colony is hard, not gummy and does not lift easily from the plate. The isolate is reddish in color and produces red pigment into the media indicated by the arrow

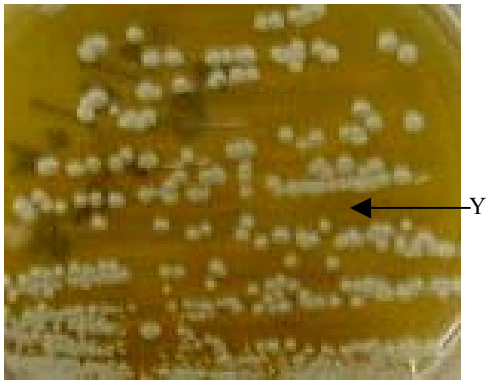


Figure 3b. A photograph of a pure plate culture of Arj 12-8 from soil number 12. The isolate secretes a yellow metabolite (arrow) but on screening, it does not have any antagonistic effect on test fungi

4.2 Bioassays

Bioassay results show that some isolates had stronger antagonism on the test fungi than others, while others showed no activity. For example in Figure 4, isolate Chy 15-5 (well 5B) showed strong activity on *Fusarium spp* than isolate Ab 7-2 (well 6C).

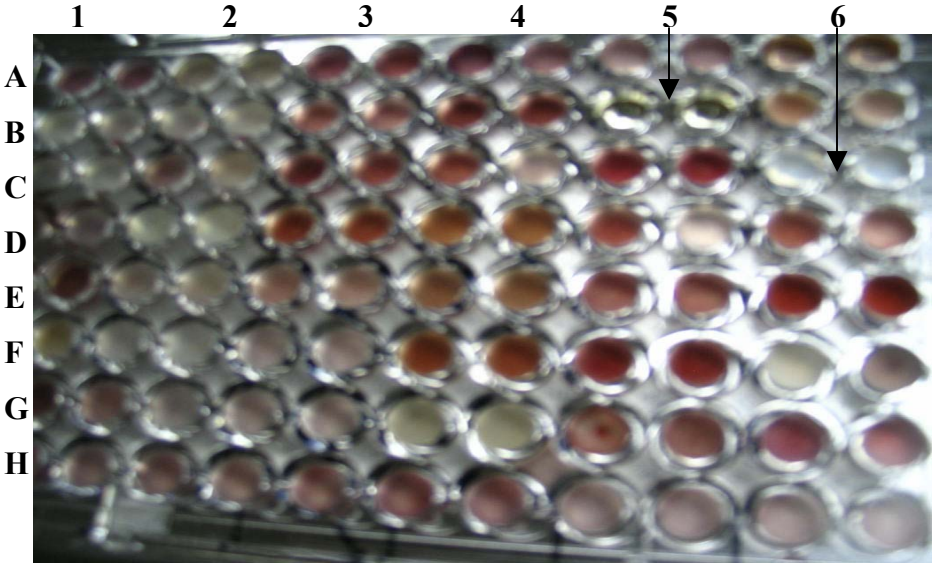


Figure 4. Antagonism of Chyulu 15-5 (well 5B) and Aberdares 7-2 (well 6C) to *F.spp* on Potato Dextrose Broth in a 96 well plate. In vitro assay was done in duplicates (two wells/sample). The clear wells (5-B) and (6-C) show that the isolates tested, Chyulu 15-5 and Aberdares 7-2 are active and suppress the growth of test fungi *F. spp* in *vitro*. The rest of the wells with turbidity show no antagonism and the test fungus grows in the wells.

Isolate Ruj 7-1 showed a very strong antagonistic activity against *F. oxysporum* (well 1A) as shown in Figure 5.

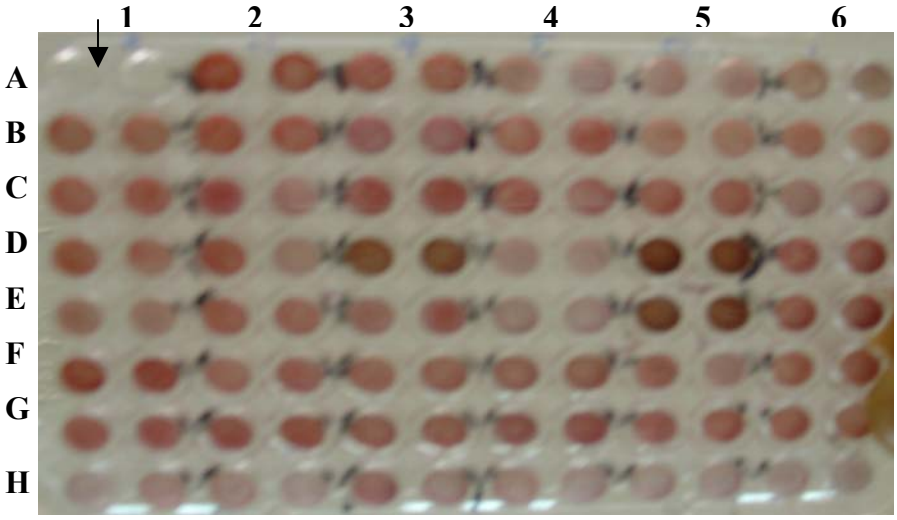


Figure 5. Antagonism of Ruj 7-1 to *F. oxysporum* (well 1A) on Potato Dextrose Broth in a 96 well plate. In vitro assay was done in duplicates (two wells/sample). The clear well (1-A) show that the isolate tested, Ruj 7-1 is active and suppress the growth of test fungi *F. oxysporum* in *vitro*. The rest of the wells with colored turbidity show no antagonism and the test fungus grows in the wells. Wells 1-G to 6G are control wells where no sample fermentation product was added and there is abundant growth of fungus.

Four hundred and twenty eight (428) isolates from the nine locations were screened for bioactivity on the three test fungi. Out of this number, eleven (11) isolates were found to have a broad-spectrum activity on all the three test fungi as shown in Table 2. They were found to suppress the growth of the three fungal phytopathogens *in vitro*. The eleven isolates were therefore selected for characterization. Two isolates out of the eleven with broad-spectrum activity showed a very strong antagonistic effect. Eighteen (18) isolates showed activity in only one or two of the test fungi. Twenty (20) isolates showed a very minor antagonism in most of the fungi tested with the activity disappearing within a week in some cases. Three hundred and seventy nine (379) isolates did not show any observable antagonism in *vitro* as shown in Table 2. The isolates that were not

antagonists, those that showed inhibition on only one or two test fungi, or those whose inhibition activity disappeared with time, were not investigated further. Six isolates out of the eleven with broad-spectrum activity were from Chyulu National Park soils. This represents about 55% of those with broad-spectrum activity from Chyulu soils. Others were Ruma (9%), Mwaluganje (9%), Aberdares (9%), Imenti (9%) and Kakamega (9%). Soils from Bogoria, Arabuko Sokoke and Mt. Kenya National Parks did not produce isolates with broad-spectrum activity on the test fungi.

