DIVERSITY, ANTAGONISTIC POTENTIAL OF ENDOPHYTES, PHYTOCHEMICALS AND ANTIMICROBIAL ACTIVITY OF SELECTED AGROFORESTRY TREES AGAINST *Xanthomonas campestris* pv. *musacearum* AND *Cercospora zeae-maydis*

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DECLARATION

This thesis is my original work and has not been presented for a degree in Maseno University or any other university. All sources of information have been acknowledged by means of references and citation.

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ABSTRACT

Maize and banana yields have continued to decline due to pests and diseases such as grey leaf spot (GLS) of maize and Xanthomonas wilt of bananas caused by *Cercospora zeae-maydis* and *Xanthomonas campestris* pv. *musacearum* respectively. Plants contain endophytes that protect them against pests and diseases. There is paucity of information regarding the diversity of endophytes of *Calliandra calothyrsus, Leucaena diversifolia* and *Sesbania sesban* are important biocontrol agents. Endophytes have antagonistic effects against plant pathogens. However, there is inadequate information on the antagonistic potential of endophytes from these plants against *C. zeae-maydis* and *X.c.* pv. *musacearum.* These pathogens have continued to lower yield of maize and bananas hence compromising food security. Plants contain phytochemical compounds with antimicrobial activity. There is lack of information on the phytochemical compounds of these plants which are vital in identifying antimicrobial properties of the plant extracts to control GLS and Xanthomonas wilt diseases. Moreover, there is little information on the antimicrobial properties of these plants extracts against *C. zeae-maydis* and *X.c.* pv. *musacearum* important in improving yield of maize and bananas. The objective of the study was to determine diversity, phylogeny, antagonistic potential of endophytes, phytochemical compounds and antimicrobial activity of *C. calothyrsus, L. diversifolia* and *S. sesban* extracts against *C. zeae-maydis* and *X.c* pv. *musacearum*. Nine plants of *C. calothyrsus, L. diversifolia* and *S. sesban* were collected randomly from an agroforestry based system comprising of maize and banana at Maseno University farm located 0° 10' 0" South, 34° 36' 0" East. Three leaves, stems and roots were surface sterilized in 4% NaOCl, rinsed with sterile water and plated on potato dextrose agar and nutrient agar for growth of endophytes. Leaf samples were dried and ground for extraction in ethanol and aqueous solvents. Test pathogens were isolated from diseased maize and banana leaves and their pathogenicity determined. Endophytes were isolated in pure cultures, characterized morphologically and molecularly and their phylogenetic relationships determined. Antagonistic potential of endophytes and activity of leaf extracts against *C. zeae-maydis* and *X.c* pv. *musacearum* was determined in dual culture, disc diffusion and food poison techniques. Treatments of 12.5, 25, 50 and 75% aqueous extracts and, 12.5, 25, 50, and 75mg/ml ethanol extracts were used. Plates were arranged in a completely randomized design. Qualitative phytochemical analysis of the extracts' compounds was determined. Morphological and molecular data was subjected to cluster analysis. Data on growth was subjected to analysis of variance and means separated using Least Significant Differences ($P \leq 0.05$). Morphological and molecular data revealed that there were Gram positive and Gram negative cocci and bacilli belonging to ten genera, while fungal endophytes belonged to four genera. Fungal endophytes clustered in three orders, and bacteria endophytes clustered in six orders. Morphological dendograms clustered isolates in two groups at 75% similarity level. Phylogenetic tree grouped the isolates into two clades at 99% similarity level. The results revealed that most of the isolates had a common ancestor. Thirteen fungal isolates showed growth inhibition against *X.c* pv. *musacearum* while twenty four inhibited growth of *C. zeae-maydis.* Nineteen bacterial isolates inhibited the growth of *X. campestris* pv. *musacearum* while eleven inhibited growth of *C. zeae-maydis.* Tannins, steroids and saponins were detected in the three plants. Terpenoids, flavonoids and alkaloids were not all present in the three plants. Leaf extracts showed significant differences in growth inhibition among the treatments and plant species. The findings revealed that endophytes and extracts from *C. calothyrsus, L. diversifolia* and *S. sesban* have the potential to control GLS and *Xanthomonas* wilt in maize and bananas. The study recommends the use of endophytes and extracts from these plants in control of GLS and Xanthomonas wilt.

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LIST OF ABBREVIATIONS, SYMBOLS AND ACRONYMS

BLAST	- Basic local alignment search tool
Bp	- Base pair
BSC	- Bacteria stem Calliandra
BLC	-Bacteria leaf Calliandra
BRC	-Bacteria root Calliandra
BLL	- Bacteria leaf Leucaena
BSL	-Bacteria stem Leucaena
BRL	Bacteria root Leucaena
BLS	-Bacteria leaf Sesbania
BSS	- Bacteria stem Sesbania
BRS	- Bacteria root Sesbania
DNA	- Deoxyribonucleic acid
F	- Forward primer
FRL	- Fungi root Leucaena
FSL	- Fungi stem Leucaena
FLL	- Fungi leaf Leucaena
FRC	- Fungi root Calliandra
FLC	- Fungi leaf Calliandra
FSC	- Fungi stem Calliandra
FRS	- Fungi root Sesbania
FLS	- Fungi leaf Sesbania
FSS	- Fungi stem Sesbania

G+	- Gram positive
G-	- Gram negative
GLS	- Grey leaf spot
Kb	- kilo base pair
µl	- Microliter
Mg/ml	- milligrams per millilitres
MEGA	- Molecular Evolutionary Genetic Analysis
NCBI	- National centre for biotechnology information
NA	- Nutrient agar
PDA	- Potato dextrose agar
PCR	- Polymerase chain reaction
R	- Reverse primer
RNA	- Ribonucleic acid
rRNA	- Ribosomal ribonucleic acid
UV	- Ultra violet
ZR	- Zymo research

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CHAPTER ONE: INTRODUCTION

1.1 Background of the study

1.1.1 Endophytic microbial community

Endophytes are microorganisms that inhabit and colonize inner organs and tissues of plants during their life-cycles without causing diseases or producing visible signs and symptoms of infection (Bamisile *et al,*. 2018; Li *et al,*. 2020; Niem *et al,*. 2020). They comprise of different communities of fungi, bacteria and actinomycetes distributed in different plant parts and plant species all over the world (Anyasi and Atagana, 2019; El-Deeb *et al.*, 2013). Naturally, bacteria and fungi enter plant tissues via germinating radicals, secondary roots, stomata, or as a result of foliar damage or by secreting hydrolytic enzymes that degrades the cell wall to gain entry (Dashyal *et al.,* 2019; El-deeb *et al.,* 2013). After entry, they can be localised at the point of entry or may systemically spread and colonise different plant parts away from the point of entry establishing a mutual relationship with the plant (Anyasi and Atagana, 2019; Coêlho *et al.,* 2011). Inside the plant, these microorganisms can be found residing within intracellular and intercellular spaces or within vascular system (Dashyal *et al.,* 2019; Khare *et al*., 2018; Suman *et al.,* 2011)

Endophytes are diversely applied in plant growth promotion, plant resistance under stressful conditions, decomposition of litter, production of bioactive compounds which are of great potential in agriculture, antimicrobial and anti-insect activity (Li *et al.,* 2020; Mahadevamurthy, *et al*., 2016; Teimoori-boghsani *et al.*, 2020; Thi and Diep, 2014). Different species of bacteria (Costa *et al.*, 2012) and fungi (Bisht *et al.,* 2016) have been isolated and characterized using both morphological and molecular characteristic in different plants and ecosystems. However, there is

little information on morphological and molecular characterization of endophytes colonising *Calliandra calothyrsus, Leucaena diversifolia* and *Sesbania sesban* that is vital in identifying the characters of these endophytes important in biocontrol of plant diseases. The knowledge of this microbiota, their interactions with host-plants and the environment, is an essential variable in the development of strategies directed to sustainable agriculture and preservation of biodiversity.

Within the endosphere of the plant, there exist diverse communities of bacteria and fungi endophytes. The microbial diversity and community structure of endophytes is influenced by soil type, plant and tissue or organ type they inhabit as well as abiotic factors (Li *et al.,* 2020; Correa-galeote *et al.,* 2018; Katoch and Pull, 2017). Studies by Coêlho *et al.* (2011) revealed low genetic diversity of endophytic bacteria in arboreal species while Costa *et al.* (2012) reported high bacterial diversity in *Phaseolus vulgaris.* Furthermore, reports indicate that there is high diversity of endophytes in leaves than any other organ in plants (Chowdhary and Kaushik, 2015; Katoch and Pull, 2017). Information on endophytic diversity of *Calliandra calothyrsus, Leucaena diversifolia* and *Sesbania sesban* remains scanty, as more studies have focused on food crops like maize (Correa-Galeote *et al.,* 2019), tomatoes (Constantin *et al.,* 2019) and medicinal plants (Khan *et al.,* 2017) forgetting agroforestry trees which could reveal novel endophytes with biocontrol potential.

