ENUMERATION, IDENTIFICATION AND ANTAGONISTIC POTENTIAL OF SELECTED MICROBIAL ISOLATES FROM SUGARCANE VARIETY CO 421 RHIZOSPHERE AGAINST *Sporisorium scitamineum* IN KIBOS, KISUMU COUNTY, KENYA

BY

ANTON ELVIS OKELO JUMA

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN BOTANY (MICROBIOLOGY)

DEPARTMENT OF BOTANY

MASENO UNIVERSITY

DECLARATION

This thesis has not been previously submitted for award of a degree in Maseno University or any other University or institution. I have carried out the work reported here in and all sources of information have been specifically acknowledged by means of references.

Sign ——————————————— Date ———————————————

Juma Elvis Okelo Anton
PG/ MSC/ 064/ 2011

Supervisors:

This thesis has been submitted to Maseno University for examination with our approval as University supervisors

Sign ——————————————— Date ———————————————

Dr. David Mutisya Musyimi
Department of Botany
School of Physical and Biological Sciences
Maseno University

Sign ——————————————— Date ———————————————

Dr. George Opande
Department of Botany
School of Physical and Biological Sciences
Maseno University

ACKNOWLEDGEMENTS

First and foremost, I would like to thank the Almighty God for all the grace and blessings He has given to me. Without Him, this study could not be accomplished.

I would also like to express my sincere gratitude to my supervisors, Dr. David M. Musyimi and Dr. George Opande, for their advice, corrections and encouragement during the entire period of study. All the lecturers in the Botany department of Maseno University I say thank you for making necessary comments and corrections on my work.

I express my appreciation to Mr. Gibson Riungu the Director of Crop Development Department and Head of Pathology section of the Kenya Agricultural Livestock Research Organization - Sugar Research Institute (KALRO – SRI) for guidance, constant encouragement and making the pathology and microbiology laboratory available to carry out my research. The technical support in the pathology and soil laboratories provided by Lillian Nyongesa, Mercy Mbago, Michael, Lencer and Irine is gratefully appreciated. I also recognize Mr. Adolwa and Erick for assistance in the field.

Special thanks to my employer Teachers Service Commission for giving me the opportunity to study by offering me a study leave and all other people not mentioned who in any way contributed to the success of this study.

Finally, I cannot forget to thank KALRO through the Director SRI Dr. Japheth Jamoza for sponsoring my research work.

DEDICATION

This work is dedicated to my sons Costa Chris and David Maxwell who had to forgo my parental attention during the course of my studies. To my beloved wife Purity for her understanding, patience and encouragement during the period of study. Loving late mum and dad Mr. and Mrs. Juma for investing in me and my brothers and sisters for their moral support.

ABSTRACT

Sugarcane smut disease caused by a fungus *Sporisorium scitamineum* is a limiting factor to cane production in Kenya. It is threatening the sugar industry due to its effect on cane quality and yields. Sugarcane (*Saccharum officinarum* L.) is known to have microbial organisms associated with its rhizosphere with potential antagonistic activity against the fungus, however numerous studies on rhizosphere microbial diversity and their antagonistic activity against fungal plant pathogens have focused on other crops such as rice and wheat with little information available on sugarcane globally and underexplored in Kenya. The objectives of this study were to enumerate fungi and bacteria in the rhizosphere of CO 421 sugarcane variety, to identify the fungi and bacteria and to evaluate the antagonistic potential of selected microbial isolates against the pathogen within Kibos, an area of high percentage smut prevalence and a close vicinity to the Kenya Agricultural and Livestock Research Organization – Sugar Research Institute in Kisumu, Kenya. Variety CO 421 was selected because it is widely adapted and grown in all sugarcane growing areas of Kenya and has breaking resistance to smut. Rhizosphere soil samples were collected randomly from five different locations per field in ten fields of the sugarcane variety using a soil auger and trowel into sterile polythene bags. Colonies isolated from the soil samples in three replicates, following serial dilution and plating techniques on potato dextrose agar for fungi and nutrient agar medium for bacteria were enumerated and the microbes identified based on their morphological characters by using taxonomic guides and standard procedures. Screening for evaluation of potential antagonism against the test organism was done *in vitro* by dual culture technique in three replicates. *In vivo* screening was done in five treatments and five replicates by growing single budded sugarcane setts treated with the test organism and selected potential antagonists in plastic pots with steam sterilized soil in green house and in the field in micro plots. Setts were treated with four antagonists and distilled water as control. The experimental design was a completely randomized design. Data was collected on colony forming units per soil sample, colony and cell morphological characteristics, inhibition of mycelia growth of the test pathogen and the number of smut whips per treatment from November 2013 to November 2014. Data on microbial count, inhibition and disease incidence were subjected to analysis of variance. Treatment means were separated and compared using Fishers Least Significance Difference at p=0.05. The study indicated an average population of 1.30×10^7 cfu/g and 4.88×10^4 cfu/g bacteria and fungi respectively in the rhizosphere soil samples. Bacteria had a higher population and rhizosphere effect than Fungi. *Aspergillus, Penicillium, Trichoderma, Rhizopus* and *Alternaria* genera of Fungi and *Bacillus, Pseudomonas* and *Azobacter* genera of bacteria were identified. The four selected potential antagonists of *Trichoderma viride* and *Trichoderma herzanium* inhibited pathogen growth by 61% and 59% *in vitro* and showed 20% and 27% disease incidence *in vivo* respectively while AJB9 (unidentified) and *Pseudomonas* sp. showed inhibition zones of 25.6mm and 24.3mm *in vitro* and 13% and 17% disease incidence *in vivo* respectively. The selected isolates had evident antagonistic activity against the pathogen *in vitro* hence recommended as potential biocontrol agents of *Sporisorium scitamineum* which affects sugarcane plants.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS, ACRONYMS AND SYMBOLS

%:	Percentage
ANOVA:	Analysis of variance
CFU:	Colony Forming Units
cm:	Centimeter
CO:	Coimbatore sugarcane varieties
D:	Demerara sugarcane varieties
DI:	Disease Incidence
EAK:	East African Community
FAO:	Food and Agriculture Organization
I:	Incidence
g:	Grams
ISSR:	Intersimple sequence repeats
KALRO-SRI:	Kenya Agricultural and Livestock Research Organization-Sugar Research Institute
KARI:	Kenya Agricultural Research Institute
KEN:	Kenya sugarcane varieties
KESREF:	Kenya Sugar Research Foundation
LSD:	Least Significant Difference
l:	Litre
ml:	Mililitre
µl	Microlitre
NA:	Nutrient Agar
N14	Natal sugarcane variety
^{O}C:	Temperature measured in Degree Centigrade
PDA:	Potato Dextrose Agar
pH:	Potential hydrogen
R/S:	Rhizosphere Effect
RAPD:	Random amplified polymorphic DNA.

LIST OF TABLES

Table **page**

LIST OF PLATES

Plate **Page**

LIST OF APPENDICES

Appendix **Page**

CHAPTER ONE

INTRODUCTION

1.1. Background to the study

Rhizosphere is a narrow region of soil around the roots that is subjected to influence of plant roots (Kumar *et al.,* 2015; Pandey and Palni, 2007).The extent of the rhizosphere may vary with the soil type, plant species, age of the plant and microbial community composition but it generally extends from the root surface (rhizosplane) out into the soil for up to a few millimeters. According to McNear Jr. (2013), rhizosphere includes three zones, the endorhizosphere which includes portions of cortex and endodermis which microbes and cations can occupy (free space), rhizosplane which includes root epidermis and mucilage and ectorhyzosphere which extends from the rhizosplane out into the bulk soil.

The rhizosphere is an area of intense microbial activity and a 'hot spot' for microbial interactions as exudates released by plants roots are a main food source for the microbes and a driving force for their population density and activities (Deshmuk *et al*., 2013; Chanda *et al*., 2011). The population of microbes in the rhizosphere differs quantitatively and qualitatively (Damle and Kulkarni, 2012) and different plant species host specific microbial communities (Berendsen *et al*., 2012). A great majority of organisms in the rhizosphere are bacteria and fungi, together with actinomycetes, protozoa, microalgae and micro fauna (Dua and Sidhu, 2012; Alimi *et al*., 2012; Tailor and Joshi, 2012; Bello and Utang, 2011; Rocha *et al*., 2009; Buee *et al*., 2009). It has been documented by several investigators that microbial population is stimulated in the rhizosphere by the exudates released by the plant root that is the rhizo effect (Nihorimbere *et al*., 2011; Tamilarasi *et al*., 2008). Bacteria records the highest stimulation followed by fungi and actinomycetes from a comparison between the number of microorganisms per gram of rhizosphere soil to the number of microorganisms per gram of a corresponding non rhizosphere soil sample (Nihorimbere *et al*., 2011; Tamilarasi *et al*., 2008; Nanpieri *et al*., 2007; Mukerji *et al* ., 2006). These studies therefore have created the need to explore the rhizosphere microorganisms of sugarcane by unraveling their possible relationships with the sugarcane plants.

The rhizosphere of sugarcane harbours many organisms that have neutral effects on the plant, but also attracts organisms that exert deleterious or beneficial effects on the plant according to a study by Deshmuk *et al*. (2013) on fungi, in the rhizosphere of sugarcane in Pune district in Maharashtra state in India. Deshmuk *et al*. (2013) indicated that *Aspergillus, Altenaria and Rhizopus* species of fungi dominated the soil. Similar studies on specific microorganisms associated with sugarcane rhizosphere are rare in Kenya.

Rhizospheric microorganisms play an important role in many processes of crop production such as decomposition, mineralization, biological nitrogen fixation, denitrification and promote growth (Pisa *et al*., 2011). From a study by Dua and Sidhu (2012) on effectiveness of rhizosphere bacteria for control of root rot disease and improving plant growth of wheat (*Triticum aestivum*), antagonistic rhizosphere microbes which inhibit the growth of pathogenic microorganisms have been found to colonize the plant's rhizosphere. *Pseudomonas* and *Bacilli* bacteria were found to be predominant showing growth promoting ability and antagonistic activity of the one hundred and thirty isolates obtained. The study was however not done in sugarcane and under field conditions which would validate the microbe's effectiveness before adoption as biocontrol agents in commercial agriculture.

Microorganisms that grow in the rhizosphere are ideal for use as biocontrol agents since the rhizosphere provides front line defense for root against attack by pathogens (Suprapta, 2012). The pathogens encounter antagonism from rhizosphere microorganisms before and during primary infection and during secondary spread (Suprapta, 2012). According to Dua and Sidhu (2012) and Alimi *et al*. (2012), soil microorganisms may stimulate, inhibit or completely suppress growth of soil borne pathogens. However knowledge on sugarcane rhizosphere microorganisms as potential antagonists against plant pathogens such as *Sporisorium scitamineum* is lacking in Kenya.

Sporisorium scitamineum the smut pathogen can be found in the soil as spores (teliospores) however, the spores can only survive for a short time under normal soil moisture regimes. Although it has been reported in a few other members of the grass family, there are probably no

important naturally occurring alternative hosts outside *Saccharum* species (Comstock and Lentini, 2005).

Sugarcane (*Saccharum officinarum* L.) is a perennial grass in the family of Poaceae cultivated for its stem (cane) which is primarily used to produce sucrose (cane sugar). Sugarcane plays a major role in the economy of sugarcane growing areas worldwide. Globally it is an important source of commercial sugar accounting for nearly 70 percent of the world's sugar production (Food and Agriculture Organization - FAO, 2008). Sugarcane is a multipurpose crop whose other products include paper, ethanol, animal feed, biofertilizer, alcohol derived chemicals, antibiotics, particle board and raw material for generating electricity. About twenty countries in Asia Pacific region grow sugarcane on a commercial basis (FAO, 2008).

In Kenya sugarcane industry is a major employer and contributor to the national economy, it is one of the most important crops alongside tea, coffee, maize and horticultural crops (Kenya Sugar Industry Strategic Plan - KSISP, 2014). Currently, the industry supports approximately 250,000 small scale farmers who supply cane to sugar companies and an estimated six million Kenyans derive their livelihoods directly or indirectly from the industry. The industry saves Kenya in excess of 19.3 billion Kenya shillings in foreign exchange annually and contributes tax revenue to the exchequer. It provides raw materials to other industries and a silent contributor to the fabric of communities in the sugar belt (KSISP, 2014).

Sugarcane smut disease caused by a fungus *Sporisorium scitamineum* was first reported in Natal South Africa in 1877. Currently the disease occurs in all sugarcane growing areas except Papua New Guinea (Magarey *et al*., 2010). The disease was first reported in Kenya in 1958 in Nyanza and Coastal provinces but since spread to all sugarcane growing areas and is considered the most important disease of sugarcane in Kenya (Ongala *et al*., 2015; Nzioki and Jamoza, 2009).

Sugarcane smut can be of epidemic proportion especially when a susceptible variety or diseased sett is planted (Nasiru and Ifenkwe, 2004). It is classified as one of the main illnesses of sugarcane and can cause total crop failure in susceptible varieties (Briceno *et al*., 2005). Smut significantly reduces the yield and quality of sugarcane (Ongala *et al*., 2015; Kavitha *et al*., 2014; Nzioki *et al*., 2010; Olweny *et al*., 2008). Yield losses of up to 38 % and 58 % have been

recorded from previous research in Kenya in plant and ratoon crops respectively (Nzioki and Jamoza, 2009), 50% and 73% in India (Viswanathan *et al*., 2009; Nasiru and Ifenkwe, 2004).

Numerous studies on rhizosphere microbial diversity and their antagonistic activity against fungal plant pathogens have focused on other crops such as rice (Kalaiselviselvaraj and Paneerselvama, 2011) tomatoes (Alwathnani *et al*., 2012) and wheat (Dua and Sidhu, 2012), little such information is available on sugarcane globally and unexplored in Kenya leading to the need for this study. Most microbial antagonistic studies have been done successfully in the laboratory with challenges under field conditions due to variations in environmental conditions from where the biocontrol agent was isolated and the point of action (Suprapta, 2012).

1.2. Problem statement

In Kenya the area under cane is approximately 169,421 hectares and sugarcane is one of the most important cash crops with an estimated six million Kenyans deriving their livelihoods directly or indirectly from the industry (KSISP, 2014).Ways of improving sugarcane productivity are subject to intense investigation in Kenya, one of them being control of sugarcane smut. Smut significantly reduces the yield and quality of sugarcane, it affects yield components such as plant girth, plant weight and millable stalks resulting in tonnage loses and juice quality by decreasing the extractability and recovery of sugar and reducing sucrose content to 3-7% (Nzioki *et al*., 2010). Recent study in Kenya on smut related yield losses by Nzioki and Jamoza (2009) revealed 21-38% loss on plant crop and 58% on ratoon crop. Economic losses due to smut could range from neglible proportions to levels serious enough to threaten sugarcane production of an area. Smut devastates large areas of susceptible varieties with a possibility of total crop failure. Sugarcane variety CO 421 is an old sugarcane variety susceptible to smut occupying 28.4% of the total area under cane in Kenya. This cultivar is favoured by most farmers at 34.6% across the sugar industry due to its low rate of deterioration after maturity, good germination and ratooning compared to new improved varieties (Ong'ala *et al* .,2015).

There are few records of the use of antagonistic rhizospheric microorganisms to control *Sporisorium scitamineum* in Kenya (Kenya Sugar Research Foundation, 2006). Numerous studies on rhizosphere microbial diversity and their antagonistic activity against fungal plant

pathogens have focused on other crops such as rice, tomatoes and wheat, with little attention given to sugarcane globally. The widely adopted control measures include the use of resistant cane varieties, hot water treatment and chemical fungicides, however none of the control method seems satisfactory alone hence the need for an integrated approach (Jamoza, 2013; Sundar *et al*., 2012; Nzioki *et al*., 2010; Amire *et al*., 1982). Therefore the use of microbial biocontrol agents against *Sporisorium scitamineum* would offer an alternative disease management strategy which is economically feasible, ecologically sound, less time consuming and environmentally safe to supplement the existing control methods hence the need to explore sugarcane's rhizosphere microorganisms to unravel their possible relationship with the plant.

1.3. Justification

Sugarcane plays major role in the economy of sugarcane growing areas, hence improving sugarcane production will greatly help in the economic prosperity of farmers and other stakeholders associated with sugarcane cultivation. However, most sugarcane producing farmers are confronted with the problem of smut disease caused by *Sporisorium scitamineum* which significantly reduces the yield and quality of sugarcane. The growing significance of this pathogen is very evident yet no control method seems satisfactory alone. Planting smut resistant varieties has proved to be the most efficient hence is the main control measure (Nzioki *et al*., 2010) however development of resistant cane varieties is very costly, time consuming and adoption of the said varieties has not been met 100% as farmers prefer the older to new varieties (Ong'ala *et al*., 2015). Varieties previously known to be resistant like CO 421, EAK 7097, CO 1148 and N 14 have shown resistance breakdown while some varieties are resistant in one zone and susceptible in others (Abd *et al*., 2010). Hot water treatment may not be practical on a large scale and its effectiveness may be subject to varietal differences. It reduces germination in most varieties. Chemical fungicides can be harmful to other living organisms besides reduction of soil microorganisms, residual problem and development of resistance in pathogen biology (Alwathnani and Perveen, 2012).

The use of antagonistic rhizospheric microorganisms have previously demonstrated antagonistic activities against plant pathogenic fungi and may serve as better biocontrol agents because they

are already associated and adapted to the plant and its rhizosphere effect as well as the environmental conditions in which they are supposed to function than organisms isolated from other plant species (Athul *et al*., 2012). This emphasizes the need for a specific host and environment for the biocontrol agent for its effective performance against the pathogen.

This research was therefore to establish the potential microbial antagonists from selected microflora in the rhizosphere of sugarcane against *Sporisorium scitamineum* causing sugarcane smut for the benefit of sugarcane industry stake holders which could provide an efficient alternative disease management approach that is cost effective and ecofriendly in the present context of sustainable agriculture to supplement the existing control methods.

The findings would allow further research on the identified microorganisms for their effect on multiple sugarcane varieties, different soil types and climatic conditions and their interaction with other microbial communities at the point of action by research scientists in microbiology and plant pathology before adoption as biocontrol agents. It will also be a source of reference information to students in these areas of study.

1.4. Objectives

1.4.1. General objective

To determine the population and identify microorganisms in the rhizosphere of sugarcane (*Saccharum officinarum* L.) variety CO 421 plants from Kibos area in Kisumu County (Kenya) and investigate the antagonistic potential of selected microbial isolates against *Sporisorium scitamineum* causing smut disease in sugarcane.

1.4.2. Specific objectives

i. To determine the population of fungi and bacteria in the rhizosphere of sugarcane variety CO 421 in Kibos.

ii. To morphologically identify the fungi and bacteria isolated from the rhizosphere of sugarcane variety CO 421 in Kibos.

iii. To determine the antagonistic potential of selected fungi and bacteria isolated from the rhizosphere of sugarcane variety CO 421 against *Sporisorium scitamineum* in Kibos.

1.5. Hypotheses

1. There is high population of fungi and bacteria in the rhizosphere of sugarcane variety CO 421.
2. There are morphologically diverse fungal and bacterial isolates in the rhizosphere of sugarcane variety CO 421.
3. Fungal and bacterial isolates from the rhizosphere of sugarcane variety CO 421 have antagonistic potential against *Sporisorium scitamineum.*

CHAPTER TWO

LITERATURE REVIEW

2.1. Sugarcane (*Saccharum officinarum* L.)

2.1.1. Biology of sugarcane

Sugarcane is a tall growing, perennial monocotyledonous crop plant belonging to;

Kingdom : Plantae,

Division : Magnoliophyta,

Class : Lilipsida,

Order : Poales,

Family : Poaceae,

Subfamily: Panicoidae,

Tribe : Androponeae

Genus : *Saccharum*

Sugarcane has a thick, tillering stem which is clearly divided into nodes and internodes. The leaves of the plant grow from the nodes of the stem, arranged in two rows on either side of the stem (Cheavegatti-Gianatto *et al*., 2011). The inflorescence of sugarcane is a terminal panicle which possesses two spikelets and seeds protected by husks (glumes) covered in silky hair. Two flowers are produced on the inflorescence, one sterile and the other bisexual. Sugarcane can reach a height of up to 6 m (3.3 ft) and once harvested, the stalk will regrow (ratoon) allowing the plant to live for between 8 and 12 years. The root system is fibrous and shallow. It has been estimated that top 25cm of soil contains 50% of the plant roots (Australian Government, 2004). The sugarcane root system consists of adventitious and permanent root types (Cheavegatti-Gianatto *et al*., 2011).

2.1.2. Geographical distribution of sugarcane

Sugarcane is grown in all tropical and subtropical regions of the world, on both sides of the equator, up to approximately 35° N and 35° S (Cheavegatti-Gianotto *et al*., 2011). In 2007, the main sugarcane-producing countries were Brazil (33% of the world's production), India (23%), China (7%), Thailand (4%), Pakistan (4%), Mexico (3%), Colombia (3%), Australia (2%), the

United States (2%) and the Philippines (2%) (Cheavegatti-Gianotto *et al*., 2011). In Kenya there are 3 sugar belts, namely the Nyando, the western sugar belt and the south Nyanza sugar zone. Most farming is in western Kenya. Previously some sugarcane was grown in parts of Coast Province (KSISP, 2014).

2.1.3. Uses of sugarcane

Sugarcane is used for sugar production, bagasse for power generation, molasses for alcohol fermentation, stock feed supplement and fertilizer in cane fields, sugarcane trash as mulching material and low grade livestock feed. (Que *et al*., 2014; KSISP, 2014).

2.1.4. Propagation and cultivars of sugarcane

Sugarcane grows best in tropical and subtropical regions with high temperature (26 - 33°C), and full sunlight (Cheavegatti- Gianatto *et al*., 2011). The plant will thrive in a variety of different soils but generally requires a deep, well-draining soil with a pH between 5 and 8 and a good supply of organic matter. Sugarcane is vegetatively propagated by planting part of a mature cane. Sections of cane, known as setts approximately 40 cm in length, each possessing 3 buds, are planted in furrows and covered in a thin layer of soil. The current commercial varieties in Kenya include: CO 617, CO 421, N 14, CO 945, CO 1148, EAK 70-97, EAK 73-335, KEN 82-247, D 8484, KEN 82-62, KEN 82-216, KEN 82-247, KEN 82-737, KEN 82-808, KEN 82-493, KEN 82-472, KEN 82-121, KEN 82 – 601, with only CO 421 susceptible to smut and the rest showing intermediate resistance (Jamoza *et al*., 2013).

