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Full Length Research Paper

Isolation and characterization of *Streptomyces* species with antifungal activity from selected national parks in Kenya

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Microorganisms and their natural products are potentially important for the biological control of crop diseases without detrimental effects to the environment. In this study, acetonitrile-methanol extracts of 361 actinobacterial isolates obtained from Aberdares, Arabuko Sokoke, Lake Bogoria, Mt Kenya, Kakamega, Ruma, Shimba Hills and Imenti forest national parks in Kenya were screened for antagonism against *Fusarium oxysporum*, *Fusarium* spp and *Colletotrichum kahawae*, which are important crop pathogens. Twenty-three isolates showed antagonistic activity to one or all of the test fungi. Five isolates that were antagonistic against all test fungi were investigated further and were also found to have antibacterial activity against *Staphylococcus aureus* and *Escherichia coli*. Morphological and physiological studies show that the isolates belong to streptomycetes. Phylogenetic analysis of amplified actinobacterial 16S rRNA gene confirmed that all the five antagonistic isolates formed close phylogenetic clusters with known members of *Streptomyces* species with a (97 - 100%) sequence identity. The results suggest that protected areas may be ideal habitats for isolation of antagonistic actinobacterial species which may have the potential for beneficial application in biological control of fungal pathogens. However, further investigation by characterization of the antifungal and antibacterial compounds produced will be necessary.

Key words: Protected areas, soil streptomyces, bio-prospecting, antimicrobial, phytopathogens.

INTRODUCTION

Our knowledge of microbial diversity, antimicrobial and biological control ability of the microbes isolated from protected areas is inadequate, yet this is critical in understanding the biotechnological application potential of this microbial diversity. A recent review (Torsvik et al., 2002) on the microbial diversity in various soil environments and sediments suggested that microbial diversity is higher in forest and pasture soils than in arable soils. National parks and reserves offer an undisturbed habitat for isolation of novel microorganisms for screening of beneficial natural products (Tinatin and Nurzat, 2006). Ecological and biotechnological features of the soil *Streptomyces* in the natural ecosystems of national parks of Kenya remains almost unexplored. This investigation is part of an ongoing research program to investigate beneficial natural products from actinobacterial species from protected areas in Kenya with potential application in biological control of fungal phytopathogens. Most soils contain 10^4 to 10^7 colony-forming units of streptomycetes

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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 (Fatope et al., 2000). In terrestrial habitats, streptomycetes are the most abundant actinomycetes (90% or more) (Demain and Davies, 1999).

Actinobacteria are important source of antibiotics, enzymes and bioactive products (Bull et al., 1992). 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) which have been used in control of plant pathogens (Liu et al., 1996). Studies have shown that actinomycetes are a promising group of the fungus-antagonistic root colonizing microbes (Crawford et a*l*., 1993). *Streptomyces* species and a few other actionmycetes have been shown to protect several different plants to various degrees from soil-borne fungal pathogens (Reddi and Rao, 1971). *Streptomyces rochei* and *Streptomyces rimosus* from the chickpea rhizosphere were found to be 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).

Natural products including those from microorganisms are now used widely in medicine and agriculture (Fatope et al., 2000). 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) because they are readily biodegradable, specific and generally have low toxicity (Newman et al., 2003). 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, produced by some *Pseudomonas* spp. Pyrrolnitrin provides 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 led to the discovery of harpin, a protein that is now being used to activate crop defenses prior to pathogen attack (Hutchinson, 1998). Indeed, a variety of pathogenic and non-pathogenic microorganisms can induce plant defenses and may be useful as biological control agents (Loon et al., 1998).

In the present study, actinobacteria isolated from soil collected from selected National Parks in Kenya were screened for antagonistic activity on three plant fungal pathogens: *F. oxysporum* sp. *lycopercisi*, a pathogen of tomato; *Fusarium* sp., a rose flower pathogen and *Colletotrichum kahawae*, a coffee berry pathogen. The isolates that showed broad spectrum antimicrobial activity against the test organisms were characterized using morphological, physiological and molecular techniques.

MATERIALS AND METHODS

Soil sampling sites

Ten soil samples were collected from 10 sites each from Kakamega, Ruma and Lake-Bogoria National Parks from western region of the country; Shimba Hills and Arabuko Sokoke from coastal region; Mt. Kenya, Aberdares and Imenti forest from the central region. These national parks represent the high altitude zones in central region, middle altitude zones in western Kenya and low altitude zones in the coastal region (Figure 1). In each national park, samples were randomly collected on sites at a distance of 2 km apart from each other within the accessible areas, which was representative of the park. Soil samples (2 g per site) were randomly collected from the open land, and under the shrubs and trees within the national park. Soil samples were taken from the top (7.5 - 10 cm) of the soil profile using a sterile sample collection corer, put in sterile soil sample bags and kept in a cool box for transportation to the laboratory, where they were subsequently stored at 4°C.

