MOLECULAR CHARACTERIZATION AND SCREENING OF SELECTED ANTAGONISTIC MICROORGANISMS AGAINST MAJOR MAIZE AND BEAN PATHOGENS

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A Thesis Submitted to the Graduate School in Partial Fulfillment for the Requirements of the Award of the Master of Science Degree in Plant Pathology of Egerton University

EGERTON UNIVERSITY

MAY, 2016

DECLARATION AND RECOMMENDATION

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This thesis is my original work and has not been previously presented in this University or any other institution for the award of any degree.

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DEDICATION

I wish to dedicate this work to my family: Mr. and Mrs. Hassan Kimandi and my siblings Lwambi, Halima and Omar for their moral and financial support throughout my studies.

ACKNOWLEDGEMENT

First, I would like to acknowledge the almighty God for his enormous blessings showered upon me and the guidance he has constantly given me. Secondly, I would like to acknowledge Egerton University for the opportunity granted to undertake my studies. I wish to express my sincere gratitude and acknowledge my supervisors: Prof I. N. Wagara and Prof J. C. Mataysoh for their insights, valuable guidance and facilitation in this research project. My special thanks to the Helmholtz Zentrum Für Infektionforschung (HZI) in Braunschweig, Germany for allowing me to use their laboratories for my molecular research work.

My deepest gratitude to Prof. Dr. Marc Stadler, head of Microbial Drugs Department and the entire MWIS group of HZI institution for the good working relationship throughout my stay at the institute.

Lastly, I wish to appreciate the technical assistance offered by technicians and colleagues at the Department of Biological Sciences especially Mr Francis Ngumbu, Mr Anderson Mwangi, Mr Geoffry Langat, Mrs Cynthia Adongo, Ms Fauzia Musasyia, Ms Velma Nasimiyu, Mr Silvance Ochola.

ABSTRACT

Various plant diseases (*Fusarium* ear rots, bean anthracnose and damping off of seedlings) caused by phytopathogenic agents are known to cause considerable crop losses to agricultural yield all over the world. The use of antagonistic microorganisms in controlling maize and bean pathogens is an area of great potential in order to increase yield, thereof mitigate food insecurity. The aim of this study was to characterize and screen selected tropical fungi and bacteria for antimicrobial activity against maize pathogens (*Fusarium moniliforme, Fusarium graminearum*) and bean pathogens (*Colletotrichum lindemuthianum, Pythium ultimum*). A total of 87 tropical fungi were collected from Kakamega tropical rain forest. Two bacterial isolates; B1 and B5 (which had already been identified as *Bacillus amyloliquefaciens*), were obtained from groundnuts and mushroom respectively. Isolation and culturing of the microorganisms was done in different media of varying pH. Extraction, amplification and purification of the tropical fungi deoxyribonucleic acid-DNA, was done using BIO BASIC EZ-10 spin column DNA mini-prep kit. Molecular characterization was done through sequencing using the internal transcribed spacer region and ß-tubulin primers. Preliminary screening of the tropical fungi against test organism; *Bacillus subtilis* and *Mucor plumbeus* was done. Fermentation and extraction of secondary metabolites was also done on the active tropical fungi isolates. Anti-microbial activity of the selected tropical fungi and bacteria against the four phytopathogens was studied *in vitro* using the dual culture assay and the resulting inhibition zones analyzed using analysis of variance. A total of 64 tropical fungi were isolated into pure and axenic cultures. The cultures grown on potato dextrose agar at pH 5.6 ± 0.2 showed better mycelial growth compared to Mueller Hinton agar, Sabouraud Dextrose Agar and Yeast Malt Glucose agar. Eight tropical fungi were active against *B. subtilis* and two were active against *M. plumbeus.* Majority of the isolates were from the genus *Xylaria* (10), *Psathyrella* (8) and *Fusarium* (7). F. *solani* had the highest inhibition of 64% while *Phaeomarasmius* sp. had the lowest inhibition of 19.1% both against *F. moniliforme*. *Epicoccum* sp. inhibited the mycelial growth of *P. ultimum* by 38% and also inhibited *C. lindemuthianum* by 58%. None of the fungal antagonists inhibited the mycelial growth of *F. graminearum*. *Bacillus amyloliquefaciens* (B1 and B5) suppressed the mycelial growth of the four phytopathogens. The results of this study indicate the potential of antagonistic tropical fungi and bacteria as possible biocontrol agents against maize and bean fungal pathogens.

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LIST OF ABBREVIATIONS/ACRONYMS

ANOVA	Analysis of Variance
BCAs	Biological Control Agents
BLAST	Basic Local Alignment Search Tool
CRD	Complete Randomized Design
EPA	Environmental Protection Agency
FAO	Food and Agriculture Organization
GLS	Grey leaf spot
HPLC	High Performance Liquid Chromatography
ITS	Internal Transcribed Spacer
LSD	Least Significant Difference
MHA	Mueller Hinton Agar
MLND	Maize Lethal Necrosis Disease
MLNV	Maize Lethal Necrosis Virus
MoA	Ministry of Agriculture
Nr- DNA	Nuclear ribosomal Deoxyribonucleic acid
PDA	Potato Dextrose Agar
r- RNA	Ribosomal Ribonucleic Acid
rcf	Relative centrifugal force
SDA	Sabouraud Dextrose Agar
TF	Tropical fungi
YMG/YM	Yeast Malt Glucose
ZM	Zucker Malt

CHAPTER ONE
INTRODUCTION

1.1 Background information

Maize and beans have been rated the main staple food crops in sub-Saharan countries (Byerlee, 1994; Smale and Jayne, 2003) with thousands of metric tonnes in output per annum. In Kenya, maize (*Zea mays*) is a principal staple food and a cash crop, with beans coming in second as a major food crop. Both maize and bean crops play an important role in the diets of millions of people worldwide due to their nutritional value. Maize provides carbohydrates and according to the Food and Agriculture Organization (FAO) statistics (2005-2007), maize contributes about 68% of daily per capita cereal consumption, 35% of total dietary energy consumption and 32% of total protein consumption. Bean on the other hand is an important source of human dietary proteins and the third most important source of calories (Sarikamis *et al*., 2009).

Maize occupies 75% or more of the production area in Kenya and the average consumption of maize per person as a food is over 94 kg/yr (Smale and Jyne, 2003). The total annual production of common beans (*Phaseolus vulgaris* L.) in Kenya, according to FAOSTAT (2012) is estimated at 613902 tons of dry seeds and covering an area of 5797.4 hectares. Trends in population growth in Kenya show that the demand for maize and beans is expected to continue increasing at 3–4% annually, supporting a case of the sustainable intensification of maize and bean production (Achieng *et al*., 2011). Field assessment in Kenya revealed that compared to 2011, production of nearly all major food crops increased in 2012. Maize production increased by 7% from 37,520,694 bags in 2011 to 40,037,090 bags in 2012 and beans by 14% from 6,418,590 bags in 2011 to 7,308,225 bags in 2012 (MoA, 2013). This increase in yields is attributed to adequate rainfall pattern in terms of temporal and spatial distribution compared to the previous year. In addition, food security interventions, proper farm and disease management also played a role. However, despite considerable research done on improving maize and bean production in terms of quantity and quality, these two crops are plagued by many diseases that reduce their potential yield thus threatening food security.

In Kenya, fungal diseases have been reported to affect the maize and bean crops. Classical examples of devastating diseases caused by maize pathogens in Kenya include acute aflatoxicosis that was attributed to maize ear rot disease in 2004 (Nyikal *et al*., 2004; Azziz-Baumgartner, 2006), the maize lethal necrosis disease (MLND) which according to the Ministry

of Agriculture affected an estimated 35000 hectares of land (MoA, 2013). These named diseases together with grey leaf spot diseases (GLS), ear rots and *Fusarium* diseases are among the most prevalent and problematic. The latter produces mycotoxin contamination in the crop and grains of maize, thereby not only reducing yield, but more importantly posing a high risk of toxicity to animals and humans (Nielsen et *al*., 2011). Yield losses in beans may range from a trace to 100 percent, especially when adverse environmental conditions persist during the early growth and flowering stages (Schwartz*,* 2013). Bean anthracnose, *Fusarium* root rot and *Pythium* root rot, are among important diseases causing severe damage to bean crop and are major constraints to bean production in Kenya. Crop losses of between 10 and 75% have been reported (www.infonet-biovision.org).

Several control measures have been put in place to mitigate the diseases affecting maize and beans. They include use of agrochemicals, cultural practices, physical control measures, use of resistant varieties, integrated pest management and use of antagonistic microorganisms like endophytes (Azevedo *et al*., 2000; Rodriguez *et al*., 2009) as biocontrol agents. Many beneficial fungi and bacteria have been isolated from the soil and tested in private and university-based laboratories as to their ability to control plant pathogens and the more promising beneficial microorganisms have been further developed and marketed to plant growers (Nameth, 2003). The word endophyte has been described as microorganism typically bacteria or fungi that reside within plant tissues (Porras-Alfaro and Bayman, 2010) and further described as asymptomatic microorganism that part of their life cycle can be found within the plant tissues (Clay and Schardl, 2002; Porras-Alfaro and Bayman, 2010). Bacterial and fungal antagonists have been reviewed by Reinhold-Hurek and Hurek (2011) and O'Hanlon *et al*. (2012) respectively. The increasing growing list of benefits that they can confer to their host has made these antagonistic microorganisms interesting as an area of research. Such benefits include tolerance to drought (Clay and Schardl, 2002), enhanced growth, resistance to herbivore attack and nematodes (Schardl *et al*., 2004) and increased tolerance to pathogens (Porras-Alfaro and Bayman, 2010). More research needs to be done on the use of antagonistic microorganisms against maize and bean pathogens.

Taxonomic identification of microorganisms and their biodiversity both *in vitro* and in natural environments is quite a difficult task, especially when using conventional methods like morphological differences (Liew *et al*., 1998; Siriwach, 2013). Since the nucleotide sequence of

rDNA changes very slowly, its sequencing is convenient as a molecular taxonomical tool for evolutionary comparison among relatively distant organisms. Approaches in using protein coding genes like β-tubulin and internal transcribed spacer (ITS) regions are more suitable for phylogenetic studies since they have less introns and depict evolution superiorly (Siriwach, 2013). However, the β−tubulin gene has a small number of gene sequences deposited in databases. Thus, use of molecular tools in identification ought to be exploited in order to accurately classify microorganisms in their right taxa even though the techniques involved are constantly being refined or modified for greater efficiency.

1.2 Statement of the problem

Plant pathogens like *Fusarium* spp., C*olletotrichum lindemuthianum* and *Pythium ultimum* cause diseases in maize and bean crops and results to extensive crop damage. For instance, the maize lethal necrotic disease has been reported to affect 60% of the maize production land. This leads to deficits in food supply for most Kenyans. Efforts to control phytopathogens and diseases have primarily relied on use of synthetic pesticides. Application of various synthetic pesticides often causes undesirable toxicological and environmental side effects. Such problems include leaving toxic residues in food, soil and water; adverse effects on non-target insects and other beneficial organisms and development of resistant strains of insects, fungal pathogens and other pests. In addition, their use is, in certain cases, economically unviable. Use of resistant varieties especially for beans has been practiced but shortage of enough certified seeds make it difficult in managing seed borne pathogens.

1.3 Objectives

1.3.1 General objective

To identify potential antagonistic microorganisms for the management of major maize and bean pathogens in Kenya.

1.3.2 Specific objectives

1. To isolate and identify targeted tropical fungi using molecular techniques.
2. To determine antimicrobial activity of tropical fungi and bacteria against the major pathogens of maize and beans.

1.4 Hypotheses

1. Molecular techniques will not reveal different species of tropical fungi
2. Tropical fungi and bacteria will not display significant antagonistic activity against the major pathogens of maize and beans.

1.5 Justification

Bean and maize diseases; bean anthracnose, *Pythium* root rots, ear rots and mycotoxin production, have caused massive damage in crop production and yields, both in pre and post-harvest. It is estimated that 1.3 billion people live on less than 1$ a day and at least 10% of global food production is lost to plant disease caused by phytopathogens. Assuring food security and high production of quality beans and maize of high nutritional value to sustain the growing population in Kenya is very crucial. The use of tropical plant fungi and antagonistic bacteria as biological control agents (BCAs) in managing these diseases will reduce the losses caused. Biological control agents like endophytes and antagonistic bacteria are known to provide the plant with antagonism against pests and diseases by inducing resistance that provides a susceptible plant cultivar with pest or disease resistance. They also promote plant growth, either through facilitation of increased nutrient uptake or through synthesis of plant hormones. Use of BCAs in managing these diseases is not fully utilized in Kenya as out of the total sales from use of pesticides only 2% is from biopesticide used primarily in high-value horticulture crops. Thus, there is need to demonstrate the efficacy of tropical fungi and antagonistic bacteria as biological control agents that are valuable components in the integrated disease management programme to mitigate food shortage. Their use may constitute important bio-control strategy that will reduce population of phytopathogens and mitigate diseases hence contribute towards food security.

CHAPTER TWO

LITERATURE REVIEW

2.1 Maize crop and its pathogens

Since the introduction of maize crop in Africa in the 16th century (FAO, 1996) and eventually becoming the staple food for most sub-Saharan countries, farmers have been looking for better ways to enhance and improve the maize grain. The nutritional value of the crop, its ease of cultivation and adaptability to different agro-ecological zones, has increased its popularity among many people. Maize provides carbohydrates (Oduor *et al*., 1998) and according to FAO statistics (2005-2007), maize contributes about 68 % of daily per capita cereal consumption, 35% of total dietary energy consumption and 32 % of total protein consumption. In Kenya, maize accounts for about 20% of total agricultural production and 25% of agricultural employment (Muasya and Diallo, 2001), thus its production is closely linked to food security in order to meet the increasing domestic demand (Odendo *et al*., 2001). An increase in the maize production will rather be achieved by improving yield per hectare than by expanding production areas (Wokabi, 1998; Schroeder *et al*., 2013). This has been adapted in several African countries like Benin and Malawi where increase in yield potential per hectare is given more emphasis more so in the marginal areas (Heisey and Smale, 1995). The levels of maize production results from; interactions among the availability of water and nutrients, occurrence of pests and diseases, and the proper cultural practices (Nyoro, 2002). Diseases and pests play significant roles in production in terms of yields in both pre and post-harvest of maize crop. Recent outbreaks in diseases worldwide and more so in Africa have led to massive losses in maize. The maize lethal necrosis disease (MLND), which has been the center of attention lately in Kenya, is estimated to have affected 65,000 hectares but has scaled down to 35,000 hectares in 2012 (MoA, 2013). Other outbreaks comprise acute aflatoxicosis in Kenya in 2004 associated with maize, chemical intoxication due to consumption of seed beans and maize in Nigeria among others (Mensah *et al*., 2012).

Common maize pathogens that cause the most damage include; *Cercospora zeae-maydis* causing grey leaf spot disease (GLS), *Aspergillus* spp. that has two strains which are known to cause severe infection of ear rot (Hell *et al*., 2010; Okoth and Kola, 2012), *Fusarium* spp. causing seedling blight and *Fusarium* stalk of maize (Gerber, 2010). Apart from the maize lethal necrosis virus (MLNV) the other three named genera of fungi cause massive damage to

maize crop both in the fields or during storage. It is reported that 50-80 % of maize loss during storage is due to fungal infection (Orsi *et al.,* 2000; Fandohan *et al*., 2003). Yield losses caused by GLS are estimated to be in the range of 30-50% in Kenya (Kinyua *et al*., 2010). This is alarming to food security considering 90% of the population in Kenya relies on maize as a staple food. On the other hand, *Fusarium* and *Aspergillus* spp. are problematic because of production of mycotoxins like fumonisins (Fandohan *et al*., 2003) and aflatoxins, respectively thereby not only reducing yield but more importantly posing a high risk of toxicity to animals and humans (Nielsen *et al*., 2011). However, not all species in these genera are harmful, infact some are beneficial biocontrol agents against phytopathogens. For instance, biological control of aflatoxin production in crops in the US is being practiced and a commercial product of *Aspergillus flavus* strain (Afla-Guard® is being marketed (Atehnkeng *et al*., 2008a; Hell *et al*., 2010). In Africa, two strains of *A. flavus* have been identified and shown to reduce aflatoxin concentrations in both laboratory and field trials, due to their atoxigenic nature, reducing toxin contamination by 70 to 99 % (Atehnkeng *et al*., 2008a). A mixture of four atoxigenic strains of *A. flavus* of Nigerian origin has gained provisional registration (AflaSafe) to determine efficacy in on-farm tests and candidate strains have been selected for Kenya and Senegal (Hell *et al*., 2010). The presence of *Fusarium moniliforme* and *F. proliferatum* (two of the most important producers of fumonisins) may be reduced significantly by the presence of *F. graminearum* (Velluti *et al.*, 2000). On the other hand, *F. verticillioides* and *F. proliferatum* can be highly competitive against *Aspergillus flavus* as well as *Penicillium* species (Marin *et al.*, 1998). A lot of research has been done on both aflatoxins and fumonisins contamination (Marin *et al.*, 1998; Velluti *et al.*, 2000; Atehnkeng *et al*., 2008a; Gerber, 2010) and strategies of controlling these two pathogens is still an economic and public health concern.

2.1.1 Ear rots

Fusarium species are the most important problem facing production of maize grains in the tropics and also cause harmful effects to both humans and animals' feeds by producing a range of toxic secondary metabolites called mycotoxins when consumed (Reid *et al*., 1999; Reid *et al*., 2002; Brennan *et al*., 2003; Dragich and Nelson, 2014; Turkington *et al*., 2014). The predominant species causing maize 'red ear rot' is *F. graminearum* (Brennan *et al*., 2003). Their prevalence is typically due to multiple yearly cropping cycles and poor land tillage allowing the

pathogens to develop large inoculum. This creates a need for public awareness in order to manage the pathogens large populations. Spores are primarily dispersed by wind and rain. Insects such as corn borers, maize earworms and thrips also significantly serve as vectors to these *Fusarium* spp. causing ear rots (Parsons, 2008). Table 1 below shows *Fusarium* species causing ear rots and their wide host range.