2.1.4.1. CO 421 variety characteristics

CO 421 is an imported sugarcane variety from India (Coimbatore). It has pale green stalks of medium thickness. CO 421 is a high cane and sugar yielding variety. It is a good germinator, good ratooning and widely adapted and grown in all sugarcane growing areas as an old variety (Ong'ala *et al*., 2015; Jamoza *et al.*, 2013). Currently it occupies 28.4 % of the total area under cane in Kenya (KSISP, 2014) and grown by most farmers at 34.6% across the sugar industry from a study by Ong'ala *et al*., 2015. It is late maturing and susceptible to smut and mosaic virus diseases (Jamoza *et al.*, 2013). CO 421 is of commercial importance in Western Kenya and its resistance to smut has broken over time (Nzioki *et al*., 2010).

2.1.5. Diseases of sugarcane and management

Bacterial diseases of sugarcane include, leaf scald (*Xanthomonas albilineans*), and ratoon stunting disease (*Clavibacter xylem*) and red stripe (*Acidovora avenae*). Fungal diseases include rust *(Puccinia melanocephala* and *Pucciniakuehni*), yellow spot (*Mycovellosiella koepkei*), pachymetra root rot (*Pachymetra chaunorhiza*), sugarcane smut (*Sporisorium scitamineum),* pineapple disease (*Ceratocytis paradoxa*), eye spot (*Bipolaris sacchari*), red rot (*Glomerella tucumanesis*), pokkah boeng *(Fusarium monoliforme* and *F.subglutinans*). Viral diseases include chlorotic streak, Fiji disease (Fiji disease phytoreovirus), mosaic disease (sugarcane mosaic virus, sorghum mosaic virus, maize dwarf mosaic virus, Johnson grass mosaic virus and striate mosaic associated virus). However in Kenya, according to Osoro, (1997) Smut, ratoon stunting disease, mosaic virus, pineapple disease and eye spot are the diseases which have received major attention and generally controlled by planting resistant varieties and disease free planting materials.

2.2. Sugarcane smut disease

Sugarcane smut is a fungal disease of sugarcane caused by the fungus *Sporisorium scitamineum,* it is an important disease of sugarcane worldwide and leads to considerable yield loss and reduction in cane quality (Kavitha *et al*., 2014; Shen *et al*., 2012; Nzioki and Jamoza, 2009).

2.2.1. Causal organism

The disease causing fungus belongs to;

Kingdom : Fungi,

Division : Basidiomycota,

Class : Basidiomycetes,

Sub class : Ustilaginomycetidae,

Order : Ustilaginales,

Family : Ustilaginaceae

Genus : *Sporisorium*

The sugarcane smut fungus is a dynamic organism, capable of mutating and hybridizing in nature to produce new virulent pathogenic races. However it does not produce new pathogenic

races as frequent as many other pathogenic fungi but can produce multiple races (Que *et al*., 2012; Amire *et al*., 1982). Several races of the sugarcane smut fungus (*Sporisorium scitamineum)* are known to exist, but the race picture is poorly defined at this time partly due to sugarcane variety - environment interactions. In Taiwan, three physiological races 1, 2, and 3 were found from a study by Shen *et al*. (2012) on genetic diversity of *Sporisorium scitamineum* in southern China revealed by combined Intersimple sequence repeats(ISSR) and Random amplified polymorphic DNA (RAPD) analysis. A recent study on characterization of physiologic races of sugarcane smut established existence of smut races in Kenya from the variability in smut reactions of cultivars used in this study (Nzioki *et al*., 2010).

2.2.2. Symptoms

The disease can be noticed in all the crop stages after germination. The most recognizable diagnostic feature of a smut infected plant is the emergence of a "smut whip" (Kavitha *et al*., 2014; Croft and Braithwaite, 2006; Comstock and Lentini, 2005; Gillaspie *et al*., 1983). A "smut whip" is a curved, pencil-thick growth, gray to black in color that emerges from the top of the affected cane plant. These "whips" arise from the terminal bud or from lateral shoots on infected stalks (appendix 1). They can vary in length from a few centimeters to large whips up to 1.5 m long extending high above the crop canopy. The emerged whips are composed of a central core of host and fungus tissue surrounded by a thin layer of black spores that is covered by a thin silver-white membrane. This membrane quickly disintegrates exposing the spores and central core of host tissue. Whips begin emerging from infected cane by 2-4 months of age with peak whip growth occurring at the 6^{th} or 7^{th} month (Meena and Ramyabharathi, 2012; Legaz *et al*., 2011). Abnormal whips that contain some flower parts can sometimes be formed. Other smut symptoms may be evident before the characteristic whip is seen that is the sugarcane plants may tiller profusely with the shoots being more spindly and erect with small narrow leaves (i.e. the cane appears "grass-like") severely stunted and thin stalks. Less common symptoms are leaf and stem galls, and bud proliferation (Sundar *et al*., 2012; Croft and Braithwaite, 2006; Comstock and Lentini, 2005; Legaz *et al*., 2011).

2.2.3. Disease cycle and pathogenicity

Sugarcane smut is disseminated via teliospores that are produced in the smut whip. These teliospores located either in the soil or on the plant, germinate in the presence of water (Sundar *et al*., 2012; Hoy and Grisham, 1988) after germination they produce promycelium and undergo meiosis to create four haploid sporidia. Sugarcane smut is bipolar and therefore produces two different mating types of sporida. For infection to occur, two sporida from different mating types must come together and form a dikaryon (Qui *et al*., 2014; Izadi and Jorf, 2007). This dikaryon then produces hyphae that penetrate the bud scales of the sugarcane plant and infect the meristematic tissue. The fungus grows within the meristematic tissue and induces formation of flowering structures which it colonizes to produce its teliopores (Kavitha *et al*., 2014; Comstock and Lentini, 2005). The flowering structures, usually typical grass arrows, are transformed into a whip like sorus that grows out between the leaf sheaths. At first it is covered by a thin silvery peridium (this is the host tissue) which easily peels back when desiccated to expose the sooty black-brown teliospores. These teliospores are then dispersed via wind and the cycle continues. The spores are reddish brown, round and sub ovoid and may be smooth to moderately echinulate that is with small spines or prickles. The size varies from 6.5 to 8 um. Sugarcane cultivars intended for distribution to other geographical areas should be tested for susceptibility to *U. scitamineum* populations in each area (Sundar *et al*., 2012; Alfieri, 1978).

2.2.4. Transmission of the disease

Standing cane becomes infected in the buds. Since many infected buds remain dormant until the cane is cut for seed and planted, the use of infected seed cane is an important way the disease is spread. Primary transmission of the smut fungus occurs through planting diseased seed cane (Meena and Ramyabharathi, 2012; Nzioki and Jamoza, 2009; Comstock and Lentini, 2005). Sugarcane smut is spread by wind dispersal of the microspores. The spores can be spread over great distances by wind currents. The whip serves as a source of spores. It has been shown that approximately one billion spores per whip per day can be released into the air. Secondary spread is through windblown spores (Nzioki and Jamoza, 2009; Comstock and Lentini, 2005).Windborne spores may settle on the soil of cropped or newly prepared fields. Disease-free seed pieces may become infected if planted in soil containing viable spores. The spores,

however, only survive for a short time in the soil under normal soil moisture regimes, for 2-3 months in moist soil but for longer periods in dry soil or other dry environments. Spores present in or on the soil surface are also carried to different fields via irrigation and rain water (Comstock and Lentini, 2005; Nasiru and Ifenkwe, 2004). Several species of insects have been consistently associated with smut whips and spores have been found on their bodies. These observations suggest insects could play a role in spore dissemination (Bowler *et al*., 1977). Although sugarcane smut has been reported on a few other members of the grass family, there are probably no important naturally occurring alternative hosts outside the *Saccharum* genus (Viswanathan *et al*., 2009; Comstock and Lentini, 2005). The fungus can survive within infected cane plants as long as the plant remains alive. Smut is generally favored by hot dry weather conditions, this increases the frequency of whip development. Under high stress conditions the cultivars may show symptoms otherwise they normally do not produce whips (Viswanathan *et al*., 2009). Teliospore survival is decreased rapidly by high soil moisture. Similarly, high rainfall reduces the severity of smut development. Disease severity usually increases in the ratoon crop (Nzioki and Jamoza, 2009). The increase in incidence of smut was found to be associated with varietal susceptibility and increasing age of the crop (Que *et al*., 2014; Sundar *et al*., 2012).

2.2.5. Prevention and control

Smut is controlled by an integration of several methods rather than using a single method. Planting resistant or tolerant cultivars is the most practical as it is cheap and reliable but the pathogen occasionally produces new race that overcomes prior host resistance (Abd *et al*., 2010; Schenck, 2004). It also takes many years to develop a resistant commercial variety against a new pathogenic race (Amire *et al*., 1982). Hot water treatment of seed cane for 20 minutes at 52-54^{o}C or 30 minutes at 50^{o}C gets rid of seed borne smut spores or dormant smut infection resulting into disease free seed cane. However, hot water treatment may not be practical on a large scale and its effectiveness may be subject to varietal differences and reduces germination in most varieties. Roguing affected plants when noticed is another control measure. As the disease is systemic, it is necessary to remove the whole clump during roguing before the emergence of the whip but if the whips have already emerged, they should first be covered with a gunny/plastic bag, removed and burned. Scattering of spores should be avoided during the rouging operation. Roguing is not

practical for severe outbreaks involving commercial acreage. Reduction of the number of ratoons is recommended in susceptible cultivars. Any plant crop which has over 10% Smut infection should not be kept for ratoon (Nzioki and Jamoza, 2006). According to Kenya Legal Notice No. 390 of the Plant Protection Ordinance, cultivars which show more than 21% stools smutted in the ratoons are not considered for commercial production and it is illegal to grow such a cultivar. Seed protectant fungicides are effective in ridding seed cane of dormant smut spores and dormant smut infections (Nzioki and Jamoza, 2009; Sundar *et al*., 2012) but they can be harmful to other living organisms, reduce soil microorganisms, develop residual problem and resistance in pathogen biology (Alwathnani and perveen, 2012). Partial replacement of chemical control by biological is becoming increasingly important to crop protection, as regulations on the use of chemicals are becoming more stringent.

2.3. Microbial diversity in the rhizosphere and the rhizosphere effect

Rhizosphere is a dynamic environment, which harbors a great diversity of microorganisms (Tamilarasi *et al*., 2008). It is adjacent to and influenced by living plant roots (Tailor and Joshi, 2012). The diversity and composition of the microbial taxa in the rhizosphere can be affected by several factors including plant species, soil management practices, soil type, microbial interactions and other environmental variables (Tamilarasi *et al*., 2008).

A study by Gaddeya *et al.* (2012) on soil mycoflora in different crop fields of sugarcane, corn, paddy, red gram and finger millet at Salur Mandal in India using soil dilution and plate technique, isolated and characterized *Aspergillus, Penicillium, Trichoderma, Curvularia, Fusarium* and *Rhizopus.* The species in the fields differed in population and diversity per crop with the mycoflora of sugarcane rhizosphere showing *Aspergillus, Penicillium* and *Trichoderma* predominant. A similar study done in Nanjangud Taluk of Mysore District, Karnataka, India on fungal diversity of rhizosphere soils in different agricultural fields by Chandrashekar *et al*. (2014) isolated *Curvularia lunata, Alternaria alternate, Penicillium fumiculosum, Penicillium chrysogenum, Fusarium solani, Rhizopus stolonifer, Mucor* sp., *Aspergillus flavus, Aspergillus terreus* and *Aspergillus Niger* from sugarcane rhizosphere where *Aspergillus, Penicillium* and *mucor* sp.were predominant.

Al-Nur and Abdulmoneim (2007) indicated that the richest taxa in abundance were *Aspergillus*, *Penicillium, Rhizopus, Curvularia* and *Fusarium* using viable plate count method from their research on the rhizosphere mycoflora of sugarcane at Kenana sugarcane estate of Sudan. Similar studies on soil bacteria and fungi have not been conducted in Kenya under similar conditions.

Sood *et al*. (2007), studied tea rhizosphere of Indian Himalayan regions for bacterial dominance and antagonism which indicated *Bacillus* bacteria of up to 45% occurrence and *Pseudomonas* of up to 85% occurrence to dominate the rhizosphere of established and abandoned tea bushes, respectively. In a study by Angel *et al*. (2013) on isolation of siderosphore producing bacteria from rhizosphere soil and their antagonistic activity against selected fungal pathogens in Porur rhizosphere of tomatoes and paddy rice revealed the presence of eleven bacterial isolates which included, Fluorescent *Pseudomonas, Bacillus, Azobacter* and non fluorescent *pseudomonas* species. Similar studies on sugarcane in Kenya have however not been reported.

A study by Nekade, (2013) in India on bacterial diversity, isolated forty three bacterial isolates from sugarcane rhizosphere. Genera *Bacillus* was found to be the most dominant followed by *Pseudomonas.* However this study was done on saline soils.

Tamilarasi *et al*. (2008) in his study of diversity of root associated microorganisms of selected medicinal plants and influence of the rhizomicroorganisms on the antimicrobial property of *Coriandrum savitum* in India indicated that bacterial population was higher in the entire root zone of the plants followed by fungal and actinomycetes population. Similarly the number of microorganisms was higher in the rhizosphere soil than in the non rhizosphere soil with greater rhizosphere effect seen in bacteria than fungi and actinomycetes. The predominant bacterium was *Bacillus* and fungus was *rhizopus.*Similar studies are lacking for sugarcane in Kenya.

A study on the mycoflora in the rhizosphere of sugarcane by Deshmuk *et al*. (2013) in India isolated the largest number of fungi from the rhizosphere soil. The Sugarcane varieties promoted fungal development in the vicinity of the root zone as compared to the soil away from rhizosphere effect. *Aspergillus niger, A. tamari, Trichoderma viride* and the dark sterile mycelia were encouraged by the plant root exudates.

2.4. Rhizosphere microorganism's antagonistic activity

2.4.1. Antagonism

It has been reported that natural rhizosphere is often inimical to pathogens because antagonists form part of the rhizosphere community (Sood *et al*., 2007). Antagonists are naturally occurring organisms with traits enabling them to interfere with pathogen growth, survival, infection or plant attack (Berg *et al*., 2006). Mechanisms responsible for antagonistic activity include: (1) inhibition of the pathogen by antibiotics, toxins and biosurfactants (antibiosis); (2) competition for colonization sites, nutrients and minerals; (3) parasitism that may involve production of extracellular cell-wall-degrading enzymes such as chitinase and b-1, 3 glucanase; and (4) mycophagy (Angel *et al*., 2011; Berg *et al*., 2006; Pal and McSpadden, 2006). The interaction between soil borne pathogens and their antagonistic counterparts are of fundamental importance for plant nutrition and health (Berg *et al*., 2006). Antagonistic microorganisms applied to seeds before planting colonize the rhizosphere and share the area of infection of the pathogen (Ha, 2010).

2.4.2. Microbial antagonists against plant fungal pathogens

Many microbial antagonists have been reported to posses' antagonistic activities against plant fungal pathogens, such as *Pseudomonas fluorescens*, *Agrobacterium radiobacter*, *Bacillus subtilis, B. cereus*, *B. amyloliquefaciens*, *Trichoderma virens*, *Burkholderia cepacia*, *Saccharomyces* sp, *Gliocadium* sp. (Suprapta, 2012). The successful control by these antagonists mainly against the diseases caused by following genera of fungi: *Alternaria*, *Pythium*, *Aspergillus*, *Fusarium*, *Rhizoctonia*, *Phytophthora*, *Botrytis*, *Pyricularia*, *Gaeumannomyces and Sporosium* (Adebayo and Ekpo, 2014; Suprapta, 2012; Ru and Di, 2012; Alimi *et al*., 2012; Dua and Sidhu 2012; Meena and Ramyabharathi., 2012; Dela- Cruz-quiroz *et al*., 2011; Prince *et al*., 2011; Heydari and Pessriki, 2010; Joshi *et al*., 2010; Ha, 2010; Heidi and Elnaga, 2006; Pal and Mcspadden, 2006; Emmert and Handelsman ,1999).

2.4.3. Common microbial antagonists

2.4.3.1. *Trichoderma* species

Antagonistic species include *T.harzianum, T.viride, T.viren, T.hamatum, T.koningi and T. longibrachiatum* (Ru and Di, 2012; Benitez *et al*., 2004). A study done by Lal *et al*. (2009) in the laboratory and field trials in India on biocontrol of sugarcane smut using plant extracts of twenty five plant species and *T.viride* indicated that filtrate (5%) *T. viride* (bioagent) inhibited mycelial growth and teliospore germination of *Sporisorium scitamineum*; it improved germination (6.2%), millable canes (27.33%) and cane yield (38.18%) in plant crop and sprouting of clumps (12.57%), millable canes (51.46%) and yield (48.75%) in ratoon crop, respectively. However the *Trichoderma* species used was not obtained from the sugarcane rhizosphere. According to Ha (2010), twenty eight research projects conducted in Vietnam for the last twenty years in laboratory, greenhouse and field works on economically important crops such as coffee, tomato, maize, rice, peanut and rubber results have proved that *Trichoderma* species could be used to control several fungal pathogens causing plant diseases. This study was not done on sugarcane crop. Inhibition of 78-79% by *Trichoderma* sp. against *Sarocladium oryzae* causing sheath rot disease in paddy was noted in India in a study by Kalaiselvis and paneerselvama (2011) on *in vitro* screening using dual culture technique to assess the potential of *Trichoderma* species on the pathogen however this study was not done *in vivo* and not in sugarcane. The success of *Trichoderma* strains as biocontrol agent is due to their high reproductive capacity, ability to survive under very unfavorable conditions, efficiency in the utilization of nutrients, capacity to modify the rhizosphere, strong aggressiveness against phytopathogenic fungi, and efficiency in promoting plant growth and defense mechanisms. These properties have made *Trichoderma* genus to be found everywhere (Benitez *et al*., 2004).

2.4.3.2. *Pseudomonas fluorescens*

Pseudomonas fluorescens is adapted to survival in soil and colonization of plant roots (Couillerot *et al*., 2009). In a study by Reddy *et al*. (2007) in Andhra Pradesh, *P. fluorescens* effectively inhibited mycelia growth of three fungi species of rice *Magnapotha grisen, Rhizoctonia solani* and *Dreschelaria oryzae* in dual culture tests by 62-85%. According to a study by Anand and

Kulothugan (2010) on *Pseudomonas fluorescens* antagonistic activity towards the crown rot pathogen *Aspergillus niger* in *Arachis hypogea* in India, *in vitro* assay revealed that 3% (5/60) of the isolates were antagonistic in nature therefore it can be explored as one among the best biocontrol agents against phytopathogens. The mechanisms of antagonism involves, first rate of growth, ability to colonize the rhizosphere aggressively, ability to produce acidic substances, production of fluorescent pigments that are inhibitory to other organisms, production of antimicrobial compounds such as 2,4 diacetylphloroglucinal, hydrogen cyanide and surfactants, production of siderophores, competition with root pathogens for nutrients and root surface colonization giving it a competitive advantage over pathogens (Couillerot *et al*., 2009). Other antagonistic pseudomonades include *P.viridiflava, P.syringae, P.putida, P.marginalis, P .corrugata and P. resinovorans* (Gravel *et al*., 2005).

2.4.3.3. *Bacillus* species

B. subtilis, B. cereus, and B. amiloliquefaciens are some of the antagonistic species. A study of *Bacillus* isolates from potato rhizosphere by Calvo *et al*. (2010) in Peru, found that sixty three *Bacillus* strains isolated from the rhizosphere of native potato varieties growing in the Andean highlands of Peru screened for *in vitro* antagonism against *Rhizoctonia solani* and *Fusarium solani* recorded a high prevalence (68%) of antagonism against *R. solani*. Ninety one percent of those strains also inhibited the growth of *F. solani*. (Calvo *et al*., 2010). Inhibition by *Bacillus* species of 90.5% against *Fusarium* species, 85.8% against *Sclerotium* species and 41.8% against *Altenaria* species were recorded in a study by Angel *et al.* (2013) on isolation of siderophore producing bacteria from rhizosphere soil and their antagonistic activity against selected fungal plant pathogens in Porur India. *Bacillus* sp. suppresses plant pathogens by antibiotics production, inducing host resistance and siderophore production (Pal and McSpadden, 2006).

2.4.3.4. *Aspergillus* species

Antagonistic species include *A. fumigatus, A. repens, A. nigers, A. luchuwensis, A. flavus* and *A .terreus.* A study done by Adebola and Amadi (2010) in Nigeria on the potential of three *Aspergillus* species isolated from cocoa rhizosphere and rhizoplane as biological control agents of *phytophthora palmivora*, the pathogen of cocoa black pod disease revealed that all effectively

checked the growth of the pathogen where *A.repens* was the most antagonistic in solid medium. According to Dwivedi and Dwivedi (2012) a study on *in- vitro* bio-efficacy of some selected fungal antagonists against guava wilt pathogen in Kanpur revealed that *A.luchuwensis* and *A. flavus* were effective in controlling the growth of the test pathogen by 93.85% and 87.29% respectively. Their mode of action is by production of antifungal metabolites (Adebola and Amadi, 2010).

2.4.3.5. *Penicillium* species

A number of *Penicillium* sp. have been reported as antagonists of plant pathogens this includes *P.citrinum, P. restrictum, P. putida and P.chrysogenum.*According to a study by Alwathnani *et al*. (2012) on biocontrol of *Fusarium* wilt on tomatoes in Saudi Arabia, *P. citranum* inhibited radial growth of the test pathogen *Fusarium oxyporum* recording 53.5% antagonism. A review paper by Nicoletti and De Stefano (2012) on *penicellium restrictum* as an antagonist of plant pathogenic fungi underlines its competitive ability in the rhizosphere of plants, capacity to adapt to extreme environmental conditions and utilize almost any kind of organic substrate. Production of fungi toxic extrolites, induction of resistance and mycoparasitism introduces it as a plausible and effective antagonist (Nicoleti and De Stefano, 2012).