Isolation and maintenance of actinobacterial cultures

Each soil sample (0.5 g) was air dried for about six hours and subsequently suspended in 49.5 ml of sterile distilled water and shaken for one hour in a shaker incubator (200 rpm, 30°C). Twenty microlitres of the supernatant was plated on differential agar media (Demain and Davies, 1999; Kieser et al., 2000). This media is poor in organic carbon, which effectively controlled eubacterial and fungal growth and aided in isolating the more slowly growing actionmycetes (Reddi and Rao, 1971). The plates were incubated at 28°C for six days. Individual colonies of the cultures were isolated and subcultured into freshly prepared agar plates in differential media until pure cultures were obtained. The isolates were streaked on differential agar slants and maintained at 4°C and subcultured after every three months (Demain and Davies, 1999).

Growth in broth cultures and extraction of secondary metabolites

For isolation of bioactive compounds, isolates were inoculated into differential broth medium (Demain and Davies, 1999; Kieser et al., 2000) and incubated for six days in a shaker incubator at 28°C and 200 rpm. The broth media was centrifuged for 20 min at 12,000 rpm to remove the microbial cells. The supernatant was passed through waters Oasis HLB extraction cartridges (Cat. no. 186001740) 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°C (Demain and Davies, 1999).

Anti-fungal screening assays

In vitro assays were carried out to test the ability of individual actinomycete isolates to inhibit the growth of *F. oxysporum*.sp. *lycopersici* (KB 01), the causative agent of tomato wilt and *C.*

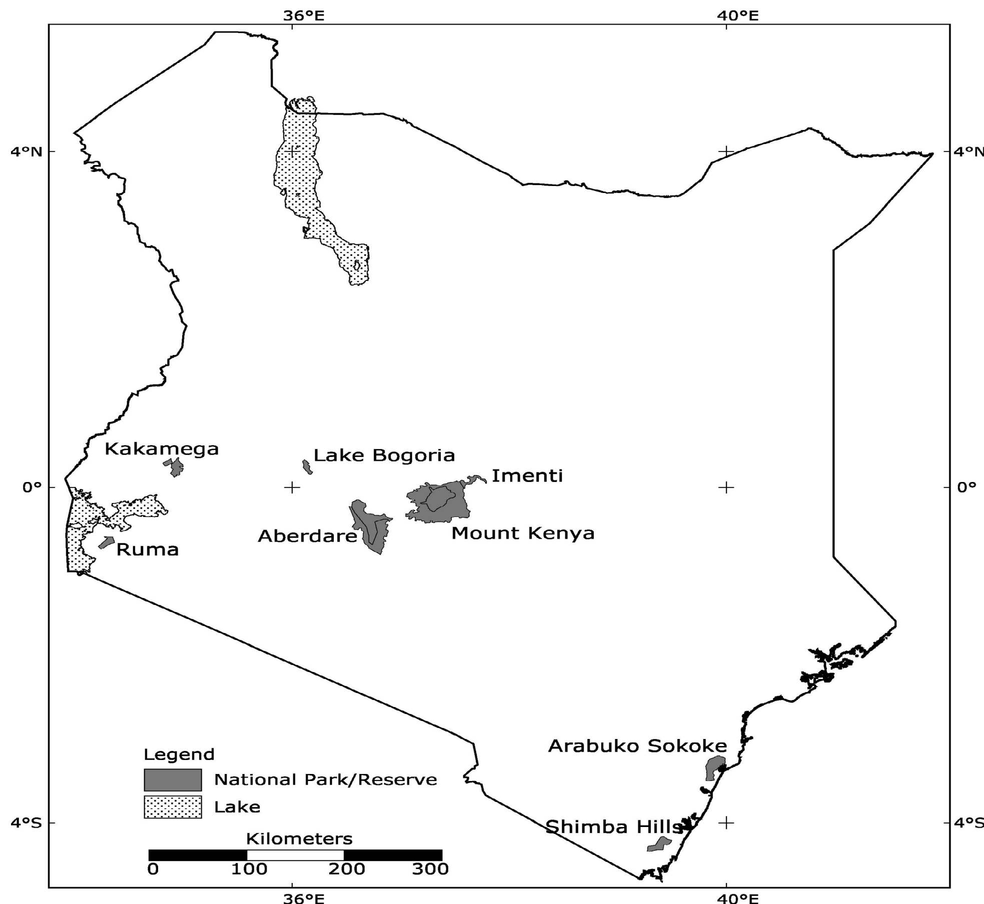

Figure 1. A map of Kenya showing the National Parks where soil samples were collected.