Table 1: *Fusarium* species causing ear rots and their host range

Sexual stage	**Asexual stage**	**Host**
Gibberella zeae	*F. graminearum*	Maize (*Zeae maydis)*, wheat (*Triticum* spp), barley (*Hordeum vulgare)*, oats (*Avena sativa)*, rye (*Secale cereal)*, pisum, trifolium and solanum like potatoes.
Gibberella fujikuroi	*F. moniliforme*	Maize, rice (*Oryza sativa*), wheat, sorghum, sugarcane (*Saccharaum officinale*), cotton (*Gossypium hirsutum*) and banana.

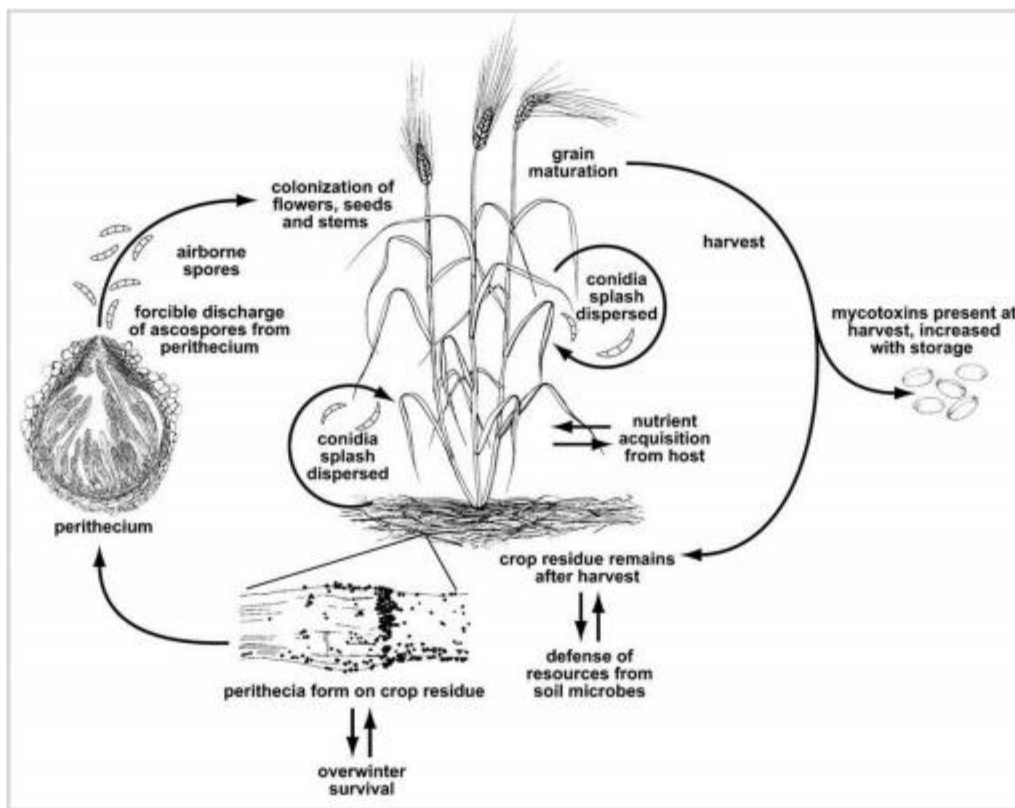
Source: Dragich and Nelson (2014).

Several control measures have been practiced but crop rotation between maize and wheat and poor tillage practices are the high risk factors that render the control of these pathogens unacceptable (Eckard *et al*., 2011). The use of host resistance is an economically and environmentally effective strategy but however to date, only a few highly resistant maize cultivars have been identified from different geographic regions (Hao *et al*., 2012; Zhao *et al*., 2014). Due to the growing cost of chemical pesticides and increasing awareness about their negative effect, the farmers are looking for alternative substitutes for these products to fulfil the consumers demand on pesticide-free food while maintaining environmental safety.

2.1.2 *Fusarium graminearum*

Fusarium graminearum (anamorph), also known as *Gibberella zeae* (telemorph) causes the Gibberella ear rot of maize. It is widely distributed in almost all the corn growing regions (Dragich and Nelson, 2014; Turkington *et al*., 2014) which are conducive to the pathogen to thrive i.e. warm temperatures with persistent wetness (Reid *et al*., 1999; Reid *et al*., 2002). The

sexual stage of the ascomycete fungus produces ascospores usually ovoid (Figure 1) while the asexual stage mainly produces large spores called macroconidia. Chlamydospores may also be present serving as long term survival structures for the fungus (Leslie and Summerell, 2006). *Fusarium graminearum* infect through the maize ear and proceeds basipetically from the tip to the base of the corn ear. A diseased maize cob has a distinct pinkish-red mould throughout the infected area. The pathogen produces a variety of mycotoxins but of the key importance is the deoxynivalenol (DON) in which prolong intake of the toxins results to diarrhoea, lethargy, intestinal haemorrhage and increased susceptibility to other diseases (Willyerd *et al*., 2010).



Source: Trail, (2009)

Figure 1*: Fusarium graminearum* lifecycle

2.1.3 *Fusarium moniliforme*

Fusarium ear rot of maize is caused by *Fusarium moniliforme* as well as its sexual stage which is a different mating type of *Gibberella fujikuroi.* It has a worldwide distribution in all climates where corn is grown (Dragich and Nelson, 2014). Both the asexual and sexual stages of

this pathogen have the microconidia and macroconidia spores but lack chlamydospores. Infection is mainly through wounds especially those made by insects hence do not damage the entire maize ear but remain localised around the insect infested area. *Fusarium* ear rot produces white, pale pink or pale lavender mycelia. It produces mycotoxins called fumonisins associated with cancer in humans (Das, 2014). The fumonisin that is frequently found in maize is fumonsin B1 (FB1) (Venturini *et al*., 2011). *Fusarium moniliforme* can exist endophytically within maize under ordinary plant growth conditions asymptomatic and benefit the plants by increasing their size and productivity. However, under abiotic and/or biotic stress conditions, this relationship may convert to a disease and/or fumonisin–producing interaction (Bacon and Nelson, 1994; Abbas *et al*., 2006). Both symptomatic and asymptomatic kernel infections by *F. moniliforme* can result in decreased grain quality and economic losses due to contamination by FB1 (Glenn *et al*., 2004).

2.2 Bean crop and its pathogens

Bean crop was introduced in Africa centuries ago from Latin America and since then cultivation of this crop has been a major practice, becoming the second major food crop after maize. It is largely grown for subsistence farming and its high nutritional content has made it popular in regions in Africa, especially the eastern and southern part (Katungi *et al*., 2009). Common bean (*Phaseolus vulgaris* L.), is also referred to as dry beans, bush beans, dwarf beans, field beans, French beans, garden beans, green beans, haricot beans, kidney beans, pole beans, snap beans or string beans (www.infonet-biovision.org). It is a major source of protein, good source of energy and provides folic acid, dietary fibre and complex carbohydrates (Ferris and Kaganzi, 2008; Katungi *et al*., 2009). Food and nutritional insecurity in Sub-Saharan Africa is feared to increase due to global climate change, hence research effort on common bean production, which is strategic in alleviating malnutrition and minimizing food insecurity by increasing yields, is a key point to be focused on (Katungi *et al*., 2009). Cultivation of common bean in Africa is widespread, but approximately 80% of the production is concentrated in 10 countries (Table 2). Kenya, being the number one producer of snap beans was among the countries selected for the project entitled: “Enhancing Grain Legumes’ Productivity, and Production and the Incomes of Poor Farmers in Drought Prone Areas of Sub-Saharan Africa and South Asia (Katungi *et al*., 2009).

Table 2: Common bean producers in Africa in 2000-2007

Country	Average area (Ha)	Average production (Tons)
Kenya	910478	412381
Uganda	794375	478625
Tanzania	373125	285414
Rwanda	340055	231882
Angola	290391	92786
Burundi	249375	229607
Democratic Republic of Congo	205958	110404
Malawi	197605	87593
Ethiopia	188000	143414
Madagascar	82096	77

Source: FAO stat at www. fao.org

Bean production in Kenya is done in highlands and midlands particularly Nyanza, Rift valley and Eastern regions. In terms of output, the Rift valley contributes the biggest share, accounting for 33% of the national output followed by Nyanza and Western province accounting for 22% each (Katungi *et al.,* 2009). Diversity of common bean seed types exists in Kenya with six popular varieties namely Red and red/purple mottled (occurring in different local names such as Rosecoco, Nyayo, Wairimu, Kitui etc.), Purple/grey speckled (locally known as Mwezi moja) and Pinto sugars (locally known as Mwitemania) (Katungi *et al.,* 2009; Nzungize *et al*., 2011). In tropical regions, the common bean is characterized by low and unstable grain yields due to various ecological and agronomic parameters. Among these parameters, diseases like bean root rots caused by *Pythium* spp. and a decline in soil fertility have been cited as being among the major causes leading to bean yield losses when susceptible varieties are grown under favorable environmental conditions for the pathogen development (Otsyula *et al*., 2003; Miklas *et al*., 2006; Buruchara *et al*., 2007). This food legume is cultivated intensively under poor conditions of crop rotation due to the exiguity of the land in the region. A study carried out by Nzungize *et al*. (2011) showed that out of 16 species of *Pythium* isolated only *P. ultimum* was most severe in causing bean root rot in Uganda.

Foliar diseases like the angular leaf spot (ALS) caused by a fungus: *Pseudocercospora griseola* (Sacc.) is among the most destructive diseases of common bean (Ddamulira *et al*., 2014). The disease is ranked second among biotic and abiotic factors that constrain bean production in Africa (Aggarwal *et al*. 2004) and yield losses of upto 80% have been reported (Stenglein *et al*., 2003). Countries within the great lakes; Kenya, Uganda, Tanzania, Malawi and Ethopia have reported an estimated 374,800 tonnes annual losses due to ALS (Wagara *et al*. 2003). Soil borne fungal pathogens like; *Colletotrichum lindemuthianum, Macrophomina phaseolina*, *Rhizoctonia solani* and *F. solani* f.sp. *phaseoli* are some of the major causes of low yields (Isutsa *et al*., 2006). The latter has been reported to cause bean root rot worldwide and in Kenya where losses of 10% to 100% have been reported. In an experiment by Mwan'gombe *et al*. (2011) all 52 isolates of *F. solani* f.sp. *phaseoli* were found to incite disease to varying levels of virulence.

2.2.1 *Pythium ultimum*

Pythium ultimum is a member of the Oomycota (kingdom Chromista), which are part of the heterokont/chromist clade (Cavalier-Smith and Chao, 2006; Kirk *et al*., 2008 and Riisberg, *et al*., 2009). The species is distinguished by the filamentous vegetative body called mycelium which is sometimes colourless, yellowish or grayish lilac in colour. *Pythium ultimum* is a ubiquitous plant pathogen and one of the most pathogenic *Pythium* spp. on crops (Martin *et al.,* 1999). It is also an opportunistic pathogen of young seedlings and plant roots with little or no cuticle or heavily suberized tissue, consistent with lack of cutinase encoding genes. It does not require another mating type for sexual reproduction as it is self-fertile that is, homothallic but outcrossing has been reported. *Pythium* species are spread worldwide (Paul, 2004).

Pythium root rot constitutes a highly damaging constraint on common bean grown in several areas of Eastern and Central Africa. This food legume is cultivated intensively under poor conditions of crop rotation due to the exiguity of the land in the region. Yield losses of up to 70% in traditional local bean cultivars have been reported in Kenya and Rwanda. Over the last 20 years, there has been an increase in the importance of *Pythium* bean root rots in several countries of Eastern and Central Africa, such as Burundi, the Democratic Republic of Congo, Kenya and Uganda (Otsyula *et al*., 2003). In Western Kenya and in Rwanda, many farmers stopped growing beans between 1991 and 1993 due to a severe outbreak of root rots, which

caused serious food shortages and price increases beyond the reach of many resource-poor households (Nekesa *et al*., 1998). Root rot symptoms include poor seedling establishment, damping-off, uneven growth, leaf chlorosis, premature defoliation, death of severely infected plants, and lower yield (Abawi *et al*., 2006; Schwartz *et al*., 2007).

2.2.2 *Colletotrichum lindemuthianum*

Bean anthracnose caused by *Colletotrichum lindemuthianum* is widely spread in the tropics and sub-tropics regions, including Kenya (Amin *et al*., 2014). It is an economically important seed borne pathogen where infection of susceptible cultivars of common bean could lead to an epidemic resulting to 100% yield loss (Liu *et al*., 2013). *Colletotrichum lindemuthianum* deploys a complex lifecycle which has various development phases for the fungus to survive. Independent of the fungus development phases, the spores produced show a biphasic behaviour which means two life styles, as a saprophyte and biotroph; therefore, the fungus has been classified as hemibiotrophic. It establishes an initial asymptomatic biotrophic phase during the infection process, in which it invades the tissues of its host (*Phaseolus vulgaris*) undetected (Zavala-Paramo *et al*., 2014). It has been known to have slow growth rate and dark pigmentation colonial characteristic. *C. lindemuthianum* presents a considerable variation in colonial morphology forming reddish droplets with abundant setae in culture that are often absent on the host plant (Liu *et al*., 2013). The pathogen also infect other leguminous plants like *Dolichos lablab* (Zhuang, 2001). The management of anthracnose disease in common bean has been mainly through intergrated crop production strategies involving various inputs, practices and means of managing biotic and abiotic stresses (Amin, *et al*., 2014). The use of fungicides which dominates all other inputs leads to fungicidal resistance of the pathogens and also degradation of the environment. It is important therefore to find an alternative to avoid these risks.

2.3 Management of pathogens of maize and bean crops

Disease management of both maize and beans crops has continuously and heavily relied on the use of chemicals like fungicides, bactericides and pesticides rather than biological control. Various cultural practices are currently employed in the management of pathogens of maize and bean crops (Karavina, 2014; Eshte *et al*. 2015). These include crop rotation, field hygiene, early

planting, cultivar choice and intercropping. The latter has been extensively practiced by small scale farmers in Kenya. The combination of chemical and biological pesticides has proven to reduce pathogens in these two crops (Paparu *et al*. 2014; Yule and Srinivasan, 2014). However, application of various synthetic pesticides often causes undesirable toxicological and environmental side effects. For example, there are many specific pesticides such as benomyl, captafol, captan, carboxin, metalaxyl, propamocarb hydrochloride and etridiazole unable to control *Pythium* species as it has got resistant against these synthetic fungicides (Schwartz *et al*. 2007; Parveen and Sharma, 2015**).** Hence, there is need to replace the chemical fungicides with bio-fungicides, prepared from plant extracts and antagonistic microorganisms. Bio-fungicides will also be economical to the farmers and besides this their use will not leave any ill effect in the soil, water, as well as in the environment. It is possible that by combining these approaches, (use of plant extracts, antagonistic micro-organisms, and organic manure) an economically viable alternative for crop production system can be developed (Parveen and Sharma, 2015**)**.

2.4 Biocontrol of phytopathogens

The control of pests and diseases by means of biological processes, i.e. use of microorganisms that inhibit/antagonize other microorganisms pathogenic to plants, is an alternative that may contribute to reduce or eliminate the use of chemical products in agriculture. A lot of bio-control agents have been isolated in the recent years all over the world that are used to control plant diseases. As research has shown, not all isolates of these microorganisms show antagonistic behavior and it has been established by Bandyopadhyay and Cardwell (2003), Atehnkeng *et al*. (2008b) and Gerber (2010) that isolates from the same genera can be used to control the same microorganisms. Endophytes that are proficient to control plant diseases include *Trichoderma harzianum, Bacillus subtilis, Aspergillus* isolates, *Microbacterium maritypicum, Agrobacterium radiobacter, Pseudomonas fluorescens, Pseudomonas aureofaciens, Streptomyces griseoviridus* among others (Gerber, 2010). Use of biocontrol agents' i.e isolates of *Trichoderma* spp. and *Gliocladium* spp. antagonistics to *Pythium*-induced soil-borne diseases and several strains are already commercially available for the biological control of *Pythium* root rots (Fravel, 2005). Bacteria effective against *Pythium* are found in various genera including *Enterobacter*, *Erwinia*, *Bacillus*, *Burkholderia*, *Stenotrophomonas*, and *Rhizobium* but the most

extensively studied group of bacterial biological control agents are *Pseudomonas* spp. (Chin-A-Woeng *et al*., 2003; Bardin *et al*., 2004).

Different types of interaction among microorganisms as bio-control agents exist and can occur through different mechanisms, which are generally classified as: parasitism/predation, antibiosis, competition, lytic enzymes, and induced resistance (Pal and Gardener, 2006). There is a shift toward the important role of biological control in agriculture in the future. Several companies now have programs to develop bio-control agents as commercial products (Suprapta, 2012). Table 3 shows bacterial and fungal bio-control agents that are commercially available as well as their target pathogens (Scala *et al.*, 2007; Whipps and McQuilken, 2009; EPA, 2010).

It is clear that, not many bio-control agent formulations on maize and bean pathogens are available in Africa as shown in table 3. However, research on these agent formulations is still ongoing. Sometimes a good bio-control agent under *in vitro* conditions fails in field trials because of variations in environmental factors from one place to another. To achieve success, the environmental factors should be similar to those from which the bio-control agents were isolated. Likewise, the method of application can influence the success of field trials. In general, there are three means of applying the antagonists for bio-control namely, seed inoculation, vegetative part inoculation and soil inoculation (Suprapta, 2012).