CHAPTER THREE

MATERIALS AND METHODS

3.1. Field site characteristics

The study was carried out at Kenya Agricultural and Livestock Research Organization - Sugar Research Institute (KALRO – SRI) headquarters, Kibos area (Kisumu, Kenya) (Appendix 3) at an altitude of 1184 a.s.l. 0^0, 34^0 latitude and 04'S 48'E longitude. Kibos has a sub humid climate, characterized by high day temperatures, cool nights and bimodal rainfall pattern. Mean annual rainfall is 1464mm, while mean daily temperature is 23^0C.The long rains start in March and end in June, while short rains start in September and end in November. Average temperature, day lengths, evaporation and radiation vary very little throughout the year (KALRO - SRI Agro - Metrological Department, Appendix 4).

3.1.2. Soil physical and chemical characteristics

The soils are characterized by high clay content (over 60%), pH range of 5 – 6, high water holding capacity of 213mm/m, organic content of 0.5 - 0.75% and negligible permeability (KALRO - SRI Agro - Metrological Department, Appendix5).

3.2. Sampling

Sugarcane rhizosphere soil samples were collected from 10 experimental fields with long term sugarcane cropping history (Table 3.1) at KALRO-SRI Kibos with *Saccharum officinarum* L. cultivar CO 421 between 45-315 days old. Below 45 days the plants are still adapting to the environment and the exudates may not have reached the soil and beyond 315 days fungal activity drops as the plants are approaching senescence (Deshmuk *et al*., 2013). Selection of the CO 421 variety was based on the fact that it is widely adapted and grown in all sugarcane growing areas covering 28.4% of the total area under cane in Kenya, has breaking resistance to smut disease, is a good germinator and has lower rate of deterioration after maturity compared to new improved varieties hence is of commercial importance in western Kenya (Ong'ala *et al*., 2015; KSISP, 2014; Jamoza *et al*., 2013; Nzioki *et al*., 2010). Five soil samples were collected randomly from each study site at the center and the four corners along 5-25cm depth within the rhizosphere after

removing top 5cm litter layer using an auger and trowel. Soil sample Collection was along the roots and the soil particles closely adhering to the roots were transferred to sterile polythene bags with the help of a brush as described in Tamilarasi *et al*. (2008). Non rhizosphere (bulk) soil was also sampled corresponding to each rhizosphere soil sample with the help of a sterilized cork borer pushed horizontally to the ground same depth as in rhizosphere after removing 5cm litter layer using aseptic procedures ten centimeters away from the sugarcane root. The soil samples were emptied into sterilized polythene bags to act as control (Chanda *et al*., 2011). The soil samples were appropriately labeled then transported in a cool box to the plant pathology laboratory at Kibos (KALRO-SRI Headquarters) for processing.

Table 3.1: **Soil samples collected from fields of sugarcane variety CO 421 in Kibos**

Composite Soil sample	**Field**	**Sampling location**
1	1	F12
2	2	F24
3	3	F17
4	4	F10
5	5	F7
6	6	F6
7	7	F4
8	8	F25
9	9	F23
10	10	F1

3.2.1. Preparation of the soil samples

The five soil samples randomly collected from each field were bulked to form one composite sample by mixing thoroughly, air dried for two hours at room temperature then sieved using a 2ml mesh sieve to remove plant debris. Ten grams subsample of soil from each of the ten composite samples was used for isolation of soil microorganisms. Ten grams of non rhizosphere

soil subsample (control) was also obtained and prepared in a similar manner from each field and all the prepared samples were stored at 4^{o}C until further analysis (Chandrashekar *et al*., 2014; Shiny *et al*., 2013; Chanda *et al*., 2011; Al-Nur and Abdulmoneim, 2007).

3.3. Determination of the population of fungi and bacteria in the sugarcane rhizosphere variety CO 421.

Isolation of microorganisms from the soil samples were conducted in the plant pathology laboratory at Kibos (KALRO-SRI headquarters) Kisumu, following soil dilution and plating techniques as described by Kumar *et al.* (2015), Shiny *et al*. (2013) and Gaddeya *et al*. (2012) on different selective media and enumerated to estimate microbial population per gram of the original soil sample before sub culturing to obtain pure cultures.

3.3.1. Media preparation

The following media were prepared according to manufacturer's instructions, sterilized and poured in sterilized petri dishes.

(i) Potato dextrose agar (PDA) was prepared by suspending 39.0g in 1000mls of distilled water in a conical flask, heated to boil to dissolve the media completely and sterilized by autoclaving at 15lbs pressure (121^{o}c) for 15 minutes. (HIMEDEA Laboratories Pvt. Ltd).

(ii) Nutrient agar (NA) was prepared by suspending 28g in one litre of distilled water, heated to boil to dissolve the media completely and sterilized by autoclaving at 121^{o}c for 15 minutes (OXOID Ltd. Basing stoke, Hampshire) according to Gowsalya *et al.* (2014) and Abdulkadir and Waliyu (2012).

The media were well mixed before dispensing. One percent tetracycline solution was added to the PDA medium that is just above setting temperature before pouring into Petri plates to prevent bacterial growth. Fifteen milliliters of each media was transferred into sterilized disposable petri dishes, 90mm in diameter and allowed to cool under aseptic conditions in the laminar flow chamber before being used. The media were used since PDA was selective for fungi and NA for bacteria and their simple formulation. PDA medium is the most commonly used media as it is the best for mycelia growth and has a potential to support a wide range of fungal growth (Kumar *et al* ., 2015).

3.3.2. Isolation and enumeration procedure

Ten grams of soil sample was suspended in 90 ml of double distilled water to make a total of 100 ml suspension. The suspension was stirred and poured into a sterile 250 ml Erlenmeyer flask and shaken thoroughly for thirty minutes to a homogeneous solution. One ml of the suspension was pipetted aseptically and dispensed into dilution test tubes with 9 ml of sterilized distilled water to make microbial suspensions (10^{-1} to 10^{-5}). Dilutions of 10^{-2}, 10^{-3} and 10^{-4} were used to isolate fungi and bacteria in order to avoid crowding of colonies. One mililitre aliquot of microbial suspension of each concentration was added to sterile petri dishes containing solidified 15-20ml of sterile potato dextrose agar. Three plates were provided for each dilution (Triplicate). One percent tetracycline solution was added to the medium that is just above setting temperature before pouring into Petri plates to prevent bacterial growth. The plates were rotated by hand in a broad and slow swirling motion to disperse the soil suspension. The Petri dishes were covered, sealed with para film, turned upside down and incubated at 25 ± 2^0C in the dark for daily observation up to 5-10 days for fungal growth. For bacteria 0.1 ml aliquot of microbial suspension of each concentration was added to sterile petri dishes containing solidified 15-20ml of nutrient agar medium. Sterilized bent glass rod was used to evenly spread and distribute the aliquot. Three plates were provided for each dilution and incubated at 30 ± $2^{.0}$C to be observed for 2-5 day for bacterial growth after plating. The dilution with plates of countable number of colonies were selected and counted after 72 hours for fungi and 48 hours for bacteria. The number of microorganisms per gram of the original sample was calculated using the formulas;

a**) Number of microbes /ml** = $\dfrac{\textbf{Number of colonies (CFUs)}}{\textbf{Amount plated} \times \textbf{Dilution}}$ …… Eqn – 1

b) **Number of microbes / gram of soil =** $\dfrac{\textbf{Num.} \times \textbf{Vol. 2}}{\textbf{Mass}}$ ..……..Eqn – 2

Where **CFU** is Colony Forming Units; **Num.** is the number of microbes/ml calculated in(a) above; **Vol. 2** is the volume of the original sample; and **mass** is mass of the solid material added to the original suspension according to Reynolds, (2011).

The quantitative rhizosphere effect of the plants was calculated using the formula;

$$R/S = \frac{\text{Number of microorganisms per gram of rhizosphere soil}}{\text{Number of microorganisms per gram of non rhizosphere soil}} \quad \text{....} \ldots\ldots\ldots \text{ Eqn – 3}$$

according to Sule and Oyeyiola, (2012) and Tamilarasi *et al*. (2008). Where **R/S** is the rhizosphere effect.

3.3.2.1 Purification of fungal and bacterial isolates (pure culturing)

Morphologically different fungal colonies were selected from the petri dishes for pure culturing. Purification was done by cutting the mycelia tips with a sterile inoculating needle, transferring to a new PDA medium (sub culturing) repeatedly to obtain a pure culture (Kumar *et al.,* 2015; Sule and Oyeyiola, 2012; Al-Nur and Abdulmoneim, 2007).

Distinct individual bacterial colonies were selected from the plates and purified by streaking repeatedly on new nutrient agar plates (re-inoculation) with the aid of a sterile wire loop until all colonies were identical (Gowsalya *et al.,* 2014; Nandhini and Josephine, 2013; Kimberly and Elsa, 2003; Cappuccino and Sherman, 2008). The pure cultures were maintained in PDA slants and plates in a refrigerator at $4^{O}C$ for identification and antimicrobial tests.

The percentage frequency of occurrence of each isolate was calculated using the formula;

A/B x 100…….. Eqn – 4 according to Makut and Owolewa (2011).

Where **A** is the number of sites in which the species was observed and **B** is total number of sites**.**

3.4. Morphological identification of fungi and bacteria in the sugarcane rhizosphere variety CO 421

3.4.1. Fungi

Identification was done macroscopically by visual observation of petri dishes for the colony characteristics (color, shape, diameter, margin, elevation and presence of aerial mycelium) (Kumar *et al*., 2015; Afzal *et al*., 2013) and microscopic observations in slide culture, by wet mounting using lacto phenol cotton blue staining technique (LPCB) for shape, size, conidia,

conidiophores and arrangement of spores according to a method described by Ibrahim and Rahma (2009).

3.4.1.1. Lacto phenol cotton blue staining procedure

A drop of the stain was placed on clean slide with the aid of a sterile mounting needle, a small portion of the mycelium from the fungal cultures was removed and placed in the drop of lacto phenol stain. The specimen was teased carefully using inoculating wire loops to avoid squashing and over-crowding of the mycelium and with the aid of the needle, a cover slip was gently applied with little pressure to eliminate air bubbles. The slide was mounted and observed with x10 and x40 objective lenses respectively under a phase contrast microscope, model: Carl zeiss (Appendix 6). Identity was confirmed with the help of literature (Cappuccino and Sherman, 2008; Ellis *et al*., 2007; Alexopoulos *et al*., 2002; Williams, 2001; Fassatiova, 1986).

3.4.2. Bacteria

Identification was done microscopically by observing colony features (Surface, shape, pigmentation, margin, elevation and opacity) for characteristics that may be unique to it hence preliminary identification (Tshikudo *et al*., 2013) and cell features (shape, arrangement and gram reactivity according to Kimberly and Elsa (2003) and Cappuccino and Sherman (2008) with reference to Bergey's manual of determinative bacteriology identification flow chart for identity confirmation.

3.4.2.1. Gram staining procedure

Heat fixed bacterial smear on a slide was flooded with crystal violet stain for one minute, then washed off with tap water. Gram iodine was applied for one minute and washed off with tap water. 95% alcohol was added drop by drop until it ran almost clear then washed off with tap water and counterstained with safranin and allowed 30 seconds staining then washed off with tap water, drained and blotted to dry. The slide was then examined under an oil immersion microscope for purple (G+) or pink (G-) color according to Gowsalya *et al* (2014).

3.5. Determination of the antagonistic potential of fungi and bacteria isolated from sugarcane rhizosphere variety CO 421 against *Sporisorium scitamineum*

3.5.1. Pathogen isolation and identification

Spores of viable sugarcane smut pathogen of CO 421 variety were obtained from the Kenya Sugar Research Foundation plant pathology laboratory sourced from freshly collected culture of teliospores having more than 70% germination from naturally infected plants. The teliospores were surface disinfected with sterile distilled water and a loop of the fungal suspension streaked on a Petri plate containing PDA with tetracycline to prevent bacterial growth and incubated at 30^0C for 10 days. Tips of the fungal mycelia were cut and recultured to obtain pure cultures (Abd *et al*., 2010). Identification was done macroscopically and microscopically in reference to Cappuccino and Sherman, (2008) Ellis *et al*. (2007), Alexopoulos *et al*. (2002), Williams, (2001) and Fassatiova, (1986). The identity was confirmed by comparison with the reference strain in the Sugar Research Institute laboratories in Kibos for the macroscopic and microscopic features (Appendix 2,).

3.5.2. Inhibition tests

Inhibition tests were done at Kibos (KALRO-SRI) plant pathology laboratory. Inhibition of the pathogen growth by the fungal Isolates was carried out on PDA medium using dual culture technique as described in Paramdeep *et al*. (2014), Alwathnani and Perveen (2012) and Selvaraj and Panneerselvam (2011) where 5mm diameter mycellial plugs of pathogen and isolated fungi (test antagonist) were cut with the help of a sterilized cork borer from the edge of 5 days old culture. The 5mm plug from each test antagonist was placed 2cm inside of the periphery of different culture plates and each plate was doubly inoculated with 5 mm diameter mycelia of the pathogen and placed 5 cm opposite the test antagonist. The dual plates were incubated at 25 ± 2^0C. Control was PDA plates inoculated with pathogen only. The experiment was performed in three replicates in a complete random design. The radial growth of the test pathogen in treated and control plates were recorded after 7 days of incubation and percentage inhibition of mycelial growth of the pathogen calculated using the formula below;

$$\mathbf{I = C - T / C \times 100} \quad \text{…… Eqn – 5 according to Alwathnani and Perveen (2012).}$$

Where, **I** is the percent inhibition; **C** is the pathogen colony diameter (mycelia growth) in control plate and **T** is the pathogen colony diameter (mycelia growth) in treated plate.

For bacterial isolates, a loopful of test antagonist bacteria culture was streaked 5cm. away from the plug of the pathogen by line inoculation on the same dish. Zone of inhibition was recorded as the smallest distance between the fungal pathogen and the antagonist after seven days of incubation at 28 ± 2^0C (Morang *et al*., 2012). The Experiment was done in three replications in a completely randomized design. The isolates that rendered highest inhibition/antagonism of pathogen mycelial growth and highest zone of inhibition in the *in vitro* assay were selected for pot experiment in green house and micro plots in the field. Four isolates, two each from fungi and bacteria were selected for green house and field experiments.

3.5.3. Greenhouse experiment

Plastic pots of 30 × 30 cm (20 l) with holes drilled at the bottom to facilitate drainage were surface sterilized with sodium hypochlorite and filled with steam sterilized top soil from where sugarcane has not been grown for the past five years. One budded sets of CO 421 variety were surface sterilized and dipped for 30 minutes in suspension of smut spores(4g spores/litre of sterile water) from freshly collected culture of teliospores having more than 70% germination and then held overnight in polythene sacks (Paramdeep *et al*., 2014; Nzioki and Jamoza, 2009; Olweny *et al*., 2008). Plates of antagonists cultures grown on PDA at 25^0C for 14 days and nutrient agar medium for 2 days were scrapped using a sterilized spatula and mixed with sterilized distilled water, filtered through a nylon mesh then adjusted to get a final cfu of 1.0×10^6 spores/ml and 1.0×10^4 cfu/ml respectively is using a modified calibrated microscope slide (haemocytometer).The suspension was pippeted into the counting chambers of the haemocytometer using a clean pipette tip and the cell or spores counted in the grids of known volume (0.1µl) under a microscope to be used to estimate its concentration per ml.The smut infected one budded setts were dip inoculated for 15 minutes in the respective antagonist suspensions and the control setts dipped into sterile distilled water for similar duration. Three setts were then planted individually in each pot and irrigated regularly, after every two days up to

the second month where they were exposed to water stress to enhance development of smut fungi in the vascular system and produce symptoms.

The treatments were:

(i) Sett treatment with antagonist 1(Fungi/AJF7) + pathogen.

(ii) Sett treatment with antagonist 2 (Fungi/AJF8) + pathogen.

(iii) Sett treatment with antagonist 3 (Bacteria/AJB4) + pathogen.

(iv) Sett treatment with antagonist 4 (Bacteria/AJB9) + pathogen.

(v) Sett treatment with distilled water + pathogen (control).

The treatments were in five replicates and the pots were arranged in a glass house in a completely randomized design (Appendix 7). Data was collected on smut disease incidence on all treatments and control by scoring the number of smut whips appearing per treatment monthly for six months starting from two months after planting. Plants with smut whips were recorded, whip removed until the trial was completed. The data was then converted into disease incidence (DI) per treatment according to Dua and Sidhu (2012) and Morang *et al*. (2012) using the formulas below;

$$\textbf{(i) DI} = \frac{\textbf{Total no. of diseased plants.}}{\textbf{Total no. of observed plants}} \times \textbf{100} \quad \text{... Eqn. - 6}$$

Where **DI** is disease incidence.

3.5.4 Field Experiment in microplots

This trial was established in field 7 at (KESREF), Kibos which is well isolated to limit spread of the innoculum to commercial sugar fields. Plastic pots of 30 × 30 cm were surface sterilized with sodium hypochlorite and filled with top soil from where sugarcane has not been grown for the past five years. One budded sets of CO 421 variety were surface sterilized and dipped for 30 minutes in suspension of smut spores containing 4grams spores/litre of sterile water then held overnight in polythene sacks (Nzioki *et al*., 2010; Olweny *et al*., 2008). The treatments were as in green house in five replicates and completely randomized design but the pots were buried three quarters underground in the soil to constitute a micro plot and relied on rainfall water

(Appendix 7). Data was collected on smut disease incidence on all treatments and control as in green house experiment above.

3.6. Data analysis

Statistical analysis of data was conducted using SAS 9.1 package to determine effect of fields on microbial population and the effect of antagonists on radial growth of pathogen mycelia, inhibition of growth percentage of the pathogen mycelia and smut disease incidence in the green house and field.

Treatment means separation was accomplished by Turkey LSD and significance level tested at P= 0.05.

CHAPTER FOUR

RESULTS

4.1. Population of fungi and bacteria in the rhizosphere of sugarcane variety CO 421.

4.1.1. Fungal count

There was a significant difference at P = 0.05 between the populations of fungi among the ten fields. Field ten had the highest population of fungi (6.75×10^4 cfu/g) significantly different from all other fields except field 3. Field six had the least population (3.42×10^4 cfu/g) significantly different from fields 2, 3, 5, 7, 9 and 10 but not different from fields 1, 4 and 8 (Table 4.1).

Fungal count in cfu/g of the rhizosphere and non rhizosphere soil samples of sugarcane variety CO 421 collected and enumerated from mixed culture colonies (Plate 1) between the month January and March 2014 showed a higher average total population of 4.89×10^4 cfu/g for rhizosphere soil ranging from the least population of 3.42×10^4 cfu/g to the highest of 6.75×10^4 cfu/g compared to an average total population of 3.14×10^4 cfu/g for non rhizosphere soil ranging from the least of 2.25×10^4 cfu/g to the highest of 5.97×10^4 cfu/g making variation in population between the two regions evident. The mean rhizosphere effect was 1.7 indicating that the population in the rhizosphere was twice more than the non rhizosphere (Table 4.1).

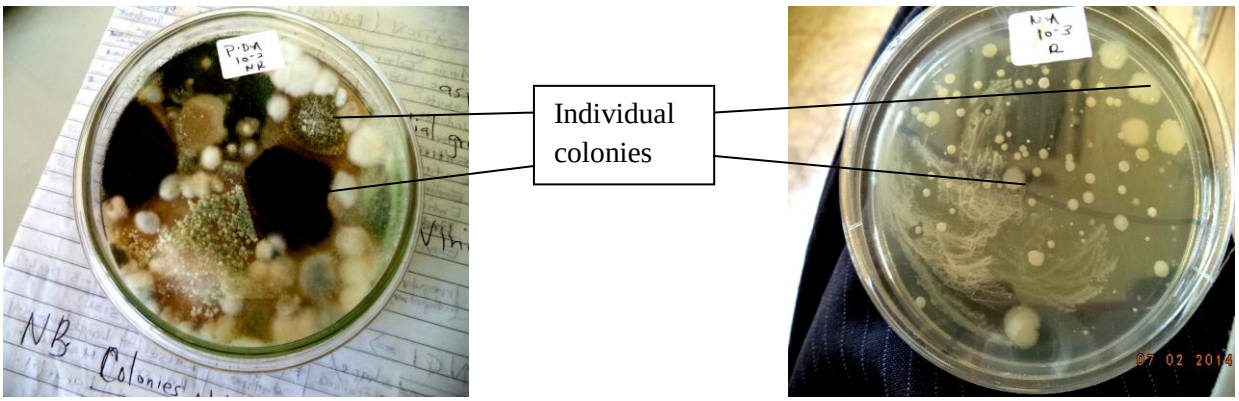
4.1.2. Bacterial count

There was a significant difference at P = 0.05 between the populations of bacteria among the ten fields. Field one had the highest population of bacteria (2.18×10^7cfu/g) significantly different from all the other fields. Field 2 had the least population of 7.92×10^6cfu/g significantly different from fields 1, 5, 7 and 9 but not different from fields 3, 4, 6, 8 and 10 (Table 4.1).

Bacterial count in cfu/g of the rhizosphere and non rhizosphere soil samples of sugarcane variety CO 421 collected and enumerated from mixed culture colonies (Plate 1) between the months January and March 2014 indicated a higher average total population of 1.265×10^7 cfu/g for rhizosphere soil ranging from the least population of 7.92×10^6 cfu/g to the highest of 2.18×10^7 cfu/g compared to an average total population of 6.23×10^6 cfu/g for non rhizosphere soil ranging from the least of 4.05×10^4 cfu/g to the highest of 9.57×10^6 cfu/g of soil. The mean

rhizosphere effect was 2.2 indicating that the population in the rhizosphere was twice more than the non rhizosphere (Table 4.1). The population of bacteria in the CO 421 sugarcane variety rhizosphere was much higher than the population of fungi. Bacteria had a higher mean population of 1.27×10^{7}cfu/g compared to fungi's of 4.89×10^{4}cfu/g (Table 4.1).

A total of sixteen pure fungal colonies and twelve pure bacterial colonies were tentatively identified as AJF-: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 and16 for fungi and AJB-: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12 for bacteria were reisolated from mixed culture colonies (Plate 1) in the period of January to April 2014. Fungal isolate AJF 15 was present only in one site with the least percentage frequency of 9% and isolates AJF 2, 4, 8 and 13 were present in all the fields 100% frequency (Appendix 8). Bacterial isolate AJB 12 showed the least percentage frequency of 36% and isolates AJB 1, 2, 4, 7 and 8 were present in all fields hence 100% frequency (Appendix 9).