Phylogenetic studies in clustering of endophytes show that distribution and abundance of endophytic microorganisms from different geographical locations is a function of prevailing environmental conditions. Chowdhary and Kaushik (2015) reported clustering of fungal isolates from *Osmium sanctum* in the same clade as a function of temperature. Report by Costa *et al*.

(2012) indicates that there is a high degree of relationship of endophytes from related plants than unrelated ones. Currently there is no enough evidence on the phylogenetic relationship of endophytes colonizing *Calliandra calothyrsus, Leucaena diversifolia* and *Sesbania sesban* which is useful in understanding the ancestral origin of endophytes with antimicrobial potential. Knowing the phylogenetic relationships of organisms helps us understand their ancestral origin and the divergence that they have undergone over a period of time in relation to their use as biocontrol agents.

Endophytes isolated from some plants including agroforestry tree have great antimicrobial activity against plant pathogens as they have been identified as reservoirs of novel bioactive secondary metabolites (Bisht *et al.,* 2016; Pawthong *et al.,* 2013). *Trichoderma koningii* and *Alternaria alternata* isolated from maize roots reduced growth of *Fusarium* pathogen (Orole and Adejumo, 2009) while some banana endophytic bacteria inhibited growth of fungal pathogens of bananas (Souza *et al.,* 2014).

Similarly endophytic fungi isolated from *Sesbania grandiflora* exhibited great antimicrobial potential against *Xanthomonas axonopodis* pv. *citri, Xanthomonas axonopodis* pv. *glycines*, *Xanthomonas campestris* pv. *campestris* and *Acidovorax avenae* subsp. *avenae* (Pawthong *et al.,* 2013). However, there is paucity of information on the antimicrobial properties of endophytes of *C. calothyrsus, L. diversifolia* and *S. sesban* as potential biocontrol agents against Grey leaf sport (GLS) and *Xanthomonas* wilt diseases of maize and bananas caused by *Cercospora zeae-maydis* and *Xanthomonas campestris* pv. *musacearum* respectively. Control of plant diseases using

natural enemies like endophytes reduces the effect of synthetic chemicals that results to biodiversity degradation leading to increased harvestable products.

1.1.2 Grey leaf spot and *Xanthomonas* wilt diseases

Grey leaf spot (GLS) and *Xanthomonas* wilt diseases of maize and bananas caused by *Cercospora zeae-maydis* and *Xanthomonas campestris* pv. *musacearum* respectively contributes to low yield of bananas and maize in western Kenya (Bekeko *et al.,* 2018; Ocimati *et al.,* 2019). Gray leaf spot is one of the deadly diseases of maize in western Kenya which has been reported to play a major role in lowering maize production when interacting with other environmental factors (Adam *et al.,* 2017; Bekeko *et al.,* 2018). The disease is characterized by the formation of necrotic rectangular lesions on maize leaves separated by the leaf margins, which reduce the photosynthetic potential and ultimately yield of the crop. Symptoms of grey leaf spot develop starting from the lower leaves upwards on a maize plant and reach their optimum intensity after flowering (Berger *et al*., 2014; Sibanda *et al.,* 2019**;** Dhami *et al*., 2015).

Xanthomonas wilt disease severely affects some cultivars of bananas resulting into heavy losses. The disease is caused by *Xanthomonas campestris* pv. *musacearum* and may result to up to 100% yield loss thereby compromising food security and income of the farmers (AATF, 2003; Ocimati *et al.,* 2019; Nkuba *et al*. 2015). *Xanthomonas campestris* pv. *musacearum* infects bananas resulting into yellowing and wilting of leaves, which starts with the youngest leaf (Uwamahoro *et al.,* 2019). This is then progresses to withering of male buds, premature ripening and fall off of the fruits and yellow bacterial ooze will be observed in about 15 minutes after the

pseudostem is cut (Uwamahoro *et al.,* 2019; Nakakawa *et al.,* 2017). Since the bacterium attacks all cultivars, it has become almost impossible to control the disease using the proposed mechanisms such as planting healthy suckers, breaking of male buds with a forked stick, disinfection of farm tools and removal of infected plants (Uwamahoro *et al.,* 2019b; Kubiriba and Tushemereirwe 2014).

Control of these diseases is by use of cultural practices, synthetic chemicals, and genetic breeding to obtain resistant varieties but farmers still experience heavy losses (Gang *et al.,* 2013; Uwamahoro *et al.,* 2019b). Considering limitations of the different strategies for the management of these diseases and awareness about human health and environment, biological method is preferred for management of diseases. Consequently there is inadequate information on the use of endophytes as biological control against these pathogens to increase yield of maize and bananas. Use of endophytes and their products as control measures for plant pathogens alleviates the negative effects of synthetic chemicals while promoting increased yield there by leading to increased food security.

1.1.3 Agroforestry and disease control

In agroforestry system, food crops are intercropped with non food crops to improves soil health (Sileshi *et al.,* 2014) and disease and pest control by increased biodiversity of the ecosystem (Lasco *et al.,* 2014). This is achieved through allelochemical and phytochemical compounds with antimicrobial properties produced as volatile organic compounds by both host and non host plants to fight against the pathogens (Zhu and Morel 2019; Luo *et al.,* 2021). The antimicrobial

compounds exudeted by plants are as a result of phytochemical compounds synthesised by both plants and their associated endophytes which can be released on decomposition of the litter. According to Zhu and Morel (2019), yield in intercropping systems such as in agroforestry is always elevated compared to monoculture systems due to reduced disease incidence.

Environmental factors of a given ecological zone in which the plant is growing affect the type, content and concentration of phytochemical compounds present in that plant (Liu *et al.,* 2017; Liu *et al.,* 2016). Some species of genus *Leucaena, Calliandra* and *Sesbania* such as *Leucaena leucocephala*, *Calliandra tergemina* and *Sesbania sesban* have phytochemical compounds including flavonoids, tannins, triterpenoids, carbohydrates and vitamins (Chew *et al.,* 2011; Gomase *et al.,* 2012) with antimicrobial properties. Considering the role of phytochemicals in plants, there is lack of adequate information on the phytochemical compounds of *Calliandra calothyrsus, Leucaena diversifolia* and *Sesbania sesban* which is vital in identifying antimicrobial properties of the plant extracts to control grey leaf spot and *Xanthomonas* wilt diseases. Understanding the type of phytochemicals present in these trees is central in explaining their role in disease control in agaroecosystem when they are released either on decomposition of litter or as volatile organic compounds.

Studies shows that *Leucaena leucocephala* (Abu *et al.*, 2016; Aderibigbe *et al*., 2011), *Calliandra tergemina* (Chew *et al.*, 2011) and *Sesbania sesban* (Gomase *et al.,* 2012; Kathiresh *et al.,* 2012) extracts have antimicrobial activity against Gram positive and Gram negative human pathogens as well as fungal pathogens. Even though some species of *Calliandra, Leucaena* and *Sesbania* have been reported to have antimicrobial activities, the antimicrobial

activity of *Calliandra calothyrsus, Leucaena diversifolia* and *Sesbania sesban* leaf extract against *Cercospora zeae-maydis* and *Xanthomonas campestris* pv. *musacearum* pathogens has not been exposed and is important for improved yield of maize and bananas. The use of botanicals in control of plant diseases is essential to ecosystem sustainability as they are affordable, easily available and biodegradable compared to synthetic chemicals which are expensive and unfriendly to the environment.

This research aimed at determining the diversity and phylogenetic relationship of endophytes from *Calliandra calothyrsus, Leucaena diversifolia* and *Sesbania sesban* and evaluate their antagonistic potential against *Cercospora zeae-maydis* and *Xanthomonas campestris* pv. *musacearum* pathogens of maize and bananas which can be an alternative to chemical compounds. Phytochemical compounds and antimicrobial activity of *Calliandra calothyrsus, Leucaena diversifolia* and *Sesbania sesban* extracts were also assessed.