Table 2. A summary of biological activity results for all the 428 isolates screened against *F. oxysporum, Fusarium. spp. and C. kahawae*

Source	N	Biological activity of isolates on culture				
		+++	++	+	+-	-
Chy	67	2	4	6	9	46
Arj	63	0	0	3	3	57
Ab	49	0	1	0	0	48
Mws	35	0	1	1	1	32
Imt	70	0	1	1	2	66
Boj	53	0	0	1	5	47
Ruj	50	1	0	3	0	46
Kkj	24	0	1	3	0	20
Mkj	17	0	0	0	0	17
Total	428	3	8	18	20	379

Inhibition of *C.kahawae*, *F.spp*. and *F.oxysporum*, defined as growth suppressed and growth less abundant in the wells where the isolate's extract has been added;
+++ very strong activity on all the three test fungi
++ strong inhibition on all the three test fungi with no fungal growth observed
+ inhibition on only one or two of the test fungi
+- very minor inhibition with activity disappearing with time (some hyphal growth observed)
- no inhibition;
N number screened.

The bioassay period was extended to more than one week for all the isolates for each test fungi. This was done to validate the bioactivity of the bioactive secondary metabolites from each isolate over an extended period of time. After the confirmatory assays, the eleven (11) isolates were found to suppress the growth of the three test fungi and the suppression activity was persistent even after three weeks as shown in Table 3. These results therefore gave a strong indication about the biological control potential of the isolates, justifying their further characterization. The 11 isolates are: (1) Chy 15-6; (2) Ab 7-2; (3) Mws 1-3; (4) Chy 15-5; (5) Ruj 7-1; (6) Chy 2-8; (7) Kkj 5-1; (8) Chy 15-10; (9) Chy 4-10; (10) Imt 7-3 and (11) Chy 2-3. These isolates were therefore used for further studies.

Table 3. Validation of in *vitro* antagonism of the active isolates beyond one week

Biological activity after one week				Biological activity after three weeks			
Isolate	Test organism			Isolate	Test organism		
	C.k	F.spp	F.oxy		C.k	F.spp	F.oxy
Chy 15-6	++	++	++	Chy 15-6	++	++	++
Chy 15-10	++	++	++	Chy 15-10	++	++	++
Chy 2-3	++	++	++	Chy 2-3	++	++	++
Chy 2-8	++	++	++	Chy 2-8	++	++	++
Chy 15-5	+++	+++	+++	Chy 15-5	+++	+++	+++
Chy 4-10	+++	+++	+++	Chy 4-10	+++	+++	+++
Imt 7-3	++	++	++	Imt 7-3	++	++	++
Mws 1-3	++	++	++	Mws 1-3	++	++	++
Ab 7-2	++	++	++	Ab 7-2	++	++	++
Ruj 7-1	+++	+++	+++	Ruj 7-1	+++	+++	+++
Kkj 5-1	++	++	++	Kkj 5-1	++	++	++
Control	G	G	G	Control	G	G	G

Inhibition of *C.kahawae*, *F.spp*. and *F.oxysporum*, defined as growth suppressed and growth less abundant in the wells where the isolate's extract has been added;
+++ very strong inhibition on all the three test fungi
++ strong inhibition on all the three test fungi with no fungal growth observed
G abundant growth

To confirm that the antagonistic ability of the isolates was not only on target test fungi but also on bacteria, type culture collection (TCC) specimens of Gram+ and Gram- bacteria were also introduced. Their extracts were assayed on known characterized bacteria as shown in Table 4. The eleven isolates with broad-spectrum activity were also tested on type culture collections of *Escherichia coli* (NCTC 10418) and *Staphylococcus aureus* (NCTC 10788) as control test organisms. The assay was done first on the two control organisms in 96-well plate and observed for growth suppression after incubation. The results obtained ranged from strong to weak antagonism as shown in Table 4. The second assay on the two-control organism was done using disc assay method and it gave inhibition zones ranging from 0.1 cm to 2.0 cm (Table 4).

Table 4. Sensitivity test of the active isolates on Type Culture Collection of Gram +ve (*Staphylococcus aureus*) and Gram –ve (*Escherichia coli*)

Isolate	A. 96 well plate assay		B. Disc assay (diameter in cm)	
	S. aureus (NCTC 10788)	*E. coli* (NCTC 10418)	*S. aureus* (NCTC 10788)	*E. coli* (NCTC 10418)
Chy 15-6	++	++	1.2 cm	1.2cm
Chy 15-10	++	-	1.0 cm	-
Chy 2-3	++	-	1.6 cm	-
Chy 2-8	++	+	2.0 cm	0.2cm
Chy 15-5	++	++	1.2 cm	1.4 cm
Chy 4-10	++	++	1.8 cm	0.3 cm
Imt 7-3	++	+	1.4 cm	0.2 cm
Mws 1-3	++	+	1.6 cm	0.4 cm
Ab 7-2	+	+	0.5 cm	0.1 cm
Ruj 7-1	++	++	1.7 cm	1.2 cm
Kkj 5-1	++	++	1.4 cm	0.3cm

A: Inhibition of *S. aureus and E. coli*, defined as growth suppressed and growth less abundant in the wells where the isolate's extract has been added;
++ strong inhibition with no growth
+ minimal inhibition
- no inhibition.
B: Zones of inhibition for the two bacteria measured in cm.

Extracts of isolates were tested for antagonistic effects on the type culture collection of both Gram-positive bacteria (*S. aureus*) and Gram-negative bacteria (*E. coli*). Results indicate that some isolates showed more antagonistic activity on *S. aureus* than on *E. coli.* For example isolate Chy 15-5 was more active on *S. aureus* than on *E. coli* as shown in Figure 6. Different isolates also demonstrated different antagonistic activity on the same test bacteria. For example isolate Chy 2-8 was more active on *S. aureus* than isolate Chy 4-10 as shown in Figures 7a and 7b.

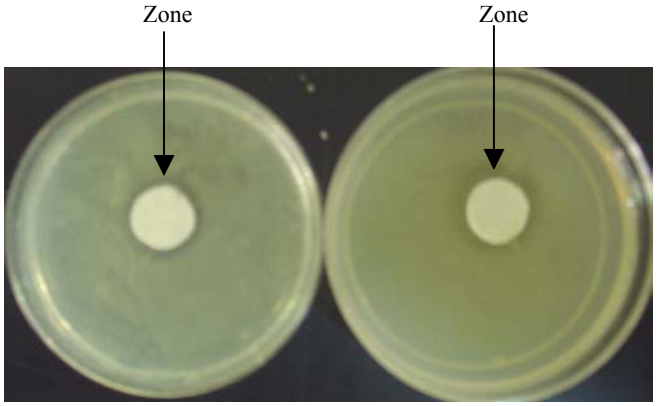


Figure 6 Antagonistic activity of Chy 15-5 on *E. coli* and *S. aureus*. The antagonistic activity can be seen by the clear zone of inhibition around the paper disc

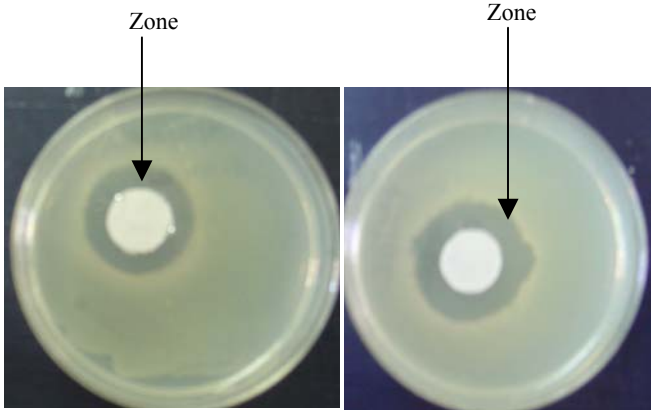


Figure 7a Antagonistic activity of Chy 4-10 on *S.aureus*. The zone of inhibition around the paper disc show antagonistic effect

Figure 7b. Antagonistic activity of Chy 2-8 on S. aureus. The zone of inhibition around the paper disc show antagonistic effect

4.3 Biochemical characterization

The isolates were taken through a series of biochemical tests to determine their physiological characteristics. All the isolates except two, Imt 7-3 and Mws 1-3 hydrolyzed starch suggesting that majority of these isolates play a role in degradation of organic matter in the environment. None of the isolates liquefied gelatin but all of them were catalase positive. A few isolates for example, Chy 2-3, Imt 7-3, Mws 1-3 and Ruj 7-1 were nitrate reducers.