1a **1b**

Plate 1: Samples of mixed fungal colonies on PDA medium (1a) and mixed bacterial colonies on NA medium (1b).

Table 4.1: Fungal and bacterial counts in the rhizosphere and non rhizosphere soil samples from ten fields of sugarcane variety CO 421.

Field	Fungi			Bacteria		
	Rhizosphere (cfu/g)	**Non Rhizosphere (cfu/g)**	**Rhizosphere effect**	**Rhizosphere (cfu/g)**	**Non Rhizosphere (cfu/g)**	**Rhizosphere effect**
1	3.48×10^4 e	2.25×10^4 c	1.61	2.18×10^7 a	7.02×10^6 bc	3.11
2	5.04×10^4 cd	3.18×10^4 bc	1.61	7.92×10^6 c	4.05×10^6 e	1.98
3	6.48×10^4 ab	5.97×10^4 a	1.08	1.26×10^7 bc	9.57×10^6 a	1.31
4	4.65×10^4cde	2.79×10^4 bc	1.67	1.15×10^7 bc	5.22×10^6 de	2.22
5	4.77×10^4 cd	3.15×10^4 bc	1.54	1.49×10^7 b	8.4×10^6 ab	1.79
6	3.42×10^4 e	2.49×10^4 bc	1.40	1.13×10^7 bc	4.11×10^6 e	2.79
7	5.1×10^4 cd	3.48×10^4 b	1.50	1.41×10^7 b	7.86×10^6 b	1.79
8	3.9×10^4 de	2.88×10^4 bc	1.35	9.0×10^6 c	5.43×10^6cde	1.87
9	5.28×10^4 bc	2.31×10^4 c	2.33	1.47×10^7 b	6.03×10^6 cd	2.57
10	6.75×10^4 a	2.88×10^4 bc	2.43	8.82×10^6 c	4.56×10^6 de	2.78
Mean	4.89×10^4	3.14×10^4	1.65	1.27×10^7	6.23×10^6	2.22
LSD	1.23×10^4	1.10×10^4		4.84×10^6	1.67×10^6	

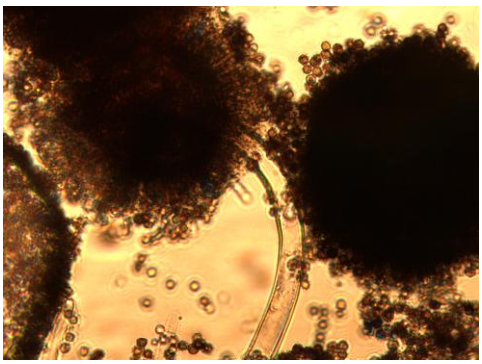
Means followed by different letters down the columns differ significantly at P=0.05.
Each value is an average of three replicates.

4.2. Morphological identification of fungi and bacteria in the rhizosphere of sugarcane variety CO 421.

Fungal identification

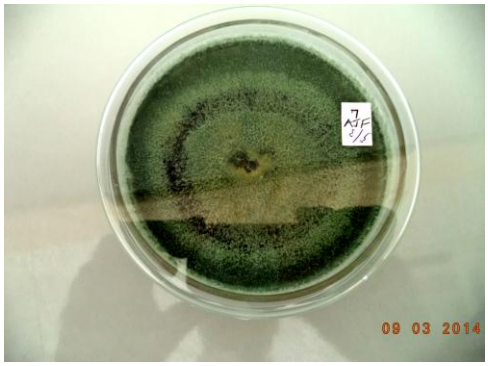
Sixteen pure fungal isolates tentatively identified as AJF1 - AJF 16 (Plates 2, 3, 4 and 5, Appendix 13) with varied morphological characteristics at day seven on PDA medium and the image of mycelia tip as observed under a phase contrast microscope magnification ×400 were described based on colony diameter, shape, margin, elevation, top and bottom colour, surface mycelia, hyphae and conidiophores shape (Table 4.2). The morphologically described fungal isolates were identified in reference to Ellis *et al*. (2007), Alexopoulos *et al*. (2002) and Williams *et al*. (2001). Five of the isolates were identified to species level (AJF 4, 7, 8, 11 and 16) and six isolates to genus level (AJF 1, 2, 3, 6, 10 and 13) and five isolates unidentified (AJF 5, 9, 12, 14 and 15). *Trichoderma* was predominant with five isolates (AJF 3, 6, 7, 8 and 10) followed by *Aspergillus* three isolates (AJF 4, 11, and 16) then *Rhizopus* (AJF 2), *Penicillium* (AJF 1) and *Alternaria* (AJF 13) one isolate each (Table 4.3).

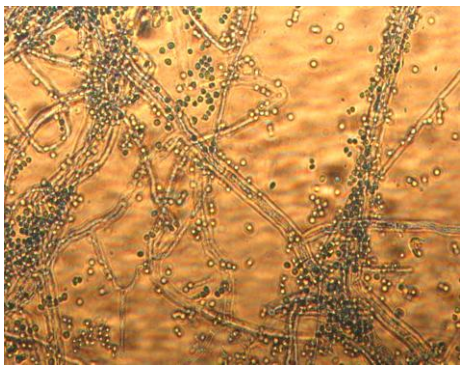
a



b

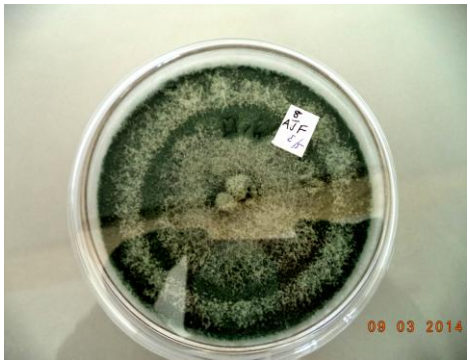
Plates 2: Pure fungal isolate AJF2 on PDA medium at day 7 (2a) and the mycelia tip mg. ×400 (2b) showing morphological characteristics.

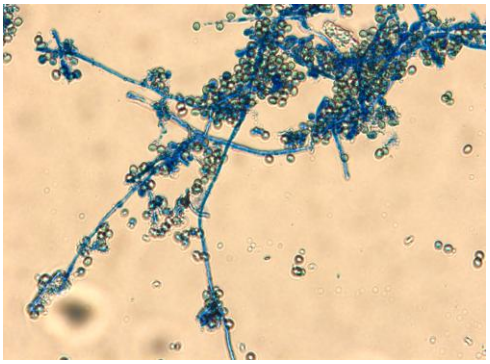




a **b**

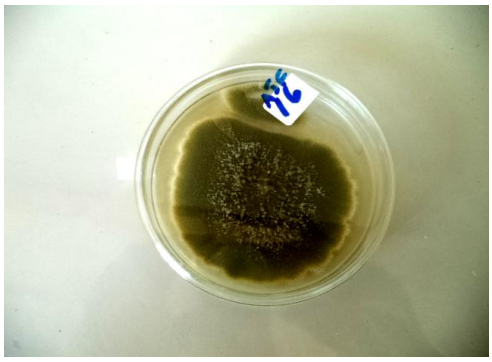
Plates 3: Pure fungal isolate AJF7 on PDA medium at day 7 (3a) and the mycelia tip mg. ×400 (3b) showing morphological characteristics.

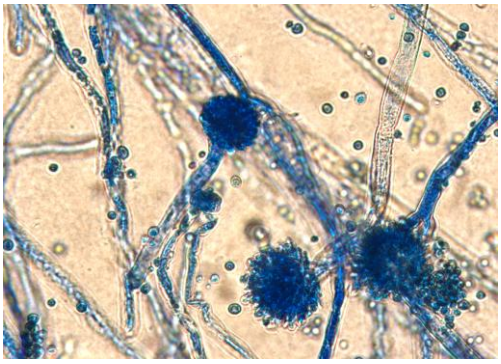




a **b**

Plates 4: Pure fungal isolate AJF8 on PDA medium at day 7 (4a) and the mycelia tip mg. ×400 (4b) showing morphological characteristics.





a **b**

Plates 5: Pure fungal isolate AJF 16 on PDA medium at day 7 (5a) and the mycelia tip mg. ×400 (5b) showing morphological characteristics.

Table 4.2: Morphological characteristics of fungal colonies from the rhizosphere of sugarcane variety CO 421.

Isolate	**Diameter(mm)**	**Shape**	**Margin**	**Elevation**	**Color and surface**		**Hyphae**	**Conidia shape and conidiophores**
					Top	Bottom		
Penicillium sp. AJF 1	10	Circular	Entire	Flat	Grayish green White bushy mycelia	Pale yellow	Septate	Globose/ Spherical
Rhizopus sp. AJF 2	60	Pyramid	Lobbed	Raised	Grayish black and powdery	Pale white	Aseptate	Globose
Trichoderma sp. AJF 3	90	Circular	Entire	Flat	Green White mycelia	yellowish	Septate	Spherical
Aspergillus aureus. AJF 4	45	Circular	Lobbed	Raised	Yellow and brown at the centre Grooved	Purplish Red	Septate	Spherical
AJF 5 unidentified	90	Circular	Entire	Raised	Yellow green Grooved and white mycelia	Yellow	septate	Spherical
Trichoderma sp. AJF 6	90	Circular	Entire	raised	Light green White mycelia	Yellow	septate	Spherical Green Branched conidiophores

Isolate	**Diameter(mm)**	**Shape**	**Margin**	**Elevation**	**Color and surface**		**Hyphae**	**Conidia shape and conidiophores**
Trichoderma viride AJF 7	90	Circular	Entire	Flat	Dark green, yellowish at the centre White mycelia	Yellowish pale	Septate	Globose numerous and Green in colour
Trichoderma herzanium AJF 8	90	Circular	Entire	Raised	Dark green from the centre Many white mycelia	yellow	Septate	Ellipsoidal/ovalish Green in color
AJF 9 Unidentified	90	Circular	Entire	Flat	White	cream	-	Spherical
Trichoderma sp. AJF 10	90	Circular	Entire	Raised	Green with grey centre	Pale yellow	Septate	Globose and green
Aspergillus niger AJF 11	40	Circular	Filamentous	Raised	Black with white margin Hairy	Yellowish	Septate	Spherical
Unidentified AJF 12	5	circular	Lobbed	Raised	White Smooth	Cream	Aseptate	Spherical
Alternaria sp. AJF 13	35	Circular	Filamentous	Raised	Grey with white	Brown to black	Septate	Oval Club like

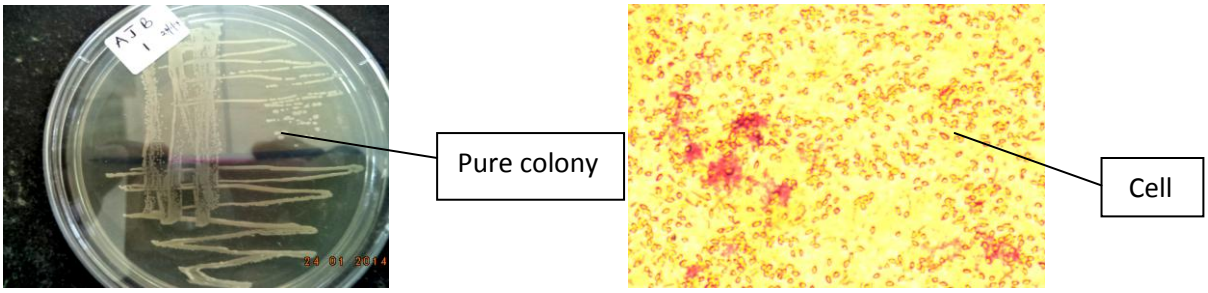
					towards the margin Hairy			
AJF 14 (Plate 15) Unidentified	63	Irregular	Lobbed	Raised	White hairy with grooves	Yellow	Aseptate	Spherical
AJF 15 (Plate 16) Unidentified	80	Irregular	Lobbed	Raised	White Rings curled	Cream	Aseptate	Spherical
Aspergillus flavus AJF 16 (Plate 17)	65	Circular	Filamentous	Raised	Yellow green White mycelia	Yellow brown	Septate	Spherical Conidia head

Table 4.3: Identity of Fungal isolates from rhizosphere of sugarcane variety CO 421.

Isolate	Kingdom `	Phylum	Class	Order	Family	Genus	Species
AJF 1	Fungi	Ascomycota	Euascomycetes	Eurotiales	Trichomaceae	*Penicillium*	-
AJF 2	Fungi	zygomycota	zygomycetes	Mucorales	Mucoraceae	*Rhizopus*	-
AJF 3	Fungi	Ascomycota	Euascomycetes	Hypocreales	Hypocreaceae	*Trichoderma*	-
AJF 4	Fungi	Ascomycota	Eurotiomycetes	Eurotiales	Trichocomaceae	*Aspergillus*	*A.aureus*
AJF5	Fungi	-	-	-	-	-	
AJF 6	Fungi	Ascomycota	Euascomycetes	Hypocreales	Hypocreaceae	*Trichoderma*	-
AJF 7	Fungi	Ascomycota	Euascomycetes	Hypocreales	Hypocreaceae	*Trichoderma*	*T.viride*
AJF 8	Fungi	Ascomycota	Euascomycetes	Hypocreales	Hypocreaceae	*Trichoderma*	*T.herzanium*
AJF 9	Fungi	-	-	-	-	-	-
AJF 10	Fungi	Ascomycota	Euascomycetes	Hypocreales	Hypocreaceae	*Trichoderma*	-
AJF 11	Fungi	Ascomycota	Eurotiomycetes	Eurotiales	Trichocomaceae	*Aspergillus*	*A.niger*
AJF 12	Fungi	-	-	-	-	-	-
AJF 13	Fungi	Ascomycota	Euascomycetes	Pleosporales	Pleosporaceae	*Alternaria*	-
AJF 14	Fungi	-	-	-	-	-	-
AJF 15	Fungi	-	-	-	-	-	-
AJF 16	Fungi	Ascomycota	Eurotiomycetes	Eurotiales	Trichomaceae	*Aspergillus*	*A.flavus*

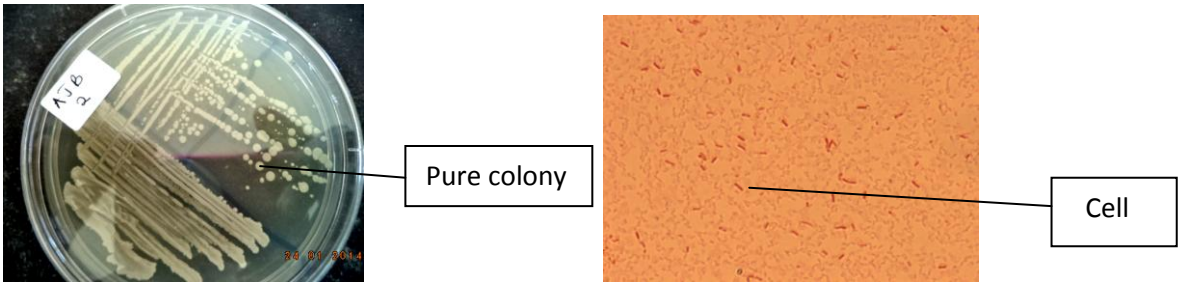
Bacterial identification

Twelve pure bacterial isolates were tentatively identified as AJB1 – AJB 12 (Plates 6, 7, 8, 9 and 10, Appendix 14) with varied colony and cell morphological characteristics on NA medium. The isolates were morphologically described based on colony shape, elevation, margin, surface, opacity and colour followed by Cell shape, arrangement and gram reactivity as observed under a phase contrast microscope magnification ×1000 (Table 4.4). All the isolates were gram negative except isolate 3 and 12. The morphologically described isolates were identified in reference to Bergey's manual of determinative bacteriology identification flow chart. Six of the isolates identified to genus level (AJB 3, 4, 5, 6, 7 and 12) and six isolates unidentified (AJB 1, 2, 8, 9, 10 and 11). *Pseudomonas* was predominant with three isolates (AJB 4, 5 and 6) followed by *Bacillus* two isolates (AJB 3 and 12) and *Azobacter* (AJB 7) one isolate (Table 4.5).



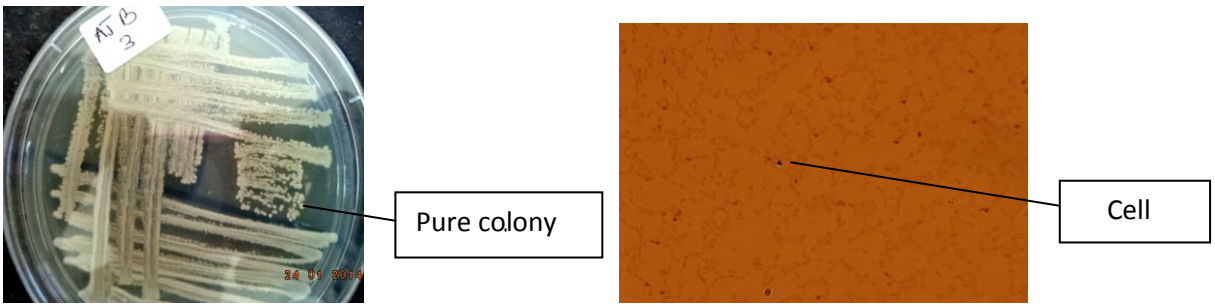
a **b**

Plates 6: Pure bacterial isolate AJB1 on NA medium (6a) and the cells mg. ×1000 (6b) showing morphological characteristics.



a **b**

Plates 7: Pure bacterial isolate AJB2 on NA medium (7a) and the cells mg ×1000 (7b) showing morphological characteristics.



a **b**

Plates 8: Pure bacterial isolate AJB3 on NA medium (8a) and the cells mg ×1000 (8b) showing morphological characteristics

a **b**

Plates 9: Pure bacterial isolate AJB4 on NA medium (9a) and the cells mg ×1000 (21b) showing morphological characteristics.

a **b**

Plates 10: Pure bacterial isolate AJB5 on NA medium (10a) and the cells mg ×1000 (10b).showing morphological characteristics.

Table 4.4: Morphological characteristics of bacterial colonies and cells from rhizosphere of sugarcane variety CO 421

Isolate	**Colony shape**	**Elevation**	**Margin**	**Surface**	**Opacity**	**Color**	**Cell shape**	**Cell arrangement**	**Gram reactivity**
AJB1	Circular	Raised	Entire	Glistening	Translucent	Cream	Comma	Single	Negative
AJB2	Circular	Flat	Entire	Glistening	Opaque	Cream	Short rods	Single	Negative
AJB3 *Bacillus sp.*	Irregular	Flat	Entire	Glistening	Translucent	Cream	Rods	Chain	positive
AJB4 *Pseudomonas sp.*	Filamentous	Raised	Filiform	Glistening	Translucent	Cream	Short rods	Chain	Negative
AJB5 *Pseudomonas sp.*	Circular	Raised	Entire	Glistening	Opaque	Cream	Rods	Single	Negative
AJB6 *Pseudomonas sp.*	Circular	Raised	Entire	Glistening	Transparent	Cream	Short rods	Single	Negative
AJB7 *Azobacter sp.*	Circular	Flat	Irregular	Glistening	Opaque	Cream	Short rods	Single	Negative
AJB8	Circular	Flat	Irregular	Glistening	Opaque	White	Short rods	Double	Negative

Isolate	**Colony shape**	**Elevation**	**Margin**	**Surface**	**Opacity**	**Color**	**Cell shape**	**Cell arrangement**	**Gram reactivity**
AJB9	Circular	Flat	Irregular	Glistening	Translucent	White	Circular	Bunches	Negative
AJB10	Circular	Flat	Entire	Glistening	Opaque	Yellow	Circular	Bunches	Negative
AJB11	Circular	Flat	Wavy	Dull/dry	Translucent	Cream	Long rods	Single	Negative
AJB12 *Bacillus sp.*	Circular	Raised	Wavy	Glistening	Translucent	Cream	Short rods	Double	Positive

Table 4.5: Identity of Bacterial isolates from rhizosphere of sugarcane variety CO 421

Code name	Kingdom	Phylum	Class	Order	Family	Genus
AJB 1	Monera	-	-	-	-	-
AJB 2	Monera	-	-	-	-	-
AJB 3	Monera	Firmicutis	Bacilli	Bacillales	Bacillaceae	*Bacillus*
AJB 4	Monera	Proteobacteria	Gamma proteobacteria	Pseudomonadales	Pseudomonadaceae	*pseudomonas*
AJB 5	Monera	Proteobacteria	Gamma proteobacteria	Pseudomonadales	Pseudomonadaceae	*Pseudomonas*
AJB 6	Monera	Proteobacteria	Gamma proteobacteria	Pseudomonadales	Pseudomonadaceae	*Pseudomonas*
AJB 7	Monera	Proteobacteria	Gamma proteobacteria	Pseudomonadales	Azobacteraceae	*Azobacter*
AJB 8	Monera	-	-	-	-	-
AJB 9	Monera	-	-	-	-	-
AJB 10	Monera	-	-	-	-	-
AJB 11	Monera	Proteobacteria	-	-	-	-
AJB 12	Monera	Firmicutis	Bacilli	Bacillales	Bacillaceae	*Bacillus*

4.3. Antagonistic potential of fungi and bacteria isolated from rhizosphere of sugarcane variety CO 421 against *Sporisorium scitamineum.*

4.3.1. *In vitro* screening for potential antagonism by dual culture technique

4.3.1.1. Fungi

The pathogen colony radial growth in the dual plates (Plate 11, Appendix 11) ranged between 12.3mm - 30mm and percentage inhibition ranged between 6 % - 61% (Table 4.6). The tested antagonists inhibited radial colony growth of the pathogen *Sporisorium scitamineum* at varying degrees. Isolate AJF 7 (*Trichoderma viride*) showed the lowest radial growth (12.3mm) and highest growth inhibition percentage of the pathogen (61%) while AJF 15 (unidentified) showed the lowest growth inhibition of 6% and highest radial growth of the pathogen (30mm). The results produced by the test antagonists were significantly different from control except AJF 15 that was not significant at (P=0.05). There was no significant difference between isolates AJF 7, 8, 9, 10 and 3 which were the best potential fungal antagonists under the conditions of this study followed by isolates AJF 1, 2, 4, 5, 6, 13, 14 and 16 were not significantly different from each other on suppression of radial growth of the pathogen. (Table 4.6).