1.2 Statement of the problem

Production of maize and bananas which are some of the staple foods in western Kenya has been on the decline due to infestation by pests and diseases. Gray leaf spot (GLS) caused by *Cercospora zeae-maydis* has been reported to cause over 60% loss of maize yield in western Kenya (Bekeko *et al.,* 2018) while in bananas, bacterial wilt incited by *Xanthomonas campestris* pv. *musacearum* can cause up to 100% crop loss if not controlled (Ambachew, 2019). Farmers are encouraged to intercrop maize and bananas with agroforestry trees to increase habitat diversity there by lowering disease infestation. Agroforestry trees harbour diverse endophytic

fungi and bacteria with antimicrobial properties (Birhanu *et al.*, 2020; Vargas *et al.*, 2018). These endophytes have protective function for the host and intercropped crops against disease pathogens. There is inadequate information regarding morphological and molecular characterization of endophytes of *C. calothyrsus, L. diversifolia* and *S. sesban* trees which can be used in identifying potential biocontrol agents of grey leaf spot of maize and *Xanthomonas* wilt of banana. Molecular phylogenetic analysis is important in grouping microorganisms into their taxonomic groupings in relation to their evolutionary relationship but there is little information on phylogenetic analysis of endophytes colonizing *C. calothyrsus, L. diversifolia* and *S. sesban* which can be vital in controlling grey leaf spot disease of maize and *Xanthomonas* wilt of bananas. Furthermore, endophytes have the ability to inhibit the growth of plant pathogens but there is inadequate information on antagonistic potential of endophytes of *C. calothyrsus, L. diversifolia* and *S. sesban* against *C. zeae-maydis* and *Xc.* pv. *musacearum* which are known to lower yield of maize and bananas. Plants contain phytochemical compounds with antimicrobial properties. Phytochemical compounds present in plants are influenced by climate and prevailing environmental conditions, therefore there is need to determine phytochemical compounds of *C. calothyrsus, L. diversifolia* and *S. sesban* which can be used to help in identifying antimicrobial properties of the plant extracts. Little is known on the antimicrobial activity of *C. calothyrsus, L. diversifolia* and *S. sesban* plant extracts against *Cercospora zeae-maydis* and *Xanthomonas campestris* pv. *musacearum* pathogens of maize and bananas. Knowledge on the antimicrobial activity of extracts from these plants is vital in controlling grey leaf spot and Xanthomonas wilt to improve on the yield of maize and bananas.

1.3 Justification

Maximum yield of food crops is an essential element in attaining food security and nutrition for any given population in a country. Maize and banana yields have greatly dropped due to diseases and pests which have continued to destroy the plants. This is as a result of inadequate knowledge and information on how best plant diseases can be managed in order to have increased yield. This study therefore is important in identifying endophytic bacteria and fungi of *C. calothyrsus, L. diversifolia* and *S. sesban* as biocontrols for effective control of *Cercospora zeae-maydis* and *Xanthomonas campestris* pv. *musacearum* in order to improve yield of maize and bananas. Similarly, knowing the phytochemical compounds of these plants is essential in understanding the antimicrobial activities of plant extracts to control grey leaf spot and *Xanthomonas* wilt leading to increased yield. The study would be of great benefit to the sustainability of the ecosystem as it seeks to use more environmentally friendly methods of controlling GLS and *Xanthomonas* wilt while avoiding synthetic chemicals that have caused disturbances in ecological balance to increase food security.

1.4 Significance of the study

The study therefore aimed at adding new lineages to the fungal and bacteria trees of life by describing new species of fungi and bacteria inhabiting *C. calothyrsus, L. diversifolia* and *S. sesban*. This will improve knowledge not only in fungal and bacterial diversity but also in their evolution. The DNA sequences of the identified endophytes will be deposited and be available in NCBI GenBank, where they can be accessed through public online databases. Both morphological and molecular data will be useful in identification of endophytic fungi and bacteria by other researchers studying microbial diversity and will aid in the dissemination of

information about fungal and bacterial diversity, ecology and systematics, to scientists and other interested individuals working in various fields. Antagonistic studies will document endophytic species that are promising candidates for future biocontrol against *C. zeae-maydis* and *Xc pv. musacearum*. Growth inhibition assays will uncover potential endophytes for biocontrol strategies, improving our understanding on the role endophytes play within their host. In addition, the use of extracts as growth inhibitory agents would expand knowledge of integrated pest management of maize and bananas.

1.4 Objectives

1.4.1 Main objective

To determine the diversity, antagonistic potential of endophytes, phytochemical compounds and antimicrobial activity of *Calliandra calothyrsus, Leucaena diversifolia* and *Sesbania sesban* leaf extracts against *Cercospora zeae-maydis* and *Xanthomonas campestris* pv. *musacearum.*

1.4.2 Specific objectives

1. To determine morphological and molecular profile of fungal and bacterial endophytes of *Calliandra calothyrsus, Leucaena diversifolia* and *Sesbania sesban.*
2. To determine molecular phylogenetic relationships of endophytic bacteria and fungi of *Calliandra calothyrsus, Leucaena diversifolia* and *Sesbania sesban.*
3. To evaluate growth inhibition potential of endophytic bacteria and fungi of *Calliandra calothyrsus, Leucaena diversifolia* and *Sesbania sesban* against *Cercospora zeae-maydis* and *Xanthomonas campestris* pv. *musacearum.*

4. To determine the phytochemical compounds of *Calliandra calothyrsus, Leucaena diversifolia* and *Sesbania sesban* leaf extracts used as antimicrobial agents.
5. To determine growth inhibition activity of *Calliandra calothyrsus, Leucaena diversifolia* and *Sesbania sesban* leaf extract against *Cercospora zeae-maydis* and *Xanthomonas campestris* pv. *musacearum.*

1.5 Hypotheses

1. There are no morphological and molecular differences between bacterial and fungal endophytes colonizing *C. calothyrsus, L. diversifolia* and *S. sesban.*
2. There is no molecular phylogenetic relationship between endophytic bacteria and fungi isolates from *C. calothyrsus, L. diversifolia* and *S. sesban*.
3. Endophytic bacteria and fungi from *C. calothyrsus, L. diversifolia* and *S. sesban* have no significant growth inhibition potential against *C. zeae-maydis* and *Xanthomonas campestris* pv. *musacearum*.
4. There is no difference in phytochemical compounds of *C. calothyrsus, L. diversifolia* and *S. sesban* leaf extracts used as antimicrobial agents.
5. *Calliandra calothyrsus, Leucaena diversifolia* and *Sesbania sesban* leaf extracts have no growth inhibitory activity against *C. zeae-maydis* and *Xc pv. musacearum*.

1.6 Scope and limitations of the study.

The study involved isolation of endophytes from *C. calothyrsus, L. diversifolia* and *S. sesban* collected from intercrop of maize banana and the three agroforestry trees. Endophytes were

characterized using morphological and molecular characteristic and the phylogenetic relationships analyzed. The antagonistic activity of the endophytes as well as antimicrobial activity of the leaf extracts from the three plants against *C. zeae-maydis* and *Xc* pv. *musacearum* was also determined. Phytochemical compounds in the leaf extracts used as antimicrobial agents were also determined. However, isolation of endophytes was based on only culturable bacteria and fungi although unculturable endophytes are also found in the plant. Media used for isolation were nutrient agar and potato dextrose extracts which could not have supported all the culturable endophytes. In this study, actual endophytes were used for antagonistic study without harnessing the active chemicals they synthesize as antimicrobial agents. Phytochemical compounds were determined qualitatively without considering their quantitative nature in the leaf extracts. Similarly there was no consideration of volatile organic chemicals produced by these plants. Only leaves were used for antimicrobial activity without considering the effects of other plant parts like roots and stem.

CHAPTER TWO: LITERATURE REVIEW

2.1 Endophytes

Endophytes are microorganisms that reside inside the plant for the part or all of their lifetime without causing any visible disease symptoms (Niem *et al.,* 2020; Li *et al.,* 2020; Anyasi and Atagana, 2019), but may become pathogenic with changes in environmental condition or when the plant senesce (Duan *et al.,* 2019). They colonize different organs and tissues in plants and can be isolated from the plant after surface sterilization or disinfection of the plant tissues or can be extracted from internal plant organs (Bamisile *et al.,* 2018; El-Deeb *et al*., 2013). They are distributed in all parts of the plant including roots, leaves, stems, flowers and seeds but they do not cause any disease or visible external manifestations of disease infestation (Chowdhary and Kaushik, 2015; El-deeb *et al.,* 2013; Coêlho *et al.,* 2011).

Endophytes gain entry into the host plant via stomata, wounds, or areas of lateral root development, or may even be facilitated by hydrolytic enzymes they produce that are capable of degrading the cell wall of the plant cells to create entry point (Dashyal *et al.,* 2019; Khare *et al.,* 2018). Once inside the plant, they may lodge in specific tissues, or may even systemically colonize the plant, thereby establishing a relationship that may be symbiotic, mutualistic, commensal or tropobiotic (Dashyal *et al.,* 2019; Khare *et al.,* 2018; Suman *et al.,* 2011).