2.5 Biocontrol of phytopathogens using endophytes

In the past decades the use of endophytic microorganisms as a management strategy against plant pathogens has been shown (Quecine *et al*., 2008; Gangwar *et al*., 2011; Moussa *et al*., 2011, Lopes *et al*., 2012). Endophytes are plant organisms living inside another plant in symbiotic associations without causing any external signs of damage and contamination (Arnold and Lutzoni, 2007). The symbiotic relationship between endophytes and plants and their rich biodiversity makes them unique and most potent sources for discovery of novel bioactive molecules (Selim *et* al., 2012). For instance the discovery of the strobilurines, that were first isolated from *Strobilurus* sp. and served as lead compounds for synthetic fungicides such as trifloxystrobin (Kjer, 2009). Also, three new metabolites isolated from the culture of *Colletotrichum* sp. in *Artemisia annua* were detected to be fungistatic against plant pathogenic fungi (Guo *et al*., 2006). However, only a handful of natural products from endophytic microorganism of the nearly 300000 plant species in the earth have been reported which means

the opportunity to find new potent and target natural products from interesting endophytic microorganisms among myriads of plants in different niches and ecosystems is great (Sturz *et al*., 2000; Zhou, 2003; Kjer, 2009).

For this reason, the scarcely explored ecological niche that offer a plenitude of novel bioactive compounds (Kjer, 2009) inhabiting distinct biotopes such as medicinal plants has become the focus of attention in the past two decades. Fungal endophytes have been categorized into two major groups; the clavicipitaceous and the non-clavicipitaceous endophytes and were reviewed by Rodriguez *et al*. (2009) on the basis of evolutionary relatedness, taxonomy, host plant range and ecological function. Bacterial endophytes likewise can be classified as 'facultative' or 'obligate'. Obligate endophytes are strictly dependent on the host plant for their growth and survival and transmission to other plants occurs vertically or via vectors. Facultative endophytes have a stage in their life cycle in which they exist outside host plants (Sturz *et al*., 2000). Bacterial endophytes belonging to the genera *Bacillus* and *Pseudomonas* are most predominant. They are easy to culture, and cultivation dependent studies have identified them as frequently occurring endophytes (Haas and Keel, 2003).

Examples of endophytes that have been showed to act as bio-control agents include *Bacillus subtilis* as biological control agent of maize and sunflower diseases in Germany (Schmiedeknecht *et al*., 2001). The bacterial endophyte had antifungal activity in both *in vitro* and *in vivo* trials towards *F. oxysporum*. Gangwar *et al.* (2011) reported that eight isolates out of the 40 endophytic *actinomycetes* from *Aloe vera*, *Mentha* and *Ocimum sanctum* inhibited growth or were antagonistic to one or more phytopathogenic fungi. Endophytic streptomyces have also exhibited potential for bio-control against phytopathogenic fungi (Quecine *et al*., 2008). Another endophytic strain of *Paraconiothyrium brasiliens* LT161 isolated from the healthy stems of *Cinamonum camphora* collected from Nanjing, China, showed strong growth inhibition activity *in vitro* against fungal phytopathogens such as *Rhizoctonia solani, Alternaria alternaae, Glomerella glycines, Phytophthora capsici, Fusarium oxysporum, Fusarium graminearum* and *Cryphonectria parasitica* (Han *et al.,* 2012). Fungal endophytes like *Fusarium* spp., *Xylaria* spp. and *Pestaliopsis* spp. are among the common endophytes encountered (Joseph and Priya, 2011). The fungal endophyte *Acremonium zeae* is antagonistic to kernel-rotting and mycotoxin-producing fungi *Aspergillus flavus* and *Fusarium verticillioides* in cultural tests for antagonism

and interferes with *A. flavus* infection and aflatoxin contamination of preharvest maize (*Zea mays*) seed (Wicklow and Poling, 2009).

In Kenya, biopesticide-specific registration regulations have been developed, representing a proportional and reasonable system that correctly assesses the safe and risks associated with microbial pesticides like endophytes (Wabule *et al*., 2004). Not much has been done on the use of antagonistic microorganisms against maize and bean pathogens though. Seed treatment with *Trichoderma* spp. against *Fusarium* root rot has been practiced and a commercial biopesticide is registered in Kenya under the name Rootgard (Kabaluk *et al*., 2010). *Trichoderma harzianum* has been used to control fungal root diseases and the commercial products include ECO-T and Promote (temporary registered). Trichotech from *Trichoderma asperellum* has been used to control soil fungal pathogens.

Table 3: Commercially available bio-pesticides products

Biological control agents	**Country of registration**	**Name of product**	**Target pathogen or diseases**	**Crops**
Bacillus pumillus GB34	United States	Yield shield concetrate, GB34 biological fungicide-	Soil borne fungal pathogen causing root diseases	Soybean
Bacillus subtilis MB1600	United States and Mexico	HiStickN/T, Pro-mix, SubtilexHB.	*Fusarium, Aspergillus, Rhizoctonia, Alternaria*	Soybean, peanuts, alfafa & dry/ snapbeans, cotton
Burkholderia cepacia type Wisconsin	USA	Intercept	*Rhizoctonia solani*, *Pythium* and *Fusarium* spp	Maize, vegetables, cotton
Microbacterium maritypicum isolate DB107	South Africa	Bismarck	*Xanthomonas*, *Pseudomonas* and *Erwinia*.	Maize, tomato, potato
Trichoderma harzianum	South Africa	Tri-Cure	*Fusarium* spp, *Rhizoctonia* spp. Stem canker, blackscurf	Maize, wheat, dry beans, peanuts, soybean

Source: Gerber, (2010)

CHAPTER THREE
MATERIALS AND METHODS

3.1 Collection of tropical fungi

A total of 87 tropical fungi were collected from Kakamega rain forest located at 00° 16’ N, 34° 53’ E, where there is both the primary and secondary forests and indigenous plant species acceptable by the neighbouring communities as medicinal. Random sampling of the tropical fungi was done in September 2014 based on ethno botanical information obtained from the local community. The sampled materials were colected in khaki bags in order to free moisture from the sample material and transported back to the biotechnology laboratory (Egerton University) in a cooler box.

3.2 Collection of phytopathogens

This study involved four phytopathogens; *Fusarium graminearum, Fusarium moniliforme, Pythium ultimum,* and *Colletotrichum lindemuthianum*, which cause ear rots, root rots and bean anthracnose diseases respectively. Infected plant samples with disease symptoms, were collected from Nakuru, Narok and Bomet counties, and sandwiched with newspapers for transportation back to Egerton University (Biotechnology laboratory). Identification on basis of symptomatological evidence was done at the Department of Biological Sciences of Egerton University and were then isolated at the same institution in Biotechnology laboratory. Plate 1 below shows bean crop affected by *Colletotrichum lindemuthianum* in Field 7 and Biological Sciences Department, Egerton University Nakuru.

3.3 Isolation of tropical fungi, antagonistic *Bacillus amyloliquefaciens* and phytopathogens

3.3.1 Media preparation

A concentration of 28 g/L of yeast malt extract agar (YMG), 39 g/L of potato dextrose agar (PDA) and 38 g/L of Sabouraud dextrose agar (SDA) were prepared. For YMG, the agar was added later when the media had boiled up as it is hardly soluble in cold water and sinks at the bottom of the flasks. The pH was adjusted to 6.3 using a pH meter. The media was autoclaved and sterilized at 15 lbs 121° C for 15 minutes. It was cooled to $45^{o}C$ and a concentration of 250 mg/L of streptomycin sulphate, and 50 mg/L of ivermectin were added to inhibit bacterial growth and mites respectively. The media was poured into sterile petri dishes in

the laminar flow and left to cool. For liquid media like essential bacterial media: EBS (peptone marcor 0.5%, meat extract 0.1%, Agar 1.5% and Hepes 1.19%), the same procedure was repeated only that there was omission of adding agar and the cooled media was left in the flasks on a cooling chamber for further use.

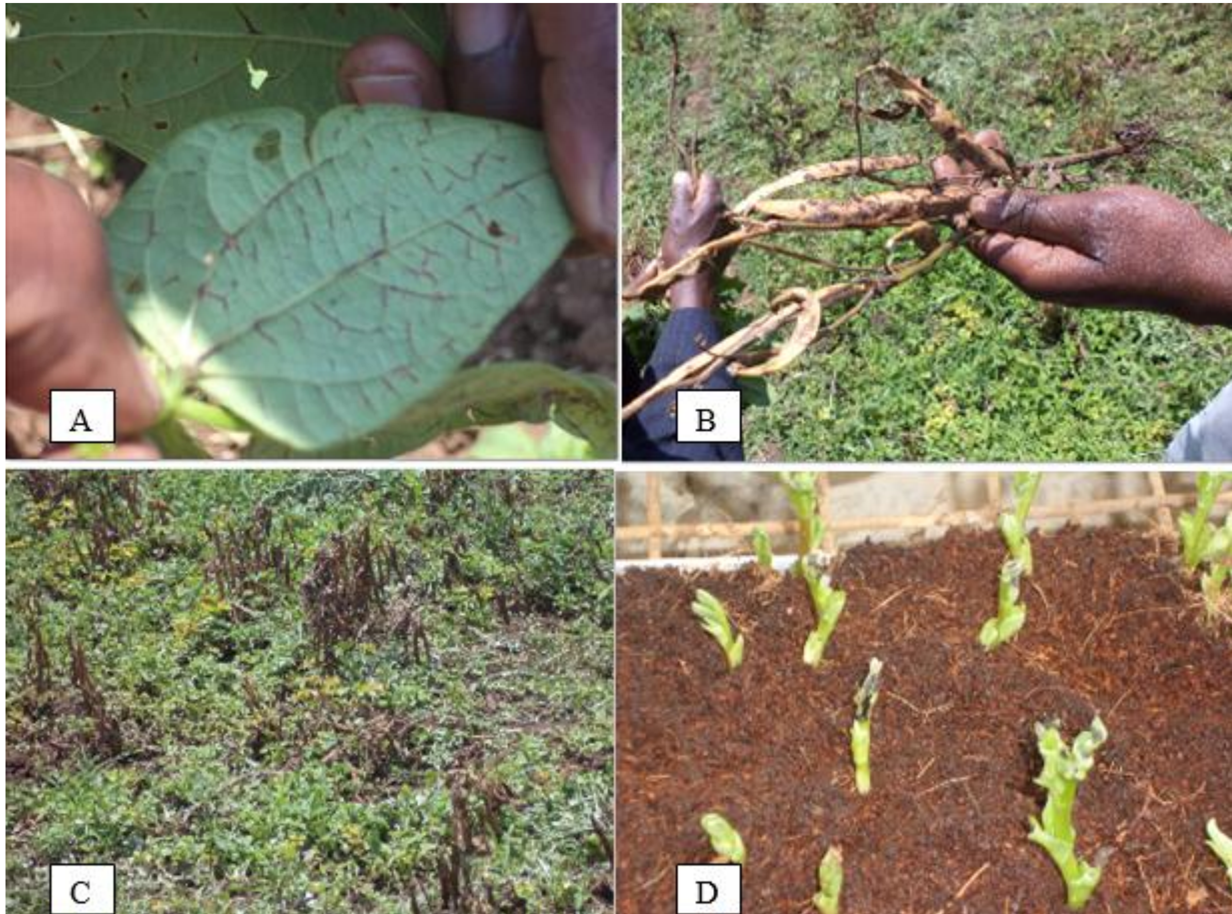


Plate 1: Symptoms of bean anthracnose manifesting itself on leaves, pods and seedlings.

A- Leaf showing necrotic venation as one of the symptoms of the disease

B- Dry bean pod with sunken necrotic lesions

C- A small plot showing the disease severity, with all the plants infected

D- Green pea seedlings showing infection in a greenhouse

From plate 1 above, it is evident that the pathogen infects the bean crop at an early stage (D) from seedlings to mature pods (B) which results in massive destructions of the crop (C) hence reducing yields.

3.3.2 Isolation of tropical fungi

Isolation of the tropical fungi was done immediately upon collection. The inner part of the Basidiomycete (mushroom) sample was picked with a fine sterile forceps, and stuck onto the inner top side of the petri dish containing PDA media, by the help of silicone high vacuum grease, where the spores were left to drop on the media plate overnight. Thereafter, a sterile spatula was used to cut out the stamp of spores and placed inverted on a media plate. For the ascomycetes, the perithecia were cut open under a dissecting microscope with a sterile scalpel to release spores, which were picked with a fine needle, and plated on AB- media plates. Both instances were followed by sub-culturing at Egerton University, Kenya and Helmholtz Zentrum Für Infektionforschung in Braunschweig, Germany. The subcultered isolates were transferred to sterilized YMG media containing streptomycin sulphate, where they were incubated at 25°C. The bacteria free isolates were then transferred to pure YMG media at pH of 6.3, afterwhich they were transferred to ivermectin plates. The mite free isolates were finally plated on pure YMG media.

3.3.3 Isolation of *Bacillus amyloliquefaciens* (B1 and B5)

Bacillus amyloliquefaciens isolate B5 was isolated as an opportunistic bacteria during isolation of tropical fungus TF4 (mushroom) whereas *B. amyloliquefaciens* B1 was obtained from the Biotechnology Laboratory and had earlier been isolated from groundnut seed and found to have antifungal activity (A. M. Kiburai, personal communication).

3.3.4 Isolation of the Plant Pathogens

In the laboratory, the infected plant materials were washed under running tap water to remove any soil and blotted dry. Small sections were cut and surface sterilized for 10 seconds with 2% sodium hypochlorite containing 0.1% Tween 20. The plant tissues were rinsed three times each in two washes of sterile distilled water and blotted dry with sterile paper towels. Thereafter, they were plated on PDA and SDA plates amended with streptomycin sulphate to inhibit any bacterial growth (Whiteside, 1986). The plates were then incubated at 25°C for 4-7 days and monitored for mycelial growth. The cultures obtained were sub-cultured till a pure and axenic culture was obtained. Morphological identification of the cultures was accomplished by use of a compound microscope.

3.4 Preliminay screening of the tropical fungi against test organisms (*Bacillus subtilis* and *Mucor plumbeus)*

Preliminary screening was done against *Bacillus subtilis* strain DSM10 and *Mucor plumbeus* strain MUCL49355 to identify the active antagonists. Overnight cultures of the test organisms were prepared in Erlenmeyer flasks containing 200 mL of media and incubated in a rotary shaker (140rpm) for approximately 24 hours at 37°C and 30°C for *B. subtilis* and *M. plumbeus,* respectively. The media used was Yeast- Malt- Glucose medium (YMG/YM) pH 6.3 for *M. Plumbeus* and EBS medium of pH 7.0 for *B. subtilis.* A concentration of 1×10^6 cells/ml of *B. subtilis* from the overnight culture was calculated using a Haemocytometer under a compound microscope to count bacterial cells. The bacteria cells were then inoculated into EBS media with agar, and poured into sterile petri dishes in the isolation chamber to solidify in a cool room at 4°C. A spore suspension of *M. plumbeus* at 2.7×10^7 spores/ml were inoculated into YMG media with agar and poured into sterile petri dishes under the isolation chamber to solidify in a cool room at 4°C.

3.4.1 Agar diffusion assay

This method was used to screen the tropical fungi against the test organisms. A cork borer (7 mm in diameter) was used to plunge into the culture plate with the tropical fungi (7 days old) and was carefully placed on the media plates previously inoculated with *B. subtilis* and *M. plumbeus.* For controls, 10 mg/ml of both penicillin and streptomycin sulphate was spread evenly by glass spreader until they were dry in the plates previously inoculated with *M. plumbeus* and *B. subtilis*. The inoculated plates were incubated in the culture room at 23°C in the dark room. Monitoring for any growth inhibition was done after 24 hours and 48 hours for *B. subtilis* and *M. plumbeus* respectively. The diameter of the growth inhibition zone was measured and results recorded.

3.4.2 Fermentation and Extraction of secondary metabolites

Tropical fungi which showed any antimicrobial activity were selected for fermentation and testing of their secondary metabolites. The fungi were grown under submerged shake-flask conditions in YM 6.3 and Sugar Malt (ZM½) liquid medium. Erlenmeyer flasks (500 ml) containing 200 ml of specific media were inoculated with well-grown cultures of the respective

strains. Five mycelia plugs were cut using a cork borer (7 mm in diameter) from well grown culture plates and used to inoculate the liquid media. The cultures were grown on a rotary shaker at 140 rpm at 25°C in the dark. The level of free glucose was monitored from 5 days onwards by glucose test strips as described by Stadler *et al*. (2001).

Upon depletion of the glucose content as indicated by change of colour of the glucose test strips, the pH of the culture was determined. Subsequently, mycelia and supernatant were separated by filtration through a siphon. For the slimy mycelia, gauze was used or centrifugation (30 min, 4000 rpm) was done for sufficient separation. After successful separation, the supernatant was transferred to a separating funnel. The same volume of ethyl acetate (for a 200 ml culture, 200 ml of ethyl acetate) was added and shaken out while ventilating the separating funnel regularly to avoid too much pressure. When the phases had separated properly, the lower (watery/ hydrophilic) phase was discarded while the lipophilic (organic) phase was transferred into a beaker. The extract was then stirred on a magnetic stirrer while some amounts of sodium sulfate was added to it until it was over saturated as small particle of the sodium sulfate were seen moving freely in the extract. The extract was then transferred to a round bottom flask over a filter. A little ethyl acetate was added to the filtrate to get all the extract in the beaker. The ethyl acetate was steamed up with a rotary evaporator. The extract was then resolved with 1-4 ml methanol where the methanol was added stepwise with 500 µL added each time for better solution and put into a 4 ml vial (without lid but with the label). The methanol was then steamed up with heat and nitrogen over a speed vacuum and the vials weighed again to determine the amount of extract.

For extraction of the mycelia, 50 mL of acetone was added and the mixture was placed in an ultrasonic bath at 40°C for 30 minutes. The organic solvent was removed in vacuum until an aqueous phase was left and 50 mL of distilled water was added (Kuhnert *et al*., 2015). Afterwards, an extraction with 50 mL ethyl acetate following the same procedure as described for the extraction of the filtrate was carried out. The samples were stored dry in -20°C for further use.