Table 5. Biochemical test results for all the eleven antagonistic isolates

	Biochemical tests									
Isolate	Starch	Gelatinase	Catalase	Indole	Urease	MR	VP	Nitrate	H_2S	TSI
Chy 15-6	+	-	+	-	-	+	-	-	+	+
Chy 15-5	+	-	+	-	+	+	-	-	+	+
Chy 4-10	+	-	+	-	+	+	-	-	+	+
Chy 2-8	+	-	+	-	+	+	-	-	-	+
Chy 2-3	+	-	+	-	-	+	-	+	+	+
Imt 7-3	-	-	+	-	+	-	-	+	-	-
Mws 1-3	-	-	+	-	+	+	-	+	-	+
Ruj 7-1	+	-	+	-	+	+	-	+	-	+
Kkj 5-1	+	-	+	-	+	+	-	-	-	-
Ab 7-2	-	-	+	-	-	+	-	-	-	-
Chy 15-10	+	-	+	-	-	-	-	-	+	+

Biochemical test results for the 11 isolates with broad-spectrum activity on test fungi defined as; (+) a positive result for the reaction and (-) a negative test for the reaction
MR- Methyl Red; VP- Voges Proskauer; H_2S- Hydrogen Sulphide

4.4 Morphological characterization of isolates

Morphological studies of the isolates were done using the dissecting (x160) and inverted microscopes (x1000). All the eleven isolates were Gram positive and grew well on differential agar media. Growth on the media was moderate to abundant for most of the isolates (Table 6). All the isolates displayed a branched network of mycelia with conidiospores arrangement that is characteristic of streptomycetes.

Table 6. Morphological characteristics of the isolates as observed under dissecting microscope (x160 and enlarged two-fold) and compound microscope (x1000 and enlarged two-fold).

Isolate	**Characteristics**			
	Colony color	Growth form	Pigment color	Spore forms
Ruj 7-1	Pale-brown	Abundant and rhizoid	Yellow pigment	Chainlike at the terminal of aerial mycelia (Rectiflexibiles)
Chy15-5	Dark-gray	Moderate, wrinkled, tough and leathery	Dark-gray pigment	Round at the terminal of aerial mycelia (Rectiflexibiles)
Chy15-6	Brown-gray	Moderate with big, tough, leathery and concentric	Dark-brown pigment	Spiral at the terminal of aerial mycelia (Spiral)
Kkj 5-1	White	Oval, tough and leathery	Pale-brown pigment	Spiral at the terminal of aerial mycelia (Retinaculum-Apertum)
Imt 7-3	White	Abundant, tough, leathery and round	Yellow pigment	Branched network on either side of aerial mycelia (Spiral)
Chy 4-10	Red	Abundant, round and wrinkled with raised margins	Red pigment	Spiral at the terminal of aerial mycelia (Spiral)
Chy 2-8	Red	Abundant round, smooth and rhizoid	Red pigment	Spiral at the terminal of aerial mycelia (Spiral)
Ab 7-2	Dark-gray	Moderate, complex, tough	Dark-gray pigment	-
Chy 15-10	Brown	Moderate, concentric and tough	Brown pigment	Spiral with a branched network (Spiral)
Chy 2-3	Gray	Moderate with	Brownish- yellow	Open spirals form

		complex tough and leathery	pigment	at the terminal of aerial mycelia (Spiral)
Mws 1-3	Deep-dark	Abundant, rhizoid, tough and leathery	Dark pigment	Oval spores at the terminal of aerial mycelia (Spiral)

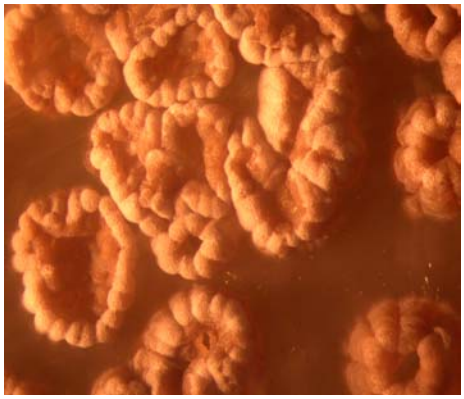


Figure 8a. Chy 15-6 colonies under dissecting microscope (x160) and enlarged two-fold. Notice the concentric growth on media

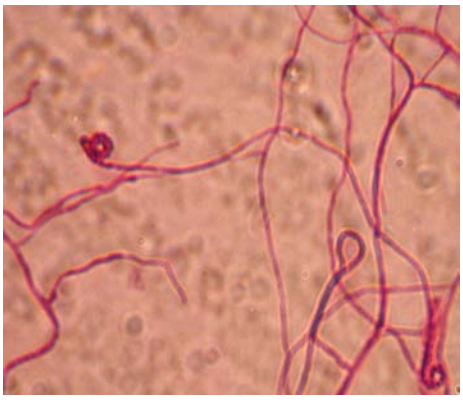


Figure 8b. Chy 15-6 spores at the terminal of mycelia under compound microscope (x1000) and enlarged 2 fold. Closed spirals with gnarled sporophores (Spiral).

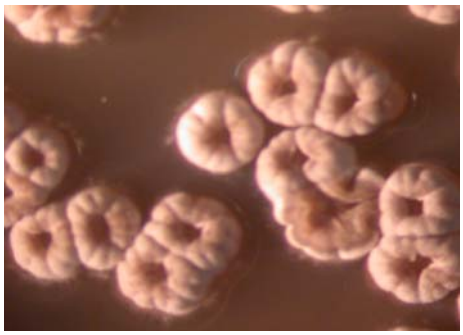


Figure 9a. Chy 15-5 colonies under dissecting microscope (x160) and enlarged two-fold. Notice the wrinkled margins.

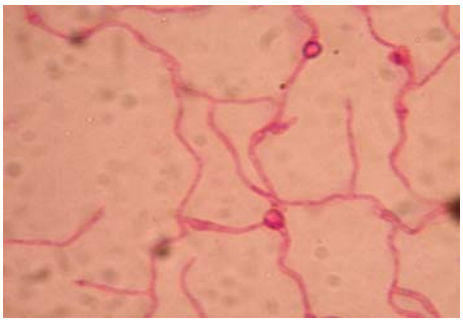


Figure 9b. Chy 15-5 spores with a branching network of mycelia under compound microscope (x1000) and enlarged 2 fold. Notice the flexous sporophores (Rectiflexibiles).