kahawae (Isolate KB 02) a coffee pathogen, both obtained from the Plant Science and Crop Protection Department of the University of Nairobi and *Fusarium* spp (ICIPE 06), a rose flower pathogen isolated at (ICIPE). The modified procedure of DeFrank and Putnam (1985) was used to assay each isolate for its effect on germination of test fungi spores. Freeze-dried extracts were dissolved in 1 ml of sterile distilled water and mixed thoroughly. The fungal spores were collected by scrapping with a sterile inoculating loop crossing the surface of sporulating plate. Concentrated suspensions were made in half strength potato dextrose broth (PDB) medium (Difco) prepared according to manufacturer's instructions and were counted using a Neubauer chamber. Fungal spore suspensions were diluted to give a concentration of 250 spores per 90 µl of half strength PDB. Ten microlitres of extract from the test *Streptomyces* isolates being tested, prepared in sterile distilled water was applied to the 96 well microtitre plates (Demain and Davies, 1999). Ninety microlitres (90 µl) of fungal spore suspension of each test fungi was added to each well and thoroughly mixed. The plates were covered and incubated for 48 h at 28°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 the wells where the growth of the test fungi was observed were recorded as negative and were not investigated further as well as those that were inhibitory to one or two test pathogens. The samples that inhibited fungal growth for all the three test fungi after 48 h were scored as positive and were investigated further.

Anti-bacterial assays

Extracts of the five active isolates, Ruj 7-1 from Ruma; Ab 7-2 from Aberdares; Mws 1-3 from Shimba Hills; Imt 7-3 from Imenti and Kkj 5-1 from Kakamega Forest National Parks, respectively, were also tested for antagonistic ability on type culture collection (TCC) specimens of *S. aureus* (NCTC 10788) to represent gram-positive bacteria and *E. coli* (NCTC 10418) to represent gram-negative bacteria. The assay was done first on the two control organisms in 96 well plates and observed for growth inhibition after incubation (DeFrank and Putnam, 1985). The second assay on the two control organism was done using disc assay method (Thongchai et al., 2005).

Characterization of isolates

Actinobacterial cultures that showed antagonistic activity against all the test pathogens were further characterized using a polyphasic approach. Morphological and cultural characteristics were observed on 8 day cultures of isolates grown on differential agar medium and inorganic salts glycerol medium, and observed under the dissecting compound microscope for colony and cell characteristics (Janssen et al., 2002). The cover slip technique (Zhou et al., 1998; Kawato and Shinobu, 1959) was used to observe the hyphae and

Table 1. A summary of the National Parks where soil samples were collected, number of isolates screened and the number showing broad-spectrum activity on test fungi.

Location	Soil code	No. of isolates screened	No. of isolates active on 3 test fungi
Arabuko	Arj	63	0
Aberdares	Ab	49	1
Imenti	Imt	70	1
Mwaluganje	Mws	35	1
Ruma	Ruj	50	1
Bogoria	Boj	53	0
Kakamega	Kkj	24	1
Mt. Kenya	Mkj	17	0
Total	8	361	5

spore chain characters by light microscopy. Spore chain morphology and spore surface ornamentation were studied by examining gluteraldehyde fixed ethanol dehydrated specimens with a compound microscope (Williams et al., 1983; Shirling and Gottlieb, 1966). 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).

Phylogenetic analysis of 16S rRNA of the isolates

Pure subcultures of the antagonistic isolates were inoculated in freshly prepared LB broth and incubated for six days in a shaker incubator at 28°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). The DNA was semi quantified on a 1% agarose gel in 1 x TAE buffer and visualized under UV by staining with ethidium bromide (Sambrook et al., 1989).

Total DNA from each isolate was used as a template for amplification of the 16 S rDNA gene. 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 *E. coli* gene sequence (Embley and Stackebrandt, 1994; Lane, 1991). Amplification was performed using a model PTC-100 thermal cycler (MJ research inc., USA) according to the procedure described by Roux (1995). Amplification products (20 µl) were separated on a 1% agarose gel in 1 x TAE buffer and visualized by ethidium bromide staining (Sambrook et al., 1989).

Sequencing of purified PCR products was done in both directions 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.nlm.nih.gov/) in order to determine similarity between sequences in the GenBank 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 used in the construction of the phylogenetic tree. 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 (Felsenstein, 1989; Saitou and Nei, 1987). Nucleotide sequences for the five isolates were submitted to the NCBI database and were assigned accession numbers from GQ222215 - GQ222219.