The test antagonists grew faster occupying more space and showing dominance over the pathogen *Sporisorium scitamineum* in dual culture plates thereby limiting its growth in most of the petri plates. Isolate AJF 3, 7, 8, 9 and 10 almost completely covered the growth of the pathogen within seven days of incubation (Plate 11). Isolates AJF 1, 2, 4, 5, 6, 13, and 16 inhibited the pathogen growth at varying levels. Isolate AJF 15 was dominated by the pathogen (Appendix 11).

Table 4.6: Radial growth and inhibition percentage of *Sporisorium scitamineum* in dual culture plates with fungal test antagonists.

Fungal test antagonists and pathogen	Radial growth(mm)	Growth inhibition %
Control	32a	-
AJF15	30ab	6fg
AJF12	28.3b	11.3f
AJF14	25c	25.3e
AJF5	23.3cd	27e
AJF13	23cd	28.3de
AJF6	21.3de	33.3cde
AJF4	20ef	37.7bcd
AJF1	19.7ef	38.7bc
AJF16	19ef	40.7bc
AJF11	18.7ef	41.7bc
AJF2	18f	43.7b
AJF3	14g	56a
AJF10	14g	56a
AJF9	13.7g	57a
AJF8	13g	59a
AJF7	12.3g	61a
LSD	2.9	9.3

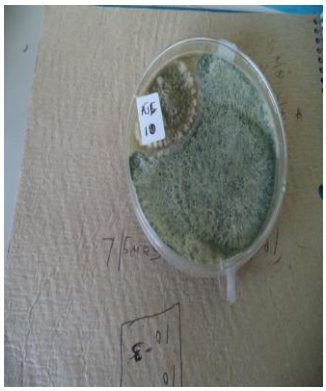
Means followed by different letters down the columns differ significantly (P=0.05).
Values presented are means of three replicates.

11a

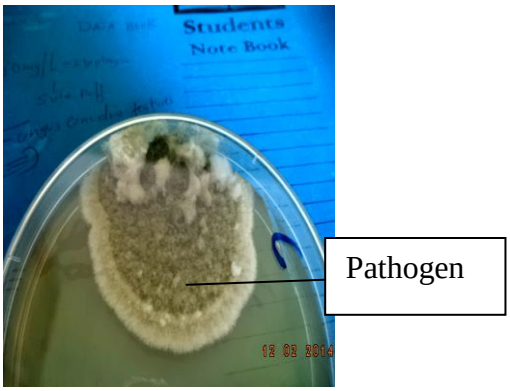
11b

11c

11d



11 e



11f

Plates 11: Radial growth of *Sporisorium scitamineum* in dual culture plates by isolates AJF 3 (11a), AJF 7 (11b), AJF 8 (11c) ,AJF 9 (11d), AJF10 (11e) on PDA medium at day seven of incubation and *Sporisorium scitamineum* alone / control (11f).

4.3.1.2. Bacteria

The inhibition zones in dual culture plates (Plate 12, Appendix 12) on solid NA medium of bacterial isolates and pathogen *Sporisorium scitamineum* ranged from 1mm – 25.6mm after seven days (Table 4.7). The test antagonists inhibited growth of the pathogen at varying degrees. AJB 9 showed the highest inhibition zone of 25.7mm and AJF 11 the lowest zone of 1mm.The results produced by the test antagonists were significantly different from control except AJB 1, 2, 5, 7 and 11 at (P=0.05). There was no significant difference between isolates AJB 9 and AJB 4 which showed the highest inhibition followed by isolates AJB3, AJB4, AJB8, and AJB 12 that

were not significantly different from each other. The test antagonists significantly reduced the growth of the pathogen except AJB1, 2, 7, 5 and 11. The antagonists grew faster than the pathogen and produced inhibition zones (plate 12, Appendix 12) at varying levels there by limiting the growth of the pathogen under the conditions of this study.

Table 4.7: Inhibition zones between bacterial isolates and *Sporisorium scitamineum* using line inoculation method in a dual culture plate at seven days.

Bacterial isolates and *Sporisorium scitamineum*	**Inhibition zone (mm)**
AJB9	25.7a
AJB4	24.3ab
AJB3	23abc
AJB8	23abc
AJB12	22.3bc
AJB6	21.3c
AJB10	21bc
AJB5	20.3cd
AJB7	20.3cd
AJB1	20.3cd
CONTROL	18ed
AJB2	16e
AJB11	1f
LSD	2.9

Means followed by different letters down the column differ significantly (P = 0.05).
Values presented are means of three replicates

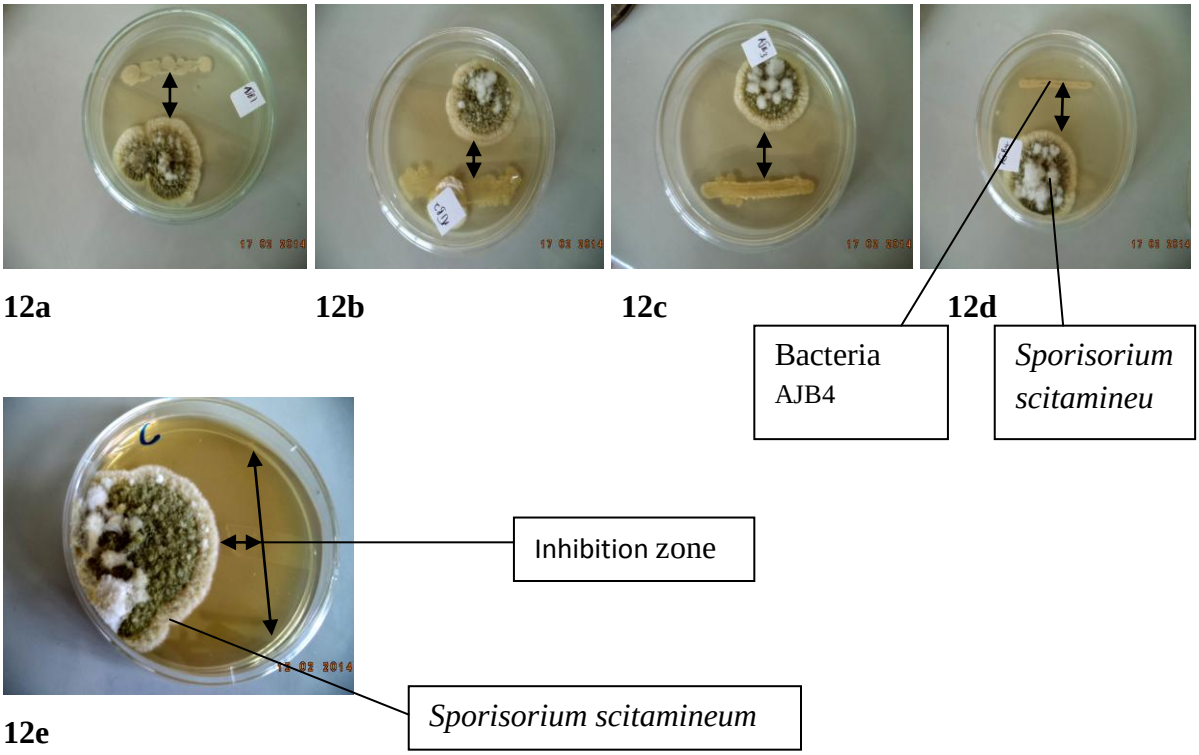


Plate 12: Inhibition zones between *Sporisorium scitamineum* and bacterial test antagonist AJB1 (12a), AJB 2 (12b), AJB 3 (12c), AJB 4 (12d), in dual culture plate on solid PDA medium after seven days of incubation and control (12e) with pathogen alone.

4.3.2. *In vivo* screening for potential antagonism

AJF 7 (*Trichodema viride*), AJF 8 (*Trichodrema herzanium*), AJB 4 (*Pseudomonas* sp.) and AJB 9 (unidentified) were selected based on their high antagonistic potential against *Sporisorium scitamineum* from *in vitro* inhibition for potential antagonism in green house and micro plots in the months of April – November 2014.

4.3.2.1. Green house

The four selected antagonists showed varying degree of disease control from number of infected plants and percentage disease incidence in the green house. Maximum disease incidence of 27% was observed in AJF 8 (*Trichoderma herzanium)* and minimum of 13% in AJB9 (unidentified) in the green house compared to 30% incidence of control (Table 4.8). There was no significant difference at P=0.05 on the number of smut whips, infected plants and percentage disease incidence between the treatments (Table 4.8).

Table 4.8: Percentage disease incidence, infected plants and number of smut whips produced by sugarcane plants treated by selected antagonists and grown under green house conditions.

Selected antagonist	Infected plants	Number of whips	Disease incidence (%)
Control	0.8a	2.8a	30a
AJB 4	0.4a	0.6a	17a
AJB 9	0.4a	0.4a	13a
AJF 7	0.6a	2.6a	20a
AJF 8	0.6a	1.6a	27a
LSD	0.98	2.69	33.9

Means followed by same letters down the column do not differ significantly (P = 0.05).
Values presented are means of five replicates.

4.3.2.2. Field in microplots

The four selected antagonists showed varying degree of disease control in the field micro plots from number of infected plants and percentage disease incidence (Table 4.9). Maximum disease incidence of 13.3% was observed in AJB 4 (*Pseudomonas* sp.) and minimum of 6.7% in AJF7 (*Trichoderma viride*), compared to 36.6% in control. There was no significant difference at P=0.05 on percentage disease incidence, number of whips and percentage disease incidence between the treatments (Table 4.9).

Table 4.9: Percentage disease incidence, infected plants and number of smut whips produced by sugarcane plants treated with selected antagonists and grown under field conditions.

Selected antagonist	Infected plants	Number of whips	Disease incidence (%)
Control	0.6a	3.6a	36.6a
AJB 4	0.4a	3.4a	13.2a
AJB 9	0.2a	1.4a	10a
AJF 7	0.2a	3.0a	6.7a
AJF 8	0.4a	2.2a	13.3a
LSD	0.79	6.80	35.60

Means followed by same letters down the column do not differ significantly (P = 0.05).
Values presented are means of five replicates.

CHAPTER FIVE

DISCUSSION

5.1. Population of fungi and bacteria in the rhizosphere of sugarcane variety CO 421.

The findings of this research indicate that both fungi and bacteria were present in the rhizosphere of sugarcane variety CO 421 in the ten fields. High population of fungi and bacteria in the rhizosphere of sugarcane has been reported by many researchers (Kumalawati *et al*., 2014; Deshmuk *et al*., 2013; Rajasankar and Ramalingam, 2012; Tailor and Joshi, 2012; Chanda *et al*., 2011; Pankhurst *et al.,* 2000) confirming its ability to host numerous and diverse microbes than bulk soil.

The population of the micro flora was higher in the rhizosphere than non rhizosphere in all the ten fields this is in agreement with previous studies by Kelechi and Chiaka (2014) that showed higher population of bacteria in the rhizosphere (1.03×10^6 – 1.37×10^6 cfu/g) than bulk soil (2.56×10^5 – 3.12×10^5 cfu/g) in soils with *Desmodium trifolium, Phaseolus* and *Solestemon* sp. plants. Deshmuk *et al*. (2013) on rhizosphere of sugarcane varieties CO 86032 and CO 0265 also reported higher fungal development in the vicinity of the root zone as compared to the soil away from rhizosphere effect (bulk soil). The disparity in the rhizosphere and bulk soil microbial population could be due to sugarcane plant roots releasing exudates containing different organic and inorganic compounds in to the surrounding soil that stimulated development of active microbial population (Chanda *et al*., 2011). The nature and concentration of these organic constituents and the corresponding ability of the microorganisms to utilize them as sources of energy may also be a contributing factor to the disparity in population of the two regions (Nekade, 2013).

Bacterial population was more than that of fungi in all the fields which is in agreement to the findings by Tamilarasi *et al*. (2008) where bacteria recorded higher population of 2.8×10^6 cfu/g of soil than fungi 1.0×10^4cfu/g of rhizosphere soil on selected medicinal plants in and around Bharathiar university in India and the findings of Athul *et al*. (2012) on rhizosphere soils of vanilla crop that recorded 4.1×10^5 cfu/g for bacteria to 3.45×10^3 cfu/g for fungi in Kevala State

India. These numbers were lower than the population in this study (bacteria: 1.265×10^7 cfu/g and fungi: 4.89×10^4 cfu/g) of sugarcane rhizophere soil probably due to disparity in soil type, plant species, root type, soil management practices, microbial interactions, level of pollution and other environmental variables in the study areas (Kelechi and chiaka, 2014; Nihorimbere *et al*., 2011). Bello and Utang (2011) also recorded higher population of bacteria than fungi in all their locations of study of plantain rhizosphere microorganisms at cross river state in Nigeria. The high bacteria population may be attributed to greater rhizosphere effect on bacteria than fungi (Nihorimbere *et al*., 2011).

The significant differences in microbial population among the ten fields from which rhizosphere soil was obtained in this study could have been due to variations in sugarcane plant age in the fields and pH of the soils. Microbial activity increases with plant age and declines towards maturity probably due to the plants secreting exudates in reduced quality and quantity that may contain antimicrobial metabolites (Deshmuk *et al*., 2013; Nihorimbere *et al*., 2011). The pH of a particular soil reflects the chemical and mineralogical environment in that soil, and thus the pH is of great importance to plant roots and microbial activity (Sule and Oyeyiola, 2012).

The present study isolated 28 pure isolates (16 fungi and 12 bacteria). The dominant fungal genera in all the fields were *Trichoderma, Rhizopus, Aspergillus* and *Alternaria* and bacteria were *Bacillus, Pseudomonas* and *Azobacter* similar to the findings of other scientists (Chandrashekar *et al*., 2014; Deshmuck *et al*., 2013; Gaddeya *et al*., 2012; Rajasankar and Ramalingam ,2012; Ashraf *et al*., 2011). The isolation technique could have favoured the recovery of fast growing isolates hence the predominance of *Trichoderma*. High sporulation in *Rhizopus, Aspergillus* and *Alternaria may* have contributed to their dominance (Chandrashekar *et al*., 2014). *Aspergillus* is known to produce toxins that may prevent growth of other fungal species (Gaddeya *et al*., 2012) hence the predominance in all the studies.

5.2. Specific fungi and bacteria in the rhizosphere of sugarcane variety CO 421.

Morphology of single cells or colony characteristics remains a reliable parameter for bacterial and fungal species identification and still has a significant taxonomic value (Tshikudo *et al*., 2013).The isolates exhibited diverse morphological characteristics based on macroscopic

characteristics such as colony diameter, colony surface and reverse colour, colony shape and microscopic features including cell shape, gram reactivity, conidia shape, conidia colour and nature of hyphae this is in accordance to a study by Afzal *et al*. 2013 on morphological identification of *Aspergillus* sp. in Pakistan soil. In the present study, *Aspergillus, Penicillium, Trichoderma, Rhizopus* and *Alternaria* genera of fungi in the rhizosphere of CO 421 sugarcane variety were identified. These findings are similar to the results of Chandrashekar *et al.* (2014) that identified *Aspergillus, Penicillium, Rhizopus, Alternaria, Fusarium* and *Mucor* genera of fungi on his study of sugarcane rhizosphere mycoflora among other crops at Karnataka India. The findings are also similar to those reported by Deshmuk *et al.* (2013) who identified *Aspergillus, Rhizopus, Alternaria* and *Fusarium* and Mahamuni *et al.* (2012), *Aspergillus, Penicillium, Trichoderma, Rhizopus, Alternaria* and *Curvularia* in their studies of sugarcane rhizosphere soil fungi. Gaddeya *et al.* (2012) identified *Aspergillus, Penicillium, Trichoderma, Rhizopus, Alternaria* and *Curvularia* as he studied mycoflora in a sugarcane field at Salur Mandal in India. Some of the fungal genera identified in the previous studies mentioned were not in the present study that is *Curvularia, Fusarium* and *Mucor* and there were disparities among the studies in microbial diversity and composition. This could be attributed to differences in environmental variables in the areas of study such as pH and temperature (Kelechi and chiaka, 2014; Nihorimbere *et al*., 2011). Kumar *et al*. (2015) isolated and identified using morphological features *Aspergillus, Alternaria, Curvularia, Fusarium, Penicillium* and *Rhizopus* from rice rhizosphere soils in India similar to the microbes isolated in sugarcane rhizosphere probably because they belong to the same family Poaceae. Sule and Oyeyiola,(2012) on cassava crop rhizosphere isolated and identified *Byssochamys fulva*, followed by *Geotrichum candidum* and then *Papulospora coprophila* as the dominat fungal species in the soil which were absent in all the other crop fields studied including this study. This could be attributed to the fact that different plant species host specific microbial communities (Berendsen *et al*., 2012) and that diversity and composition of the microbial taxa in the rhizosphere can be affected by plant species (Tamilarasi *et al*., 2008).

This study also identified *Bacillus, Pseudomonas* and *Azobacter* genera of bacteria in the rhizosphere of sugarcane similar to the findings of Tailor and Joshi, (2012) that identified

Pseudomonas fluorescence in sugarcane rhizosphere but it isolated siderophore producing bacteria only and involved a series of biochemical characterization. Rajaskar and Ramalingam, (2012) identified five genera of bacteria in sugarcane rhizosphere that is *Pseudomonas, Bacillus, Azotobacter,* similar to the results of this study except for *Azomonas and Mesorhizobium* that were absent probably due to environmental variables in the study areas (Kelechi and chiaka, 2014). *Pseudomonas* and *Azotobacter* genera of bacteria were also identified based on the basis of colony and cell morphology by Ashraf *et al*. (2011) from sugarcane rhizosphere of unkown variety in accordance to this study. Prashar *et al*. (2012) on pearl millet rhizosphere identified Streptomyces (predominant), *Pseudomonas, Flavobacterium, Bacillus, Streptococcus* and *Staphylococcus* genera of bacteria four of which were not identified in this study may be because of the difference in plant species, environmental variations and performance of some biochemical tests for identification which was not done in this study.

5.3. Antagonistic potential of fungi and bacteria isolated from sugarcane rhizosphere variety CO421 against *Sporisorium scitamineum*.

5.3.1. *In vitro* screening

Results from this study revealed that the test antagonists checked the growth of the pathogen at varying degree. Fungal test antagonists grew faster than the pathogen considerably hindering its radial growth and bacterial test antagonists produced inhibition zones thereby limiting the growth of the pathogen in solid media. This indicated that the isolates had a considerable antagonistic effect against the pathogen *Sporisorium scitamineum* hence are potential biocontrol agents of the pathogen. The antagonistic effect of Fungi and Bacteria against plants fungal pathogens have already been reported by other investigators (Suprapta, 2012; Athul *et al*., 2012; Morang *et al*., 2012; Prajapati *et al*., 2012 Prince *et al*., 2011; Lal *et al*., 2009). Mechanisms of inhibition include mycoparasitism, antibiosis, metabolite production and competition for available nutrients as confirmed by a study done by Adebola and Amadi (2010) in Nigeria on the potential of three *Aspergillus* species isolated from cocoa rhizosphere and rhizoplane as biological control agents of *Phytophthora palmivora* in solid media and a study done by Alwathnani and Perveen (2012) on biocontrol of *Fusarium* wilt on tomatoes in Saudi Arabia, where *P. citranum* by 24.4% ,

A.niger, 35.6%) , *T. herzanium* ,44.4% *and Penicillium* sp. 0.9% inhibited radial growth of the test pathogen *Fusarium oxyporum in vitro.*These studies were however not done on sugarcane hence the disparities. Pankhurst *et al*. (2000) confirms that *Pseudomonas* sp., *Bhukolderia* sp. and *Bacillus* sp. in the rhizosphere of sugarcane showed *in vitro* inhibition of the growth of *Pachymetra chunorhiza* and *Pythium graminicola* plant fungal pathogens but not on *Sporisorium scitamineum.* A study done by Usha *et al*. (2012) confirms that *Aspergillus* sp. and *Trichoderma* sp. effectively suppressed *F.oxysporum* causative agent of fusarial wilt in common vegetables *in vitro* by 42.5% and 45% respectively indicating they have antagonistic effect against the plant pathogen however the antagonists were sourced from vermicompost and not from the vegetables rhizosphere. Gawade *et al.* (2012) reported that different strains of *Trichorderma* isolated from sugarcane rhizosphere inhibited mycelia growth of *Fusaruim moniliformae* causing wilt disease of sugarcane *in vitro* in dual culture method indicating *trichoderma's* potential as biocontrol agent recording 41.98% inhibition of mycelia growth. The antagonistic potentiality of some soil fungi against *Colletotrichum falcatum* a pathogen causing Red rot disease in sugarcane was also studied by dual culture method. The pathogen and individual species of the soil fungi showed varying degrees of percentage inhibition of mycelia growth that is *Botrytis cinera* 75%, *Penicillium chrysogenum* 69%, *P.citrinum* 23%, *Gliocladium virens* 56%, *Trichoderma glaucum* 72%, *T.harzianum* 53%, *T.koeningii* 73% and *T.viride* 70% grown on PDA medium (Prince *et al.*, 2011).

The varying degrees of antifungal activity in the various studies could be due to differences in environmental parameters, media and assay methods (Prince *et al.,* 2011). The degree of effectiveness of the various antagonists varied according to the nature, quality and quantity of the inhibitory substances secreted by the antagonists (Alwathnani and Perveen, 2011). The results of this study were confirmed by a study done by Paramdeep *et al*. (2014) on management of smut *Sporisorium scitamineum* with fungicides and bioagents *in vitro* where *Trichoderma herzanium* showed mycoparasitsm and completely covered the growth of the pathogen in 7 days.