Figure 10a. Chy 2-3 colony under dissecting microscope (x160) and enlarged two-fold. Notice the tough, leathery brown mycelia

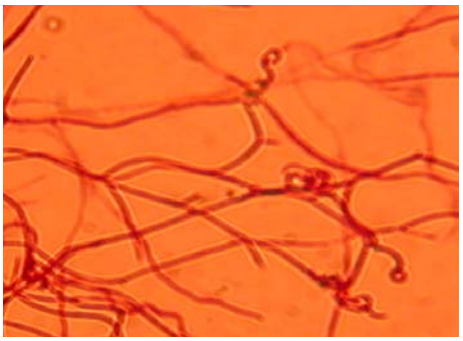


Figure 10b. Chy 2-3 spores under compound microscope (x1000) and enlarged 2 fold. Notice the long branching mycelia with closed spirals (Spiral).

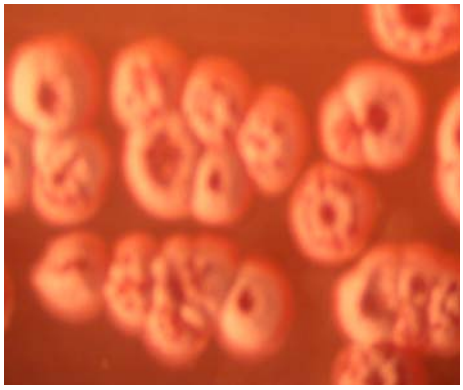


Figure 11a. Chy 4-10 colonies with round raised margins and wrinkled surface under dissecting microscope (x160) and enlarged.

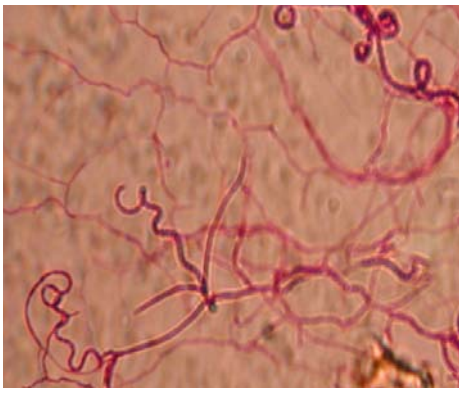


Figure 11b. Chy 4-10 spores observed under compound microscope (x1000) and enlarged. Notice the closed spiral nature of mycelia at the terminal of the filaments (Spiral)

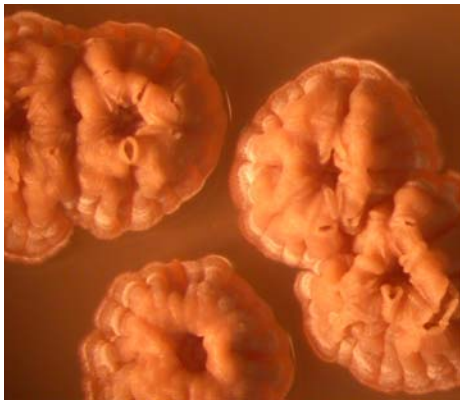


Figure 12a. Chy 15-10 colonies under dissecting microscope (x160) and enlarged two-fold. Notice the wrinkled margins

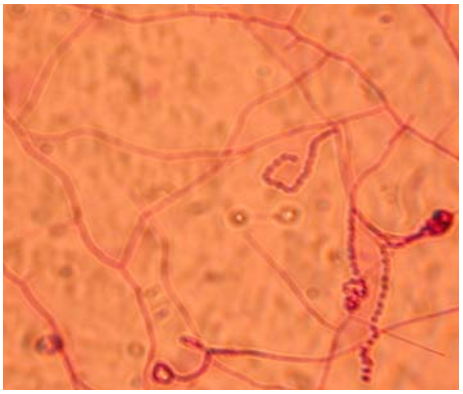


Figure 12b. Chy 15-10 spores under compound microscope (x1000) and enlarged. Notice the chain of spores at tip of mycelia in a closed spiral form (Spiral).

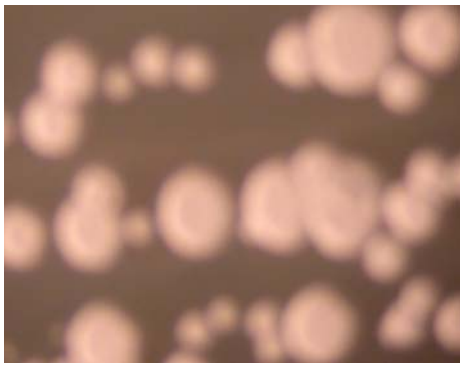


Figure 13a. Imt 7-3 colonies under microscope (x160) and enlarged two-fold. Notice the smooth round colonies

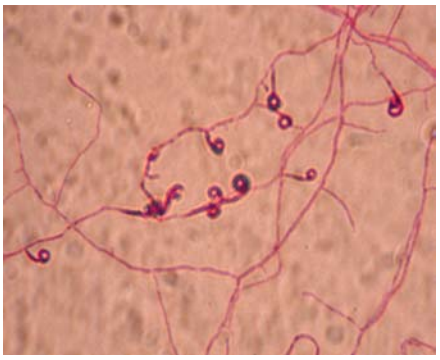
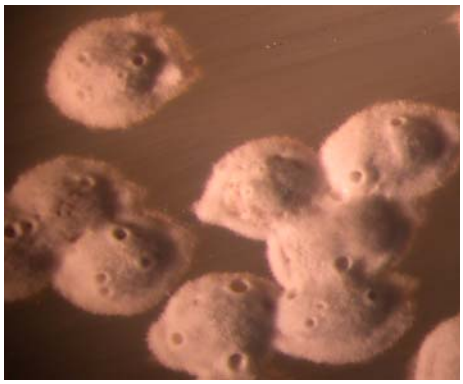


Figure 13b. Imt 7-3 spores under compound microscope (x1000) and enlarged. The spores are arranged on either side of mycelia (Closed spirals).



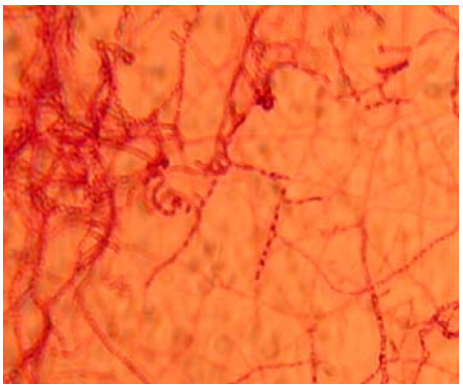


Figure 14b. Kkj 5-1 spores under compound microscope (x1000) and enlarged. Spores appear in chains at the tip of mycelia in an open loop, primitive spiral form (Retinaculum-Apertum).

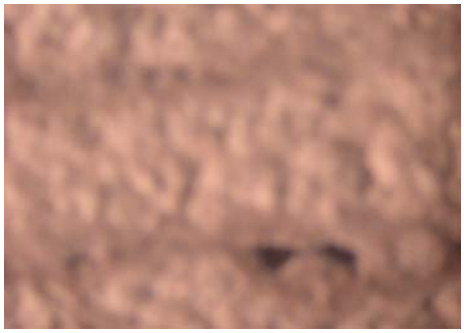


Figure 15a. Ruj 7-1 colonies under dissecting microscope (x160) and enlarged two-fold. It has abundant growth of mycelia covering the whole plate.