3.4.3 Testing the secondary metabolites for antimicrobial activity

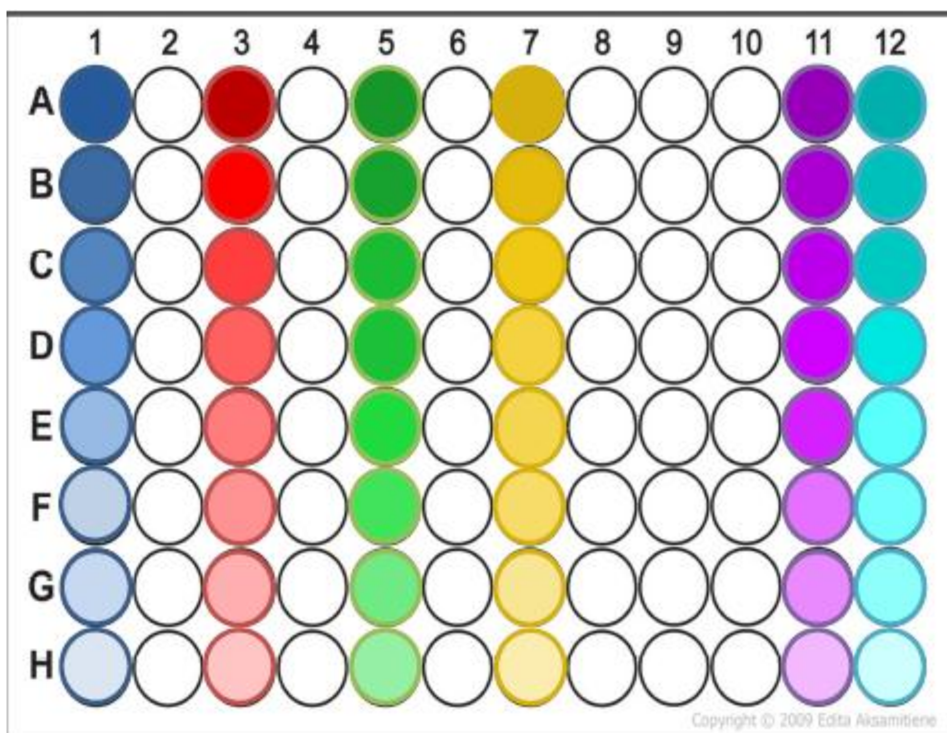
The serial dilution assay was used to determine the lowest concentration of the tropical fungl extract inhibiting growth of microorganisms. Overnight cultures of the test organisms were

prepared and incubated. A flask with EBS-medium was inoculated with 150µL of the *B. subtilis* in a cryo-preservative. The inoculated flask was incubated overnight on a shaker at 37°C. After 18-20 hours a haemocytometer (Neubauer) was used to determine the cell number. The required concentration was set to 3.6 x 10^5 cells per mL (Table 4). In order to test one 96 well plate, approximately 20 mL of the particular medium (EBS) with the required cell concentration was used. For *M. plumbeus* a spore suspension with 2.7 x 10^7 spores per mL was prepared.

Table 4: Types and concentration of test organisms, and antibiotics used for Minimum inhibitory concentration (MIC) test.

Test organism	**Strain**	**Type**	**Antibiotic**	**Concentration of test organism**
Bacillus subtilis	DSMIO	Gram positive	penicillin	3.6 $\times 10^5$ cells/ml
Mucor plumbeus	MUCL49355	Filamentous fungi	cycloheximide	2.7 $\times 10^7$ spores/ml

A volume of 20 µL of 4.5 mg/ml of the tropical fungi extract was prepared, and transferred by a multichannel pipette in to the 96 well plate. A volume of 150 µL of the bacterial suspension was added to all the wells, followed by an additional 130 µL only on the first row (A1-A12) as shown in Plate 2. The tropical fungi extracts (20µL) were then systematically added in the wells (i.e extract 1: A1; extract 2: A2; extract 3: A3, and so on). A concentration of 1.5 mg/ml of penicillin was applied to the second last well as the positive control. Methanol served as negative control and was applied in the last well. The well plates were incubated on a plate shaker for 24 hours at 30°C and monitored for any reaction by use of a reflecting mirror. The same procedure was repeated for *M. plumbeus* and the plate incubated for 48 hours before analysis. Cycloheximide (fungicidal) was used as the positive control and methanol as the negative control.



Extract 1 Extract 3 Extract 5 Extract 7 MeOH-control AB-control

Plate 2: A 96 well plate showing how the extracts from tropical fungi were placed during serial dilution assay against test organisms.

3.5 Antimicrobial assays of the tropical fungi and bacteria against phytopathogens

All the tropical fungi were subjected to bioassay *in vitro* (dual culture method) against each of the four phythopathogens i.e. *Fusarium graminearum, Fusarium moniliforme, Pythium ultimum* and *Colletotrichum lindemuthianum*. Mycelia agar blocks were cut by use of inoculating needle from the actively growing tropical fungal strains and inoculated opposite the phytopathogens approximately 4 cm apart on PDA media. The plates were incubated at 25°C and monitored for growth inhibition. Inhibition zones between the test organisms and phytopathogens (C-T) were measured in a period of 7-21 days and the resulting percentage inhibition zones were determined as follows:

$$L = \frac{(C - T)}{C} \times 100\%$$

L= inhibition of radial mycelial/colony growth; C= radial growth measurement of pathogen in control; T= radial growth measurement of pathogen in the presence of antagonist (Hajieghrari *et al*., 2008).

The same procedure was repeated with bacteria B1 and B5 against the four phytopathogens but instead of putting a plug of mycelia as the antagonist, the bacteria were streaked in a single line. Nystatin was used as negative control whereas for positive control the pathogens were left to grow in absence of the antagonists.

3.6 Molecular characterization of Tropical fungi

Molecular characterization of the tropical fungi and confirmation of the phytopathogens was done by sequencing of the ITS region (rDNA ITS) and the more conserved protein coding ß-tubulin gene for the bioactive strains and phytopathogens. For this purpose three schemes were followed to carry out molecular characterization.

3.6.1 DNA extraction

DNA extraction was done according to BIO BASIC INC. EZ-10 Spin column genomic DNA minipreps kit (Protocol for Plant material) with the following exception; instead of using 300 µL of wash solution, 500 µL was used. Six steps were followed in this procedure as shown in figure 2 below.

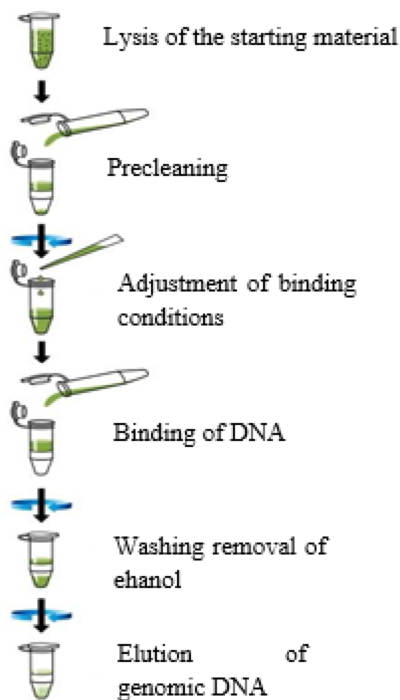


Figure 2: BIO BASIC INC. EZ-10 Spin column genomic DNA extraction Protocol

About 60 mg of clean well grown fungal hyphae were scrapped out from an actively growing culture plate using a plastic sterile scalpel and put in a 1.5 ml screw cap reaction tube. Precellys ceramic beads: 6-10 (1.4 mm in diameter) were added into the reaction tube and the samples covered with 150 µL of lysis buffer called PCL solution. The samples were homogenized in a homogenizer and incubated at 65°C for 20 minutes followed by addition of 25 µL of another special buffer: PP solution. The samples were incubated for 15 minutes on ice and centrifuged at 13 400 relative centrifugal force (rcf), at 4°C for two minutes. The clear lysate from centrifugation was transferred to an EZ-10 Spin Column and 300 µL phosphate buffer (PB) added to the samples. They were incubated for three minutes and mixing was done occasionally. The samples were centrifuged for 30 seconds followed by a series of washing whereby 500 µL of wash solution was added to the EZ-10 Spin Column tubes containing the samples. This was followed by centrifuging the samples at 13 400 rcf, at room temperature for 30 seconds. The process was repeated twice but in the last centrifuge it was left for one minute. Elution of genomic DNA was done by addition of 50 µL of 65°C warm Elution buffer to the column and was incubated at room temperature for 2-3 minutes. The samples were lastly centrifuged at 13 400 rcf at room temperature for two minutes and the EZ-10 Spin Column discarded. The DNA was stored at -20°C for further use.

3.6.2 DNA amplification

The ITS region was selected to be amplified for all the samples with the following primers: ITS1F 5'-CTT-GGT-CAT-TTA-GAG-GAA-GTA-A-3' as forward primer (Gardes & Bruns, 1993) and ITS4 5'-TCC-TCC-GCT-TAT-TGA-TAT-GC-3'as reverse primer (White *et al*. 1990). The process was also performed for ß-tubulin sequencing with T1 as forward primer and T22 as the reverse primer (O`Donnell and Cigelnik, 1997) for the active strains. In addition ß-tubulin sequencing for the phytopathogens was done with different primers as shown in Table 5 below. A reaction mixture was prepared containing 0.5 µL of each primer, 12.5 µL of JumpStart™ Taq ReadyMix™ (Sigma-Aldrich Chemie GmbH) and 9.5 µL of PCR pure water per sample for ITS and a modification of 8.5 µL of PCR pure water per sample for β-tubulin. The DNA amplification was done using the PCR programme in Table 6 below.

Table 5: β-tubulin primers used for amplification of the phytopathogens' DNA

S/N	Pathogen	Primers
1	*Fusarium* species	Btu-F-F01 (5'-CAGACCGGTCAGTGCGTAA-3'
		Btu-F-R01 (5'-TTGGGGTCGAACATCTGCT-3')
2.	*Pythium ultimum*	Forward primer
		BT5 (5'-GTATCATGTGCACGTACTCGG-3')
		Reverse primer
		BT6 (5'-CAAGAAAGCCTTACGACGGA-3')
3.	*Colletotrichum lindemuthianum*	T1 - Forward primer (5'AACATGCGTGAGATTGTAAGT-3')
		T22 - Reverse primer (5' - TCTGGATGTTGTTGGGAATCC-3')

Table 6: Polymerase chain reaction (PCR) program for DNA amplification

ITS Primers			
Program	**Temperature°C**	**Time (min)**	**Cycles**
1. Denaturation	94°	5	
2. Denaturation	94°	0.5	34
3. Annealing	52°	0.5	
4. Elongation	72°	1	
5. Elongation	72°	10	
β-tubulin Primers			
1. Denaturation	94°	5	
2. Denaturation	94°	0.5	38
3. Annealing	47°	0.5	
4. Elongation	72°	2.5	
5. Elongation	72°	10	

The PCR products were let to cool at 10°C and later stored at 4°C for purification. Gel electrophoresis was carried out to ascertain successful amplification of the samples whereby the DNA fragments were separated in an electric field based on their size and were compared to a molecular weight marker. The PCR products (3 µL) were mixed with 2 µL of the loading dye (midori green dye) and run on a 0.8% agarose gel in 1% TAE-buffer. For reference, 3 µL of 1kb DNA-Ladder was used which was likewise mixed with the loading dye. For the negative control a "no template control" (NTC) was mixed with pure water and also added to the gel. The electrophoresis was set to 30 minutes at 100 volts. The bands were visualized in a UV-transluminator. The PCR products were purified further with the BIO BASIC INC. EZ-10 Spin column PCR Product Purification Kit for sequencing.

3.6.3 PCR product Purification

The PCR products were purified in four main steps (Figure 3). The first step was the adjustment of the bonding conditions, whereby 110 µL of binding buffer (B3) was added to 22 µL of the PCR products and the samples mixed. The mixture was transferred to an EZ-10 Spin Column, incubated for two minutes and thereafter centrifuged at 9000 rcf for 30 seconds and the flow discarded. Wash solution (500 µL) was added to the spin column and centrifuged at 9 000 rcf for 30 seconds and the flow discarded. The process was repeated for one minute. Elution of the genomic DNA was the last step where the collection tube was replaced with clean 1.5 ml reaction tube. Then 15 µL of 65°C elution buffer was added and the mixture incubated at room temperature for two minutes. The mixture was centrifuged at 9 000 rcf for one minute followed by removal of the EZ-10 Spin Column. The samples were stored at -20°C for further use.

3.6.4 DNA sequencing

The purified DNA products were submitted to an internal sequencing service at the HZI with the same primers used for PCR amplification. Sequencing was done using DNA sequencer: Illumina genome analyser IIX, and the resulting raw data was edited with Geneious Sequence Assembler software (version R7) for generation of a consensus sequence. The sequences were upto 0.1% trimming error probability. The consensus sequences in FASTA format were used for identification of the microorganisms using the BLAST Tool (Basic Local Alignment Search

Tool) to compare the consensus sequences with published sequences of the "GenBank" (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

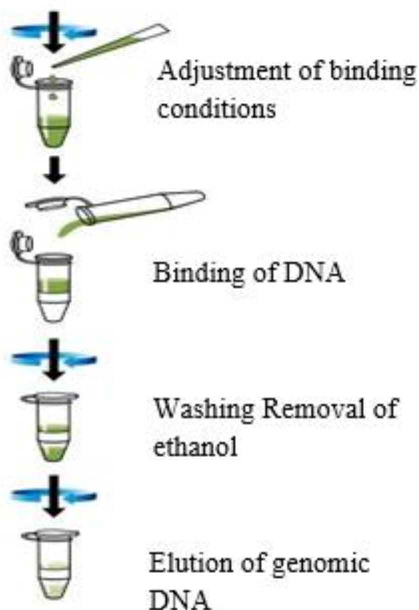


Figure 3: BIO BASIC INC.EZ-10 Spin column PCR Product Purification protocol

3.7 Data Analysis

Consensus sequences generated out of the raw data were compared to the Sequences from the BLAST programme. The tropical fungi isolates examined were considered authentic if the best hits of the BLAST search (depending on query coverage and max identity) were nearly related to the strain presumed or at least belonged to the same family. The mean mycelial radial growth data collected from inhibition zones as a result of antagonistic tropical fungi and the bacteria were subjected to analysis of variance (ANOVA) using Statistical Analysis System (SAS Institute, 2001) software. Treatment means were separated using Turkey's HSD test whenever ANOVA showed significant treatment effects.

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 Isolation of tropical fungi, phytopathogens and antagonistic *Bacillus amyloliquefaciens*

A total of 64 tropical fungi out of the 87 samples collected, two bacteria and four phytopathogens were isolated. For the tropical fungi, it was a 74% success isolation of clean pure axenic cultures. In general all the cultures grown in the dark room with temperatures maintained at 25°C showed rapid growth compared to the ones kept on clean bench at room temperature. Majority (38) of the tropical fungal isolates took 7-21 days to attain maturity while a few (10) took more than a month for the same process under the same conditions. It was observed that plates that had been amended with antibiotics and ivermectin affected the growth of the fungal strains as opposed to those without. Fungal cultures grown on PDA media at a pH of 5.6 ± 0.2 exhibited better mycelial growth compared to YMG. The growth of *B. amyloliquefaciens* isolates B1 and B5 was not affected by any change in temperature or bacterial media.

Temperature is an important factor regulating microbial activity especially growth rate of microorganisms (Pietikainen *et al*., 2005; Barcenas-Moreno *et al*., 2009; Rousk and Baath, 2011). In this study, fungal cultures maintained at temperatures of 25 °C showed faster growth in comparison to the ones left at room temperatures which fluctuated from as low as 19 °C and maximum of 23°C. The bacterial isolates however grew best irrespective of the adjustment in temperatures. These results are in agreement with Pietikainen *et al*., (2005); Rinnan *et al*., (2009) and Rajashekhar and Kaveriappa (2000) who reported that both fungal and bacterial growth had an optimum temperatures of around 25–30 °C. A shift in low temperatures could be seen to negatively affect fungal growth more compared to bacterial growth. Fluctuations in temperature which in turn affect growth rate of microorganisms has also been reported by Duarte *et al*., (2006). Some of the fungal cultures took a longer time to mature, perhaps due to the regulated environmental conditions *in vitro* as oppose to their natural habitats where variation among different plant systems in which they reside influence their growth. The influence of pH for fungal isolates was noticed. Studies have shown lower fungal growth rate at higher pH and vice-versa (Arao, 1999). In the current study, the acidic nature of the medium of pH 5.6 ± 0.2 can be one of the reasons for fast growth of fungal isolates as compared to the cultures kept in a media of pH 6.3 which is slightly alkaline. In a study by Rousk and Baath, (2011) also concluded a 30 fold increase in fungal growth rate between the pH of 4-5.

4.2 Preliminary screening of the tropical fungi against test organisms (*Bacillus subtilis* and *Mucor plumbeus*)

Overall the tropical fungi (TF) showed better antimicrobial activity against *B. subtilis* than against *M. plumbeus* in the agar diffusion assay. Eight of the isolates inhibited the growth of *B. subtilis* in contrast to only two strains that showed activity against *M. plumbeus* (Plate 3). *B. subtilis* has been tested extensively for its ability to produce antimicrobial substances (Bernal *et al*., 2002). Many fungal isolates are sensitive to *B. subtilis* or its culture filtrate which has been known to produce atleast five different antimicrobial compounds: subtillin, bacitracin, bacillin, subtenolin and bacilonycin (Killani *et al*., 2011).