5.3.2. *In vivo* screening

All the selected four potential antagonists *in vitro* showed varied percentage of smut disease incidence and suppression of smut whip occurrence however there was no significant difference between the treatments in the green house and also between the treatments in the field indicating none was superior to the other *in vivo.* There was no significant difference (p=0.05) between the treatments and control suggesting that the selected potential antagonistic microorganisms failed to effectively control sugarcane smut infection both in the field and green house. This was probably due to variations in environmental factors between the point of microbe isolation and point of action and the method of antagonist application which often leads to failure *in vivo* as observed by Suprapta (2012). These findings are in accordance to an investigation done by Paramdeep *et al*. (2014) that recorded no significant difference between *Trichoderma herzanium* (49.99%) a bioagent treatment and 53.36% of control treatment for smut disease incidence. Meena and Ramyabharathi (2012) also recorded 8.1% sugarcane smut disease incidence against 20% of control which indicated non effectiveness of the bioagents (*Pseudomonas fluorescence* and a combination of *Trichoderma viride*, *Bacillus subtilis* and *Pseudomonas fluorescence*) in reducing smut disease infection. Lal *et al.* (2009) observed 8.89% smut disease incidence against 15.1% of control with *Trichorderma viride.* In general, the potential antagonistic microorganisms selected from *in vitro* tests often fail to effectively control plant disease *in vivo* (greenhouse or field trials), particularly to soil borne pathogens, this is attributed to several factors such as the type and the content of organic matter, pH, nutrient level, and moisture level of the soil that often influence the efficacy of the biocontrol agents (Suprapta, 2012). *Trichoderma herzanium* effectively controlled *Fusarium oxyporium; Rhizoctonia solani* and *Alternaria alternate* in chili *in vivo* but this was after formulation with talcum powder and applied as foliar spray. Formulation maintains the inoculation level of the bioagent while non formulation reduces the level (Subash et al., 2013) and species of *Trichoderma* have been shown to be selective to different fungi (Adebola and Amadi, 2012).

The fungal isolates AJF 7(*Trichorderma viride*) and AJF 8 (*Trichordema herzanium*) have been identified as active rhizosphere colonizers with a noteworthy effect against various plant pathogens due to their high reproductive capacity, ability to survive under very unfavorable

conditions, efficiency in the utilization of nutrients, capacity to modify the rhizosphere, strong aggressiveness against phytopathogenic fungi and efficiency in promoting plant growth and defense mechanisms (Benitez *et al*., 2004).

AJB 4 (*Bacillus* sp.) and AJB 9 (*Pseudomonas* sp.) mechanisms of antagonism are first rate of growth, ability to colonize the rhizosphere aggressively, ability to produce acidic substances, production of fluorescent pigments that are inhibitory to other organisms, production antimicrobial compounds (2,4 diacetylphloroglucinal, hydrogen cyanide and surfactants), production of siderophores, competition with root pathogens for nutrients and root surface colonization. These give them a competitive advantage over the pathogen (Gravel *et al*., 2005; Pal and Mc Spadden, 2006).

CHAPTER SIX

CONCLUSIONS, RECOMMENDATIONS AND SUGGESTIONS FOR FUTURE RESEARCH

6.1. Conclusions

This study has confirmed that;

There was high population of fungi and bacteria in rhizosphere soil samples of CO 421 sugarcane variety crop. Rhizosphere has a stimulatory effect on the population of the micro flora making the population higher in the rhizosphere than in the non rhizosphere soil.
Bacteria were more stimulated than fungi hence had a greater number of colonies and population per gram of soil.

Aspergillus, Penicillium, Trichoderma, Rhizopus and *Alternaria* genera of Fungi and *Bacillus, Pseudomonas* and *Azobacter* genera of Bacteria were found in the rhizosphere of CO 421 sugarcane variety plants. *Trichoderma* and *Pseudomonas* genera were predominant.

In vitro studies on potential antagonism revealed that most of the isolated microorganisms had evident antagonistic activity against *Sporisorium scitamineum* with regards to dominance and inhibition of the pathogen's mycelia growth on the dual culture plates presenting them as potential microbiological control agents against the pathogen.

In vivo studies on smut disease control in the green house and micro plots revealed that the selected four potential antagonists AJF 7 (*Trichoderma viride*) and AJF 8 (*Trichoderma herzanium*) for Fungi and AJB 4 (*Pseudomonas* sp.) and AJB 9 (unidentified) for Bacteria failed to effectively control sugarcane smut disease both in the field and green house hence may be used as microbiological control agents against *Sporisorium scitamineum* subject to further studies on the best inoculation method, formulation and their ecological fitness in the actual field conditions.

6.2. Recommendations

The results of this study demonstrated diversity of the sugarcane rhizosphere microbial community and have a broader implication for improving the ability to manipulate them for improved sugarcane growth and health.

The existence of high population of rhizosphere microorganisms in sugarcane variety CO 421 plants is an indication that there are diverse exudates produced by the roots of sugarcane, hence an opportunity to optimize the biological functions of the plant soil ecosystem, which can lead to increased benefits of sugarcane production.

Isolates AJF7, AJF8, AJB4 and AJB9 may be recommended for use as potential biocontrol agents in Kibos area, Kisumu, Kenya after exploring further their formulations for performance under actual field conditions.

6.3. Suggestions for further research

Further studies should explore the possibility of isolating and identifying more antimicrobial agents of *Sporisorium scitamineum* by involving molecular techniques and newer automated systems such as fatty acid profiling, sequence analysis of the 16S rRNA gene, protein profiling using matrix-assisted laser desorption ionization time of flight (MALDI-TOF), metabolic finger profiling using (BIOLOG) and ribotyping.

Further research should be done on the efficacy of the microbial biocontrol agents in other sugarcane varieties over a longer period of time and their ecological fitness.

Further research work is needed to investigate the best antagonistic strain in relation to time of application, inoculation method and carrier material in an effort to control *Sporisorium scitamineum in vivo.*

References

Abd, E.F., Alamri, A.I., Abou-Shanab, R.A.I. and Hafez, E.E. (2010). Fingerprinting of *Sporisorium scitamineum* (Sydow) in Egypt using differential display technique: chitinase gene the main marker**.** *Research Journal of Agriculture and Biological Sciences*, **6** (1), 8-13.

Abdulkadir, M. and Waliyu, S. (2012). Screening and isolation of the soil bacteria for ability to produce antibiotics. *European Journal of Applied Sciences* **4** (5), 211-215.

Adebayo, O.S and Ekpo, E.J.A. (2014). Efficacy of fungal and bacterial biocontrol organisms for the control of Fusarium wilt of tomatoes. *Nigerian Journal of Horticultural Science,* **9**, 63-68.

Adebola, M.O. and Amadi, J.E (2010). Screening three *Aspergillus* species for antagonistic activities against the cocoa black pod organism (*Phytophthora palmivora*). *Agriculture and Biology Journal of North America*, **1**(3), 362-365.

Afzal, H., Haq, A., Shazad, S. and Qamar, S. (2013). Morphological identification of *Aspergillus* species from soils of Thata district Pakistan. *International Journal of Agriculture and Applied Sciences,* **5**(2), 105-117.

Alexopoulos, C.J., Mims, C.W and Blackwell, M. (2002). *Introductory mycology* (4th ed.). India – Wiley, 370 – 869.

Alfieri, S.A.Jr. (1978). Sugarcane smut. *Plant pathology circular* No. 185.

Alimi, M., Javad, M. S. and Darzi M.T. (2012). Characterization and application of microbial antagonists for control of *Fusarium* head blight of wheat caused by *Fusarium graminearum* using single and mixture strain of antagonistic bacteria on resistance and susceptible cultivars**.** *African Journal of Microbiology Research*, **6**(2), 326-334.

Al-Nur El-Amin and Abdulmoneim M.A. (2007). Contribution to the knowledge of soil fungi in Sudan rhizosphere mycoflora of sugarcane at Kenana sugar estate. *International Journal of Botany*, **3**, 97-102.

Alwathnani, A. H. and Perveen, K. (2012). Biological controls of Fusarium wilt of tomato by antagonist fungi and cyanobacteria. *African Journal of Biotechnology*, ***11***(5), 1100-1105.

Alwathnani, A. H., Perveen, K., Tahmaz, R and Alhaqbani, S. (2012). Evaluation of biological control potential of locally isolated antagonist fungi against *Fusarium oxysporum* under *in vitro* and pot conditions**.** *African Journal of Microbiology Research*, **6**(2), 312-319.

Amire, O. A., Trione, E. J. and Schimit, R. A. (1982). Characterization of pathogenic races of sugarcane smut fungus by nutron activation analysis. *Journal of Radioanalytical Chemistry*, **75**,195-203.

Anand, R. and Kulothungan, S. (2010). Antifungal metabolites of *Pseudomonas Fluorescens* against crown rot pathogen of *Arachis hypogaea. Annals of Biological Research*, **1***(*1*)*, 199-207.

Angel, J.M., Reena, A., Aysha, O.S., Valli, S., Nirmala, P and Vinothkumar, P. (2013). Isolation of siderophore producing bacteria from rhizosphere soil and their antagonistic activity against selected fungal plant pathogens. *International Journal of Current Microbiology and Applied Sciences*, **2**(1), 59-65.

Ashraf, A.A., Rasool, M. and Mirza, M.S. (2011). Nitrogen fixation and indole acetic acid production potential of bacteria isolated from rhizosphere of sugarcane (*Saccharum officinarum* L.*) Advances in Biological Research* **5** (6), 348-355.

Athul, S. R., Aju K., Asok and Jisha, M.S. (2012). Biocontrol of *Fusarium* wilt of vanilla (v*anilla planifolia*) using combined inoculation of *Trichoderma* sp. and *Pseudomonas* sp. *International Journal of Pharma and Bio Sciences*, **3**(3), 706 – 716.

Australian Government, (2004). The biology and ecology of sugarcane (*Saccharum sp*).Office of the gene technology regulation, www.OGTR.GOV.AU pdf. September 2012.

Bello, O.S. and Utang, G.A. (2011). An ecological survey of microorganisms associated with plantain roots rhizosphere. *American Journal of Agriculture and Biological Sciences,* **6**(4), 567-570.

Benitez, T., Rincon, A.M., Limon, A.M. and Codon, A.C. (2004). Biocontrol mechanisms of *Trichoderma* strains. *International Microbiology*, **7**, 249 - 260.

Berendsen, R.L., Pieterse, M.J.C. and Bakker, A.H.M. (2012). The rhizosphere micro biome and plant health. *Trends in Plant Science*, **17**(8), 478-486.

Berg, G., Opelt, K., Zachow, C., Lottmann, J., Gotz, M., Costa, R. and Smalla, K. (2006). The rhizosphere effect on bacteria antagonistic towards the pathogenic fungus *Verticillium* differs depending on plant species and site. *FEMS Microbiology Ecology*, **56**, 250–261.

Bergey's Manual of Determinative Bacteriology: Identification flow chart (n.d.). Retrieved from www.viweb.vidaho edu/micr/IDFlowchart.pdf. March 2014.

Bowler, P.A., Trujillch, E.E. and Beardsley-jr, J.W. (1977). Insect Feeding on Sugarcane Smut in Hawaii. *Proceedings, Hawaiian Entomological Society*, **22**(3), December, pp. 451-456.

Briceno, R.O., Vieira De souse, O. and Rea, R. (2005). Reaction of twenty sugarcane clones to smut disease *Sporisorium scitamineum. Revista Dela Facultad de Agronomia*, **22**, 399-406.

Buee, M., De Boer, W., Martin, F., Overbeek, L. and Jurkevitch, E. (2009).
The rhizosphere zoo: An overview of plant-associated communities of microorganisms, including phages, bacteria, archaea, and fungi, and of some of their structuring factors. *Plant Soil*, **321**,189–212.

Calvo, P., Ormeno-Orrillo, E., Martinez-Romero, E. and Zuniga, D. (2010). Characterization of *bacillus* isolates of potato rhizosphere from Andean soils of Peru and their potential PGPR characteristics. *Brazilian Journal of Microbiology*, **41**, 899-906.

Cappuccino, J. G and Sherma, N (2008). *Microbiology: A laboratory manual* (8th ed.).San Francisco: Pearson Education, pp 4-24.

Chanda, H., Nivedita, G., Pravin, T. and Kondiram, D. (2011). Influence of sugarcane monocropping on rhizosphere microflora, soil enzymes and NPK status. *International Journal of Pharma and Bio sciences*, **2** (1), 188-202.

Chandrashekar, M.A., Soumya Pai, K. and Raju N.S. (2014). Fungal diversity of rhizosphere soils in different agricultural fields of Nanjangud Taluk of Mysore district, Karnataka, India. *International Journal of Current Microbiology and Applied Sciences*, **3**(5), 559-566.

Cheavegatti-Gianotto, A., Couto de Abreu, H. M., Arruda, P., Filho, J. C. B., William Lee Burnquist, L.M., Creste, S., … Figueira, A.V. (2011). Sugarcane (*Saccharum officinarum* L.): A reference study for the regulation of genetically modified cultivars in Brazil. *Tropical Plant Biology*. **4**(1), 62-89.

Comstock, J.C and Lentini, R.S (2005). Sugarcane smut disease. University of Florida. *Institute of Food and Agricultural Sciences Extension SSAGR* 208.

Couillerot, O., Combaret- Prigent, C., Caballero-Mellado, J. and Moe nne-Loccoz, Y. (2009). *Pseudomonas fluorescens* and closely-related fluorescent pseudomonads as biocontrol agents of soil-borne phytopathogens. *Applied Microbiology*, **48**, 505–512.

Croft, B. J. and Braithwaite, K.S. (2006). Management of an incursion of sugarcane smut in Australia. *Australian Plant Pathology*, **35**,113-122.

Damle, N .R. and Kulkarni, S.W. (2012). Enzymatic potential of bacteria isolated from the rhizosphere of *Aegle marmelos* (Bael Tree). *World Journal of Environmental Biosciences,* **1**(2), 86-89.

De La Cruz-Quiroz, R., Chavez, C., Hernandez., Rodriguez, R., Hernandez, D., and Noe Aguilar, C. (2011). Antagonist capacity of Newly Isolated Strains of *Pseudomonas Fluorescens* against three important Phytopathogenic Bacteria. *American Journal of Agricultural and Biological Science*, **6**, 267-272.

Deshmukh, R.B., Dange, S.S., Jadhav, P.V., Deokule, S.S and Patil, N.A. (2013). Studies on the mycoflora in the rhizosphere of sugarcane (*Saccharum officinarum* L.). *International Journal of Bioassays*, **2**(4), 674-676.

Dua, S. and Sidhu, S. S. (2012). Effectiveness of rhizosphere bacteria for control of root rot disease and improving plant growth of wheat (*Triticum aestivum* L.). *Journal of Microbiology Research*, **2**(2), 26-35.

Dwivedi, S.K and Dwivedi, N. (2012). In- vitro bio-efficacy of some selected fungal antagonists against guava wilt pathogen. *IOSR Journal of Engineering,* **2**(5), 1217-1223.

Ellis, D., Davis, S., Alexious, H., Handke, R., and Bartley, R. (2007). *Description of medical fungi***.** (2nd ed.). Retrieved from http:// www.mycology.adelaide.edu.av. June 2013.

Emert, A.B. E. and Handelsman, J.O (1999). Biocontrol of plant disease: a (Gram-) positive perspective. *FEMS Microbiology Letters*, **171**(1), 1-9.

Fassatiova, O. (**1986***). Moulds and filamentous fungi in technical microbiology: Progress in industrial microbiology*: Vol. 22. New York: Elsevier Science, 1-233.

Food and Agriculture Organization of the United Nations (2008). Micro propagation for quality seed production in sugarcane in Asia and the Pacific. Retrieved from *http://apps.fao.org/*acessed and www.appari .org. May 2013.

Gaddeya, G., Niharika, S.P., Bharathi, P. and Kumar Ratna, P. K. (2012). Isolation and identification of soil mycoflora in different crop fields at Salur Mandal. *Advanced Applied Sciences Research*, **3**(4), 2020-2026.

Gawade, D.B., Pawar, B.H., Gawande S.J. and Vasekar, V.C. (2012). Antagonistic effect of *Trichoderma* against *Fusarium moniliformae* the causal of sugarcane wilt. *American-Eurasian Journal of Agriculture & Environmental Science*, **12** (9), 1236-1241.

Gillaspie, A. G., Mock, R. G., and Dean, J. L. (1983). Differentiation of *Sporisorium scitamineum* isolates in greenhouse tests. *Plant Disease*, **67**,373-375.

Gowsalya, A., Ponnusami, V., and Sugumaran, K.R. (2014). Isolation of bacteria from soil sample for exo- polysaccharide production. *International Journal of ChemTech Research*, **6**(5), 2925-2928.

Gravel, V., Martinez, C., Antoun, H. and Tweddell, R.J. (2005). Antagonist microorganisms with the ability to control Pythium damping-off of tomato seeds in rockwool. *Biological Control*, **50**,771–786.

Ha, T.N. (2010). Using *trichoderma* species for biological control of plant pathogens in Viet Nam. *Journal International Society of South East Asian Agricultural Sciences*, **16**(1), 17-21.

Heidi, I.G., and Abo-Elnaga (2006). *Bacillus subtilis* as a biocontrol agent for controlling sugar beet damping-off disease. *Egyptian Journal of Phytopathlogy*, **34**(1), 51-59.

Heydari, A. and Pessarakli, M. (2010). A review on biological control of fungal pathogens using microbial antagonists. *Biological Sciences.* **10** (4), 273-290.

Hoy, J.W. and Grisham, M.P. (1988). Spread and increase of sugarcane smut in Louisiana. *American Phytopathological Society*, **78**(10), 1371-1376.

Ibrahim, and Ramha, M.A. (2009). Isolation and identification of fungi associated with date fruits sold at Bayero University, Kano Nigeria. *Bayero Journal of Pure and Applied Sciences*, **2**(2), 127-130.

Izadi, M.B. and Jorf, M.A. (2007). Isolation and identification of yeast like and mycelial colonies of *Ustilago scitaminea* using specific primers**.** *Asian Journal of Plant Sciences*, **6**(7), 1131-1142.

Joshi, B.B., Bhatt, R.P. and Bahukhandi, D (2010). Antagonistic and plant growth activity of *Trichoderma* isolates of Western Himalayas. *Journal of Environmental Biology*, **31**, 921-928.

Jamoza, J., Kirungu, C., Amolo, R., Wanyama, P., Riungu, G and Wafubwa, J. (2013). Sugarcane growers guide. *Kenya Sugar Research Foundation*.3rd edition.pp. 26-27.

Kalaiselviselvaraj, S. and Panneerselvam, A. (2011). Invitro assessment of antagonistic activity of *Trichoderma* sp. against *Sarocladium oryzae* causing seath rot in paddy. *International Journal of Applied Biology and Pharmaceutical Technology* **2**: 179-183.

Kavitha, M., Sundar, R.A., Padmanaban, P., Viswanathan, R. and Malathi, p. (2014). Comparative study on early detection of sugarcane smut (*Sporisorium scitamineum*) by polymerase chain reaction (PCR) and microscopy. *African Journal of Biotechnology*, **13**(51), 4635-4638.

Kelechi, M.O and Chiaka, M. A. (2014). The rhizosphere effect on the bacterial genera associated with crude oil polluted soil ecosystem. *Current Research in Microbiology and Biotechnology,* **2**, (6), 495-500.

Kenya Sugar Industry strategic plan, (2014). pp. 1-5. *Retrieved from.* http://www.kenya sugar.co.ke / downloads / KSI % 20 strategic % 20plan. February 2015.

Kenya Sugar Research Foundation, (2005). Annual report. pp. 16-18.

Kimberly, C. and Elsa, B. (2003). Identification of bacterial species. *Tested studies for laboratory teaching,* **24**,103-130.

Kumalawati, Z., Musa, Y., Amin, N., Asrul, L. and Ridwan, I (2015). Exploration of arbuscular mycorrhizal fungi from sugarcane rhizosphere in South Sulawesi. *International Journal of Scientific & Technology Research,* **3**(1), 201-203.

Kumar, P.K.R., Hermanth, G., Niharika, P.S. and Kolli, S.K. (2015). Isolation and identification of soil mycoflora in agricultural fields at Tekkali Mandal in Srikakulam district. *International Journal of Advances in Pharmacy, Biology and Chemistry*, **4**(2), 484-490.

Lal, J. R., Sinha, O.K., Sanjay, B., Sunita, L. and Awasthi, S.K. (2009). Biological control of sugarcane smut (*Sporisorium scitamineum*) through botanicals and *Trichoderma viride. Sugar Tech*nology, **11**(4), 381-386.

Legaz, M.E., Santiago, R., Armas, R., Alarcon, B., Diaz, E., Sánchez-Elordi, M., Sacristán, B., Fontaniella, A. M., Millanes, M., Blancha, M. and Vicente, C. (2011). Molecular defence responses of sugarcane (*Saccharum officinarum* L.) to smut (*Sporisorium scitamineum* (Syd.) Piepenbr & Oberw. 2002. Spain – Formatex microbiology series. pp 1244-1250.

Magarey, R.C., Bull, J.I., Sheahan, T. and Denney, D. (2010).Yield losses caused by sugarcane smut in several crops in Queensland. *Proceedings of the Australian Society of Sugar Technologists*, **32,** 347-354**.**

Mahamuni, S. V. Wani, P. V. and Patil, A. S. (2012). Isolation of phosphate solubilizing fungi from rhizosphere of sugarcane & sugar beet Using Tcp & Rp Solubilization. *Asian Journal of Biochemical and Pharmaceutical Research*, **2**(1), 237-244.

Makut, M.D., and Owolewa, O.A (2011). Antibiotic producing fungi present in the soil environment of keffi metropolis, Nasarawa state of Nigeria. *Trakia Journal of Sciences*, **9**, (2), 33-39.

McNear Jr, D. H. (2013). The rhizosphere - roots, soil and everything In between. *Nature Education Knowledge*, **4**(3), 1 – 3.

Meena, B. and Ramyabharathi, S.A. (2012). Effect of fungicides and biocontrol agents in the management of sugarcane smut disease. *Journal of Today's Biological Sciences*: *Research and Review*, **1**(1), 96-103.

Morang, P., Dutta, B.K., Dileep Kumar, B.S and Kashyap, M.P. (2012). Growth promotion and bi-control approaches of brown root rot disease of tea by *Pseudomonas Aeruginosa* (PM 105). *Journal of Plant Pathology and Microbiology*, **3**(5), 1 - 4.

Mukerji, K.G., Manoharachary, C. and Singh, J. (2006). Microbial activity in the rhizosphere. Rhizosphere biology overview. *Soil Biology*, **7**, 1- 6.

Nandhini, B. and Josephine, M. R. (2013). Study of bacterial and fungal diversity in potted soil**.** *International Journal of Current Microbiology and Applied Sciences***, 2***(*2), 1-5.