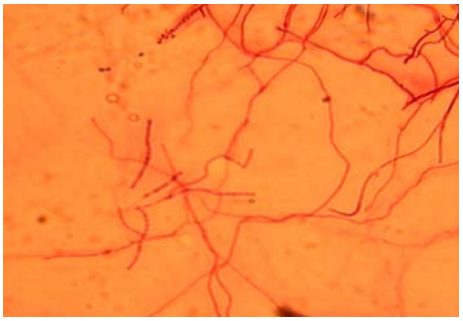


Figure 15b. Ruj 7-1 spores under compound microscope (x1000) and enlarged 2 fold. Spores are round and disseminate from mycelia at the terminal. Rectiflexibiles sporophores.

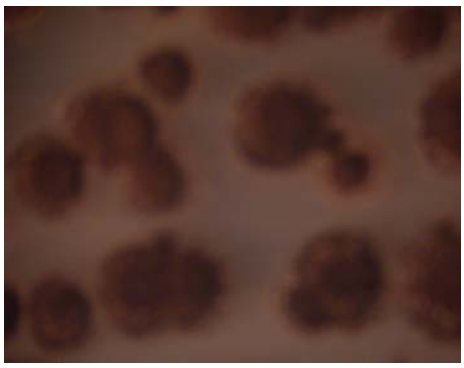


Figure 16a. Mws 1-3 colonies under dissecting microscope (x160) and enlarged two-fold. The dark, gray and irregular colonies secrete dark metabolite into the media

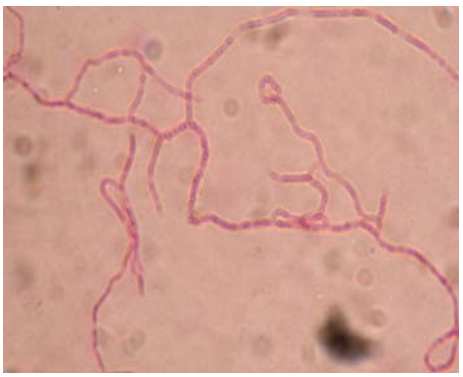


Figure 16b. Mws 1-3 oval spores at the terminal of aerial mycelia as observed under compound microscope (x1000) and enlarged two-fold (Closed spirals).

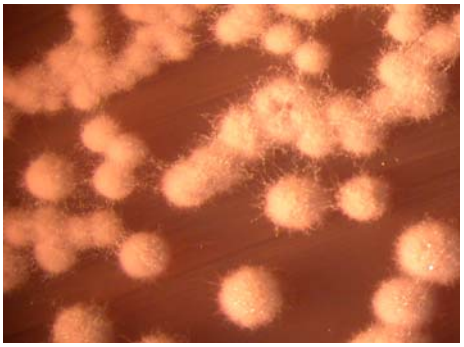


Figure 17a. Chy 2-8 colonies under dissecting microscope (x160) and enlarged two-fold. Notice the rhizoid colonies

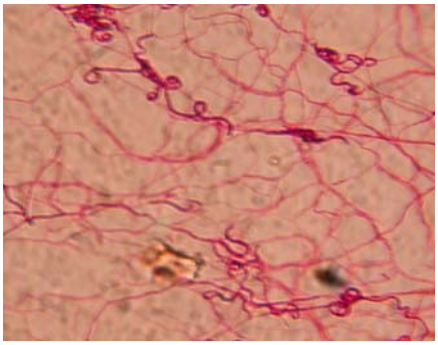


Figure 17b. Chy 2-8 spores under compound microscope (x1000) and enlarged two-fold. Spores form in an open spiral form at the terminal of mycelia (Spiral).

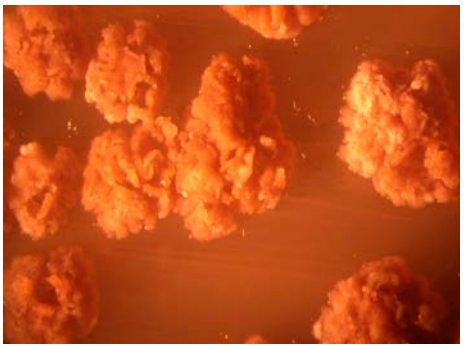


Figure 18. Ab 7-2 colonies under dissecting microscope (x160) and enlarged two-fold. The colonies form a complex shape and secrete dark pigment into the media

4.5 PCR amplification of 16S rDNA gene from isolates

Genomic DNA was successfully extracted from all the 11 active isolates. 16S rDNA amplification with bacterial based primers specific for this region of DNA yielded an amplification product of approximately 1365 bp from all the 11 samples (Fig. 19).

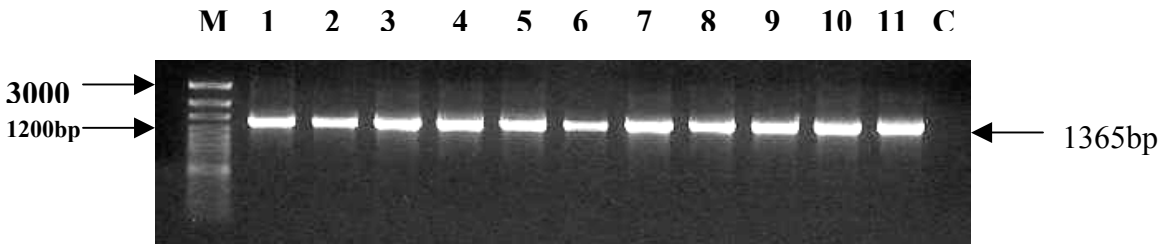


Figure 19. A 1% agarose gel showing PCR amplification of 16S rDNA of the isolates visualized after ethidium bromide staining;
(1) Chy 15-6; (2) Ab 7-2; (3) Mws 1-3; (4) Chy 15-5; (5) Ruj 7-1; (6) Chy 2-8; (7) Kkj 5-1; (8) Chy 15-10; (9) Chy 4-10; (10) Imt 7-3; (11) Chy 2-3 and (C); Control respectively. M is 100bp plus DNA ladder (Fermentas) used as a molecular marker

4.6 Phylogenetic cluster analysis of sequences

The 16SrDNA amplified product from the eleven isolates (Figure 19) were sequenced. Similarity searching using BLAST showed that they belong to the domain Bacteria. A phylogenetic tree showing the phylogenetic position of each of the members of isolates studied is shown in Figure 20. The phylogenetic tree showed five main clusters. All the clusters comprised the genus *Streptomyces* and all the eleven isolates clustered with this genus. The isolates shared sequence identity of between 97-100% with known *Streptomyces* species (Figure 20). In the first cluster, isolate Kkj 5-1 clustered with the soil actinobacterium *Streptomyces tubercidicus* and this was supported with a bootstrap value of 52% and high sequence identity of 99%. Isolates Imt 7-3 and Mws 1-3 clustered together with a high bootstrap value of 100% and this was supported by a sequence