RESULTS

Isolation

There was a high diversity of actinobacterial isolates in soils from Arabuko, Ruma and Imenti Forest National Parks, while in Mount Kenya Forest soils, only very few isolates were recovered. Pure isolates grew well on the differential agar media forming well isolated colonies. The isolates obtained formed colored tough, leathery and filamentous colonies that were hard to pick from the culture media, as a characteristic of actinomycetes and also produced colored pigments, which were secreted into the culture media within a few days of incubation. Three hundred and sixty one (361) isolates (Table 1) were obtained and screened for antifungal activity on the three test fungi.

Bioassays against test fungi and bacteria

Twenty eight (28) out of the three hundred and sixty one (361) isolates screened, showed antagonistic activity to one or more of the three test fungi. Five (5) isolates out of the 28 antagonists suppressed the growth of all the three test fungi *in vitro* (Table 2), and were investigated further based on broad spectrum *in vitro* activity. The twelve isolates that showed activity on only one or two of the test fungi, 11 isolates that showed very minor antagonism and the 333 isolates that did not show any antagonistic activity against the three test fungi at all, were not investigated further. The five (5) isolates (Table 2) which were active against all the three test fungi were identified

Table 2. 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. kahawae	*Fusarium spp*	*F. oxysporum*		*C. kahawae*	*Fusarium spp*	*F. oxysporum*
Imt 7 - 3	++	++	++	Imt 7-3	++	++	++
Mws 1 - 3	++	++	++	Mws -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*, *Fusarium 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; ++ strong inhibition with no fungal growth observed; G abundant growth.

Table 3. Antimicrobial assays of extracts from the active isolates against *S. aureus* and *E. coli* using the 96 well assays and the disc diffusion assay.

Isolate	Inhibition of Growth (Turbidity)		Zone of inhibition (cm) (mean diameter)	
	S. aureus (NCTC 10788)	*E. coli* (NCTC 10418)	*S. aureus* (NCTC 10788)	*E. coli* (NCTC 10418)
Imt 7-3	++[a]	+[b]	1.40	0.25
Mws 1-3	++	+	1.60	0.40
Ab 7-2	+	+	0.45	0.10
Ruj 7-1	++	++	1.70	1.25
Kkj 5-1	++	++	1.40	0.35

[a] Strong inhibition with no growth (wells not turbid); [b] Minimal inhibition (slight turbidity).

with the following codes: Ruj 7-1 from Ruma; Ab 7-2 from Aberdares; Mws 1-3 from Shimba Hills; Imt 7-3 from Imenti and Kkj 5-1 from Kakamega Forest National Parks, respectively. Among these five isolates, isolate Ruj 7-1 from Ruma National Park showed a better antagonistic activity against all the three test fungi than the rest. Isolates with broad range activity against the entire test fungi were not recovered in soils from Bogoria, Arabuko Sokoke and Mt. Kenya National Parks. Extended incubation showed persistency in *in vitro* inhibition by the five isolates against all the three test fungi, even when the assay period was extended to three weeks (Table 2).

Antibacterial activity assay on 96 well plates gave results ranging from strong to weak antagonism and the findings were confirmed using the paper disc assay (Table 3). Extracts from all the five isolates were antagonistic against both *S. aureus* and *E. coli*. However, extracts of isolates Mws 1-3 and Imt 7-3 appeared to be more active on *S. aureus* than on *E. coli* using both assaying methods (Table 3).

Characterization of isolates

Morphological studies of the isolates revealed that all the five 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 4). All the five isolates showed profuse sporulation on solid media and had rapid growth in liquid culture. The isolates were characterized by their tough, leathery and frequently pigmented colonies and filamentous growth (Mayfield et al., 1972). They formed a branched network of mycelia with conidiophores forming at the terminal of aerial mycelia, which is characteristic of streptomycetes (Watve et al., 2001).

Physiological studies showed that all the isolates were catalase positive, all except Imt 7-3 and Mws 1-3 hydrolyzed starch and none liquefied gelatin. Isolates Imt 7-3, Mws 1-3 and Ruj 7-1 were nitrate reducers. Physiological and cultural characteristics (Table 5) also showed that the five isolates were aerobic, gram-positive and mesophilic. The isolates grew well on minimal media and utilizing arginine as a nitrogen source and glycerol as a carbon source.