Evaluation of the potential biocontrol ability of any antagonistic microorganism is important in selecting potential biocontrol agents (Yang *et al*., 2008). In this work, preliminary screening of the tropical fungi and also their extracts was carried out to determine whether they had any antimicrobial potential. *Bacillus subtilis* and *Mucor plumbeus* are known to produce spores (Ithnin, 2007; Wu *et al*., 2014) and have antagonistic properties making it easier for studies of antagonism *in vitro*, hence were chosen as test organism for this study. Similar studies have been carried out whereby *Bacillus* sp. and *Mucor* sp. have been used as test organism in biocontrol experiments (Fenice, 2010; Sathishkumar *et al*., 2012; Thenmozhi *et al*., 2013). Antimicrobial activities of the tropical fungi against the test microorganisms were qualitatively assessed by measuring zone of inhibition: agar diffusion assay (Plate 3), and minimum inhibitory concentration: serial dilution assay (Figure 4 and Plate 4).

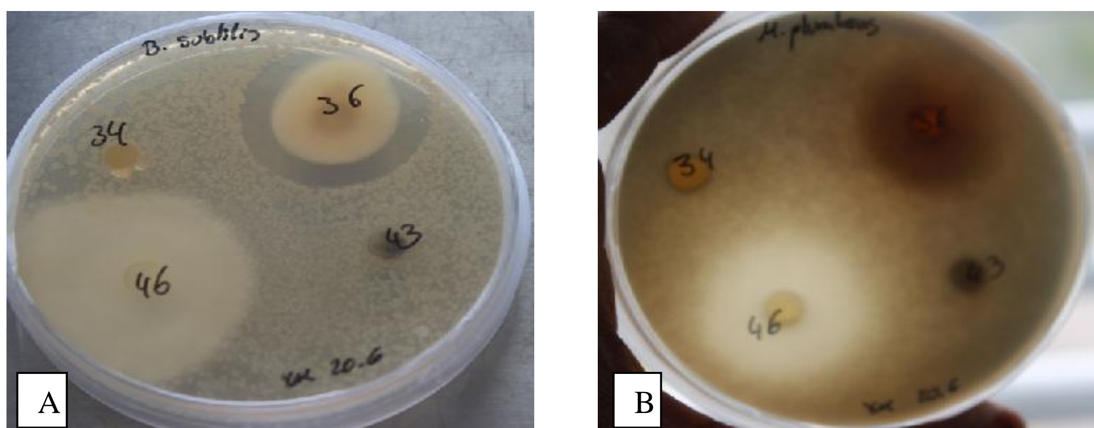
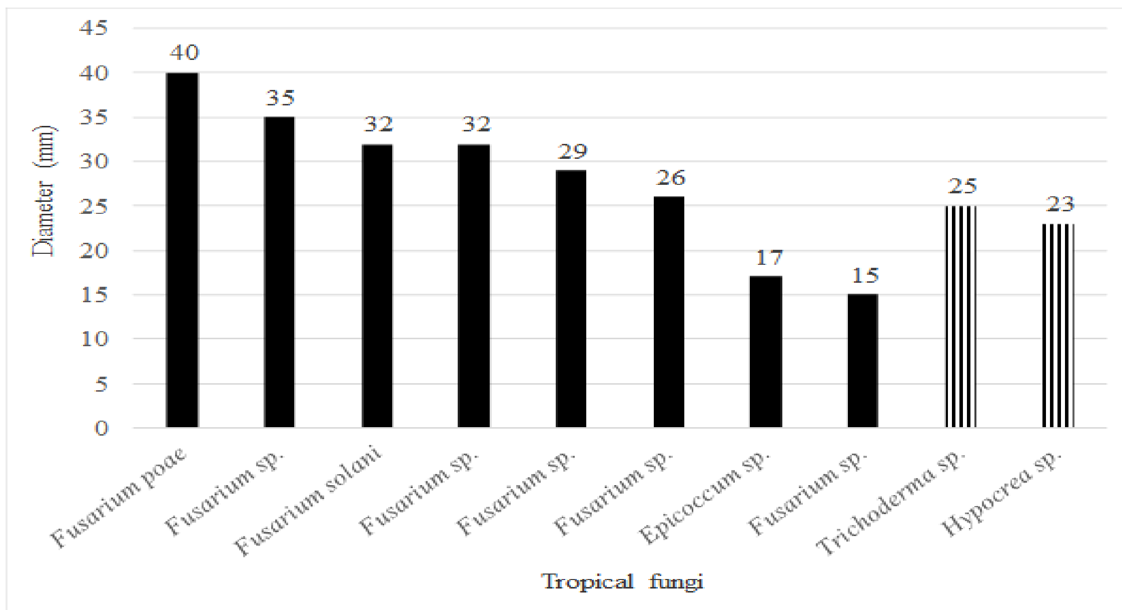


Plate 3*:* Antimicrobial activity displayed by tropical fungi against *Bacillus subtilis* (A) and *Mucor plumbeus* (B).

36 = *Fusarium solani,* 46 = *Hypocreae* sp, 34 = *Psathyrella* sp., 43 = *Daldinia* sp.

The two plates show two different tropical fungi having anti-bacterial and anti-fungal activity against *B. subtilis* and *M. plumbeus* respectively.

From the serial dilution assay results (Table 7), the MIC for the five most active tropical fungi (extracts) showed high activity against *B. subtilis* (DSM10) compared to *M. plumbeus* (MUCL 49355). Lowest MIC values (2.34 µg/ml) observed for the two test organisms and in both media were for TF59 in YMG 6.3 and TF 59 and TF 85 in ZM ½. The TF isolates incubated for eight days in YMG 6.3 media displayed more anti-microbial activity in contrast to those incubated for 11 days in ZM ½ media. Most of the TF supernatant extracts showed very high activity in both media TF 59 YMG 6.3 (2.34 µg/ml), ZM ½ (4.69 µg/ml); TF 62 ZM ½ (4.69 µg/ml). This was comparable to the low MICs value of 2.34 µg/ml in ZM ½ (TF 85) from the mycelial extracts.



KEY

- Tropical fungi active against *Bacillus subtilis*

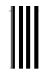
 - Tropical fungi active against *Mucor plumbeus*

Figure 4: Zones of inhibition (diameter in mm) of active tropical fungi against *Bacillus subtilis* and *Mucor plumbeus*.

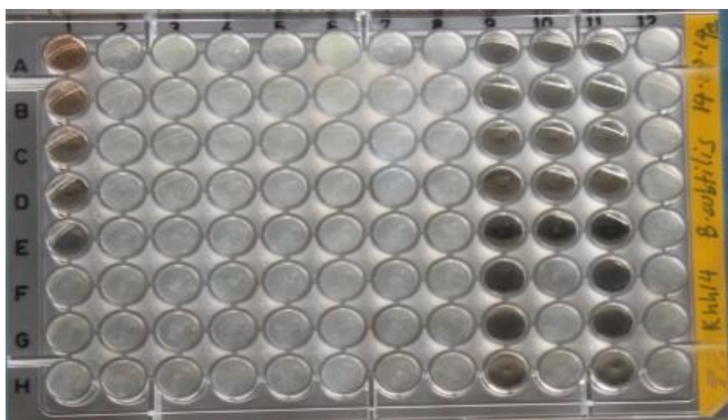


Plate 4: Serial dilution assay (MIC) of crude extracts of active tropical fungi against *Bacillus subtilis* on a 96-well plate.

The first row had a concentration of 300 µg/ml and it decreased by half in the subsequent rows thus in row H column nine the MIC of the extract was as low as 2.34 µg/ml. Column 11 and 12 were positive and negative controls respectively.

The varying degree of inhibitory effect of the tropical fungi towards the test organisms may be due to specificity of bacterial and fungal strains (Pandey *et al*., 2011). These antimicrobial activities against *B*. *subtilis* and *M*. *plumbeus* may be due to the presence of active metabolites in the fungi like those reported by Vinale *et al*. (2006), Tayung *et al*., (2011), Ramanathan *et al*. (2013), and Vinale *et al*. (2014). Variations in the MIC also could be as a result of differences in phytochemical (terpenoids, flavonoids, lactonases etc.) composition and sensitivity of microorganisms tested (Bhardwaj and Laura, 2009; Sathishkumar *et al*., 2012). Further, the presence of some antimicrobial secondary metabolites such as trichodermin (Ramanathan *et al*., 2013); chitinase and β-1, 3- glucanase (Dubey *et al*., 2011; Vipul *et al*., 2014); fumonisins, fusarins, trichothecenes and zearalenones (Desjardins and Proctor, 2007; Popiel *et al*., 2008; Ma *et al*., 2013). Epicoccoides (Talontsi *et al*., 2013) may also be explained as a factor for suppressing the colonial and mycelial growth of tested microorganism.

Table 7: Minimum Inhibitory Concentration of crude extracts of selected active tropical fungi against *Bacillus subtilis* and *Mucor plumbeus* in serial dilution assay.

Code	Name	Medium	Incubation	pH	Extracts	**MIC value** [µg/ml])	
						B. subtilis **(DSM10)**	***M. plumbeus*** **(MUCL 49355)**
TF 36	*Fusarium* sp.	YMG 6.3	8 days	3.68	S	18.75	No activity
					M	No activity	No activity
		ZM 1/2	11 days	3.49	S	No activity	No activity
					M	No activity	No activity
TF 46	*Hypocrea* sp.	YMG 6.3	8 days	4.4	S	No activity	No activity
					M	No activity	No activity
		ZM 1/2	11 days	3.08	S	No activity	No activity
					M	No activity	18.75
TF 59	*Fusarium* sp.	YMG 6.3	8 days	5.61	S	2.34	2.34
					M	18.75	No activity
		ZM 1/2	11 days	7.6	S	4.695	No activity
					M	No activity	No activity
TF 62	*Fusarium* sp.	YMG 6.3	8 days	5.27	S	18.75	No activity
					M	9.38	37.5
		ZM 1/2	11 days	7.11	S	18.75	4.69
					M	18.75	No activity
TF 85	*Epicoccum* sp.	YMG 6.3	8 days	4.78	S	150	No activity
					M	150	No activity
		ZM 1/2	11 days	7.09	S	150	No activity
					M	No activity	2.34

M-Mycelial extract; S- supernatant

4.3 Molecular characterization of the tropical fungi

The ITS rDNA region for the 64 TF isolates and four phytopathogens were successfully amplified and sequenced except for two TF isolates (STMA 14326 and 14327), providing the generated consensus sequence listed in appendix 5. In addition, this was also effectively done for the β- tubulin regions of the phytopathogens and active strains of the tropical fungi. The two isolates were not taxonomically identified because they did not sporulate well in YMG media used. In addition, because they did not show any antagonism to the test organisms no further characterization was carried out. From the scores attained from the BLAST analysis for the ITS region, 27 genera were confirmed (Appendix 4). Majority of the isolates were from the genus *Xylaria* (10), *Psathyrella* (7) and *Fusarium* (5). The isolates belonged to division Ascomycota (34) which is the largest phylum of fungi followed by division Basidiomycota (28). All the isolates belonging to Ascomycota could be divided into five classes: 22 isolates into *Sordariomycetes*, eight isolates into Ascomycetes, two isolates into *Dothideomycetes*, while *Euascomycetes* and *Pezizomycetes* both had one isolate each. The isolates belonged to five orders with the majority (21 isolates) belonging to *Xylariales* and the least (two isolates) belonging to order *Pleosporales*. For Basidiomycota, the isolates could be grouped into three classes: 26 isolates into *Agaricomycetes*, one *Basidiomycete* and one to *Dacrymycete*. The isolates belonged to four orders with the majority in *Agaricales* (23 isolates), three isolates in *Polporales* while *Auriculariales* and *Dacrycetales* each had one isolate. The generated sequences showed a high identity value and query coverage for most of the TF isolates from the database. Results of the BLAST search for β- tubulin sequences for the active isolates revealed a high identity value compared to their ITS results. The ITS and β-tubulin gel ectrophoresis of the purified genomic DNA showed close relationship among the tropical fungi isolates. The molecular weight from the ITS gel pictures ranged from 500 kbp to 800kbp as shown in Plate 5. This was in comparison with the β-tubulin amplification results (Plate 6) where their molecular weights were heavier and ranged from 1000kbp to 3000kbp. This was in accordance to the results from geneious sequence search software where the sequences lenghts ranged from 500-800 for ITS and 1000-2800bp β-tubulin respectively for the tropical fungi.

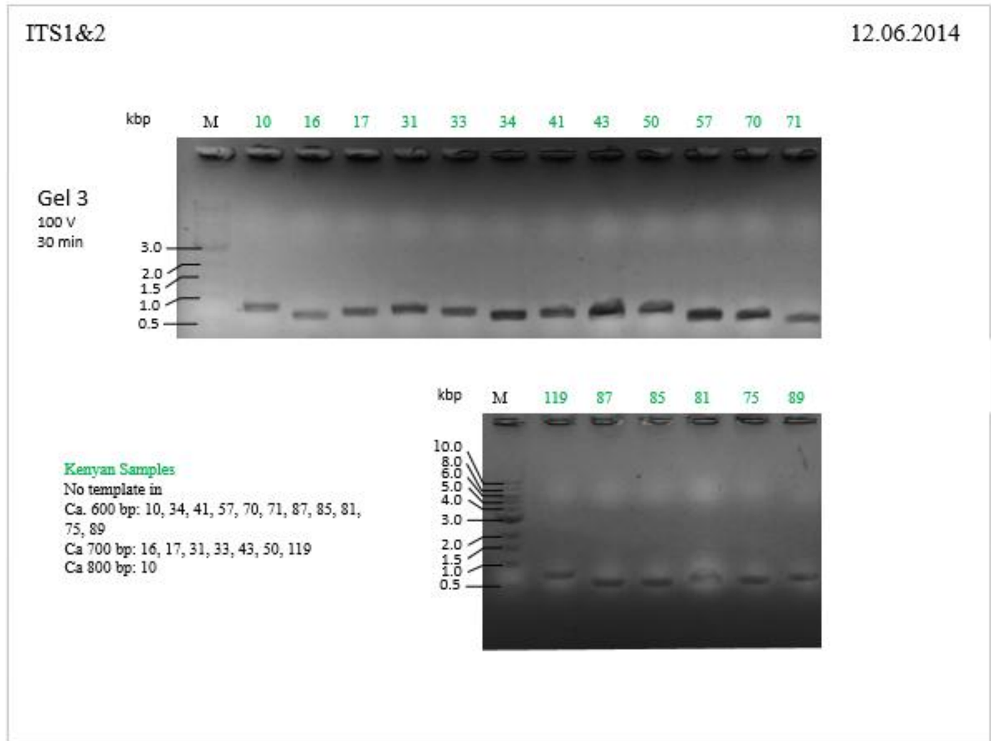


Plate 5: ITS amplification products of the tropical fungi isolates and their molecular weights

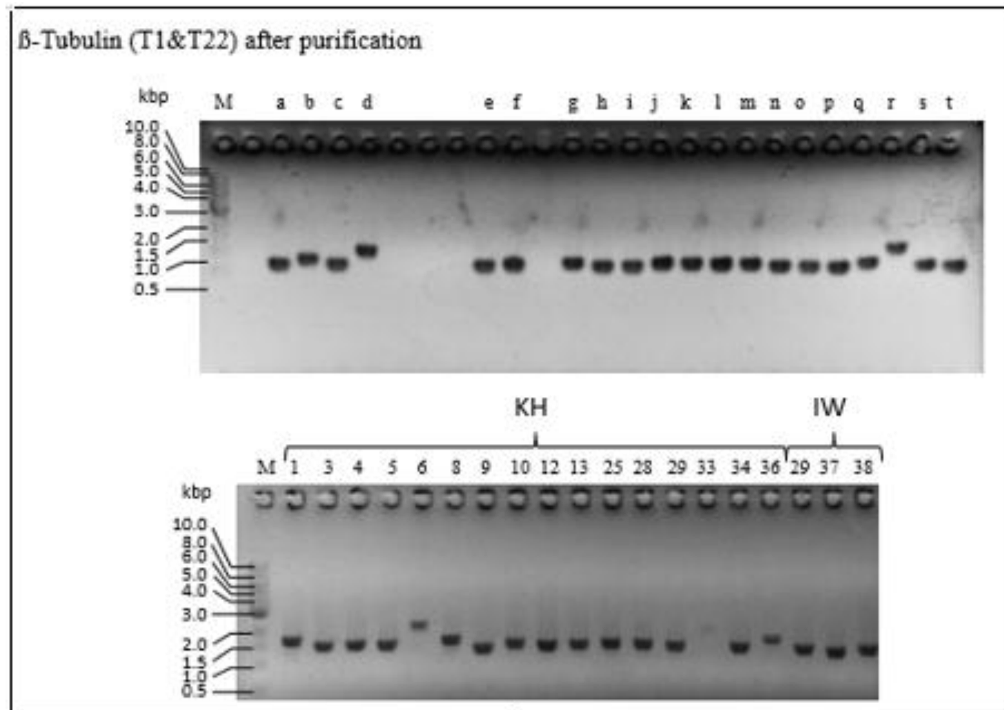


Plate 6: β-*tubulin* amplification products of tropical fungi isolates and their molecular weights

BLAST analysis revealed that the most isolated tropical fungi belonged to the family Xylariaceae. These results are in agreement with the reports that *Xylariaceous* fungi are widespread wood decomposers and are particularly common as plant endophytes in the tropics (Petrini *et al*. 1995; Rodrigues and Petrini 1997). Similar work by Crozier *et al*. (2006) also reported *Xylaria* to be the most common species among ascomycetes from a range of ecological environments including natural forest and agroforestry. *Fusarium* spp. on the other hand are ubiquitous and studies have shown them to be among the most common isolated ascomycetes in the tropics (Latiffah *et al*., 2010; Chipinga, 2012; Luo *et al*., 2014). This was likewise true in our study. It is reported that species of *Phoma*, *Phomopsis* and *Fusarium* were isolated with high frequency from dry thorn, dry deciduous and a stunted montane evergreen forest (Murali *et al*., 2013). Most of the genera from phylum Ascomycota have been isolated from the tropical forest worldwide by other researchers. For instance, *Pestalotiopsis* is one of the most commonly isolated endophytes associated with rainforests. *Pestalotiopsis* and *Phomopsis* were most frequently isolated by Cannon and Simmons (2002) in Iwokrama forest reserve. As noted by Guo *et al*. (2003) and Crozier *et al*. (2006) there are limitations to the identification of basidiomycetes with molecular data only. The current sequence data for basidiomycetes available in public databases constitute less than 10% of the known species and without the production of representative fruiting structures systematic placement is problematic (Crozier *et al*. 2006; Thomas *et al*., 2008). However, this was not the case in our study where all the basidiomycetes were identified into their respective taxa. Despite the high fungal diversity in tropical regions, in the present study all the taxa were identified into their genera or species level and no new taxa were detected.