Phylogenetic tree showing phylogenetic position of the isolates

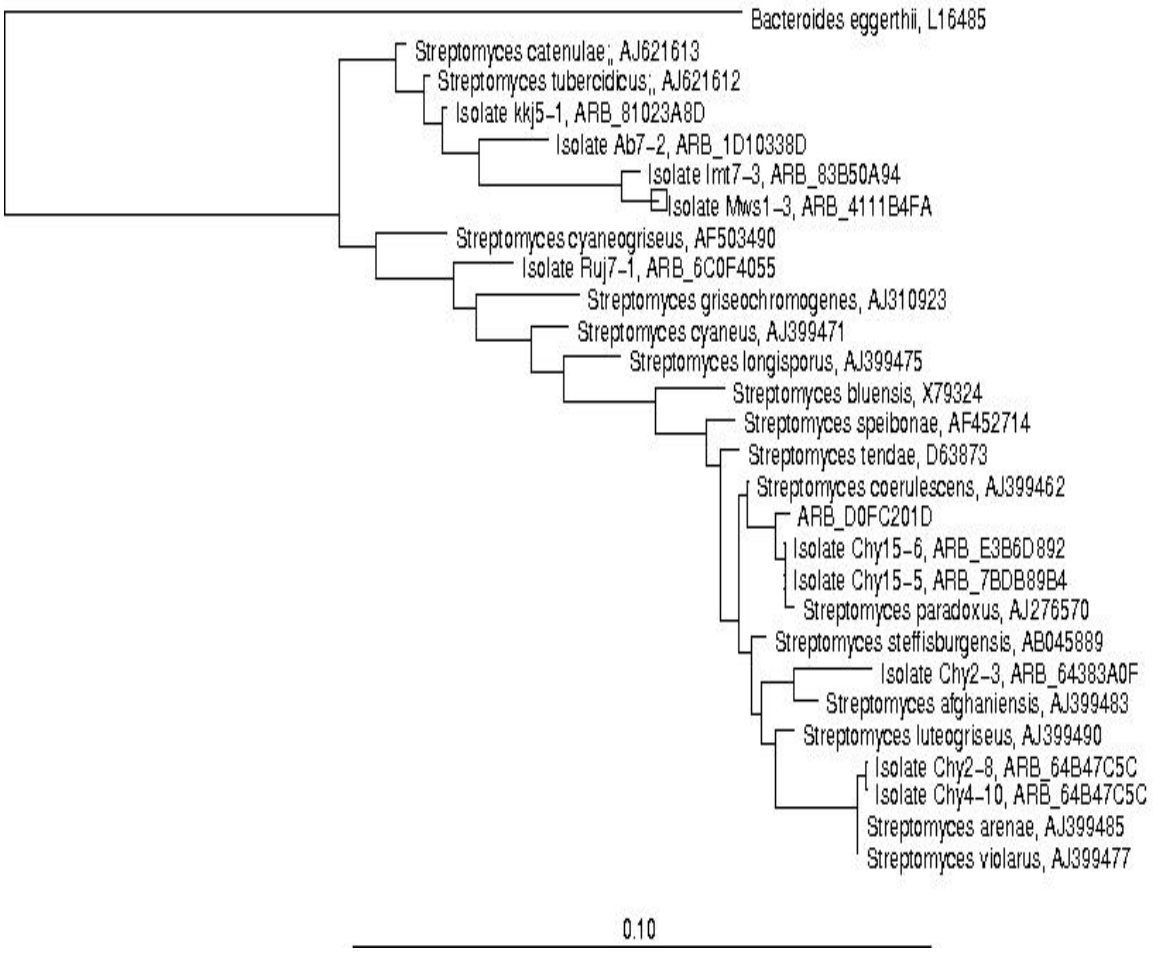


Figure 20. Phylogenetic position of isolates with broad-spectrum antifungal activity against the test fungi. The scale bar indicates approximately 10% sequence difference. The 16S rDNA sequence of *Bacteroides eggerthii* was used as an outgroup

identity of 99% showing that the two isolates are very closely related. They did not however cluster with any known isolate in the database. The two clustered distantly with Ab 7-2 with a bootstrap value of 51% and a sequence identity of 97%. Isolate Ab 7-2 also did not cluster with any known isolate in the database. The second cluster consisted of isolate Ruj 7-1, whose closest relative was *Streptomyces griseochromogenes* and this was supported by a bootstrap value of 59% and a sequence identity of 98%. It also clustered together with *Streptomyces cyaneus* in the database with a sequence identity of 98%. In the third cluster, isolates Chy 15-5 and Chy 15-6 clustered together with a sequence identity of 100% indicating that the two could be the same isolate and they clustered together with *Streptomyces paradoxus* with a sequence identity of 100%. They also clustered distantly with isolate Chy 15-10 with a sequence identity of 99%. In the fourth cluster, isolate Chy 2-3 clustered with *Streptomyces afghaniensis* with a sequence identity of 98%. The fifth cluster consisted of isolates Chy 2-8 and Chy 4-10, which clustered together with a bootstrap value of 94% and a sequence identity of 99% indicating that they are very closely related. They clustered together with the antibiotic producing soil actinobacterium *Streptomyces arenae* and this was supported with a bootstrap value of 93% and a sequence identity of 99%. They also clustered closely with *Streptomyces violarus* with a sequence identity of 99%.

5.0 CHAPTER 5: DISCUSSION

Microbial biodiversity in protected areas are often not easily accessible for sample collection for research study, limiting our understanding of the ecosystem structure. Our knowledge of microbial diversity, antifungal and biological control ability of the microbes isolated from these areas is therefore inadequate, yet this is critical in understanding the biotechnological potential of this microbial diversity. This investigation is part of an ongoing research program to investigate beneficial natural products that can be used biotechnologically for biological control of fungal pathogens from microbial sources in protected areas. The main goal of this research was to isolate a large number of soil actinobacteria from selected National Parks (Figure 1) in Kenya, to screen for antagonistic potential of the culturable isolates on selected fungal pathogens and to characterize those with broad-spectrum antifungal activity using morphological, biochemical and molecular methods. The results of this work demonstrate that soils from different national parks, from different ecological and climatic zones, harbor different actinomycetes with antagonistic ability. A rich biodiversity and antagonistic ability was found in some soils.

The total number of actinomycetes prospected was 428, and only 11 of them (about 2.6%) had evident broad-spectrum antagonistic ability (Table 2). The 11 isolates were found from soils collected from different national parks studied but most of the isolates were from Chyulu National Park. Out of the eleven, 6 isolates were from Chyulu soils (55% of the active isolates), and one each from Ruma (9%), Imenti (9%), Kakamega 9%), Aberdares (9%) and Shimba Hills National Park (9%). Moreover, the majority of

the cultures from Chyulu soils have shown a wide range of biological activity. Out of the 67 isolates screened from Chyulu soils, 21 of them showed different antagonistic activities on the test fungi with 6 showing strong antagonistic activity on all the three test fungi (Table 1 and Table 2). The study also demonstrated that soils from Imenti National Park had a very high diversity of actinomycetes where 70 isolates were screened for potential antagonistic ability (Table 1) but very few of them (4 isolates) had antagonistic ability with only one of the four (Imt 7-3) showing broad-spectrum activity on the three test fungi (Table 2). These results are in line with those of previous studies (Tinatin and Nurzat, 2006) that reported that some soils have high diversity of actinobacteria but only a few of them possess antimicrobial antagonistic abilities. By contrast, strains obtained from soils of high altitude zones such as Mount Kenya and Aberdares National Parks had low diversity of actinomycetes probably due to the fact that high altitude zones have low temperatures, which are unfavorable for growth of most species of actinobacteria. The results also indicate that very few isolates from these regions show antagonistic abilities. Only 17 isolates were screened from Mt. Kenya National Park with none of them showing any antagonistic ability (Table 1).