16S rRNA gene sequences of the isolates

BLAST analysis of partial 16S rRNA gene sequences showed that all the five isolates were closely affiliated

Table 4. 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			
	Aerial mycelia	Growth and colony form	Soluble pigment	Spore forms
Ruj 7-1	Pale-brown	Abundant and rhizoid	Yellow pigment	Chainlike in rectiflexous form
Kkj 5-1	White	Moderate, Oval, tough and leathery	None	Ornamented in open primitive-spiral form
Imt 7-3	White	Abundant, tough, leathery and round	Yellow pigment	Smooth and round in spiral chains
Ab 7-2	Gray	Moderate, complex, tough	Dark-gray pigment	-
Mws 1-3	Dark-Gray	Abundant, rhizoid, and leathery	Dark brown pigment	Oval spores in spiral chains

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

	Biochemical tests						
Isolate	Starch	Gelatinase	Catalase	Indole	Urease	Nitrate	H_2S
Imt 7-3	-	-	+	-	+	+	-
Mws1-3	-	-	+	-	+	+	-
Ruj 7-1	+	-	+	-	+	+	-
Kkj 5-1	+	-	+	-	+	-	-
Ab 7-2	-	-	+	-	-	-	-

Biochemical test results for the 5 isolates with broad spectrum activity on test fungi defined as (+) a positive result for the reaction and (-) a negative test for the reaction.

with members of the genus *Streptomyces*. A phylogenetic tree showing the phylogenetic position of each of the members of isolates studied is shown in Figure 2. The isolates shared sequence identity of between 97 - 100% with known *Streptomyces* species (Figure 2). Isolates Imt 7- 3 (Acc. GQ222215) and Mws 1-3 (Acc. GQ222216) clustered together with a high bootstrap value of 100% and this was supported by a sequence identity of 99.1%. These isolates clustered closely with *S. malaysiensis* with isolate Imt 7-3 having a sequence identity of 98.6% while isolate Mws 1-3 had 99.2% sequence identity to the organism. Isolate Ruj 7-1 (Acc. GQ222219) clustered together with *S. griseochromogenes* and this was supported by a bootstrap value of 59% and a sequence identity of 97.7%. Isolate Kkj 5-1 (Acc. GQ222217) clustered closely with *S. platensis* with a sequence identity of 99%. Isolate Ab 7-2 (Acc. GQ222218) also clustered together with *S. platensis* with a sequence identity of 98.8%. The two clustered together with a bootstrap value of 51% and a sequence identity of 98.2%.

DISCUSSION

We reported the findings of the first study in which *Streptomyces* isolates from different national parks in Kenya have been investigated for their potential in biological control as fungal pathogens. The main goal of this research was to bioprospect for soil actinobacteria with crop pest control potential from selected National Parks in Kenya, and to characterize those with broad spectrum antifungal activity using morphological, physiological and molecular methods. The resulting data showed that different species of streptomyces were isolated from different national parks and the isolates varied in their antagonistic ability against test organisms.

The predominance of *Streptomyces* species among the actinobacterial isolates from protected areas as observed in this study was also noted by Tinatin and Nurzat (2006) on soils from reserved areas in Pakistan where soils were shown to have a high diversity of actinobacteria but only a few of them with antagonistic abilities. A few cultures from Ruma, Arabuko and Shimba Hills National Parks showed wide spectrum biological activity against the test organisms. By contrast, those from Mount Kenya and Aberdares National Parks were low in species diversity and strains obtained were low in antagonistic potential. None of the 17 isolates screened from Mt. Kenya National Park showed antagonistic effect on any of the test fungi.

Phylogenetic analysis demonstrates that most of the isolates shared a sequence identity of between 97 - 100% with known *Streptomyces* species. The study confirms the findings by other researchers (Tinatin and Nurzat, 2006) who also isolated antagonistic