It is estimated that every plant species on the planet is a potential host of endophytes but only a few have been studied based on their economic relevance. Some plants in which endophytes have been isolated and characterized either morphologically or molecularly include; *Cupressus torulosa* (Bisht *et al.,* 2016), *Ocimum sanctum* (Chowdhary and Kaushik , 2015), *Boerhaavia*

diffusa L. (Mahadevamurthy, 2016) and tomato plant (Nawangsih *et al.,* 2011). However, for agroforestry trees like *Calliandra calothyrsus, Leucaena diversifolia* and *Sesbania sesban*, there is inadequate information on morphological and molecular characteristics of endophytic fungi and bacteria that inhabit them, which is important in identifying characters that may be important in biocontrol of plant diseases. Majority of endophytic communities are bacteria and fungi (Coêlho *et al.,* 2011) but the described populations of endophytic bacteria and fungi are still few. Therefore, there is still an opportunity to find new strains of endophytic microorganisms that colonize plants in different niches and ecosystems.

Endophytes, which are estimated to be up to one million species (Bisht *et al.,* 2016), colonize different plants in different ecological zones. A plant species may harbour diverse species of both fungi and bacteria endophytes which may be closely or distantly related based on phylogenetic analysis. Within the endosphere of the plant, there exist diverse communities of bacteria and fungi microorganisms (Katoch *et al.,* 2017). Soil type, plant and tissue or organ type determines and influence the microbial diversity and community structure of endophytes (Correa-Galeote *et al.,* 2018; Coêlho *et al.,* 2011). Studies by Coêlho *et al.* (2011) revealed low genetic diversity of endophytic bacteria in arboreal species while Costa *et al.* (2012) reported high bacterial diversity in *Phaseolus vulgaris.* Similarly, Katoch *et al*. (2017) reported high diversity of fungal endophytes of *Monarda citriodora.* Despite the availability of various studies on molecular phylogenetics of endophytes, there is paucity of information on phylogenetic relationships of endophytes of *C. calothyrsus, L. diversifolia* and *S. sesban* which is useful in understanding the ancestral origin of endophytes with antimicrobial properties.

2.1.1 Bacterial endophytes

These are bacteria that live in or on plant tissues without causing any substantive harm or gaining benefit other than residency (Maggini *et al.,* 2019; El-Deeb *et al*., 2013). Bacterial endophyte enables plant to cope up with both biotic and abiotic stresses as they confer survival advantage to the plant (Khare *et al.,* 2018; Liu *et al.,* 2017; Tidke *et al.,* 2017). Endophytic bacteria are usually found in intercellular spaces and vascular bundles and comprises of both Gram positive and Gram negative bacteria which can be isolated from plant tissues that have been surface sterilized (Tidke *et al.,* 2017). They have been isolated in different plant species including Grapevine (Niem *et al.,* 2020), *Tectona grandis* Linn. (Singh *et al.,* 2017), tomato plant (Nawangsih *et al.,* 2011) and many more.

Endophytic bacterial communities in many plants are dominated by Proteobacteria, Actinobacteria, Firmicutes and Bacteroidetes (Liu *et al.,* 2017). Other bacterial phyla, such as Chloroflexi, Cyanobacteria, Armatimonadetes, Verrucomicrobia, Planctomycetes, and Nitrospirae are also found majorly in root endosphere, but in smaller fraction (Liu *et al.,* 2017; Suman *et al.,* 2011). Furthermore, several different bacterial species have been isolated from a single plant but their entry into the plant is primarily through the root zone. However, some bacteria may enter the plant through aerial portions such as flowers, stems, and cotyledons (Suman *et al.,* 2011).

Different plant organs may harbour different endophytic bacterial communities in terms of diversity and composition and as reported by Liu *et al.* (2017), the diversity and density of bacteria in roots is high and decrease in stems, leaves, and reproductive organs. Some of the

common bacterial genera that have been isolates in plants include *Achromobacter*, *Azoarcus*, *Burkholderia*, *Enterobacter*, *Gluconacetobacter*, *Herbaspirillum*, *Klebsiella*, *Microbispora*, *Micromonospora*, *Nocardioides*, *Pantoea*, *Planomonospora*, *Pseudomonas*, *Serratia*, *Streptomyces* and *Thermomonospora* (Suman *et al.*, 2011).

2.1.2 Fungal endophytes

Endophytic fungi are part of inner plant microbial communities which are found in all plant species, plant organs and tissues and across every ecosystem (Ofek-lalzar *et al.*, 2016). They are defined as the microbial fungi that spend whole or part of their life cycle colonizing inner environment of healthy tissues of the host plant, without causing any apparent symptoms of disease (Mahadevamurthy *et al.*, 2016). Of the 420, 000 plant species in nature, only a few have been studied in relation to their endophytic fungi (Chowdhary and Kaushik, 2015). Endophytic fungi are worldwide and they have been isolated from a variety of plant types including mosses, liverworts, ferns and higher plants (Mahadevamurthy *et al.*, 2016; Chowdhary and Kaushik, 2015).

In the plant, they have been isolated from leaves, bark, stem and roots and those that are in a symbiotic association with roots are called mycorrhiza (Meenatchi *et al.*, 2016). Scientists have classified endophytic fungi into two main groups based on host range, colonization, transmission patterns, tissue specificity and ecological function as clavicipitaceous and non-clavicipitaceous fungi (Bamisile *et al.*, 2018; Khirella *et al.*, 2016).

Clavicipitaceous fungal endophytes are associated with warm and cool climate season and are mostly common in grasses. They are host specific and found mainly in grass family poaceae and rarely are they found in cyperaceae. They are vertically transmitted through seeds with maternal plants passing them to the offspring (Nazir and Rahman, 2018; Khiralla *et al.*, 2016). Non-clavicipitaceous fungal endophytes are highly diverse and comprise of species from Ascomycota to Basidiomycota and are associated with vascular and non vascular plant species. They colonize either specific organ of the plant; leaves, roots, or stem or they may colonize the whole plant (Nazir and Rahman, 2018; Khiralla *et al.*, 2016). These endo-fungi protect the host plant from insect animal herbivore, nematodes, pathogenic microorganisms and abiotic stress (Khiralla *et al.*, 2016).

2.1.3 Methods of studying microbial diversity

Methods used to describe microbial diversity can be categorized into two main groups according to Fakruddin and Mannan, (2013) as conventional biochemical methods and molecular methods

a) Conventional and Biochemical Methods

These methods are of high significance as microorganisms diversity can be described using physiological measures which reduce chances of grouping similar bacteria into same species group or equivalents. Multivariate data analysis can also be used to extract relevant information from large data-sets obtained when diversity studies are carried out (Salmonová and Bunešová 2017). Early microbiologists studied metabolic properties of microorganism to differentiate between different types. Metabolic properties studied included utilization of different carbon, nitrogen and energy sources in addition to their requirements for growth factors (Salmonová and Bunešová 2017; Agrawal *et al.*, 2011).

Other conventional methods used to study diversity include plate counts, Sole-Carbon source utilization and Phospholipids fatty acid (PLFA) analysis. Although methods have been commonly used to identify microorganisms, they are insufficient to discriminate between species and strains (Ikeda *et al.*, 2013) therefore the need to get more accurate methods of determining microbial diversity.

b) Molecular methods

The conventional methods for characterizing microbial communities and their diversity were based on analysis of the culturable portion of the bacteria and fungi (Fakruddin and Mannan, 2013). Some of the microorganisms in the community are unculturable and they are always left out therefore making it difficult to understand the overall structure of the community. Due to this difficulty, recent studies to characterize and determine microbial diversity have focused on the methods that can isolate microorganisms without culturing to provide genetic diversity data (Salmonová and Bunešová 2017; Agrawal *et al.*, 2011). Several methods have been developed to study molecular microbial diversity. These include DNA re-association, DNA–DNA and mRNA-DNA hybridization, DNA cloning and sequencing and other PCR-based methods (Fakruddin and Mannan, 2013; Salmonová and Bunešová 2017; Agrawal *et al.*, 2011).

2.1.4 Benefits of endophytes

Endophytic fungi have diverse application as they play major role in physiological activities of host plants. They enhance plant resistance to stressful conditions of both biotic and abiotic factors, decompose plant litter (Mahadevamurthy *et al.,* 2016; Ofek-Lalzar *et al.,* 2016), enhance insect, nematode and disease resistance (Meenatchi *et al.,* 2016). Besides the above positive roles, endophytic fungi are also known to synthesise many useful bioactive metabolites including anti-microbial, anti-insect, anti-cancer, anti-diabetic and immunosuppressant compounds (Mahadevamurthy *et al.,* 2016; Chowdhary and Kaushik, 2015). These compounds have great applications in agriculture, medicine and food industry (Mahadevamurthy *et al.,* 2016; Bisht *et al.,* 2016).