Validation of the antagonistic ability of the eleven isolates was achieved by extending the length of the bioassay time beyond three weeks. Extracts from the isolates were confirmed to suppress the growth of the test fungi *in vitro* even after this prolonged exposure (Table 3). The eleven isolates were also tested for their *in vitro* activity on type culture collection of Gram-positive (*S. aureus*) and Gram-negative bacteria (*E. coli*) and were shown to have inhibitory effects (Table 4). All of them were antagonists, varying

from weakly to strongly antagonism with inhibition zones from 2.0 cm to 0.1 cm (Figures 6 and 7). As reported in Table 1, the number isolated from each location is not the exact number of isolates brothed or screened for antifungal activity. This is because some were isolated from the soils but were lost before the pure cultures could be obtained. Some were subcultured onto fresh pure culture plates but did not grow, while some were destroyed due to contamination. The biological control potential of the strains obtained from some of the studied protected areas was discovered in this work based on results from antifungal activity assays.

Morphological studies under both dissecting and compound microscopes showed that all the isolates form various growth characteristics on the culture media (Table 6 and Figures 8-18). The isolates exhibited a range of chemotaxonomic and phenotypic properties typical of members of the genus *Streptomyces*. They formed an extensively branched substrate mycelium, aerial hyphae which carried smooth-surfaced spores in *rectiflexibiles* or *spiral* spore chains (Table 4) and a grayish, reddish or brownish aerial spore mass on culture media (Watve *et al*., 2001). The isolates were characterized by their tough, leathery and frequently pigmented colonies and their filamentous growth (Mayfield *et al*., 1972). *In vitro* assays demonstrated that the isolates were antagonistic against *Fusarium oxysporum, Fusarium spp* and *Colletotrichum kahawae*. These results were in line with previous studies by Tinatin and Nurzat (2006), which indicated that actinomycetes have been isolated from reserved areas in Pakistan. Many surveys of soil bacteria by other workers also have identified strains of *Streptomyces* and *Bacillus* as potential biological control agents (Crawford *et al*., 1993; Andrews, 1992).

Physiological studies showed that the eleven isolates were aerobic, Gram-positive and mesophilic. They grew well on minimal media, utilizing arginine as a nitrogen source and glycerol as a carbon source. They were also found to hydrolyze starch, a characteristic that may confirm their role in the decomposition of organic matter in the habitats (Kieser *et al*., 2000; Crawford, 1988). Based on previous results (Crawford *et al*., 1993; Knauss, 1976), the isolates described in this study may also have cellulolytic and lignin solubilizing activities but more physiological studies should be included in the tests to give more insight into their roles in the natural habitats. Biochemical test results were analyzed on PIBwin program (www.som.soton.ac.uk) using STREPMAJ software and the results indicated that all of them give characteristic typical of streptomycetes.

Molecular characterization of isolates indicate that all of them belong to domain Bacteria, Phylum Actinobacteria, class Actinobacteria, sub-class Actinobacteridae, order Actinomycetales, sub-order Streptomycineae and family Streptomycetaceae. Phylogenetic analysis demonstrates that all the isolates cluster with the genus *Streptomyces*. Most of them shared sequence identities of between 97-100% with known *Streptomyces* species. This supports the results from both morphological and biochemical test studies and the study confirm the findings of other researchers that majority of antagonists from reserved areas are streptomycetes (Tinatin and Nurzat, 2006). These results were in line with previous studies by Tinatin and Nurzat (2006), which indicated that actinomycetes have been isolated from reserved areas in Pakistan. Many surveys of soil bacteria by other workers also have identified strains of *Streptomyces* and *Bacillus* as potential biological control agents (Crawford *et al*., 1993).

Isolate Kkj 5-1, from Kakamega forest National Park clustered with the soil actinobacterium *Sterptomyces tubercidicus* (Accession AJ621612) with a sequence identity of 99%. This shows that the isolate could be a very close relative of *S. tubercidicus*. The actinobacterium *S. tubercidicus* is an antibiotic producing soil microorganism, which produces tubercidin, which is an enzyme inhibitor (Shimotsu *et al*., 1980; Goodfellow *et al*., 1987). This suggests that isolate Kkj 5-1 may also play a role in the production of antibiotics and other important metabolites. This isolate also grows well on minimal media and it hydrolyses starch, which might suggest that it plays an active role in breakdown of organic matter in the habitat. Isolates Mws 1-3 from Mwaluganje, Shimba Hills National Park and Imt 7-3 from Imenti, Meru are very closely related with a sequence identity of 99% showing that they could be sharing the same origin. Isolate Ab 7-2 from Aberdares National Park clustered together with the two with a sequence identity of 97% showing that the three are closely related. The three isolates did not cluster with any known sequence in the database. However, BLAST search results showed that the three clustered with soil actinomycete *S. hygroscopicus*. The antibiotic producing *S. hygroscopicus* has been reported to produce many bioactive metabolites some of which are used as plant protectants (Kieser, 2000). Isolate Ruj 7-1, from Ruma National Park clustered with the soil actinobacterium *Streptomyces griseochromogenes* (Accession AJ310923) with a sequence identity of 98%. *S. griseochromogenes* is an antibiotic producing soil actinobacterium producing several antibiotics such as blasticidin, which have antifungal potential (Skerman *et al*., 1980; Fukunaga *et al*., 1955). This indicates that isolate Ruj 7-1 is a close relative of *S. griseochromogenes* may also produce several bioactive natural products, some of which

may find useful application in biological control. This can be evidenced from the findings of the *in vitro* assays, that the isolate produces antifungal natural products. Isolates Chy 15-5 and Chy 15-6, both from Chyulu Hills National Park have a sequence identity of 100% indicating that they are very closely related and may belong to the same species. However, morphological studies shows a marked difference between the two with isolate Chy 15-5 having dark-gray, smaller round colonies with raised margins while isolate Chy 15-6 has larger pale-brown, oval and concentric colonies. The two isolates also do not conform in spore morphologies with isolate Chy 15-5 having rough-surfaced spores arranged in straight or slightly curved chains. These chains appear twisted. Isolate Chy 15-6 spores on the other hand, appear as smooth individual spores in a spiral configuration at the terminal of aerial mycelia. Isolate Chy 15-5 is similar to isolate Chy 15-6 in some physiological characteristics in that both isolates are positive for catalase activity, production of H_2S and mixed acid production and negative for 2,3 butanediol and indole production. For both, starch is hydrolyzed, nitrate is not reduced and gelatin is not liquefied. The two isolates, however, could be separated from each other on the basis of urea hydrolysis. Isolate Chy 15-5 is urease positive while isolate Chy 15-6 is negative. Morphologically, isolate Chy15-10 has slightly elevated brown colonies with wrinkled margins and the spores appear chain-like in a spiral configuration at the terminal of aerial mycelia. The two are likely to be strains of *S. paradoxus* as they cluster together with soil actinobacterium *S. paradoxus* (Accession AJ276570) with each sharing sequence identity of 100% to the organism. Isolate Chy 15-10, also from Chyulu Hills National Park is related to *S. paradoxus* with a sequence identity of 99% indicating that it is most likely a sub-species of *S. paradoxus*. *S. paradoxus* is an antibiotic producing soil