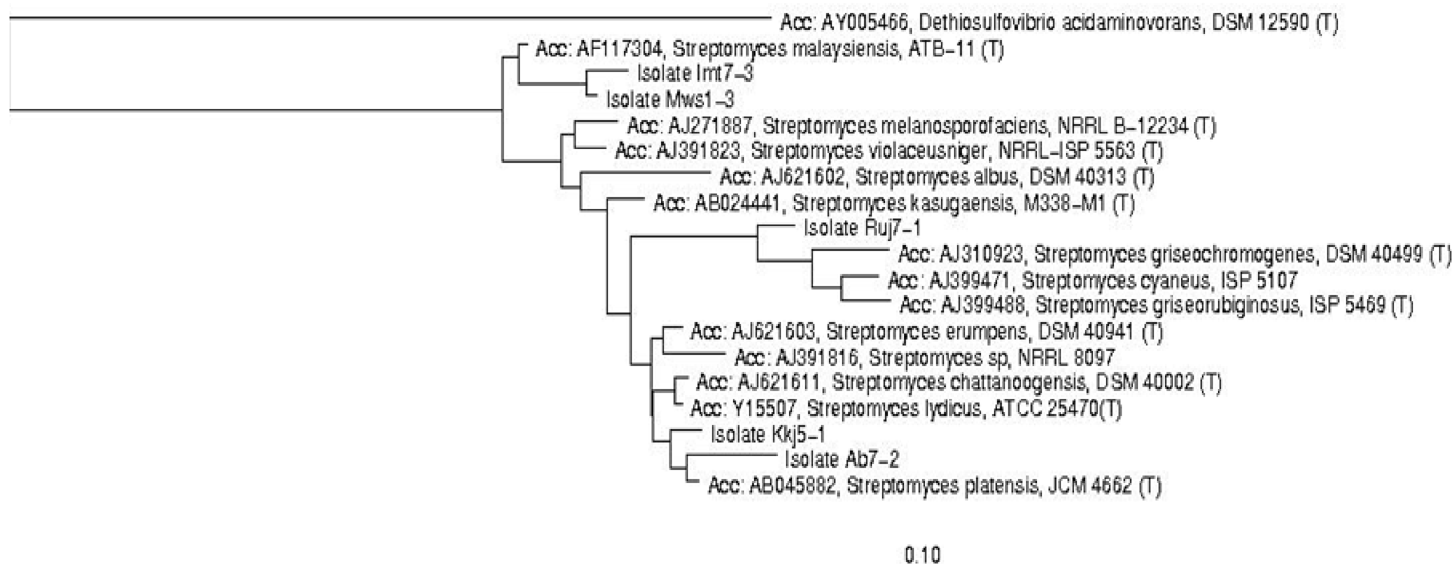

Figure 2. A maximum likelihood tree based on almost complete 16S rRNA gene sequences showing the phylogenetic relationships between the isolates with broad-spectrum antifungal activity against the test fungi and related *Streptomyces* species. Bar, 0·10 substitutions per nucleotide position. The 16S rDNA sequence of *Dethiosulfovibrio acidaminovorans* was used as an outgroup.

Streptomyces species from reserved areas in Pakistan. 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 99.1%. The two could be separated from each other morphologically by the former having white aerial hyphae and producing yellow soluble pigment while the latter formed dark gray aerial mycelium and produce dark brown soluble pigment. Each of the isolates clustered closely with soil actinobacterium *S. malaysiensis* (Accession AF117304) showing that they could be different strains with Imt 7-3 having a sequence identity of 98.6% while Mws 1-3 had 99.2% sequence identity to the organism. Al-Tai et al. (1999) reported that *S. malaysiensis* have the aerial hyphae differentiating at maturity into tight spiral chains of rugose cylindrical spores. Although both isolates Imt 7-3 and Mws 1-3 have spores arranged in a spiral form, they could be separated from *S. malaysiensis* in that Imt 7-3 has smooth round spores while Mws 1-3 has rough oval spores in tight chains. Moreover, *S. malaysiensis* was not reported to have any antagonistic activity against both *E. coli* and *S. aureus* (Al-Tai et al., 1999), while both isolates Imt 7-3 and Mws 1-3 are active against the two bacteria. The *S. malaysiensis* was isolated from a soil sample and shown to have a broad spectrum of activity against pathogenic fungi (Al-Tai et al., 1995), a characteristic shared by both isolates Imt 7-3 and Mws 1-3.

Isolate Ruj 7-1, from Ruma National Park clustered with the soil actinobacterium *S. griseochromogenes* (Accession AJ310923) with a sequence identity of 97.7%. *S. griseochromogenes* is an antibiotic producing soil actinobacterium that produces several antibiotics such as blasticidin, which have antifungal potential (Skerman et al., 1980; Fukunaga et al., 1955). Both isolate Ruj 7-1 and *S. griseochromogenes* degrade starch. However, the two could be distinguished from each other by Ruj 7-1 having brown aerial mycelia and smooth surfaced spore chains arranged in rectiflexous form while *S. griseochromogenes* spores have spiny surface ornamentation arranged in spiral form and the aerial mycelia are gray (Williams et al., 1983; Shirling and Gottlieb, 1966).

Isolate Kkj 5-1, from Kakamega forest National Park clustered closely to the soil actinobacterium *S. platensis* (Accession AB045882) with a sequence identity of 99% showing that it is most likely a strain of the organism. Both isolate Kkj 5-1 and *S. platensis* have their aerial mycelia extensively branched and appear spiral at the top with the spore chains in a spiral form (Sakai et al., 2004). The two organisms were comparably similar on glycerol salt media with both having good growth and the aerial mycelia of both appearing white. Isolate Kkj 5-1 like *S. platensis* (Sakai et al., 2004) is negative for gelatin liquefaction, Hydrogen Sulfide production and nitrate reduction, and it does not produce any soluble pigment. However, *S. platensis* hydrolyzes starch (Sakai et al., 2004) while isolate Kkj 5-1 does not.