Some of the bioactive compounds synthesized by endophytes include alkaloids, terpenoids, steroids, quinones, isocoumarins, lignans, phenylpropanoids, phenols, and lactones (Bisht *et al.,* 2016; Chowdhary and Kaushik , 2015). They also enhance plant growth and health by their ability to concentrate macro- and micro-nutrients like phosphorus, sulphur, calcium, magnesium and potassium (Waqas *et al.,* 2012). Endophytes can also accelerate plant growth by producing growth promoting hormones and enhancing nitrogen fixing capabilities of host plants (Waqas *et al.,* 2012; Orole and Adejumo, 2009).

Endophytic bacteria on the other hand, promote plant growth by producing phytohormones and increasing the ability of the plant to utilize nutrients from the soil which enhances root development, nitrate uptake or solubilisation of phosphorus and increased resistance to pathogens (El-Deeb *et al.,* 2013; Nawangish *et al.,* 2011). Studies have demonstrated that

endophytic bacteria are able to protect plants from a series of abiotic stresses including drought, low temperature and salinity. Also, it has been observed that some of the plants like *Agave tequilana* directly digest endophytic bacteria for nitrogen (N) source required for their growth (Liu *et al.,* 2017). Endophytic bacteria like rhizobium have the ability to fix nitrogen, an essential nutrient required by the plant and have been found to be able to promote root development, increase biomass and productivity, capable of inhibiting growth and sporulation of pathogenic fungi, increase plant height, leaf area, leaf number, together with fresh and dry plant matter in different plants (Szilagyi-zecchin *et al.,* 2014). Some of these benefits not only apply to the host plant but they are also beneficial to plants intercropped with them in agroforestry system.

2.2 Phylogenetic relationships of microorganisms

Phylogenetics is one of the branches of life sciences that deals with the study of evolutionary relations among different species or populations of organisms including microorganisms through molecular sequencing of data (Vinay *et al.,* 2009). Classification of microorganisms has been a major challenge especially on their phylogenetic relationships as most of the traditional methods were based on using a variety of morphological (staining), biochemical and serological procedures and grouping together those bacteria that share the greatest number of traits (Kumar *et al.* 2019; Vinay *et al.,* 2009). Phylogenetic analysis can be based on the amino acid sequence of proteins and on the presence of similar metabolic pathways but the most accurate of determining the evolutionary relationship is the comparison of DNA composition and sequence (Vinay *et al.,* 2009).

Phylogenetic studies in clustering of endophytes show that distribution and abundance of endophytic microorganisms from different geographical locations is a function of prevailing environmental conditions. Chowdhary and Kaushik (2015) reported clustering of fungal isolates from *Osmium sanctum* in the same clade as a function of temperature. Report by Costa *et al*. (2012) indicates that there is a high degree of relationship of endophytes from related plants than unrelated ones. Consequently, little is known about the phylogenetic relationship of endophytes colonizing *C. calothyrsus, L. diversifolia* and *S. sesban* useful in understanding the ancestral origin of endophytes with antagonistic potential against plant pathogens. It is therefore important to determine the phylogenetic relationship of endophytes from these important trees and ascertain whether they are only related from closely related plants or even with unrelated plant species.

2.3 Agroforestry

Agroforestry is a form of land-use systems in which woody perennials (trees, shrubs, etc.) are grown in association with herbaceous plants (crops, pastures) or livestock, in a spatial arrangement, a rotation, or both in which there are usually both ecological and economic interactions between the trees and other components of the system (Alebachew, 2012). Agroforestry has been used to mitigate drivers of climate change which directly and indirectly stress agricultural production as well as the ability of the ecosystem to provide goods and services (Lasco *et al.*, 2014). Agroforestry and conservation agriculture are being practiced in attempt to address land degradation and loss of soil fertility as they are thought to be effective

and low-cost means of minimising degradation of cultivated land and of maintaining or even increasing the productive capacity of agricultural ecosystems (Kabiru *et al.,* 2018).

The use of agroforestry systems and trees on farms provide key ecosystem services such as water conservation, improved micro-climate conditions, enhanced soil productivity, nutrient cycling and conservation and control of pests and diseases. In developing countries agroforestry improves food security for smallholder farmers by improving soil health (Sileshi *et al.,* 2014) and disease and pest control by increased biodiversity (Karp *et al.,* 2013).

The use of trees in agro-ecosystems through adoption of agroforestry increases habitat diversity, which positively correlates with abundance and diversity of natural enemies both at the field and landscape level (Tscharntke *et al.,* 2011). In some instances trees may benefit pests and disease causing agents directly by providing resources or improving microclimate, or indirectly by enhancing host plant nutritional conditions or water availability thereby reducing their effects on crops (Sileshi *et al.,* 2008). Therefore, it is still unclear to what extent different agroforestry practices can improve regulation of pests, diseases and weeds in agro-ecosystems. The success of any agroforestry system of farming depends heavily on the choice of suitable tree species that could offer diversity of benefits and show compatibility with food crops (Vignola *et al.,* 2015).

Some of agroforestry trees such as *Grevillea robusta* (Birhanu *et al.,* 2020) and *Croton lechleri* (Vargas *et al.,* 2018) have been found to harbour diverse endophytic fungi and bacteria with antimicrobial properties. Endophytes and extracts form *Croton lechleri* have antimicrobial activity against human pathogens (Vargas *et al.,* 2018; Roumy *et al.,* 2015). On the other hand

Grevillea robusta extracts have been reported to inhibit growth of Gram positive and Gram positive bacteria with no effect on fungi (Cock, 2019; Ullah *et al*., 2014). Plants such as *Calliandra calothyrsus* Meisn., *Sesbania sesban* (L.) Merrill and *Leucaena diversifolia* have been used in agroforestry to improve yields but information on their effect on disease and pest incidence reduction is scanty which is of great importance in finding new strategies to reduce disease incidences while improving yield for food security

2.3.1 Agroforestry trees

2.3.1.1 *Calliandra calothyrsus*

Calliandra calothyrsus Meisn. is a small leguminous shrub native to Central America and grows at altitudes from sea level to 1,860 m in areas where the annual precipitation ranges from 700 to 3,000 mm (Abia *et al.,* 2006). It is multistemmed shrub which can attain a height of 12 m and a trunk diameter of 30 cm when growth conditions are favourable. The colour of the bark varies from white to dark red-brown and it has both superficial and deep growing roots (Orwa *et al.*, 2009). The plant has leaves that are alternate, petiolate, bipinnately compound, 10-19 cm long and without an upper waxy sheen and red or purple flowers that are arranged in a subterminal inflorescence with numerous long, hair like stamens. Fruits are broadly linear and flattened with a pod 8-13 cm long which breaks open, each half curling back to set free 3-15 shiny, black seeds when dry (Orwa *et al.*, 2009; Chamberlain, 2001).

Its use was predominantly for production of fodder for ruminant livestock but other uses are also found within different farming systems and include the provision of green manure, fuel wood,

shade for coffee and tea, land rehabilitation, erosion control, and honey production (Chamberlain, 2001; Abia *et al.,* 2006). Tannins from this plant have been reported (Firmansyah *et al.,* 2020; Mustabi *et al.,* 2019) to have antifungal and antiparasitic activity against *Ceratobasidium ramicola* that cause damage on several types of forestry and horticultural crops. As much as its benefits are known as an agroforesry and antifungal legume, still there is inadequate information on the antimicrobial properties against *Cercospora zeae-maydis* and *Xanthomonas campestris* pv. *musacearum* plant pathogens, that is vital in lowering dieses incidences thereby increasing yields of maize and bananas.

Calliandra calothyrsus leaf extracts from other parts of the world have been reported (Setyawati *et al.,* 2019) to posses' phytochemicals such as flavonoids, alkaloids, tannins, saponnins, and phytosterol which are linked to its antimicrobial activity. To understand the antimicrobial properties of this plant growing in western Kenya, its phytochemical compounds should be screened to give an overview of the composition of the leaf extracts as an antimicrobial agent for controlling GLS and Xanthomonas wilt diseases.

2.3.1.2 *Sesbania sesban*

Sesbania sesban (L.) Merrill is one of the most productive multipurpose tree that is widely distributed in tropics and subtropics of Africa and Asia and usually planted by smallholder farmers mostly for its fodder and soil improvement values (Nigussie and Alemayehu 2014).