In the present study β−tubulin gene was also used for further species identification of the 10 active tropical fungi. However, the β−tubulin gene was not conclusive in identifying the 10 active isolates of tropical fungi into their species level. This can be attributed to the small number of gene sequences deposited in databases (Fungaro, 2000; Lazarotto *et al*., 2014). Large numbers of ITS sequences deposited in databases make this region more useful in the identification of fungal species compared to β−tubulin. However, reports by Hu *et al*. (2007) and Lazarotto *et al*. (2014) suggest that a combination of both β−tubulin and ITS genes gives a better phylogenetic resolution.

4.4 Antimicrobial activity of tropical fungi against the four phytopathogens

Nine different isolates of tropical fungi inhibited the mycelial growth of *F. moniliforme* (Table 9) while one isolate (*Epicoccum* sp.) inhibited the growth of *P. ultimum* and *C. lindemuthianum*. However, none of the tropical fungal isolates inhibited *F. graminearum*. After 10 days of incubation the *Epicoccum* sp. inhibited radial growth of *P. ultimum* and *C. lindemuthianum* by 14% and 63% respectively, and at 21 days the percentage inhibition for *P. ultimum* increased to 38% while for *C. lindemuthianum* it reduced to 58%.

Table 8: Mean nhibition zones (±SE) displayed by different tropical fungi against *Fusarium moniliforme* and their percentage inhibition after 21 days

Tropical fungi (Treatment)	***Fusarium moniliforme*** **(Inhibition zone (mm))**	**Percent Inhibition (%)**	
		10days	**21 days**
Negative control	44.50±2.01[a]	-	-
Fusarium solani	34.50±3.54[ab]	63.33	63.95
Fusarium oxysporum	32±4.40[bc]	54.17	61.90
Fusarium sp.	30.50±4.97[bcd]	46.67	55.78
Fusarium sp.	28.17±2.70[bcd]	57.5	57.14
Pezizomycetes sp.	23.83±2.50[bcde]	46.67	59.18
Phomopsis sp.	20.50±2.47[cdef]	37.5	53.06
Pestalotiopsis sp.	20.50±2.47[cdef]	37.5	46.26
Epicoccum sp.	14.83±2.15[ef]	25.83	39.46
Phaeomarasmius sp.	10±1.88[f]	26.67	19.05

Means with same letter within a column are not significantly different according to Turkey's test

Results showed that among the nine bioactive tropical fungal isolates, *Phaeomarasmius* sp., and *Epicoccum* sp. proved to be the least potent bioagents against *F. moniliforme* while *Fusarium solani* and *F. oxysporum* were the most active. Growth inhibition of the pathogen differed significantly (*P*= 0.05). The percentage inhibition was generally seen to increase as the days progressed from 10 days to 21 days (Table 9). Six of the isolates could inhibit more than 50% of the mycelial growth except for *Phaeomarasmius* sp., *Epicoccum* sp. and *Pestalotiopsis* sp. Differential biocontrol ability among the nine antagonists was noticed against *F. moniliforme*. Plate 7, 8 and 9 below show different modes of action exhibited by the tropical fungi on the three phytopathogens; *F. moniliforme*, *P. ultimum* and *C. lindemuthianum*.

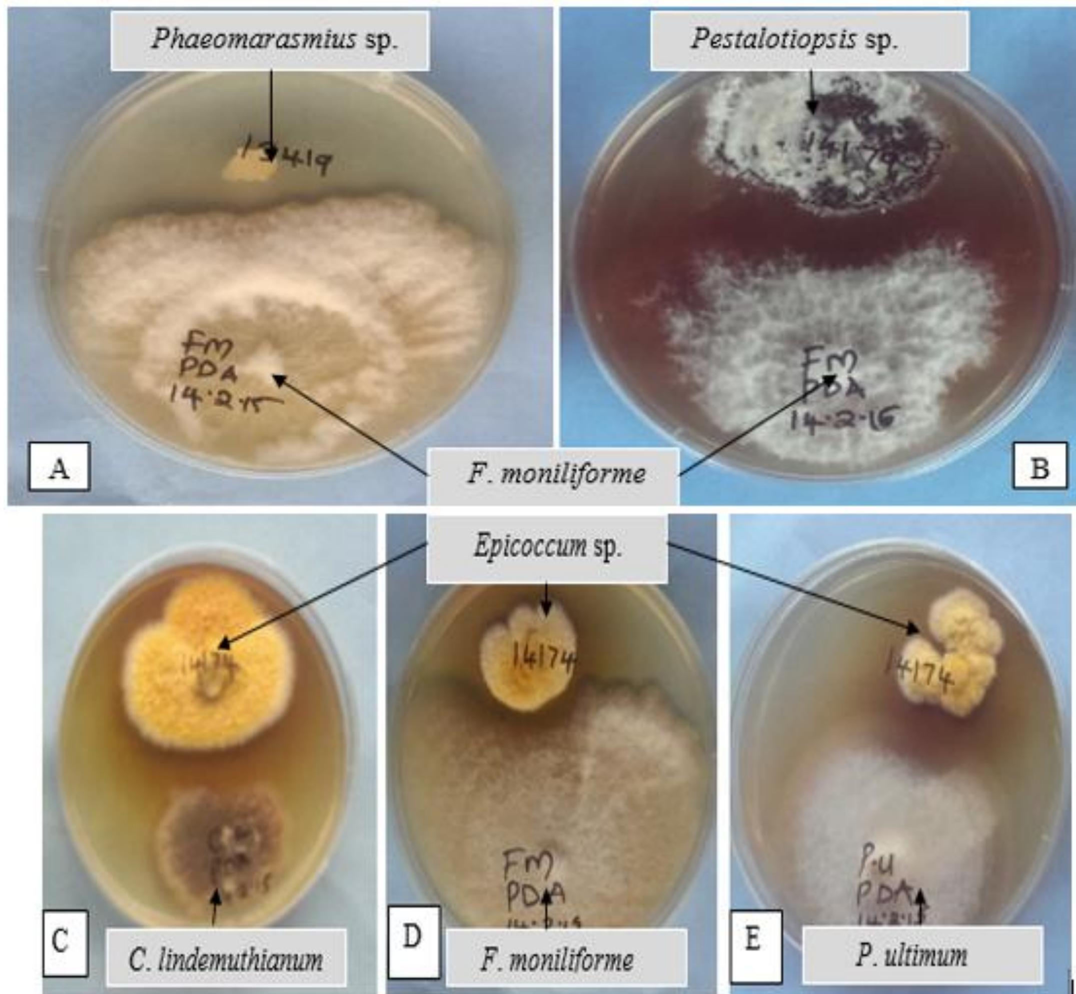


Plate 7: Different tropical fungi from Kakamega rain forest displaying antibiosis as a mechanism of inhibition against phytopathogens.

A, B and D- Tropical fungi isolates *Phaeomarasmius* sp., *Pestalotiopsis* sp. and *Epicoccum* sp. against *F. moniliforme* respectively.

C and E- *Epicoccum* sp.against *C. lindemuthianum* and *P. ultimum* respectively.

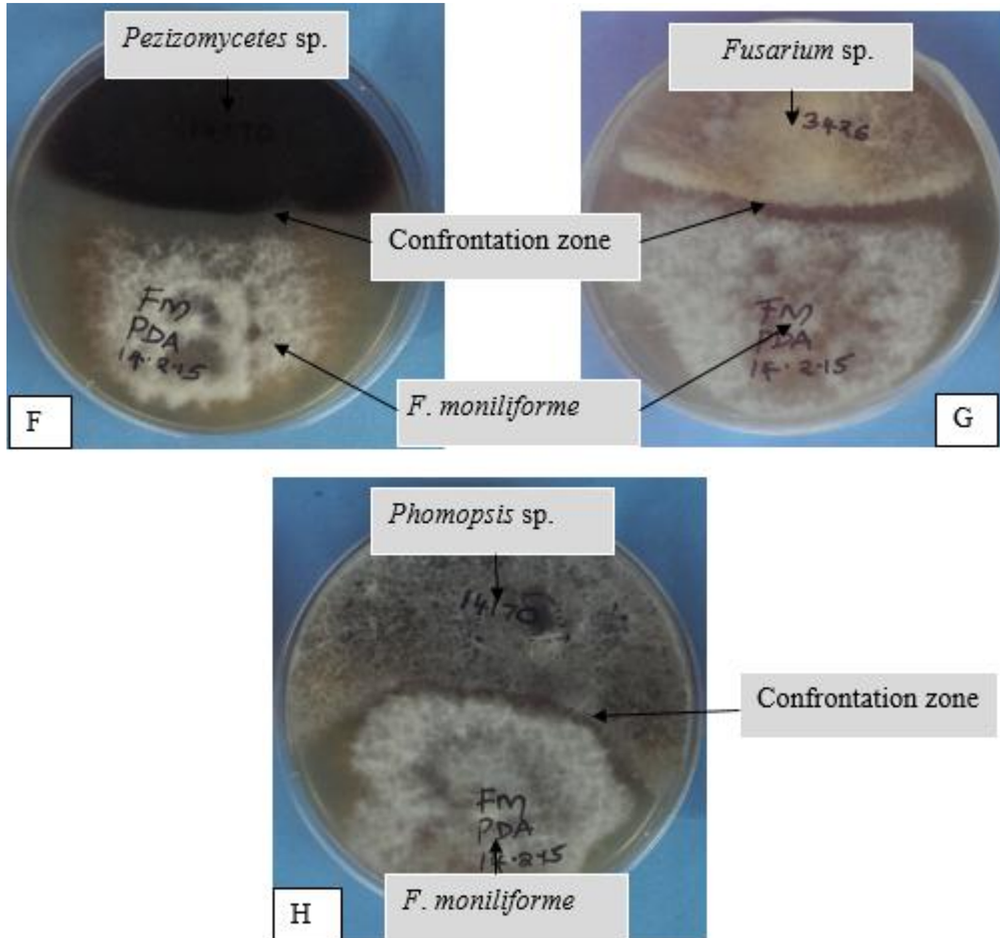


Plate 8: Dual antagonism demonstrated by different tropical fungi against *Fusarium moniliforme*

In Plate 8; F and G, both *F. moniliforme* and the tropical fungi isolate were noted to be inhibiting each other while in Plate H the tropical fungus seemed to be inhibited by the *F. moniliforme*. *Epicoccum* sp. inhibited both the growth and sporulation of *Pythium ultimum* (Plate 9). This was also evident with isolate 14327 (*Fusarium* sp.). The change of colour where both the tropical fungi isolate and pathogen almost meet could be attributed to lack of sporulation or production of chemical component responsible for inhibition. This was observed with *Epicoccum* sp. and *Fusarium* sp. as the antagonistic mechanism against *P. ultimum*.

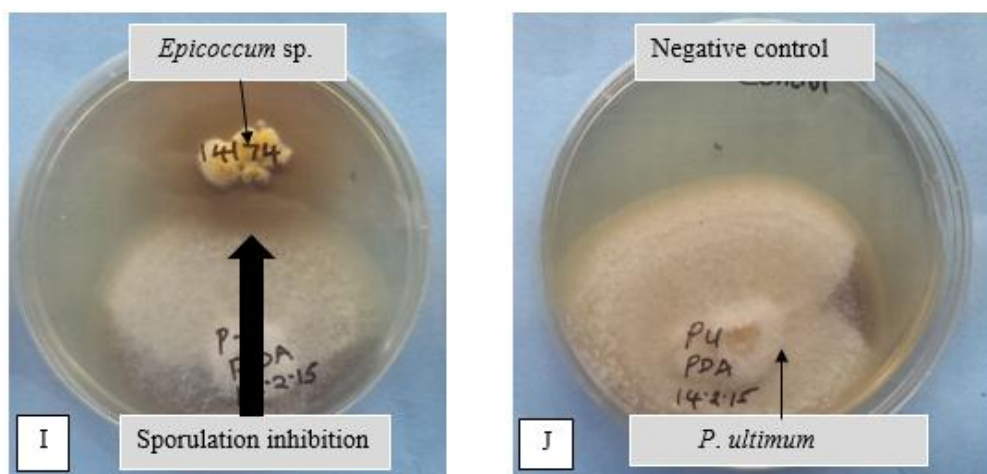


Plate 9: Tropical fungus 14174 (*Epicoccum* sp.) inhibiting sporulation of *Pythium ultimum*

I - *Pythium ultimum* inhibited by *Epicoccum* sp.

J - *Pythium ultimum* not inhibited by any tropical fungi (negative control)

Antagonistic fungal isolates have been used as bio-agents to control fungal plant pathogens. Rhizospheric, saprophytic and endophytic microoganisms are the most studied antagonists which have proven to be potent in controlling fungal plant pathogens (Schubert *et al*., 2008). Tropical fungi which include the above named types have been on the target by many researchers for the past decades due to their ability to control or suppress growth of these phytopathogens especially soil-borne and post-harvest fungal plant pathogens (Verma *et al*., 2007; Fatima *et al*., 2009; Ara *et al*., 2012). Some of the active tropical fungal isolates identified in this study have been known to exist as endophytes. For instance, isolate 14174 which was identified as *Epicoccum* sp. was effective against *F. moniliforme*, *P. ultimum* and *C. lindemuthianum*. *Epicoccum* spp. especially *E. nigrum* has been known to be an endophyte which produces active metabolites (Wang *et al*., 2014). Use of epicoccolides as antibacterial and antifungal polyketides from *Epicoccum* sp. associated with *Theobroma cacao* has been shown to have antimicrobial activity against *P. ultimum* (Talontsi *et al*., 2013). *Epicoccum* sp., *Pestalotiopsis* sp. (isolate 14179) and *Phomopsis* sp. (isolate 14170) have also been associated with antimicrobial activity against target fungal organisms (Vieira *et al*., 2014). *Pestalotiopsis* sp. has recently been reported to produce a novel compound which is antibacterial and antiyeast (Subban *et al*., 2013).

Other ascomycetes like *Trichoderma*, *Xylaria* and *Fusarium* species form the most frequent antagonistic fungi encountered (Bacon *et al*., 2001; Joseph and Priya 2011). From this current study, the named active tropical fungi isolates 14167, 14164, 13427 and 13427 we*re Fusarium* spp. Several studies involving basidiomycetes in production of bioactive compounds against microbes have been carried out and they have been proved to be effective against microorganisms (Stadler and Hoffmeister, 2015). Mushroom fruiting bodies (basidiomes) seem to be particularly talented in producing unique terpenoids, and the molecular background behind the biosynthesis of some of those compounds has only recently been elucidated (Quin *et al*., 2014).

Three antagonistic mechanisms of tropical fungi against phytopathogens were displayed in this study. Antibiotic production could have been the main mode of inhibition demonstrated by the tropical fungi isolates 14167, 14164, 14327, 14179, 14174 and 13419; identified as *F. solani*, *F. oxysporum*, *Fusarium* sp., *Pestalotiopsis* sp., *Epicoccum* sp. and *Phaeomarasmius* sp. respectively. The mechanism of antifungal antagonists could be due to the secretion of hydrolytic enzymes such as chitinase-b-3 glucanase, chitosanase, and proteases (Moreno-Perez *et al*., 2014) which degrade the fungal cell wall or the secretion of antifungal compounds (Khamna *et al*., 2009; Elamvazhuthi and Subramanian, 2013). Dual antagonism exhibited by *Phomopsis* sp., *Fusarium* sp and *Pezizomycetes* sp.; isolate 14170, 13426 and 14176 respectively was another observed mechanism of inhibition. There was a clear confrontation between the antagonist and the phytopathogen with no distinct inhibition zone (Plate 8). Competition for space and nutrients was perhaps another mode of action between the antagonist and phythopathogen: *F. moniliforme*. This is most common with Trichoderma spp. where mycoparasitism is prevalent as a mechanism of inhibition (Živković *et al*., 2010; Cuervo-Parra *et al*., 2011). Generally, either the combination of extracellular hydrolytic enzymes and secondary antifungal metabolite(s) or the secondary antifungal metabolite(s) alone can be assumed to play a major role in the inhibition of fungal growth (Prapagde*e et* al., 2008).

Inhibition of sporulation was shown by *Epicoccum* sp. (isolate 14174) against *P. ultimum* (Plate 9). A change in the mycelia colour was noticed where the antagonist was in close proximity with *P. ultimum*. Sporulation is a key component for several purposes because fungal spores are frequently used as propagules to infect plants (Rodrigues *et al*., 2009). Several studies have reported different biocontrol agents inhibiting sporulation of fungal pathogens both *in vitro*

and in field trials as a mechanism of control against phytopathogens. For instance, sporulation of *F. moniliforme* was completely inhibited by three species of *Trichoderma* and one *Penicllium* sp *in vitro (*Begum *et al*., 2015*)*. Various isolates of Trichoderma spp. have been screened against *F. oxysporum* f. sp. *lycopersici* by dual culture technique and noted to inhibit its sporulation (Sundaramoorthy and Balabaskar, 2013). Field studies have also reported success in managing fungal pathogens by inhibiting sporulation. In a farm in Panama, treatment with *C. rosea* reduced the incidence of Cacao pods with sporulating lesions of *Monillophthora roreri* by 10% (Mejía *et al.,* 2008). These examples support the potential of tropical fungi as biocontrol agents against fungal pathogens.