Isolate Ab 7-2 from Aberdares National Park clustered together with *S. platensis* (Accession AB045882) with a sequence identity of 98.8% showing that it is a close relative of the organism.

The biological control potential of isolates from selected protected areas that are antagonistic to phytopathogens was demonstrated in this work based on results from

antifungal and antibacterial assays. The isolates showed persistent antimicrobial activity and are likely to be potential candidates for discovery of novel secondary metabolites for bio-control and biotechnological application. Importantly, this study shows that protected areas may harbor *Streptomyces* species which could be useful in protecting plants against fungal pathogens such as *Fusarium* wilt, dumping-off and coffee berry disease fungi. Although the *in vitro* evaluation of the isolates for antifungal 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. Furthermore, the five isolates that were active on all the test organisms could potentially be developed as good biological control agents and as producers of novel metabolites.

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REFERENCES

Al-Tai AM, Vikineswary S, Saravana MK, Wong WH (1995). Characterization of actinomycetes from Tasek Bera-'Malaysia's first ramsar site'. International Conference on Wetlands and Development, Selangor, Malaysia. Abstract no., 273: 2.

Al-Tai AM, Bongcheol K, Seung BK, Gilson PM, Goodfellow M (1999). Streptomyces malaysiensis sp. nov., a new streptomycete species with rugose, ornamented spores. Int. J. Syst. Bacteriol., 49: 1395-1402.

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990). Basic Local Alignment Search Tool. J. Mol. Biol., 215: 403-410

Bashar MA, Rai B (1994). Antagonistic potential of root region microflora of chickpea against Fusarium oxysporum f. sp. ciceri. Bangl. J. Bot., 23: 13-19.

Bull AT, Goodfellow M, Slater JH (1992). Biodiversity as a source of innovation in biotechnology. Ann. Rev. Microbiol., 46: 219-252.

Crawford DL, Lynch MJ, Whipps MJ, Ousley AM (1993). Isolation and Characterization of Actinomycete Antagonists of a Fungal Root Pathogen. Appl. Environ. Microbiol., 59: 3899-3905.

DeFrank J, Putnam AR (1985). Screening procedures to identify soil-borne actinomycetes that can produce herbicidal compounds. Weed Sci., 33: 271-274.

Demain AL, Davies E J (1999). Manual of Industrial Microbiology and Biotechnology. 2nd Edition. American Society of Microbiology, 1325, Massachusetts Avenue, N.W. Washington DC.

Embley TM, Stackebrandt E (1994). The molecular phylogeny and systematics of the actinomycetes. Ann. Rev. Microbiol., 48: 257-289.

Fatope MO, Al-Kindi MZS, Abdulnour OA (2000). Research trends: Natural products as pest, microbial disease and tumour control agents. Sci. Technol. (Spec. Revi.)., pp. 55-71.

Felsenstein J (1989). PHYLIP-phylogeny inference package version 3.57c. Cladistic, 5: 164-166

Fukunaga K, Misato T, Ishii I, Asakawa M (1955). Blasticidin, a new antiphytopathogenic fungal substance. Part I. B. Agric. Chem. Soc. Jpn., 19: 181-188.

Hutchinson SW (1998). Current concepts of active defense in plants. Ann. Rev. Phytopathol., 36: 59-90.

Janssen HP, Yates SP, Grinton EB, Taylor MP, Sait M (2002). Improved culturability of soil bacteria and isolation in pure culture of novel members of the divisions Acidobacteria, Actinobacteria, Proteobacteria, and Verrucomicrobia. Appl. Environ. Microbiol., 68: 2391-2396.

Kämpfer P, Kroppenstedt RM, Dott W (1991). A numerical classification of the genera Steptomyces and Streptoverticillium using miniaturized physiological tests. J. Gen. Microbiol., 137: 1831-1891.

Kawato M, Shinobu R (1959). On Streptomyces herbaricolor sp. nov., supplement: a single technique for microscopical observation. Mem Osaka Unit Lib. Arts Educ. B Nat. Sci., 8: 114-119.

Kieser T, Bibb MJ, Buttner MJ, Chater KF, Hopwood DA (2000). Practical Streptomyces genetics. John Innes Centre, Norwich, England.

Lane DJ (1991). 16S/23S rRNA sequencing In: Nucleic Acid Techniques in Bacterial Systematics (Stackebrandt E and Goodfellow M, Eds.). John Wiley & Sons Inc., New York. p. 115-175.