Sesbania is an erect, branched, stout, shrubby plant which can grow to 2-3 meters high. Leave length ranges between 10 and 20 centimeters long, with 9 to 20 pairs of leaflets that are oblong and 2 to 3 centimeters long. Few flowers of the plant are yellow, about 1.5 centimeters long,

borne on axillary racemes. Sub-cylindrical or somewhat flattened pods are slightly twisted, pendulous, about 20 centimeters long, 3 millimeters wide, and depressed between the seeds (Samajdar and Ghosh, 2017).

It can grow in areas with a semi-arid to sub humid climate with a rainfall ranging between 500 and 2000 mm per year and temperature of 18 to 23°C (Degefu *et al.,* 2011; Orwa *et al.,* 2009). It can also occur in poorly drained soils which are subjected to periodic water logging or flooding as well as low temperatures (Nigussie and Alemayehu, 2013). Phytochemical screening revealed the presence of triterpenoids, starches, vitamins, amino acids, proteins, tannins, saponins glycosides and steroids (Kathiresh *et al.,* 2012; Gomase *et al.,* 2012).

The plant is usually used as a source of green manure due to its rapid foliage decomposition, forage, anti-inflammatory activities, reproduction and milk production enhancement, nitrogen fixation, bioenergy source, antibacterial and antiparasitic effect, antioxidant and mosquito repellent effects (Nigussie and Alemayehu, 2013; Degefu *et al.,* 2011). It is also used in diarrhoea, excessive menstrual flow, to reduce enlargement of spleen, in skin disease, inflammatory rheumatic swelling, as Anti-helmintic, Antidiabetic , as CNS stimulant and has antifertility effect (Gomase *et al.,* 2012; Samajdar and Ghosh 2017). Extracts from this plant have demonstrated significant antimicrobial activities against *Escherichia coli*, *Pseudomonas aeruginosa* and *Klebsiella pneumonia* (Nirosha *et al.,* 2019) but little is known on its antimicrobial activity against *Cercospora zeae-maydis* and *Xanthomonas campestris* pv. *musacearum*is which a key component in lowering development of GLS and Xanthomonas wilt

diseases of maize and bananas. Similarly, there is inadequate information on the phytochemical compounds of this plant that forms the basis of its antimicrobial potential.

2.3.1.3 *Leucaena diversifolia*

Leucaena diversifolia is an erect tree shrub of 3-20 m tall, with a single- stemmed bole of 20-50 cm in diameter, slender and grow up to 10 m tall with ascending branches that have horizontal twigs (Orwa *et al.,* 2009). It grows well in cool and seasonally wet locations with an average annual rainfall of 600-2800 mm and a mean maximum temperature of the hottest month of 18-30 °C. It has no capacity to withstand drought well, it has a strong light requirement as it tolerates shade only partially. *Leucaena diversifolia* do well in slightly acid, fertile soils, but is tolerant of leached soils (Orwa *et al.,* 2009; Bray and Sorensson, 1992).

Leucaena is planted in tropics mainly to provide crude protein for livestock. It is also planted to control soil erosion, as shelter tree over perennial crops, for reclamation of waste land and nitrogen fixation in soils with low nitrogen content (Walker, 2012; Orwa *et al.,* 2009). Some species in genus *Leucaena* like *Leucaena leucocephala* have great antimicrobial activity against human pathogens (Abu *et al.,* 2016; Aderibigbe *et al.,* 2011) while *Leucaena diversifolia* extracts are active against *Haemonchus contortus* (Pone *et al.,* 2011). However, little information is available on the antimicrobial activity of *Leucaena diversifolia* extracts against *Cercospora zeae-maydis* and *Xanthomonas campestris* pv. *musacearum* pathogens causing maize and banana diseases which is key in improving yield production for food security.

2.4 Maize and Bananas

2.4.1 Maize

Maize (*Zea mays* L.) is the main staple food particularly in western Kenya, averaging over eighty percent of total cereals grown in the country (Mbogo *et al.,* 2016; Wambugu *et al.,* 2012). However, its production has continued to lag behind and its production capacity has not kept pace with increasing demand for food. Consequently, low yields are recorded in the country and are attributed to abiotic and biotic constraints such as drought incidences, pests and diseases among other factors (Mbogo *et al.,* 2016). Poor yields of maize in Western Kenya are attributed to various factors which include unreliable rains, labour constraints at critical periods, use of recycled hybrid seed over several seasons, weeds, low and declining soil fertility, pests and disease problems (Odendo *et al.,* 2001).

2.4.1.1 Maize diseases

Maize is affected by various types of disease caused by fungi bacteria and viruses. Fungal diseases like Gray leaf spot (GLS) caused by *Cercospora zeae maydis* and Phaeosphaeria leaf spot (PLS) caused by *Phaeosphaeria maydis* (Henn.) are some of the deadly diseases of maize in western Kenya as they have been reported to play major roles in lowering maize production when interacting with other environmental factors (Bhatia and Munkvold, 2002; Bigirwa *et al*., 2001). Other fungal diseases include Brown spot (*Physoderma maydis*), Downy mildew (*Peronosclerospora species*), Common rust (*Puccinia sorghi*) Anthracnose Leaf Blight (*Colletotrichum graminicola*), Southern Rust (*Puccinia polysora*), Common Smut (*Ustilago maydis*), Crazy Top (*Sclerophthora macrospora*) and many more.

Some of important bacterial diseases of maize are Goss's Wilt (*Clavibacter michiganensis* subsp. *nebraskensis*), Holcus Leaf Spot (*Pseudomonas syringae pv. syringae*), Stewart's Disease or Stewart's Wilt (*Erwinia stewartii*), Bacterial Stalk Rot (*Erwinia chrysanthemi*) and Stewart's wilt (*Erwinia stewartii*) (Strunk and Byamukama *et al*., 2016; Pratt *et al.,* 2003).

Diseases of maize caused by viruses include; Maize chlorotic dwarf virus (MCDV), Maize chlorotic mottle virus (MCMV), Maize dwarf mosaic virus (MDMV), Maize lethal necrosis (MLN), Maize mosaic virus I (MMV), Maize stripe virus (M StV), Maize streak virus (MSV), Maize rough dwarf virus (MRDV), Maize fine stripe virus and Maize bushy stunt (MBS) (CIMMYT, 2004). Maize yield is also affected by insect pests which are destructive to the crop. They include African Maize Stalk Borer (*Busseola fusca)* (Calatayud *et al.,* 2014; Odendo *et al*., 2001), northern and western corn rootworm, European corn borer, black cutworm, corn leaf aphid, fall armyworm, true armyworm (Varenhorst *et al*., 2015).

2.4.1.2 Grey leaf spot

Gray leaf spot (GLS) is a disease that affects the foliar parts of maize and is caused by necrotrophic polycyclic fungal pathogen *Cercospora zeae maydis,* which survives as mycelium in the residues of infected maize crops after harvesting (Benson *et al.,* 2015; Sibanda *et al*., 2019). The disease was first highlighted as a threat to maize production in the USA in the 1980s, followed by South Africa in the 1990s and currently has a worldwide distribution in maize production areas, including South America and China (Berger *et al*., 2014; Dhami *et al*., 2015). In Kenya, the first incidence was reported during 1995 and small-scale farmers have continued to

experience considerable yield losses due to the effects grey leaf spot disease (Jagnani *et al.*, 2019; Kinyua *et al.,* 2010).

The disease can cause yield los of over 70% due to associated severe blighting, stalk deterioration and lodging (Benson *et al.,* 2015; Nega *et al.,* 2016). GLS disease begins with the conidia infecting the maize crops through the stomatal openings of leaves which germinate when humidity is high leading to the formation of rectangular lesions on maize leaves that reduce the photosynthetic potential and ultimately yield of the crop (Sibanda *et al*., 2019; Dhami *et al*., 2015). The symptoms of GLS disease develop from the lower leaves upwards on a maize plant leaves and reaches its maximum intensity after flowering which leads to poor grain filling hence low maize yields(Berger *et al*., 2014; Kinyua *et al.,* 2010).

In Western and Coastal Kenya, GLS disease incidences have been reported to be >50% because of high humidity that the pathogen requires to complete its infection process (Mwalugha *et al*., 2012). Several methods of managing GLS have been proposed including use of cultural control methods, chemical control (Mwalugha *et al*., 2012; Kinyua *et al.,* 2010), use of resistant maize varieties, folia fertilizers and biological control (Sserumaga *et al.,* 2020). The development and use of resistant lines has been proposed to be the most efficient and cost effective way of managing the disease, ecologically friendly and contributes to increased yield (Mwalugha *et al*., 2012; Sserumaga *et al.,* 2020) but more affordable and environmentally friendly methods of controlling GLS are still required.