4.5 Antimicrobial activity of *Bacillus amyloliquefaciens* (isolate B1 and B5) against phytopathogens

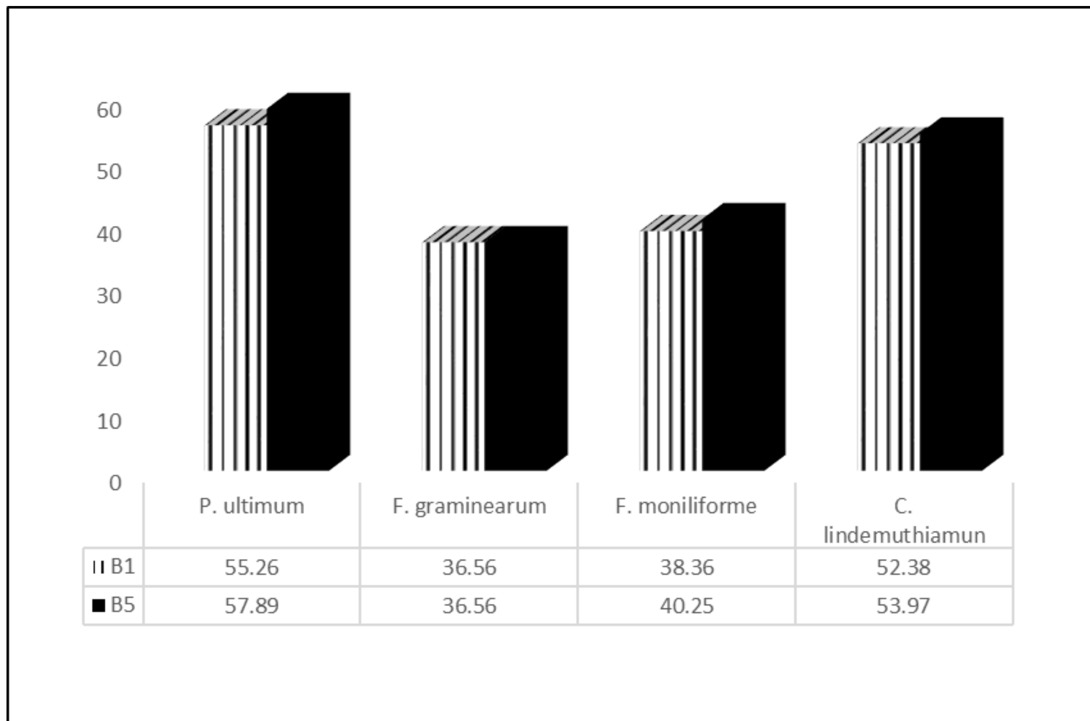
The dual culture bioassays revealed strong antagonistic activity of the *Bacillus amyloliquefaciens* isolates against *Pythium ultimum*, *Fusarium graminearum*, *Fusarium moniliforme* and *Colletotrichum lindemuthianum*. Generally, the two antagonistic isolates of *B. amyloliquefaciens* (B1 and B5) showed antagonistic activity against the four phytopathogens. However, the bacterial isolates did not differ significantly (P = 0.05) on the basis of the mean mycelial growth as shown in Table 8.

Table 9: Mean mycelial growth (mm) of antagonistic *Bacillus amyloliquefaciens* isolates B1 and B5 on the phytopathogens

Pathogen	**Treatment**		
	B1	**B5**	**Control**
Pythium ultimum	11 ± 2.25^{a}	9.44 ± 2.4^{a}	30.67 ± 2.60^{b}
Fusarium graminearum	23.22 ± 1.94^{c}	20.11 ± 1.51^{c}	47.67 ± 2.49^{d}
Fusarium moniliforme	15.11 ± 0.79^{b}	11.1 ± 0.73^{b}	38.33 ± 0.83^{c}
Colletotrichum lindemuthianum	10.11 ± 1.25^{a}	9.22 ± 1.10^{a}	20 ± 1.00^{a}

Means with same letter within a column are not significantly different at P = 0.05

Bacillus amyloliquefaciens isolate B5 produced significantly larger inhibition zones with *P. ultimum* and *C. lindemuthianum* as compared to the two *Fusarium* spp. This was also evident with B1 against the same pathogens. The percentage inhibition zone is as shown in figure 5 below.



	P. ultimum	F. graminearum	F. moniliforme	C. lindemuthiamun
B1	55.26	36.56	38.36	52.38
B5	57.89	36.56	40.25	53.97

Figure 5: Zones of inhibition (%) induced by *Bacillus amyloliquefaciens* (B1 and B5) against the phytopathogens.

Pythium ultimum and *C. lindemuthianum* had a mean percentage inhibition of above 50% as compared to the *Fusarium* species with an inhibition of between 35- 45%. From the graph above it is clear that of the two isolates of *B. amyloliquefaciens*, B5 had a slightly higher activity in contrast to B1. Figure 6 gives a summary of the results over time. As the time progressed from week one to week three the bacterial antagonists were still active and controlled further mycelia radial growth of the phytopathogens.

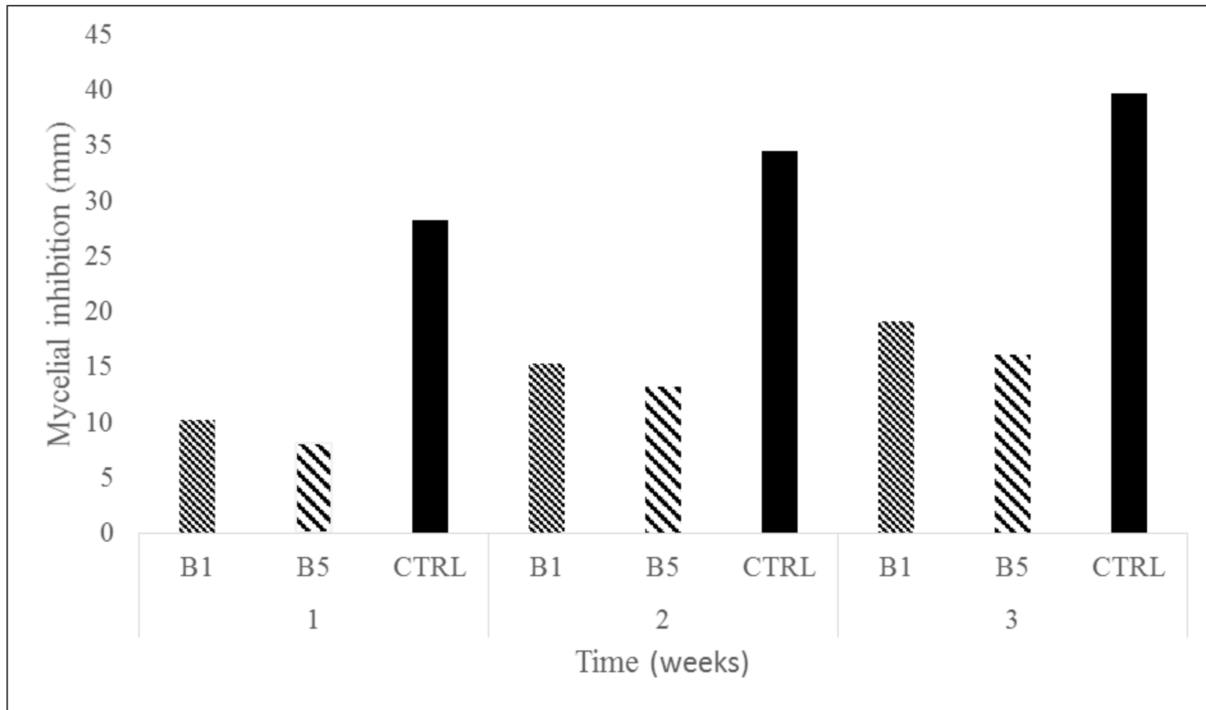


Figure 6: Effect of *Bacillus amyloliquefaciens* (B1 and B5) on mycelial growth of the phytopathogens over time

Bacteria B1 and B5 proved to be effective in controlling mycelial growth of the four phytopathogens. Different *Bacillus amyloliquefaciens* isolates have been reported to differ in their effectiveness against plant pathogens (Wu *et al*., 2014). Different species of the same family and different strains of the same species often can produce significantly different compounds which suggest that secondary metabolites express the individuality of species in chemical terms (Sivasithamparam and Ghisalberti, 1998; Sharfuddin and Mohanka, 2012). *Bacillus* spp. like *B. amyloliquefaciens* have been reported widely to be effective antagonist due to their ability to produce a wide arsenal of antimicrobial substances both *in vitro* and *in vivo* inhibitory to phytopathogens like lipopeptide and macrolactins, volatile compounds and hydrolytic enzymes (Abriouel *et al*., 2011; Seema and Devaki, 2012). For example, the application of *B. amyloliquefaciens* strain NJZJSB3 inhibited mycelial growth of *Sclerotina sclerotiorum*, a causal agent of Canola stem rot (Wu *et al*., 2014). *Bacillus amyloliquefaciens* strain PPCB004 was used as a biocontrol agent to control post-harvest fungal pathogens (Arrebola *et al*., 2010). Other strains of *B. amyloliquefaciens* producing inturin A have also been reported to suppress growth of post-harvest pathogens of fruit (Yoshida *et al*., 2001; Yu *et al*.,

2002). According to Arrebola *et al*. (2010) among the lipopeptides produced by *B. amyloliquefaciens* Inturin A is the principal inhibitor in the biocontrol activity against fungal pathogens.

The growth inhibition of *B. amyloliquefaciens* against the four phytopathogens was almost comparable except for *F. graminearum* which was significantly higher. *Fusarium* spp. have been known to be ubiquitous and having a high growth rate under optimum temeperatures (Martínez-Medina *et al*., 2014) as compared to the other two phytopathogens. *Colletotrichum lindemuthianum* actually is known for its slow growth (Liu *et al*., 2013) and perhaps that was the reason for high percentage mean inhibition compared to the other phytopathogens. The strong inhibitory activity of *B. amyloliquefaciens* isolate B1 and B5 on all the fungal phytopathogens by suppressing the mycelial growth was perhaps through production of lipopeptides, antibiotics or hydrolytic enzymes like protease, glucanase and chitinase which are normally produced by biocontrol agents to degrade fungal pathogen cell wall (Fernando *et al*., 2005; Grosu *et al*., 2014).

5.0 CONCLUSIONS AND RECOMMENDATIONS

5.1 CONCLUSIONS

1. The collected tropical fungi were effectively characterized into 62 species based on their internal transcribed spacer region and β-tubulin thus demonstrated a rich diversity of fungi in the Kakamega forest. Among the species included *Epicoccum* sp., *Fusarium* sp., and *Phaeomarasmius* sp. known to have antimicrobial properties.
2. Nine of the collected tropical fungi; *Fusarium solani*, *F. oxysporum*, two unidentified *Fusarium* spp., *Pestalotiopsis* sp., *Epicoccum* sp., *Phaeomarasmius* sp., *Phomopsis* sp. and *Pezizomycetes* sp, had antimicrobial activity against three fungal phytopathogens; *Fusarium moniliforme*, *Colletotrichum lindemuthianum* and *Pythium ultimum*. Moreover, the two *Bacillus amyloliquefaciens* isolates; B1 and B5 were effective in controlling the mycelial growth of all the four phytopathogens. Consequently, indicating that the tropical fungi and antagonistic bacteria are potential antifungal agents that can be used to manage plant diseases.

5.2 RECOMMENDATIONS

1. The antagonistic fungi and bacterial should be formulated and tested against the four pathogens and other phytopathogens *in vivo* under greenhouse and field conditions to determine their potential in management of the plant diseases.
2. More studies should be conducted to determine the active compounds within the antagonistic fungal and bacterial isolates inorder to enhance production of more efficient pesticides.
3. It is important to understand the mechanism by which these antagonists inhibit the pathogens and thereby shed light on alternative mitigation measures for many other stubborn pathogens that destroy crops.

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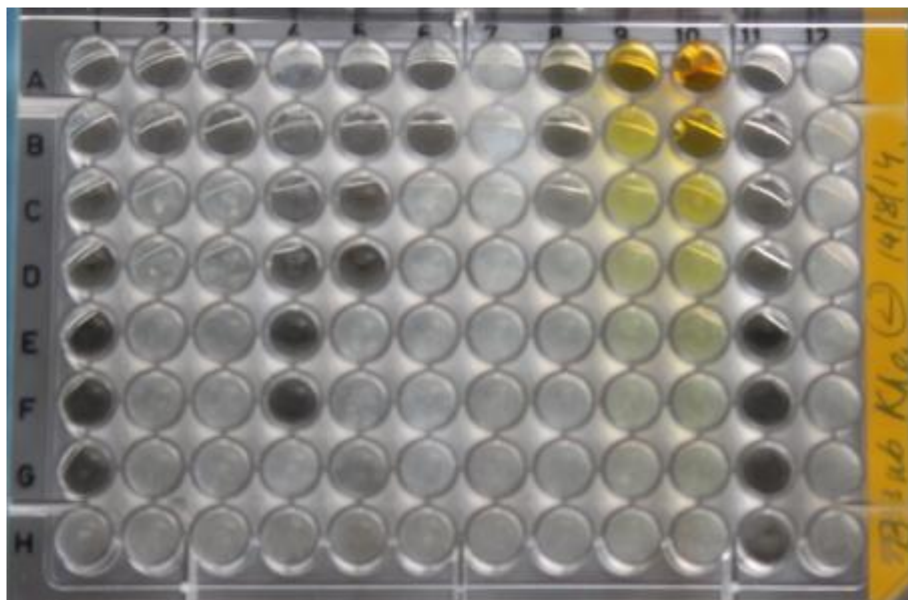
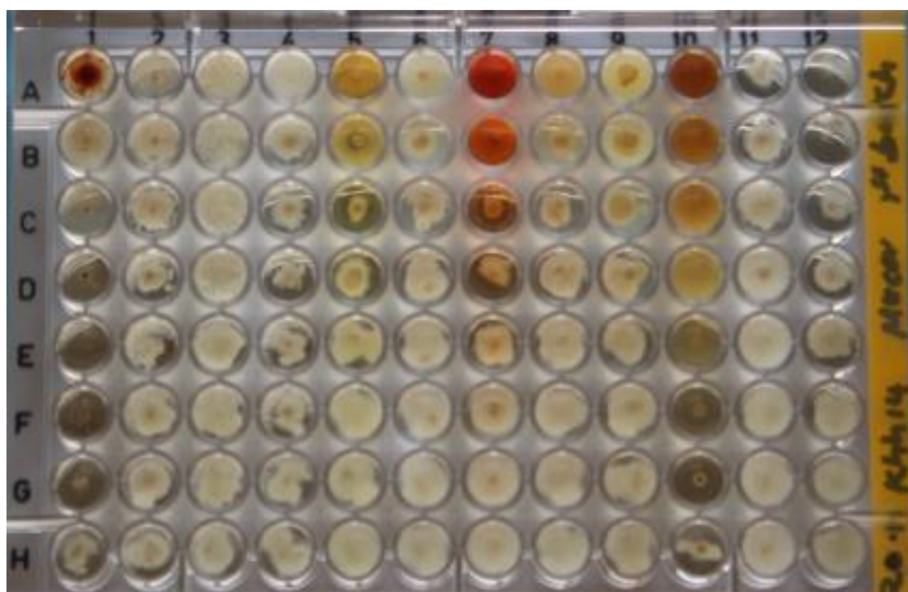
APPENDICES

Appendix 1: Agar diffusion assay of active tropical fungi against *Bacillus subtilis*

Appendix 2: Agar diffusion assay of active tropical fungi against *Mucor plumbeus*

Appendix 3: Serial dilution assay (MIC) of crude extracts of active tropical fungi against *Bacillus subtilis* and *Mucor plumbeus* on a 96- well plate.