Ligon JM, Hill, DS, Hammer PE, Torkewitz NR, Hofmann D, Kempf HJ, Van Pee KH (2000). Natural products with antifungal activity from Pseudomonas biocontrol bacteria. Pest Manag. Sci., 56: 688-695.

Liu D, Anderson NA, Kinkel LK (1996). Selection and characterization of strains of Streptomyces suppressive to potato scab pathogen. C. J. Microbiol., 42: 487-502.

Maidak BL, Cole JR, Lilburn TG, Parcker CT Jr, Saxman PR, Farris RJ, Garrity GM, Olsen GJ, Schmidt TM, Tiedje JM (2001). The RDP-II (Ribosomal Database Project). Nucleic Acids Res., 29: 173-174.

Mayfield CI, Williams ST, Ruddick SM, Hatfield HL (1972). Studies on the ecology of actinomycetes in soil. IV. Observations on the form and growth of streptomycetes in soil. Soil Biol. Biochem., 4: 79-91.

McSpadden Gardener BB, Fravel DR (2002). Biological control of plant pathogens: Research, commercialization, and application in the USA. Plant Health Progr. 10: 1094.

Newman DJ, Cragg GM, Snader KM (2003). Natural products as a source of new drugs over the period 1981-2002. A detailed analysis and description of current natural product derived therapeutic agents. J. Nat. Prod., 66: 1002-1037.

Reddi GS, Rao AS (1971). Antagonism of soil actinomycetes to some soil-borne plant pathogenic fungi. Ind. Phytopathol., 24: 649-657.

Rothrock CS, Gottlieb D (1984). Role of antibiosis of Streptomyces hygroscopicus var. geldanus to Rhizoctonia solani in soil. Can. J. Microbiol., 30: 1440-1447.

Roux KH (1995). Optimization and troubleshooting in PCR, In Dieffenbach CW and Dveksler GS (ed.), PCR primer: a laboratory manual. Cold Spring Harbor Laboratory Press, Plainview, N.Y. pp. 53-62.

Saitou N, Nei M (1987). The neighbor-joining method. A new method for reconstructing phylogenetic trees. Mol. Biol. Evol., 4: 406-425.

Sakai T, Sameshima T, Matsufuji M, Kawamura N, Dobashi K, Mizui Y (2004). Pladienolides, New substances from culture of Streptomyces platiensis Mer 11107. J. Antibiot., 57: 173-179.

Sambrook J, Fritsch EF, Maniatis T (1989). Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

Shayne JJ, Hugenholtz P, Sangwan P, Osborne C, Janssen HP (2003). Laboratory cultivation of widespread and previously uncultured soil bacteria. App. Environ. Microbiol., 69: 7211-7214.

Shirling EB, Gottlieb D (1966). Methods for characterization of Streptomyces species. Int. J. Syst. Bacteriol., 16: 313-340.

Skerman VBD, Mcgowan V, Sneath PHA (1980). Approved Lists of Bacterial Names. Int. J. Syst. Bacteriol., 30: 225-420.

Stach EM, Maldonado AL, Masson GD, Ward CA, Goodfellow M, Bull TA (2003). Statistical approaches for estimating actinobacterial diversity in marine sediments. Appl. Environ. Microbiol. 69: 6189-6200.

Thongchai T, Chunhua L, Yuemao S, Saisamorn L (2005). Secondary metabolites from endophytic Streptomyces aureofaciens CMUAc130 and their antifungal activity. Microbiol., 151: 1691-1695.

Tinatin D, Nurzat T (2006). Biodiversity of Streptomyces of high-mountainous ecosystems of Kyrgystan and its biotechnological potential. Antonie Leeuwenhoek, 89: 325-328.
Torsvik V, Øvreås L, Thingstad TF (2002). Prokaryotic diversity: magnitude, dynamics, and controlling factors. Sci., 296: 1064-1066.
Van Loon LC, Bakker PA, Pietrse CMJ (1998). Systemic resistance induced by rhizosphere bacteria. Ann. Rev. Phytopathol., 36: 453-483.
Watve MG, Tickoo R, Jog MM, Bhole BD (2001). How many antibiotics are produced by the genus Styreptomyces? Arch. Microbiol., 176: 386-390.
Williams ST, Goodfellow M, Alderson G, Wellington FMH, Sneath PHA, Sackin MJ (1983). Numerical classification of Streptomyces and related genera. J. Gen. Microbiol., 129: 1743-1813.
Zhou ZH, Liu ZH, Qian YD, Kim SB, Goodfellow M (1998). Saccharopolyspora spinosporotrichia sp. nov., a novel actinomycete from soil. Int. J. Syst. Bact., 48: 53-58.