2.4.2 Bananas

Banana (*Musa* spp) is an important crop for the people living in east and central Africa. It is regarded as a key staple food in the region and as a source of income for resource poor farmers (Nkuba *et al.,* 2015; Jogo *et al.,* 2011). The annual per capita consumption of banana in this region ranges from 400kg-600kg which is the highest in the world (Nkuba *et al.,* 2015). Despite its importance, the livelihoods of banana farmers are being threatened as the crop has been quickly losing ground as a dependable crop due to several biotic and abiotic constraints (Jogo *et al.,* 2011). These factors include poor crop production and management practices, insect pests and diseases, low and declining soil fertility, socio-economic factors such as inadequate capital, labour and marketing problems. Of all these factors, pests and diseases pose a serious threat to banana production (Wachira *et al.,* 2013).

2.4.2.1 Banana diseases

Some cultivars of banana have been severely damaged by a wide range of pests and diseases in western Kenya, resulting in heavy yield losses, for example, fields infested by *Xanthomonas* wilt or *Fusarium* wilt have reported losses of up to 100% (AATF, 2003). Other wide spread and important diseases of bananas in Kenya include Panama disease caused by *Fusarium oxysporum* f. sp. *cubense* (FOC), Black and Yellow sigatoka caused by *Mycosphaerella fijiensis* (Morelet) and *Mycosphaerella musicola* (Leach) respectively, weevils (*Cosmopolites sordidus*) and nematode (*Radopholus similis*) (Wachira *et al.,* 2013).

2.4.2.2 *Xanthomonas* wilt

Xanthomonas wilt is a disease of bananas caused by *Xanthomonas campestris* pv. *musacearum* and has been identified as the major constraint to banana production in the East and Central Africa (Nkuba *et al*. 2015; Uwamahoro *et al*., 2019). The disease was initially reported to have been identified on the banana relative enset (*Ensete ventricosum*) and bananas in Ethiopia in1968 (Adriko *et al*., 2011; Uwamahoro *et al*., 2019) from which it moved along the Great Lakes region of East and Central Africa. It was then reported in central Uganda in 2001; from where it spread to the Democratic Republic of Congo in 2004, Rwanda in 2005, Tanzania and Kenya in 2006(Adriko *et al*.,2011; Uwamahoro *et al.,* 2019).

Xanthomonas campestris pv. *musacearum* (Xcm) is a Gram negative bacteria, which invades the vascular system of banana causing wilting and death of the plant (Shimwela *et al.,* 2016). *Xanthomnas* wilt is primarily transmitted via infected planting material, contaminated garden tools, traded infected bunches covered by banana leaves that are discarded in banana fields, and vectors such as insects, birds, and bats (Shimwela *et al.,* 2016; Nakato *et al.,* 2013). Among the vectors, insects' especially stingless bees are the most important vectors when they are attracted to the male flower (Shimwela *et al.,* 2016). *Xanthomonas* wilt disease can cause up to 100% yield loss if control is delayed thereby severely compromising food and income security of households and communities (Ocimati *et al.,* 2019; Nkuba *et al*. 2015).

Once the banana plant has been infected, symptoms will include progressive yellowing and wilting of leaves, which starts with the youngest leaf (Uwamahoro *et al.,* 2019). This is then progresses to withering of male buds, premature ripening and fall off of the fruit and yellow

bacterial ooze will be observed in about 15 minutes after the pseudostem is cut (Uwamahoro *et al.,* 2019; Nakakawa *et al.,* 2017). Since the bacterium attacks all cultivars, it has become almost impossible to control the disease using the proposed mechanisms such as planting healthy suckers, breaking of male buds with a forked stick, disinfection of farm tools and removal of infected plants (Uwamahoro *et al.,* 2019b; Kubiriba and Tushemereirwe 2014). This is exacerbated by lack of knowledge by farmers and the methods being labour intensive costly (Uwamahoro *et al.,* 2019b; Nakakawa *et al.,* 2017) therefore calling for adoption of other management method such as botanicals and biological controls which are readily available and less costly.

2.5 Disease control in agroforestry system

Intercropping of food crops or food crops and non food crops has contributed considerably to the success of both traditional and modern agriculture as it provides a natural barrier to disease while increasing biodiversity (Luo *et al*., 2021; Lopes *et al.,* 2016). Similarly, planting a mixture of disease-susceptible and resistant varieties reduces the occurrence of disease incidences while enhancing ecosystem sustainability thus resulting in increased yield (Luo *et al*., 2021; Li *et al*., 2020; Chang *et al*., 2020). Chang *et al*. (2020) reported reduction of soybean root rot prevalence when intercropped with maize while Li *et al*. (2020) reported increased photosynthetic characteristics, vegetative growth and yield but decreased disease incidence of Panama disease in banana plantations intercropped with three Chinese chive cultivars.

Plant allelochemicals that reduces disease incidences and prevalence in intercropping systems results from exudation, decomposition and leaching or volatilization (Massalha *et al.,* 2017; Zhu and Morel 209). In developing countries agroforestry improves food security for smallholder farmers by improving soil health (Sileshi *et al.,* 2014) and disease and pest control by increased biodiversity (Karp *et al.,* 2013). In western Kenya maize and bananas are intercropped with *Calliandra calothyrsus, Leucaena diversifolia* and *Sesbania sesban* to address soil fertility and land degradation problems but inadequate information on the effect of these agroforestry trees on grey leaf spot and xanthomonas wilt affecting maize and bananas respectively is available.

2.6 Control of Grey leaf spot and Xanthomonas wilt.

Control of plant diseases for a long period has been based on the use of cultural methods and synthetic chemicals which have become ineffective and are known to have environmental and health problems (Gang *et al.,* 2013; Uwamahoro *et al.,* 2019b). For these reason, biological and botanical control measures of plant diseases are being sought to be used in management and control of plant diseases. Endophytes have shown great antimicrobial activity when tested against plant pathogens. For example, *Trichoderma koningii* and *Alternaria alternate* isolated from maize roots reduced growth of *Fusarium* pathogen by 25 -75% and 53 - 80%, respectively (Orole and Adejumo, 2009) while some banana endophytic bacteria inhibited growth of fungal pathogens of bananas (Souza *et al.,* 2014**).** As much as there is information on antimicrobial activity of endophytes from maize and bananas, there is no enough evidence on growth inhibition potential of endophytes of *Calliandra calothyrsus, Leucaena diversifolia* and *Sesbania sesban* against *Cercospora zeae-maydis* and *Xanthomonas campestris* pv. *musacearum* causing

grey leaf sport (GLS) and *Xanthomonas* wilt diseases of maize and bananas hence lowering their productivity.

Plants have phytochemical compounds with antimicrobial and antihelmithic activity against human pathogens. *Calliandra haematocephala* extracts have secondary metabolites that are active against selected gram positive and gram negative strains (Josephine *et al.,* 2017) and helminthes (Tiwari and Rai, 2016). *Leucaena leucocephala* extracts have been reported to poses phytochemical compounds that have antimicrobial and antioxidant activity (Zayed *et al.,* 2018). Studies by Mythili and Ravindhran (2012) and Samajdar and Ghosh (2017) shows that *Sesbania sesban* has phytochemical compounds with antimicrobial activities against human pathogens. Even though there is a lot of information on the phytochemical compounds of *Calliandra, Leucaena* and *Sesbania* species from different parts of the world, there is no enough evidence of phytochemical compounds of *Calliandra calothyrsus*, *Leucaena diversifolia* and *Sesbania sesban* which is vital in identifying antimicrobial properties of the plant extracts to control grey leaf spot and *Xanthomonas* wilt diseases.

The phytochemical compounds in plants or in the crude extracts are known to be of great biological importance. They have been reported to be directly responsible for different activity such as antioxidant, antimicrobial, antifungal and anticancer (Sheel *et al.,* 2014; Hossain *et al*., 2013). Saponins are bioactive chemical compounds which are involved in plant disease resistance because of their antimicrobial activity, as detergents, pesticides, mollucicides and are also used in healing of heart conditions (Hossein *et al*., 2013; Revathi, 2018; Nirosha *et al.,*

2019). Tannins are phenolic compound considered as primary antioxidants or free radical scavengers, antibacterial, antifungal and insecticides (Pizzi, 2019; Hossein *et al*., 2013).