Appendix 4: Internal transcribed spacer region (ITS) BLAST results of the tropical fungi

Fungal isolate	Genus name	Species name	family	order	class	division
TF31	Psathyrella	sp.	psathyrellaceae	agaricales	agaricomycetes	basidiomycota
	Clitocybe	sp.	tricholomataceae	agaricales	agaricomycetes	basidiomycota
	Resupinatus	sp.	tricholomataceae	agaricales	agaricomycetes	basidiomycota
	Psathyrella	sp.	psathyrellaceae	agaricales	agaricomycetes	basidiomycota
	Panellus	sp.	mycenaceae	agaricales	agaricomycetes	basidiomycota
	Mycena	sp.	mycenaceae	agaricales	agaricomycetes	basidiomycota
TF32	Psathyrella	sp.	psathyrellaceae	agaricales	agaricomycetes	basidiomycota
TF17	Polyporus	sp.	polyporaceae	polyporales	agaricomycetes	basidiomycota
	Phaeomarasmius	sp.	inocybaceae	agaricales	basidiomycetes	basidiomycota
TF72	Daldinia	eschscholtzii	xylariaceae	xylariales	ascomycetes	ascomycota
	Calocera	sp.	dacrymyceteceae	dacrymycetales	dacrymycete	basidiomycota
TF33	Coprinellus	sp.	psathyrellaceae	agaricales	agaricomycetes	basidiomycota
	Marasmius	sp.	marasmiaceae	agaricales	agaricomycetes	basidiomycota
	Clitopilus	sp.	entolomataceae	agaricales	agaricomycetes	basidiomycota
TF2	Fusarium	sp.	nectriaceae	hypocreales	sordariomycetes	ascomycota
	Pleurotus	djamar strain	pleurotaceae	agaricales	agaricomycetes	basidiomycota
	Psathyrella	sp.	psathyrellaceae	agaricales	agaricomycetes	basidiomycota
	Mycena	sp.	mycenaceae	agaricales	agariacales	basidiomycota
	Polyporus	sp.	polyporaceae	polyporales	agaricomycetes	basidiomycota

Fungal isolate	**Genus name**	**Species name**	**family**	**order**	**class**	**division**
	Panellus	sp.	mycenaceae	agaricales	agaricomycetes	basidiomycota
	Auricularia	polytricha	auriculariaceae	auriculariales	agaricomycetes	basidiomycota
	Polyporus	sp.	polyporaceae	polyporales	agaricomycetes	basidiomycota
	Pleurotus	djamar strain	pleurotaceae	agaricales	agaricomycetes	basidiomycota
	Pleurotus	djamar strain	pleurotaceae	agaricales	agaricomycetes	basidiomycota
	Pleurotus	djamar strain	pleurotaceae	agaricales	agaricomycetes	basidiomycota
	Pleurotus	djamar strain	pleurotaceae	agaricales	agaricomycetes	basidiomycota
	Daldinia	eschscholtzii	xylariaceae	xylariales	ascomycetes	ascomycota
	Daldinia	eschscholtzii	xylariaceae	xylariales	ascomycetes	ascomycota
	Daldinia	childiae	xylariaceae	xylariales	ascomycetes	ascomycota
	Psathyrella	sp.	psathyrellaceae	agaricales	agaricomycetes	basidiomycota
	Xylaria	schweinitzii	xylariaceae	xylariales	sordariomycetes	ascomycota
TF1	Fusarium	poae	nectriaceae	hypocreales	sordariomycetes	ascomycota
TF83	Fusarium	oxysporum	nectriaceae	hypocreales	sordariomycetes	ascomycota
TF68	Fusarium	oxysporum	nectriaceae	hypocreales	sordariomycetes	ascomycota
TF75	Fusarium	solani	nectriaceae	hypocreales	sordariomycetes	ascomycota
TF70	Fusarium	solani	nectriaceae	hypocreales	sordariomycetes	ascomycota
TF19	Psathyrella	sp.	psathyrellaceae	agariacales	agaricomycetes	basidiomycota
TF22	Psathyrella	sp.	psathyrellaceae	agaricales	agaricomycetes	basidiomycota
TF16	Phomopsis	sp.	diaporthaceae	diaporthales	sordariomycyetes	ascomycota

Fungal isolate	Genus name	Species name	family	order	class	division
TF56	Trichoderma	sp.	hypocreaceae	hypocreales	sordariomycyetes	ascomycota
TF43	Eutypella	sp.	diatrypaceae	xylariales	ascomycetes	ascomycota
TF59	Fusarium	sp.	nectriaceae	hypocreales	sordariomycetes	ascomycota
TF85	Epicoccum	sp.	incertae sedis	incertae sedis	ascomycetes	ascomycota
TF57	Bionectria	sp.	bionectriaceae	hypocreales	sordariomyectes	ascomycota
TF61	Pezizomycetes	sp.			pezizomycetes	ascomycota
TF81	Pleosporales	sp.		pleosporales	dothideomycetes	ascomycota
TF50	Clitopilus	sp.	entolomataceae	agaricales	agaricomycetes	basidiomycota
TF15	Pestalotiopsis	sp.	amphisohaeriaceae	xylariales	sordariomycetes	ascomycota
	Tremalia	sp.				
	Fusarium	moniliforme	nectriaceae	hypocreales	sordariomycetes	ascomycota
	Fusarium	graminearum	nectriaceae	hypocreales	sordariomycetes	ascomycota
	Epicoccum	nigrum	incertae sedis	incertae sedis	ascomycetes	ascomycota
	Colletotrichum	lindemuthianum	glomerellaceae	glomerellales	sordariomycetes	ascomycota
	Pythium	ultimum	pythiaceae	pythiales	oomycota	
TF10	Not characterised					
TF36	Not characterised					
X11	Xylaria	sp.	xylariaceae	xylariales	sordariomycetes	ascomycota
X11	Xylaria	sp.	xylariaceae	xylariales	sordariomycetes	ascomycota
X7	Xylaria	sp.	xylariaceae	xylariales	sordariomycetes	ascomycota

Fungal isolate	Genus name	Species name	family	order	class	division
X2	Xylaria	sp.	xylariaceae	xylariales	sordariomycetes	ascomycota
X16	Xylaria	sp.	xylariaceae	xylariales	sordariomycetes	ascomycota
X14	Xylaria	sp.	xylariaceae	xylariales	sordariomycetes	ascomycota
X8	Xylaria	sp.	xylariaceae	xylariales	sordariomycetes	ascomycota
X3	Xylaria	sp.	xylariaceae	xylariales	sordariomycetes	ascomycota
X17	Xylaria	sp.	xylariaceae	xylariales	sordariomycetes	ascomycota
	Epicoccum	nigrum	incertae sedis	incertae sedis	ascomycetes	ascomycota
	Phoma	sp.	incertae sedis	pleosporales	dothideomycetes	ascomycota
X4	Hypoxylon	sp.	xylariaceae	xylariales	euascomycotina	ascomycota

Appendix 5: Consensus sequences of bioactive tropical fungi

Fungus code and Name	**Sequence in base pair**	**QUERY COVERAGE**	**IDENTITY**	**ERROR.VA**
TF 15 *Pestalotiopsis* sp.	GAGGAAGTAAAAGTCGTAACAAGGTCTCCGTTGGTGAACCAGCGGA GGGATCATTATAGAGTTTTCTAAACTCCCAACCCATGTGAACTTACC TTTTGTTGCCTCGGCAGAAGTTATAGGTCTTCTTATAGCTGCTGCCGG TGGACCATTAAACTCTTGTTATTTTATGTAATCTGAGCGTCTTATTTT AATAAGTCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGAT GAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCA GTGAATCATCGAATCTTTGAACGCACATTGCGCCCATTAGTATTCTA GTGGGCATGCCTGTTCGAGCGTCATTTCAACCCTTAAGCCTAGCTTA GTGTTGGGAATCTACTTCTCTTAGGAGTTGTAGTTCCTGAAATACAA CGGCGGATTTGTAGTATCCTCTGAGCGTAGTAATTTTTTTCTCGCTTT TGTTAGGTGCTATAACTCCCAGCCGCTAAACCCCCAATTTTTTGTGG TTGACCTCGGATCAGGTAGGAATACCCGCTGAACTTAAGCATAT **562kb**	99-100	100	0.0
TF 61 *Pezizzomycete* sp.	GGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAG GATCATTAACTAAATCGAGACGGGCCCTTCGGGGTCCGGCCCGTAC AAACCCTCTGCGTACCCGTACCTTTGTTGCTTCCCGTCGGGGCCCTTC GGCTCCCGGCGGGGAGGTCTACCAAAACTCCTGTCTTTGCATGCAGT CTGTAGATGGCGGCCAGTCCGCGACAATTAAAAGTTAAAACTTTCA ACAACGGATCTCTTGGTTCTCGCATCGATGAAGAACGCAGCGAAAT GCGATAAGTAGTGTGAATTGCAGAATTCAGTGAATCATCGAATCTTT GAACGCACATTGCGCCCTCTGGTATTCCGGAGGGCATGCCTGTCCGA GCGTCGTCAAACCCCCTCAAGCGAGCTTTTGCTTGGTCATGGCCGGA GATCGTCCCTCTGCGGGCGTTCTCGGCTGAAAGGGATCTGGCGGAG AGCCTGGTCTCCACGGACGTAGTAAGCTTTTTGCCTATCGTCCTGTG GTAAGGCCAGTTATCCAGCCGTCGACCTCAATTATTTCTGGTTGACC TCGGATCAGGTAGGGATACCCGCTGAACTTAAGCATAT **598kb**	86-93\97-98	90\87-88	0.0
TF 83 *Fusarium oxysporum.*	TAGAGGAAGTAAAAGTCGTAACAAGGTCTCCGTTGGTGAACCAGCG GAGGGATCATTACCGAGTTTACAACTCCCAAACCCCTGTGAACATAC CACTTGTTGCCTCGGCGGATCAGCCCGCTCCCGGTAAAACGGGACG GCCCGCCAGAGGACCCCTAAACTCTGTTTCTATATGTAACTTCTGAG TAAAACCATAAATAAATCAAAACTTTCAACAACGGATCTCTTGGTTC TGGCATCGATGAAGAACGCAGCAAAATGCGATAAGTAATGTGAATT	100	100	0.0

	GCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGC CAGTATTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAA GCACAGCTTGGTGTTGGGACTCGCGTTAATTCGCGTTCCTCAAATTG ATTGGCGGTCACGTCGAGCTTCCATAGCGTAGTAGTAAAACCCTCGT TACTGGTAATCGTCGCGGCCACGCCGTTAAACCCCAACTTCTGAATG TTGACCTCGGATCAGGTAGGAATACCCGCTGAACTTAAGCATATC **559kb**			
TF 2 *Fusarium solani*.	AGGAAGTAAAAGTCGTAACAAGGTCTCCGTTGGTGAACCAGCGGAG GGATCATTACCGAGTTTACAACTCCCAAACCCCTGTGAACATACCTA TACGTTGCCTCGGCGGATCAGCCCGCGCCCGGTAAAACGGGACGGC CCGCCCGAGGACCCTAAACTCTGTTTTTAGTGGAACTTCTGAGTAAA ACAAACAAATAAATCAAAACTTTCAACAACGGATCTCTTGGTTCTGG CATCGATGAAGAACGCAGCAAAATGCGATAAGTAATGTGAATTGCA GAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAG TATTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCT CAGCTTGGTGTTGGGACTCGCGGTAACCCGCGTTCCCCAAATCGATT GGCGGTCACGTCGAGCTTCCATAGCGTAGTAATCATACACCTCGTTA CTGGTAATCGTCGCGGCCACGCCGTAAAACCCCAACTTCTGAATGTT GACCTCGGATCAGGTAGGAATACCCGCTGAACTTAAGCATATC **557kb**	100	99	0.0
Fusarium sp	TTAAGTTCAGCGGGTAGTCCTACCTGATTTGAGGTCAAATTGGTCAA GTAGATTGTCCTTGCGGACGGTTAGAAGCAAGCACGAGTCCAATCC ACGGCATAGATATTATCACACCAATAGACGGAAGCTCAGTATAAGC TCGCTAATGCATTTCAGGGAAGCAGACCAGCACTGAGGCAGCCTGC AAAACCCCCACATCCAAGCCTTCACCTGTCTCGTTACAAAACTGGTG AGGTTGAGAATTTAATGACACTCAAACAGGCATGCTCCTCGGAATA CCAAGGAGCGCAAGGTGCGTTCAAAGATTCGATGATTCACTGAATT CTGCAATTCACATTACTTATCGCATTTCGCTGCGTTCTTCATCGATGC GAGAGCCAAGAGATCCGTTGCTGAAAGTTGTATAGTGTTTTATAGGC ATGAAAGCCCATTGATTACATTCTACATTATTCAAATGGAGTGTGTA AAAGACATAGAACCTGGAAATTCAAAGAGAGCCGGCCTTATCGACA CAGCAATCCTTGCATCCGCTTCGTTACCAAAGCGAGAGGTATCCAGG CCTACACATAGTTCACAGGTGGAAAGATGATATGAATGACGGGCGT GCACAAATGCTCCTAGGAGCCAGCTACAACCAACGCCATAGATATT CGTTAATGATCCTTCCGCAGGTTCACCTACGGAAACCTTGTTACGAC TTTTACTTC **707kb**	100	98.3	0.0
Epicoccum sp	AAAGTGTCCACNGAGGGACGGCTGTAAGCAGCACCGCTGAAGAGGC CTAAGGCATTGGCGCAGATAATTATCACACCGTCGCCCAGCACTCTA AAAGCGCCAGCTAATGCATTTCAAGACGAGCCGGNCNCGGCACAGT CCAAGTCCACCGCGAGCGACTGTTACATCGCAAGGGTGAGGGTTTA	100	93.3	0.0

	CGTGACACTCAAACAGGCATGCTCCATGGAATACCAAGGAGCGCAA GATGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCACATT ACTTATCGCATTTCGCTGCGTTCTTCATCGATGCGAGAGCCAAGAGA TCCGTTGTTGAAAGTTGTTACTTTTTTTATGGTTTTGTTAACATTCGA GACTGAGTTGTTGCATTTGAAAGCGGCAGCGACCGAAGCCGCAACC GAAAAGGTGCACAGGTGTGGGGTCTTGCTCCAGCGTGCAGCCCGGT GAAGGGCGCACAGCTGAACGATCGGGTTAAAAGCCCAAAATCTTTA ATG **536kb**			
NO 5 HQ % 98.8 *Phaeomarasmius* sp	AGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAA GGATCATTATCGAGTTCTTGAAACGGGTTGTAGCTGGCCTTACGAAG GCATGTGCACGCCCTGCTCAATCCACTCTACACCTGTGCACTTACTG TGGGTCTTCGAGCAAAGGGGGTTTATAATTTCTTATAAGCCTTCGTT TGAGGCCCACGTTTACACACAAACACTATTAAGTAAAAGAATGTGT ATTGCGATGTAACGCATCCATAATACAACTTTCAGCAACGGATCTCT TGGCTCTCGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATG TGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACCTTGC GCTCCTTGGTATTCCGAGGAGCACGCCTGTTTGAGTGTCGTGTAATT CTCAAACTTACCCATCTTTGCGGATGGTGTAAGGCTTGGATGTTGGA GGTCTATTGTCGGTTTGTAATGAACCGGCTCCTCTTAAATGCATTAG CTCAGTCCTTTGTGGATCGGCTCCCAGTGTGATAATTATCTGCGCTG CGACCGTGAAGCGTTTTGTTATGGCGAGCTTCTAACGGTCTCTTCAA TGAGACAAGCAACACTTTGACAATCTGACCTCAAATCAGGCGGGAC TACCCGCTGAACTTAAGCATATCANT **680kb**	100	98.8	0.0
No 16 HQ % 74.9 *Fusarium* sp.	GATATGCTTAAGTTCAGCGGGTATTCCTACCTGATCCGAGGTCAACA TTCAGAAGTTGGGGTTTAACGGCGTGGCCGCGACGATTACCAGTAA CGATGTGTAAATTACTACGCTATGGAAGCTCGACGTGACCGCCAATC AATTTGGGGAATGCGAATTAACGCAAGTCCCAACACCAAGCTGGGC TTGAGGGTTGAAATGACGCTCGAACAGGCATGCCCGCCAGAATACT GGCGGGCGCAATGTGCGTTCAAAGATTCGATGATTCACTGAATTCTG CAATTCACATTACTTATCGCATTTTGCTGCGTTCTTCATCGATGCCAG AACCAAGAGATCCGTTGTTGAAAGTTTTGATTTATTTGTTTTTTTACT CAGAAGTTCCACTAAAAACAGAGTTTAGGGTCCTGCGGCGGGCCGT CCCGAAGGACGGGCTGATCCGCCGAGGCAACATATGGTATGTTCAC AGGGGTTTGGGAGTTGTAAACTCGGTAATGATCCCTCCGCTGGTTCA CCAACGGAGACCTTGTTACGACTTTTACTTCCTCTATTNNNNNAAGA NNNGGGNNATCCNNATCTNCNCNTANTTCACANGTGNAAAGATGAT ANNNNTNNCGGGTGTGCNCNNTGCTCCTNNNNNNCCNNCTACNNCCN NCNCCATANATATTCNTTAATGATCCTTCCGCACGTTCACCTACNGA AACCN **705kb**	100	74.9	0.0

No 18 HQ % 100.0 *Phomopsis* sp	AAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCAT TATCGAGTTTTGAAAGGGGGTTGTAGCTGGCCTTTACGAGGCATTGT GCACGCCCCGCTCAATCCACTCTACACCTGTGAACTAACTGTGGGTC TTTCGGGAGGGCTTTGTTTAAAGCCCTTGGAGAGCTCATGTTTACTTT ACAAACACTTATAAAGTAACGGAATGTGTGTTGCGATGTAACGCAT CTATATACAACTTTCAGCAACGGATCTCTTGGCTCTCGCATCGATGA AGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGT GAATCATCGAATCTTTGAACGCACCTTGCGCTCCTTGGTATTCCGAG GAGCACGCCTGTTTGAGTGTCATGTAATTCTCAACCTACGAGTCCTT GTTGACTTCGTTAGGCTTGGATATTGGAGGATCTAATTGTCGGCTCG CATGAGTCGGCTCCTCTCAAATGCATTAGCTTGGTCCTTTGCGGATC GGCTCTCGGTGTGATAAGTTTGTCTATACCGTGACCGTGAAGCACTT GTTGGGAAAGAGCTTCTAGTGGTCTCTTTATTTGAGACAATGTACTT CTTGACATCTGACCTCAAATCAGGCGGGACTACCCGCTGAACTTAA **655kb**	100	100	0.0
Fusarium sp.	GATATGCTTAAGTTCAGCGGGTATTCCTASSTGATCCGAGGTCAACA TTCAGAAGTTGGGGTTTAACGGCGTGGCCGCGACGATTACCAGTAA CGATGTGTAAATTACTACGCTATGGAAGCTCGACGTGACCGCCAATC AATTTGGGGAATGCGAATTAACGCAAGTCCCAACACCAAGCTGGGC TTGAGGGTTGAAATGACGCTCGAACAGGCATGCCCGCCAGAATACT GGCGGGCGCAATGTGCGTTCAAAGATTCGATGATTCACTGAATTCTG CAATTCACATTACTTATCGCATTTTGCTGCGTTCTTCATCGATGCCAG AACCAAGAGATCCGTTGTTGAAAGTTTTGATTTATTTGTTTTTTTACT CAGAAGTTCCACTAAAAACAGAGTTTAGGGTCCTGCGGCGGGCCGT CCCGAAGGACGGGCTGATCCGCCGAGGCAACATATGGTATGTTCAC AGGGGTTTGGGAGTTGTAAACTCGGTAATGATCCCTCCGCTGGTTCA CCAACGGAGACCTTGTTACGACTTTTACTTCCTCTATT	99	99	0.0

Appendix 6: Gel pictures (ITS & β- tubulin) of tropical fungi showing the molecular weight of the DNA and after DNA purification