actinobacterium (Skerman *et al*., 1980). It has also been reported to have suppressive activity against soil-borne pathogens (Bell *et al*., 1982). It is also known to colonize chitin, which is a major component in the soil and walls of fungi, and use it as a sole energy source (Chet, 1990). Because they are closely related to *S. paradoxus*, the three isolates, Chy 15-5, Chy 15-6 and Chy 15-10 may also have a role in the production of enzymes. Isolate Chy 2-3, from Chyulu Hills National Park clustered with taitomycin antibiotic-producing soil actinobacterium *S. afghaniensis* (Accession AJ399483) (Shimo *et al*., 1959) with a sequence identity of 98% indicating that it is a close relative of this organism hence may also produce other important natural products some of which may play an important role in biological control of crop pathogens. The isolate also hydrolyses starch and reduces nitrate, a characteristic that may describe its role in decomposition of organic matter in the soil. Isolates Chy 4-10 and Chy 2-8, both from Chyulu Hills National Park are very closely related with a sequence identity of 99% and they clustered together with the antibiotic producing soil actinobacterium *S. arenae* (AJ399485) (Pridham, 1958; Skerman *et al*., 1980) with a sequence identity of 99% signifying relatedness and may belong to same species. This indicates that they are likely to be sub-species of this organism and that they may produce antibiotics with great potential. Also, the two isolates each shared a sequence identity of 99% with the antibiotic producing soil actinobacterium *S. violarus* (Accession ARB AJ399477) indicating the role of the isolates in antibiotic production in the soil. They may also produce other important proteins and enzymes such as chitinase. On morphological basis, although the colonies of both isolates appear red in color, those of isolate Chy 4-10 are bigger with wrinkled and raised margins, while isolate Chy 2-8 colonies appear smaller, more filamentous with round

margins. For both isolates, the spore-bearing structures (sporophores) appear branched on either side of aerial mycelia. However, the spores of isolate Chy 4-10 have rough surfaces whereas those of isolate Chy 2-8 appear smooth around the conidia.

All the eleven isolates have been shown through bioassays to have good antimicrobial activity towards the test organisms and therefore have a strong biological control potential that can be exploited further. Three isolates Chy 15-5 and Chy 4-10, both from Chyulu Hills National Park and Ruj 7-1, from Ruma National Park showed stronger broad-spectrum antifungal activity than the rest of the eleven isolates. The eleven isolates investigated in this study have a high potential in biological control of test organisms studied and could offer a higher probability for discovering novel compounds. The isolates could therefore, be exploited economically for development of commercial products for management of the three fungi; *Fusarium oxysporum*, *Fusarium spp*. and *Colletotrichum kahawae*, which are important fungal pathogens. Since the isolates also showed antibacterial activity on the two indicator bacteria *E. coli* and *S. aureus*, they could also have potential to produce antibiotics that could be exploited for control of medically important bacteria.

6.0 CONCLUSIONS

The study has demonstrated that soils of the protected areas (National Parks and Reserves) are rich in actinomycetes species, with some isolates having antifungal and antibacterial potential. Eleven isolates have broad-spectrum activity with three of them likely to be novel isolates with very strong antagonistic effects. Most of the isolates with strong antagonism were obtained from soils from Chyulu National Park. It was also evidenced that isolates from low altitude (warmer) zones showed strong antifungal and antibacterial potential to test organisms than species from high altitude (cooler) zones. There was also rich diversity of actinomycetes in soils collected from low altitude ecological zones compared to high altitude areas. Results show that actinomycetes in different soils are species-diverse and therefore have different antagonistic abilities. Based on the genotypic and phenotypic evidence, the antagonistic strains warrant classification as the type strain of species of the genus *Streptomyces*. Biological control potential of the isolates on *F. species* and *C. kahawae* was demonstrated through production of antifungal and antibacterial secondary metabolites.

Importantly, this study shows that actinomycetes may be useful in protecting plants against fungal pathogens such as *Fusarium* wilt and dumping-off and coffee berry disease fungi. A significant amount of the isolates screened (11 isolates) demonstrated broad-spectrum antifungal activity against test fungi *in vitro*. Although the *in vitro* evaluation of the isolates for antimicrobial activity may not give any correlation with *in vivo* assays, they may aid in screening studies to provide positive antagonists for further testing by *in vivo* assays.

7.0 RECOMMENDATIONS

- To further expand our knowledge of microbial diversity in the protected areas, it may be necessary for further research to investigate the effect of variation of growth parameters such as temperature, pH and culture media composition for isolation of isolates to correspond with those of the natural habitats of different areas. Changes could result in differences in the abundance and diversity of isolates obtained from each area as some isolates may grow under some conditions and not others.

- Culture independent sampling directly from the soil using PCR based approaches is recommended to asses the diversity of actinobacteria in the soils to accommodate microbes that could not be cultivated.

- Further work should be carried out to establish actinobacterial diversity in relation to different soil types from protected areas and biotechnological potential of the diversity of microorganisms isolated from these areas. This is important in understanding the overall diversity of biotechnologically important bacteria from such protected areas. It may also be interesting to report on the relative abundance of antagonistic actinobacteria from rhizosphere soils in protected areas as compared to the surrounding soils.

- In order to gain more insights into the bioactivity from protected areas, all isolates showing antagonistic effects against any test fungi needs to be considered as some

may only perform best on one target organism. It may therefore be possible to follow even those isolates with antagonistic effects on individual or two test organisms as they too may posses a strong antagonistic potential that can be utilized for beneficial biotechnological purposes. Further it may be possible to enhance improved culturability of isolable actinobacteria as this would help to capture even those isolates that were obtained from the soils but were lost through subsequent subcultures. Such isolates may also posses' beneficial antagonistic abilities.

- To get a clear insight into the mechanism of antagonism, further studies should be done to characterize and identify the proteins or compounds with antifungal activity. That information would be important in the biotechnological processes such as molecular cloning of the genes coding for such proteins. It will also be necessary to evaluate the bioactivity of such proteins *in vivo* through screen house experiments.

- The most highly antagonistic isolates should be further studied to optimize the conditions of establishment, antifungal activity and to investigate dose-response activities.

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9.0 APPENDICES

1. Differential Agar (1 Litre)

   12.5g Glycerol

   1.0g Arginine

   1.0g Sodium Chloride

   1.0g Di-potassium hydrogen phosphate

   0.5g Magnesium sulphate

   0.01g Ferrous sulphate heptahydrate

   0.001g Copper sulphate pentahydrate

   0.001g Zinc sulphate sextahydrate

   0.001g Manganese sulphate sextahydrate

   20g Agar

   Add 1000 ml of distilled water, mix well and autoclave

2. LB Broth (1 Litre)

10g Sodium Chloride

10g Tryptone

5.0g Yeast extract

Add deionized water to a final volume of 1 Litre

Adjust pH to 7.5 with NaOH and autoclave

3. 1x TAE Buffer (50x)

242g Tris base

57.1 ml Glacial acetic acid

100 ml 0.5M EDTA (pH 8.0)

Dilute to 1x TAE working solution with dH_2O