Nannipieri, P., Ascher J., Ceccherini, M.T., Landi, L., Pietramellara G., Renella, G. and Valori F. (2007). Microbial diversity and microbial activity in the rhizosphere. *Cienciadel. Suelo (ARGENTINA*) **25**(1), 89-97.

Nasiru, I. and Ifenkwe, O.P. (2004). Screening of bi-parental and mutant clones of Sugarcane (*Saccharum officinarum* L.) for resistance to smut disease. *Tropicultura***, 22(**4), 173-175.

Nekade, D.B. (2013). Bacterial diversity in sugarcane (*Saccharum officinarum* L.) rhizosphere of saline soil. *International Research Journal of Biological Sciences*, **2**(2), 60-64.

Nicoletti, R. and De Stefano, M. (2012). *Penicillium restrictum* as an antagonist of plant pathogenic fungi. *Dynamic Biochemistry, Process Biotechnology and Molecular Biology*, **6**, 61-69.

Nihorimbere, V., Ongena, M., Smargiassi, M and Thonart, P. (2011). Beneficial effect of the rhizosphere microbial community for plant growth and health. *Biotechnology Agrononomy Society and Environment.* , **15**(2), 327-337.

Nzioki, H .S., Jamoza, J. E., Olweny C.O and Rono, J.K. (2010). Characterization of physiologic races of sugarcane smut (*Sporisorium scitamineum*) in Kenya. *African Journal of Microbiology Research*, ***4***(16), 1694-1697.

Nzioki, H.S. and Jamoza, J. E. (2006). Assessment of yield losses due to sugarcane smut (*Sporisorium scitamineum*) infection in Kenya. *Kenya Sugar Research Foundation, Technical Bulletin* **1**(1), 1 – 9.

Nzioki, H.S. and Jamoza, J.E. (2009). Assessment of yield losses due to sugarcane smut (*Sporisorium scitamineum*) infection in ratoon crops of sugarcane in Kenya. , *Kenya Sugar Research Foundation Technical Bulletin* **3**(1), 1 – 9.

Olweny, C.O., Ngugi, K., Nzioki, H. and Githiri. , S.M. (2008). Evaluation of smut inoculation techniques in sugarcane seedlings. *Sugar Technology*, **10**,341-345**.**

Ong'ala, J., Mulianga, B., Wawire, N., Riungu, G. and Mwanga, D. (2015). Determination of sugarcane smut prevalence in the Kenya sugar industry. *International Journal of Agriculture Innovations and Research*, **4**(1), 153- 157.

Osoro M. O. (1997). Sugar. Review of Kenya Agricultural Research (KARI), ***18,*** 1-45

Pal, K. K. and McSpadden G.B. (2006). Biological control of plant pathogens. *The Plant Health Instructor*, pp 1-25. doi: 10.1094/PHI-A-1117-02.

Pankhurst, C.E., Hawke, B.G., Holt, J.A., Magarey, R.C. and Garside, A.L. (2000). Effect of rotation break on the diversity of bacteria in the rhizophere of sugarcane and its potential impact on yield decline. *Proceeds of the Australian Society of sugarcane Technologists*, **22**, 77- 83.

Pandey, A. and Palni, S. L. (2007). The rhizosphere effect in trees of the Indian central Himalaya with special reference to altitude**.** *Applied Ecology and Environmental Research*, **5**(1), 93-102.

Paramdeep, S., **Bipen, K., Meeta, M.J. and Ritu, R. (2014).** Management of sugarcane smut *Sporisorium scitamineum* with fungicides and bioagents. *African Journal of Microbiology and Research,* **8**(51), 3954-3959.

Pisa, G., Magnani, G.S., Weber, H., Souza, E.M., Faoro, H., Monterio R.A., … Cruz, L.M. (2011). Diversity of *16S rRNA* genes from bacteria of sugarcane rhizosphere soil. *Brazilian Journal of Medical and Biological Research*, **44**, 1215-1221.

Prajapati, V.P. Joshi, D.M. Gajre, N.K.A. and Kansara, S.S. (2012). Antagonism of some well-known bioagents against *curvularia eragrostidis* an incitant of spider lily leaf tip blight. *The Bioscan*, **7**(4), 701-703.

Prashar, P., Kapoor, N., and Sachdeva, S. (2012). Structural and functional diversity of rhizobacteria of pearl millet in semi-arid agro climatic zone. *Asian Journal of Plant Science and Research,* **2** (5), 599-606.

Prince, L., Raja, A. and Prabakaran, P. (2011). Antagonistic potentiality of some soil mycoflora against *Colletotrichum falcatum*. *World Journal of Science and Technology*, **1**(4), 39-42.

Que, Y., Xu, J., Lin, J., Chen R. and Grisham, M.P. (2012). Molecular variation of *Sporisorium scitamineum* in mainland china revealed by RAPD and SRAP markers**.** *Plant Diseases***, 96,** 1519-1525**.**

Que, Y., Xu, L., Wu, Q., Liu, Y., Ling, H., Zhang, Y… and Zhang, C. (2014). Genome sequencing of *Sporisorium scitamineum* provides insights into the pathogenic mechanisms of sugarcane smut. *Biomedical Central Genomics*, **15,** 1-19.

Rajasankar, R. and Ramalingam, C. (2012). In vitro screening of *Saccharum officinarum* L. rhizophere bacteria for plant growth promotion traits. *International Journal of Advanced Life Sciences*, **2**, 40-49.

Reddy, K.R.N., Choudary, K.A. and Reddy, M. S. (2007). Antifungal metabolites of *Pseudomonas fluorescens* isolated from rhizosphere of rice crop. *Journal of Mycology and Plant Pathology* **3** (2), 1 - 5.

Reynolds, J. (2011). Counting bacteria. *Biology 2420 lab manual*. pp. 1-2. *Retrieved from.* http://www.//delrio.dcccd.edu/jreynolds/microbiology/RLC microindex.html. August 2013.

Rocha, U.N., Leonard Simon van Overbeek, S.L. and Elsas, D.J. (2009). Exploration of hitherto-uncultured bacteria from the rhizosphere. FEMS Microbiology Ecology, **69,** 313-328.

Ru, Z. and Di, W. (2012). *Trichoderma* spp. from rhizosphere soil and their antagonism against *Fusarium sambucinum***.** *African Journal of Biotechnology*, **11**(18), 4180-4186.

Selvaraj, K. S., and Panneerselvam, A. (2011). Invitro assessment of antagonistic activity of *trichoderma* sp.against *Sarocladium oryzae* causing sheath rot disease in paddy. *International Journal of Applied Biology and Pharmaceutical Technology*, **2**, (1) 179-183.

Schenck, S. (2003). New race of sugarcane smut on Maui. *Hawaii Agriculture Research Center Pathology Report* **69**, 1 – 4.

Shen, W., Xi P., Li, M. Liu, R., Sun, L. Jiang, Z. and Zhang, L. (2012). Genetic diversity of *Sporisorium scitamineum* Syd. in Southern China revealed by combined ISSR and RAPD analysis. *African Journal of Biotechnology*, **11**(54), 11693-11703.

Shiny, N.P., Gaddeyya, G., and Ratna, K. P. K. (2013). An investigation on soil mycoflora of different crop fields at Narasannapeta Mandal, Srikakulam District. *International Research Journal of Environment Sciences*, **2**(9), 38-44.

Sood, A., Sharma, S., Kumar, V and Thakur, R.L. (2007). Antagonism of dominant bacteria in tea rhizosphere of Indian Himalayan region. *Journal of Applied Science sand Environmental Management*, **11**(4), 63-66.

Subash, N., Meenakshisundaram, M., Unnamalai, N and Sasikumar, C. (2013). Effect of *Trichoderma herzanium* to control damping off disease and growth promotion in chilli (*capsicum annuum). International Journal of Pharma and Bio Sciences,* **4**(2), 1076 – 1082.

Sule, I. O. and Oyeyiola, G. P. (2012). Fungi in the Rhizosphere and Rhizoplane of Cassava cultivar TME 419. *International Journal of Applied Biological Research,* **4**(1&2):18 – 30.

Sundar, R. A., Barnabas, L. E., Malathi, P. and Viswanathan, R. (2012). A Mini-review on smut disease of sugarcane caused by *Sporisorium scitamineum. Retrieved from.* http://www.intechopen.com/books/botany. August 2013.

Suprapta, D. N. (2012). Potential of microbial antagonists as biocontrol agents against plant fungal pathogens. *Journal of International Society of South East Asian Agricultural Science*. **18** (2), 1-8.

Tailor, J. A. and Joshi, H. B. (2012). Characterization and optimization of siderophore production from *pseudomonas fluorescens* strain isolated from sugarcane rhizosphere. *Journal of Environmental Research and Development,* **6** (3), 688-694.

Tamilarasi, S., Nanthakumar, K., Karthikeyan, K and Lakshamanaperumalsami (2008). Diversity of root associate microorganisms of selected medicinal plants and influence of rhizomicroorganisms on the antimicrobial property of *Coriandum savitum.Journal of Environmental Biology*, **29**(1), 127- 134.

Tshikudo, p., Nnzeru, R., Ntushelo, K. and Mudau, F. (2013). Bacterial species identification getting easier. *African Journal of Biotechnology*, **12(**41), 5975-5982**.**

Usha, E.1., Reddy, S.A., Suba, G.A., Radha, M. and Kale, D. (2012). *In vitro* control of Fusarium *oxysporum* by *Aspergillu*s sp. and *Trichoderma* sp. isolated from vermicompost. *Journal of Bio Innovation*, **1**(5), 142-147.

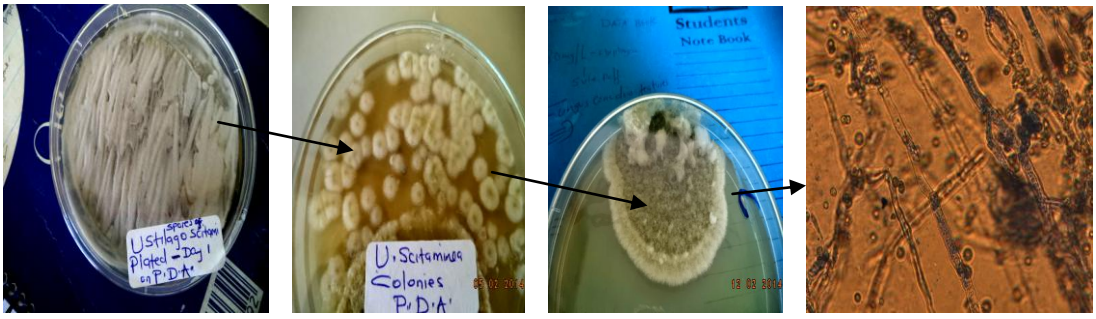
Viswanathan, R., Sundar, A.R., Malathi, P. and Padmanban, P. (2009). Sugarcane smut. *Sugarcane Breeding Institute, Indian Council of Agricultural Research Coimbatore*, Extension publication, 179.

Williams, J. (2001). Simplified fungi identification key. *The University of Georgia Co-operative Extension Service*, **37**, (1- 11).

Appendices

Appendix 1: smut whip on sugarcane variety CO 421

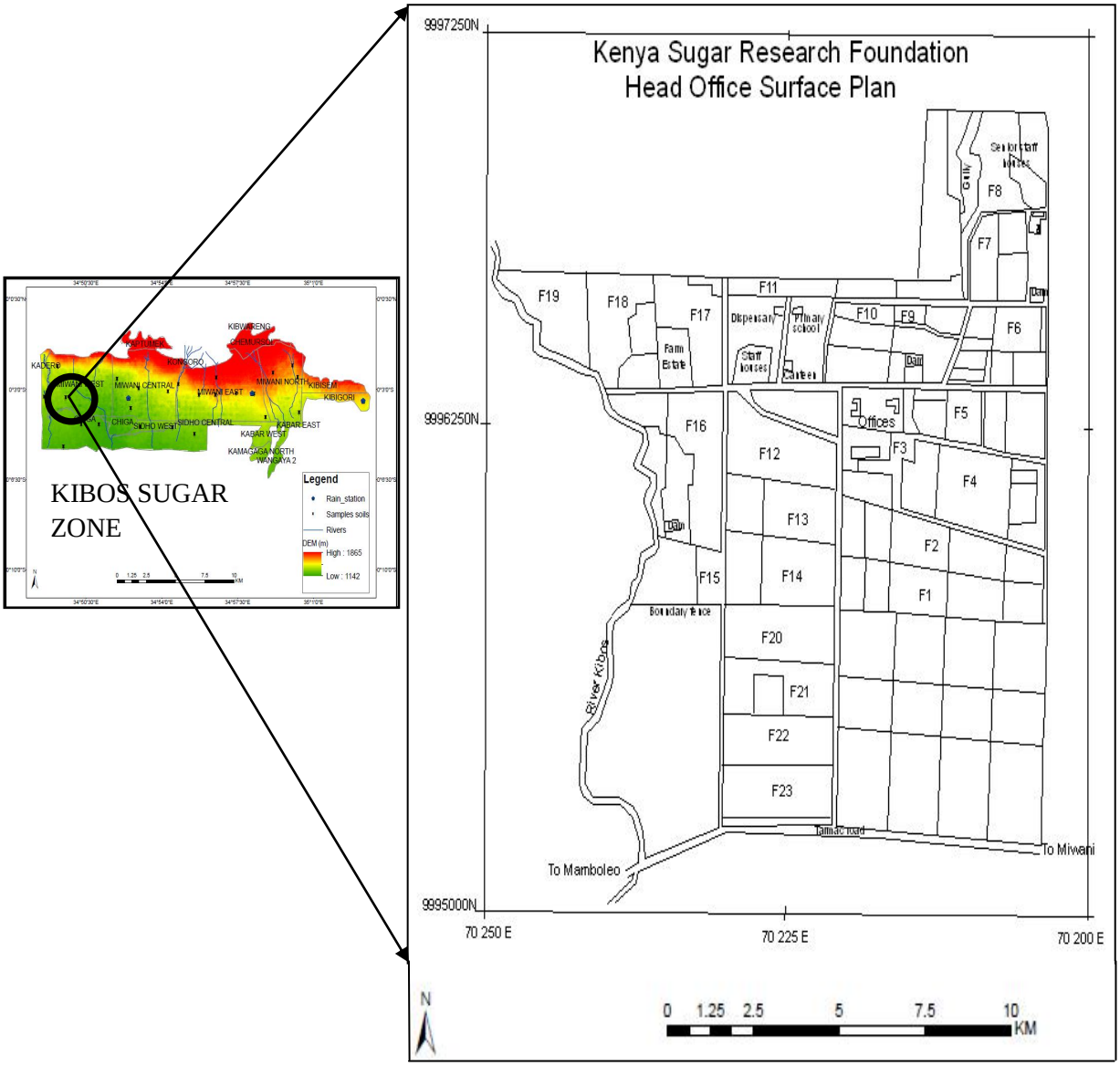
Appendix 2: *Sporosorium scitamineum* pathogen colony isolation procedure from viable spores on PDA medium.



a **b** **c** **d**

Spores after streaking on PDA medium (2a), round pure culture colonies after 3 days (2b), single pure colony after 7 days, greyish and greenish towards the centre with white floccose and erected mycelia (2c) and ×400 microscopic image of mycelia tip showing septate hyphae and globose conidia (2d).

Appendix 3: Map of kibos sugar zone and the sugarcane fields



KESREF GIS UNIT

F1- F26 – SUGARCANE FIELDS

Appendix 4: Long term weather data of Kibos area

Months / Weather parameter	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Total	Mean
Rainfall mm	97.5	85.5	155.0	207.1	107.0	67.2	74.5	112.7	111.4	101.7	138.3	106.1	1364	113.67
Evaporation (mm)	179.8	182.0	176.7	141.0	139.5	120.0	130.2	139.5	141.0	148.8	129.0	155.0	1782.5	148.54
Moisture deficit(mm)	-82.3	-96.5	-21.7	66.1	-32.5	-52.8	-55.7	-26.8	-29.6	-47.1	9.3	-48.9	-418.5	-34.88
Radiation MJ/m^2/month	858.7	910.0	880.4	852.0	756.4	720.0	747.1	771.9	807.0	864.9	801.0	871.1	9840.5	820.04
Sunshine hrs/month	269.7	257.6	226.3	201.0	145.7	183.0	204.6	220.1	195.0	201.5	195.0	241.8	2541.3	211.78
Temp max o C	31.6	30.2	28.2	26.8	28.2	27.4	25.8	25.5	26.6	27.3	27.0	27.1	331.7	27.642
Temp min o C	15.0	16.5	15.9	16.1	17.0	14.1	14.6	14.6	14.7	15.2	15.2	15.3	184.2	15.35
RH % 0900	65.7	60	72.9	74.2	77.4	75.7	64	64.1	58.6	58	68.1	66.3	80.5	67.0
RH % 1500	41.9	34.5	43.5	50.7	50.4	53.5	42.0	40.1	40.6	40.0	47.7	44.7	529.6	44.133

Appendix 5: pH (water method) of soil samples

SAMPLE	FIELD	PH 1	PH2	AVERAGE
1	12	5.82	5.79	5.81
2	24	5.40	5.46	5.43
3	17	6.26	6.22	6.24
4	10	5.69	5.67	5.68
5	7	5.25	5.26	5.25
6	6	5.68	5.62	5.65
7	4	6.10	5.80	5.95
8	25	5.38	5.37	5.38
9	23	5.97	5.94	5.81
10	1	5.62	5.65	5.64

Appendix 6: Phase contrast microscope: Model: Carl zeiss

Appendix 7: Microplot (a) and green house (b) layout

a.

b.

Appendix 8: Percentage frequency of occurrence of fungal isolates in the rhizosphere soil samples.

Site/field Isolate (AJF)	1	2	3	4	5	6	7	8	9	10	11	sites present	% Frequency
1. *Penicillium* sp.	+	+	+	+	+	+	-	+	+	-	-	08	73
2. *Rhizopus* sp.	+	+	+	+	+	+	+	+	+	+	+	11	100
3.*Trichorderma* sp	+	+	+	+	+	+	+	+	+	-	-	09	82
4. *Aspergillus aureus*	+	+	+	+	+	+	+	+	+	+	+	11	100
5. Unidentified	+	+	+	+	+	+	+	+	+	+	-	10	91
6. *Trichorderma* sp.	+	+	+	+	+	+	+	+	+	+	-	10	91
7. *Trichorderma viride*	+	+	+	+	+	+	+	+	+	+	-	10	91
8. *Trichorderma herzanium*	+	+	+	+	+	+	+	+	+	+	+	11	100
9. Unidentified	+	+	+	+	+	+	+	+	+	+	-	10	91
10 *Trichorderma* sp.	+	+	-	+	+	+	+	-	-	-	-	06	55
11 *Aspergillus niger*	+	+	+	+	+	+	+	+	+	+	+	11	100
12 Unidentified	+	+	-	-	+	+	+	-	+	+	+	08	73
13 *Alternaria* sp.	+	+	+	+	+	+	+	+	+	+	+	11	100
14 Unidentified	-	-	+	-	-	+	-	-	+	-	-	03	27
15 Unidentified	-	-	+	-	-	-	-	-	-	-	-	01	09
16 *Aspergillus flavus*	-	-	+	+	-	+	-	+	-	-	-	04	36

Key

\+ Present - Absent

Appendix 9: Percentage frequency of occurrence of bacterial isolates in the rhizosphere soil samples.

SITE/FIELD ISOLATE AJB	1	2	3	4	5	6	7	8	9	10	11	NO. of sites present	% Frequency
1.Unidentified	+	+	+	+	+	+	+	+	+	+	+	11	100
2.Unidentified	+	+	+	+	+	+	+	+	+	+	+	11	100
3. *Bacillus* sp	-	+	+	+	+	+	+	+	+	-	+	09	82
4. *Pseudomonas* sp	+	+	+	+	+	+	+	+	+	+	+	11	100
5. *Pseudomonas* sp	-	+	+	+	+	+	+	+	+	+	+	10	91
6. *Pseudomonas* sp	+	+	+	+	+	+	+	-	+	+	+	10	91
7. *Azobacter*	+	+	+	+	+	+	+	+	+	+	+	11	100
8.Unidentified	+	+	+	+	+	+	+	+	+	+	+	11	100
9. Unidentified	+	+	-	+	+	+	+	+	+	+	+	10	91
10. Unidentified	+	+	-	-	+	-	+	+	-	-	-	05	55
11. Unidentified	-	-	+	-	+	+	-	+	+	+	-	06	45
12. *Bacillus*	-	-	-	+	+	-	+	-	-	+	-	04	36

Key

\+ Present - Absent

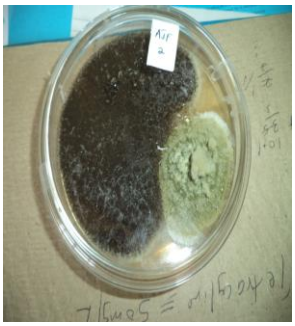
Appendix 10: Analysis Of Variance tables on sugarcane variety CO 421 rhizosphere microbial population, inhibition of pathogen *Sporisorium scitamineum* mycelia growth and smut disease incidence**.**

Parameter	**Source**	**DF**	**SS**	**MS**	**F**	**Pr > F**
Effect of field on rhizosphere fungal population	Fields Error Total	9 20 29	3.42×10^{9} 1.05×10^{9} 4.47×10^{9}	3.80×10^{8} 5.25×10^{7}	7.24	**0.0001**
Effect of field on rhizosphere bacterial population	Fields Error Total	9 20 29	4.44×10^{14} 1.61×10^{14} 6.05×10^{14}	4.94×10^{13} 8.08×10^{12}	6.11	**0.0004**
Effect of fungal test antagonists on Pathogen colony radial growth	Fungal antagonists Error Total	16 34 50	1756.31 100.67 1856.98	109.77 2.96	37.07	< **0.0001**
Effect of fungal test antagonists on pathogen growth percentage	Fungal antagonists Error Total	16 34 50	16727.92 1076.00 17803.92	1045.49 31.65	33.04	< **0.0001**
Effect of bacterial test antagonists on Pathogen colony radial growth	Bacterial antagonists Error Total	12 26 38	1372.77 78.67 1451.44	114.39 3.03	37.81	< **0.0001**
Effect of antagonists on the number of whips in green house	Antagonists Error Total	4 20 24	24.4 83.6 108.0	6.1 4.18	1.46	**0.2517**

Parameter	Source	DF	SS	MS	F	Pr > F
Effect of antagonists on the number of infected plants in green house	Antagonists Error Total	4 20 24	0.56 9.6 10.16	0.14 0.48	0.29	**0.879**
Effect of antagonists on percentage infection in green house	Antagonists Error Total	4 20 24	955.112 13205.788 14160.9	238.778 660.289	0.36	**0.8329**
Effect of antagonists on the number of whips in the field	Antagonists Error Total	4 20 24	16.64 532.4 549.04	4.16 26.62	0.16	**0.9579**
Effect of antagonists on the number of infected plants in the field	Antagonists Error Total	4 20 24	0.56 7.2 7.76	0.14 0.36	0.39	**0.8141**
Effect of antagonists on percentage infection in the field	Antagonists Error Total	4 20 24	2825.4424 14591.692 17417.1344	706.36 729.58	0.97	**0.4466**

Appendix 11: Dual culture plates of fungal isolates and *Sporisorium scitamineum* on PDA medium.

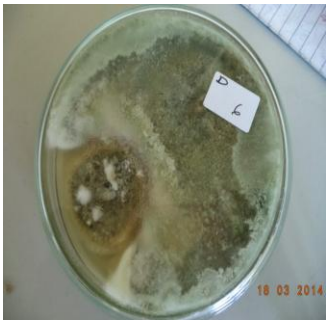
a



b

c

d



e

f

g

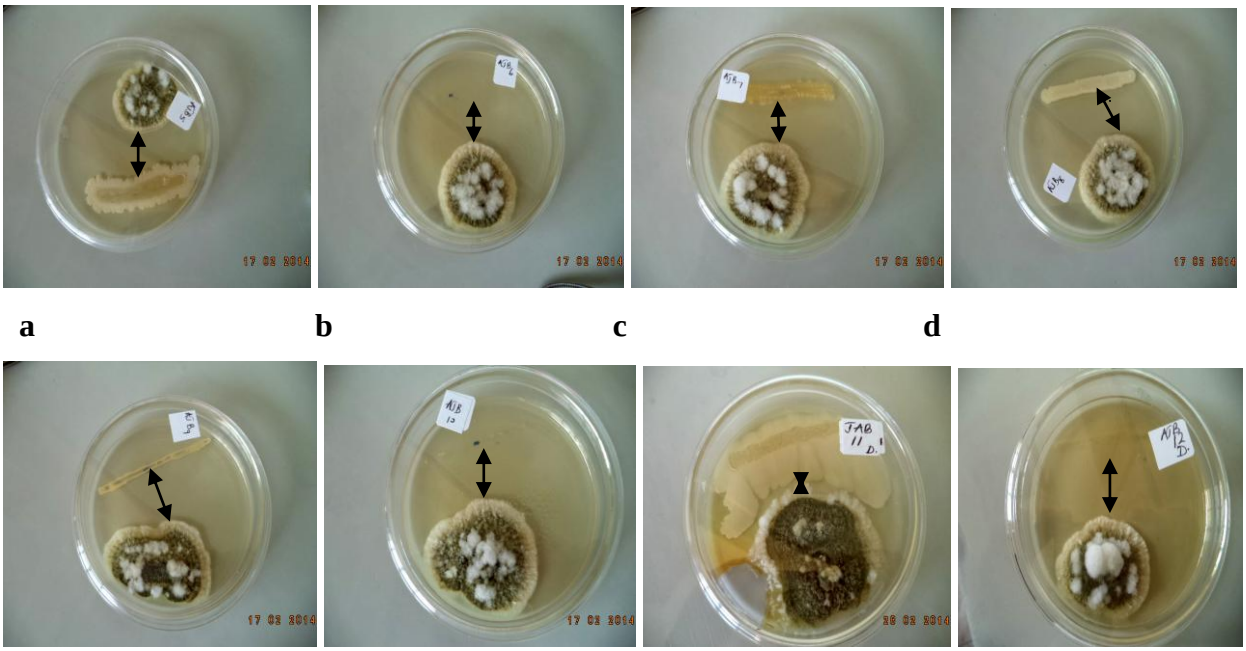
h

i

j

Inhibition of pathogen *Sporisorium scitamineum* radial growth in dual culture plates by isolates AJF 1 (a), AJF 2 (b), AJF 4 (c) ,AJF 5 (d), AJF 6 (e), AJF11(f), AJF 12(g) AJF 13(h), AJF 14(14) and AJF 15(J) on PDA medium at day seven of incubation.

Appendix 12: Dual culture plates of bacterial isolates and *Sporisorium scitamineum* on PDA medium.

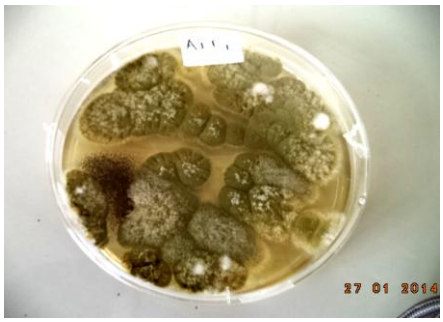


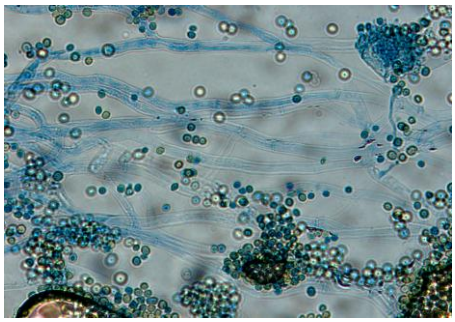
a **b** **c** **d**

e **f** **g** **h**

Inhibition zones between the pathogen and bacterial test antagonist AJB5(a), AJB 6 (b), AJB 7 (c), AJB 8 (d), AJB9(e), AJB 10(e),AJB 11(f) and AJB 12(h) in dual culture plate on solid PDA medium after seven days of incubation.

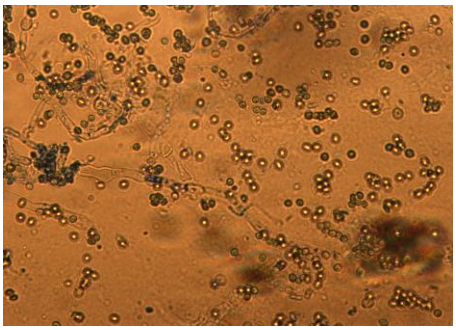
Appendix 13: Pure fungal isolates on PDA medium at day 7 and mycelia tip mg. ×400 showing morphological characteristics.





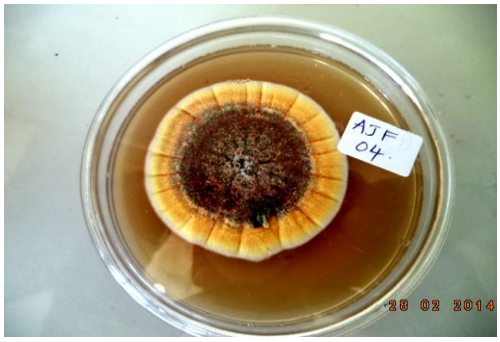
a **b**

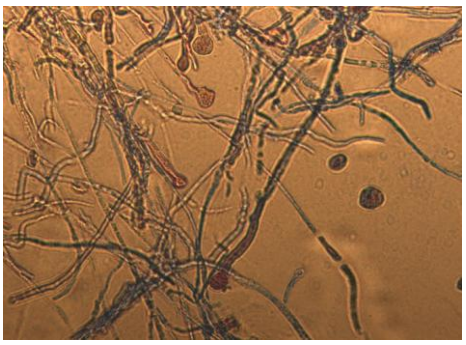
Pure fungal isolate AJF1 on PDA medium at day 7 (a) and the mycelia tip mg. ×400 (b).



c **d**

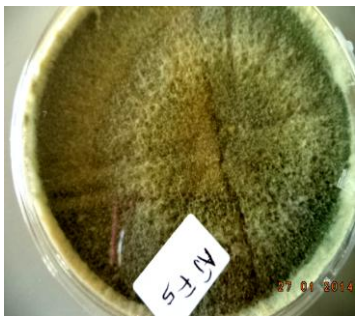
Pure fungal isolate AJF3 on PDA medium at day 7 (c) and the mycelia tip mg. ×400 (d).

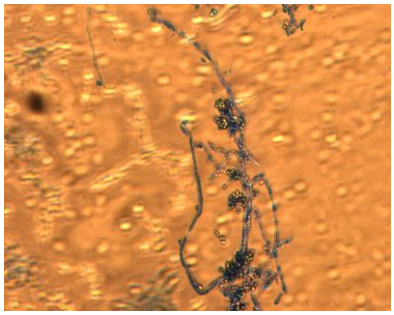




e **f**

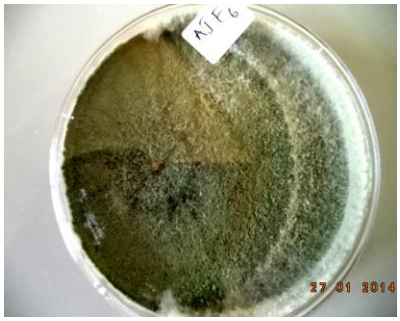
Pure fungal isolate AJF4 on PDA medium at day 7 (e) and the mycelia tip mg. ×400 (f).

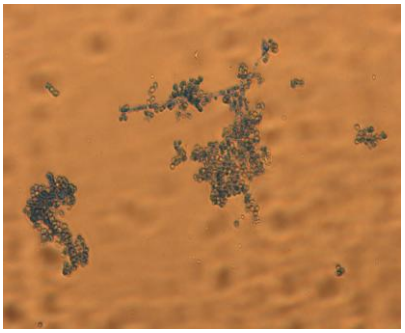




g **h**

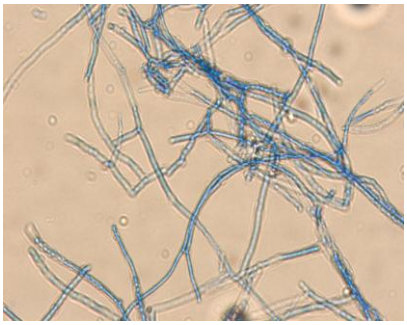
Pure fungal isolate AJF5 on PDA medium at day 7 (g) and the mycelia tip mg. ×400 (h).





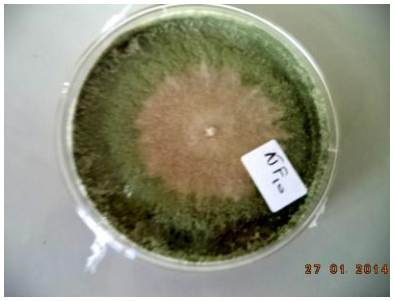
i **j**

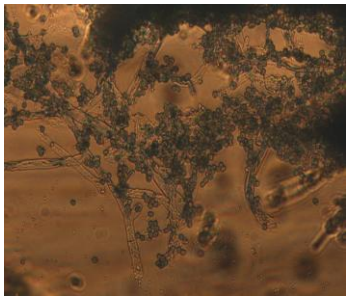
Pure fungal isolate AJF6 on PDA medium at day 7 (i) and the mycelia tip mg. ×400 (j)



k **l**

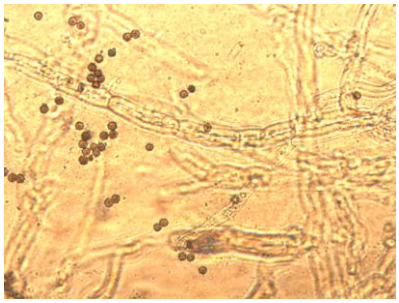
Pure fungal isolate AJF9 on PDA medium at day 7 (k)) and the mycelia mg. ×400 (l).





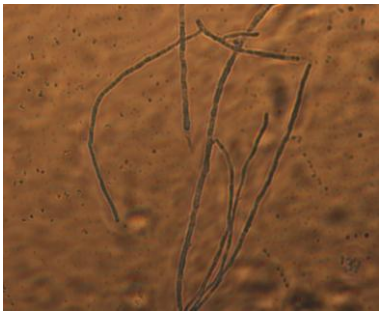
m **n**

Pure fungal isolate AJF10 on PDA medium at day 7(m) and the mycelia tip mg. ×400 (n)



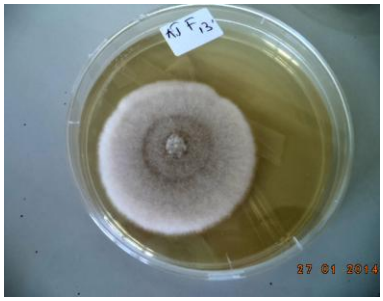
o **p**

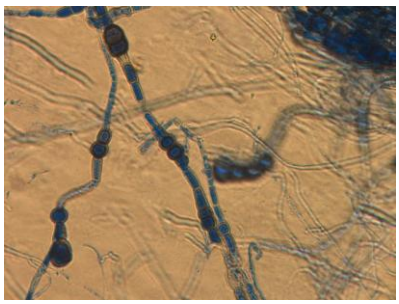
Pure fungal isolate AJF11 on PDA medium at day 7 (o) and the mycelia tip mg. ×400(p)



q **r**

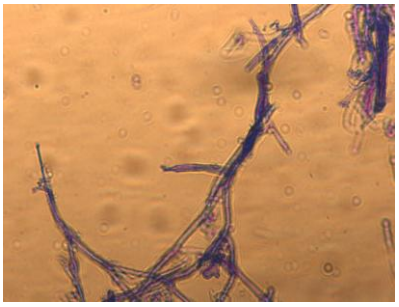
Pure fungal isolate AJF12 on PDA medium at day 7(q) and the mycelia tip mg. ×400 (r)





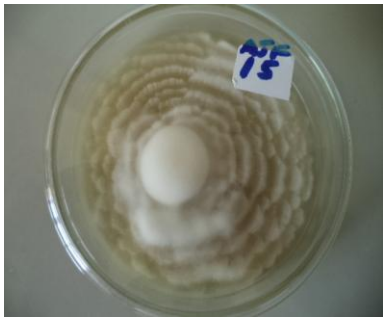
s **t**

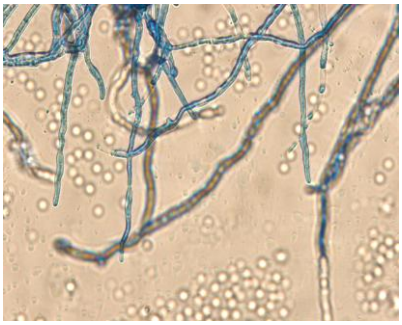
Pure fungal isolate AJF13 on PDA medium at day 7(s) and the mycelia tip mg. ×400 (t)



u **v**

Pure fungal isolate AJF14 on PDA medium at day 7(u) and the mycelia tip mg. ×400(v).

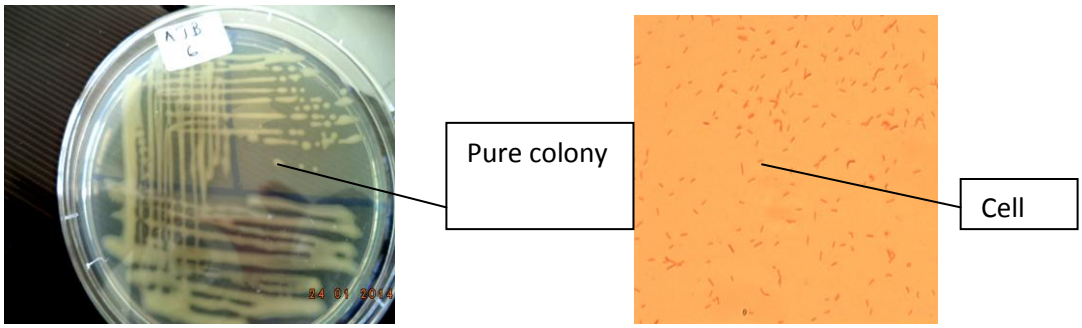




w **x**

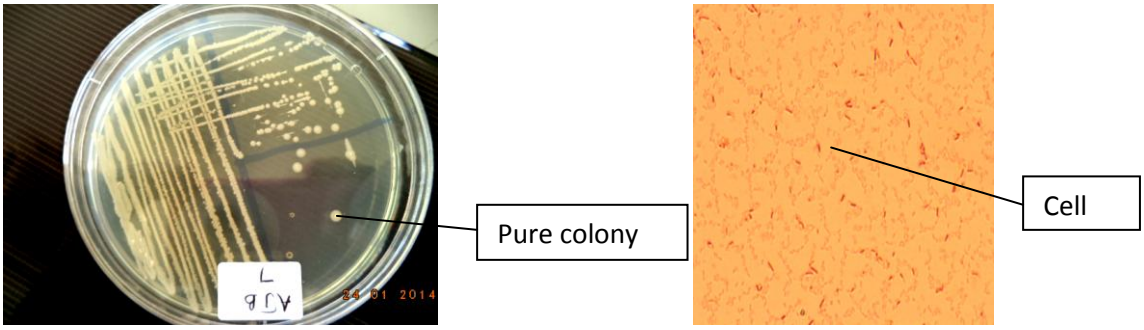
Pure fungal isolate AJF15 on PDA medium at day 7(w) and the mycelia tip mg. ×400 (x)

Appendix 14: Pure bacterial isolates on NA medium and the cells mg. ×1000 showing morphological characteristics.



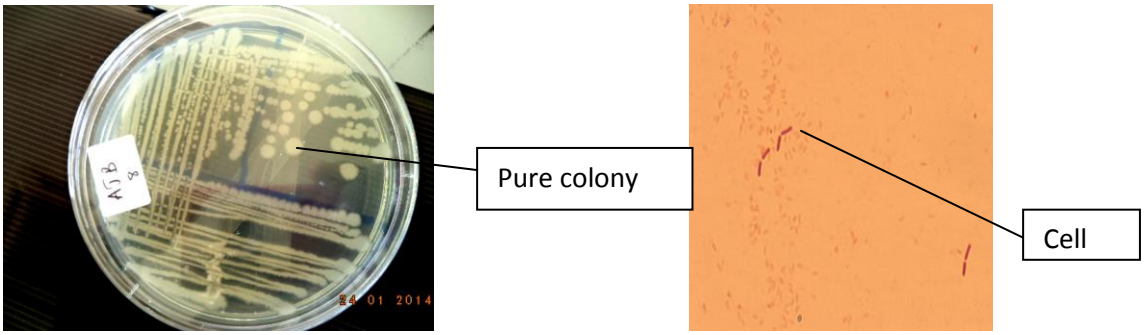
a **b**

Pure bacterial isolate AJB6 on NA medium (a) and the cells mg ×1000 (b).



c **d**

Pure bacterial isolate AJB7 on NA medium (c) and the cells mg ×1000 (d).



e **f**

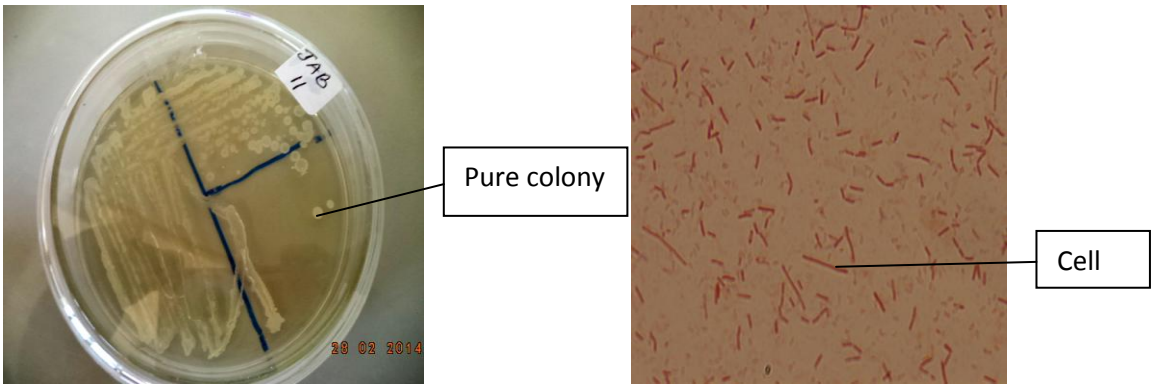
Pure bacterial isolate AJB8 on NA medium (e) and the cells mg ×1000 (f).

g **h**

Pure bacterial isolate AJB9 on NA medium (g) and the cells mg ×1000 (h).

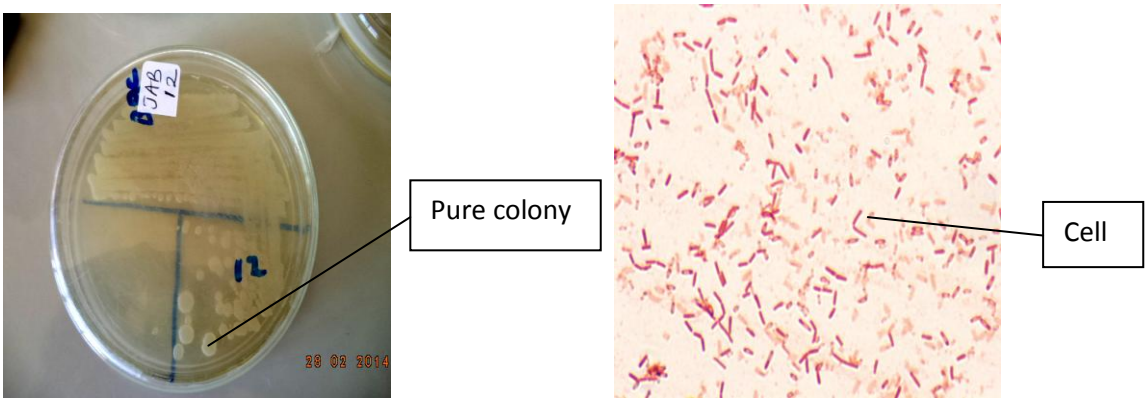
i **j**

Pure bacterial isolate AJB10 on NA medium (i) and the cells mg ×1000 (j).



k **l**

Pure bacterial isolate AJB11 on NA medium (k) and the cells mg ×1000 (l).



m **n**

Pure bacterial isolate AJB12 on NA medium (m) and the cells mg ×1000 (n).