Flavonoids have a wide range of biological activities such as scavenging for superoxide anions, anti-inflammatory, antimicrobial, anti-angionic, anticancer and anti-alergic properties (Nirosha *et al.,* 2019; Hossain *et al*., 2013). Alkaloids from medicinal plants have been reported to show biological activities like, anti-inflammatory, antimalarial, antimicrobial, cytotoxicity, antispasmodic and other pharmacological effects ((Hussain *et al.,* 2018; Matsuura and Fett-neto 2015; Iqbal *et al.*, 2015). Similarly, steroids from plants and their extracts have been reported to posses cardiotonic effect, antibacterial and insecticidal properties (Iqbal *et al.,* 2015). Terpenoids are biologically active compounds used for the treatment of many diseases and as anticancer drugs. They have been used as antimalarial drugs, cosmetics, hormones, vitamins and as pathogen and herbivore-induced resistance in plants (Bergman *et al.,* 2019; Tholl, 2015).

Plants continue to be reliable sources for discovery of useful compounds relevant to pest and disease management in food crops (Musyimi *et al*., 2008; Emitaro *et al.,* 2018; Izah, 2018; Sales *et al.,* 2015). Studies show that extracts from some species of *Calliandra* (Josephine *et al.,* 2017), *Leucaena* (Zayed *et al.,* 2018) and *Sesbania* (Samajdar and Ghosh, 2017) are active against human pathogens but little information is available on growth inhibitory activity of extracts from *C. calothyrsus, S. sesban* and *L. diversifolia* against *Cercospora zeae-maydis* and *Xanthomonas campestris* pv. *musacearum* pathogens of maize and bananas that are of great concern in lowering yields.

CHAPTER THREE: MATERIALS AND METHODS

3.1 Collection and processing of plant materials

Plant materials for isolation of endophytes and plant pathogens were collected from Maseno University farm located 0° 10' 0" South, 34° 36' 0" East along Kisumu Busia road. The location is ideal for the growing of bananas and maize as it has an average temperature of 20.6 °C and annual average rainfall of 1820 mm. This favours the development of diseases under study. Isolation of the endophytes and pathogens as well antagonistic studies and DNA extraction was carried out at Jaramogi Oginga Odinga University University of Science and Technology botany Laboratory located 0°05'38.0"S, 34°15'31.0"E along Bondo Usenge road.

Maize and banana fields intercropped with three agroforestry trees i.e *Calliandra calothyrsus, Leucaena diversifolia* and *Sesbania sesban* were used for collection of plant samples. The intercrops were of maize + banana+ *Calliandra calothyrsus,* maize + banana+ *Leucaena diversifolia* and maize + banana+ *Sesbania sesban.* Three plants were chosen randomly from each intercrop from which leaves, stems and roots were collected in triplicates for isolation of endophytes and extraction from leaves.

3.1.1 Ethanol and aqueous extraction

Leaves of *Calliandra calothyrsus, Leucaena diversifolia* and *Sesbania sesban* were collected in different cloth bags and taken to botany laboratory where they were identified and authenticated by a taxonomist. The plant materials were thoroughly washed with tap water to remove dust and other unwanted materials accumulated from their natural environment. Leaves were separated and allowed to dry under shade in the botany laboratory for 14 days turning them after every two

days to allow free and even air circulation and to avoid rotting. The dried plant materials were powdered separately using an electric motor model DS100 SDMAR. Finally, fine powder was collected from the powdered leaves by sieving through the kitchen strainer of pore size 0.25 mm and used for extraction (Okoli *et al.,* 2009; Abubakar, 2009).

Ten grams of each powdered plant leaf materials was separately kept in 500 ml conical flask and 100 ml ethanol and aqueous added respectively. The mouth of the conical flasks was covered with aluminium foil, mixed thoroughly and left to stand overnight for maximum extraction of active compounds. The extracts were filtered using muslin cloth followed by Whatman no 1 filter paper. Ethanol was evaporated using rotary vacuum evaporator with the water bath temperature of 45^{o}C. The filtrate was used to test for the presence of phytochemical compounds and test for antimicrobial activity of the extracts (Dent *et al.,* 2013). Foe aqueous extracts, the filtrate was considered to be 100% concentrated.

3.2 Study design and sampling procedure

General purpose media i.e Nutrient agar and potato dextrose agar were used for isolation of bacteria and fungi endophytes (Thi and Diep 2014; Mahadevamurthy *et al.*, 2016). From each plant, three leaves, pieces of stem and roots were collected using sterile scalpel, pooled together and leaves, stems and roots processed separately. Fungal diseases of maize common in western Kenya i.e Grey leaf spot (GLS) caused by *Cercospora zaea-maydis* and banana bacterial disease i.e bacterial wilt (*Xanthomonas* wilt) caused by *Xanthomonas campestris* pv. *musacearum* were identified using field identification manual (CIMMYT 2004; Viljoen *et al.,* 2017). Diseased banana and maize leaves were aseptically collected in polythne bags and used for isolation of

pathogens. Isolated pathogens were subjected to endophytes to assess growth inhibition potential. The plates were arranged in a completely randomized design in the oven. Phytochemical screening and antimicrobial activity of *Calliandra calothyrsus, Leucaena diversifolia* and *Sesbania sesban* ethanol and aqueous extract of leaves was carried out separately and in triplicates. Ethanol and aqueous solvents were used as controls.

3.3 Morphological and molecular profiling of endophytic bacteria and fungi of *Calliandra calothyrsus, Leucaena diversifolia* and *Sesbania sesban*

3.3.1 Isolation of endophytic bacteria and fungi

It was carried according to the procedure developed by Thi and Diep (2014) and Mahadevamurthy *et al.* (2016). Plant parts which included leaves, stems and roots were separately obtained from agroforestry trees. Roots were washed with tap water to remove attached soil. Stems, roots and leaves of each plant species were cut separately and immersed in 70% ethanol for 3 minutes then washed with 4% fresh sodium hypochlorite solution for 5 minutes and finally washed five times with sterile distilled water. To confirm that sterilisation process was successful, the aliquots of the sterile distilled water used in the final rinse was inoculated on nutrient agar (NA) medium plates. Plates were examined for presence or absence of bacterial growth after incubation at 28^{o} C for 3 days. Samples were cut into 0.5 cm pieces and macerated in 5 ml of aqueous solution (0.9 % NaCl) with a sterile mortar and pestle. The extract was allowed to stand for 30 minutes at room temperature to allow for complete release of endophytic microorganisms.

Tissue extracts was serially diluted in aqueous solution (0.9 % NaCl) and plated in triplicate on nutrient agar to recover any bacterial endophytes present in the plant tissue. Plates were incubated at 28 ^{o}C for 1-7 days or until growth was observed. Colonies were identified and isolated in pure cultures based on their morphological characteristics (Nhu and Diep *et al.*, 2017). Fungal endophytes were isolated by plating 3-5 pieces of each plant part separately on Potato dextrose agar plates incorporated with streptomycin (1.0 g/l) to inhibit bacterial growth. Plates were sealed with parafilm and incubated at 25 ± 2 ^{o}C for 7 days. The endophytic fungal colonies emerging from plant host were picked with sterile fine tip needle and sub cultured on fresh PDA plates devoid of antibiotic to obtain pure cultures which were identified based on their morphological characteristic.

3.3.2 Morphological characterisation of endophytic bacteria and fungi

Bacterial colonies recovered from plant parts were grouped based on colony morphology, colony colour, cell shape and Gram's reaction (Thi and Diep, 2014). Cell shape was determined by observing the cells in light microscope after staining with methylene blue stain. Gram's reaction was carried out according to Prasad and Dagar (2014). Thin smear of each bacterial isolate was prepared, heat fixed and flooded with crystal violet for 1minute then washed in tap water. The smear was treated with iodine for 1 minute followed by application of absolute alcohol for 30 seconds and then flooded with safranin for 1 minute. The smear was washed in tap water after every treatment and finally it was bloated, dried and observed under emulsion oil objective lens to reveal the colour and shape of the cells. Fungal isolates were identified according to Bisht *et al.* (2016) based on cultural characteristics such as top and bottom surface colour, morphology of

fruiting bodies and spores. Isolates were stained with lactophenol cotton blue and examined under a light microscope at ×40 to reveal the morphology of the spores and mycelia.

3.3.4 Molecular characterization of endophytes using 16S and ITS rDNA regions

3.3.4.1 Genomic DNA extraction

Forty two bacteria and thirty three fungi isolated in pure cultures from leaves, stems and roots of *Calliandra calothyrsus, Leucaena diversifolia* and *Sesbania sesban* were used in DNA extraction. DNA was extracted using the Zymo Research DNA Mini Prep™ kit according to the manufacturer's specifications (Zymo Research Corp, South Africa) Appendix V. The concentration and purity of extracted DNA was estimated using a Nanodrop™ Lite Spectrophotometer (Thermo Scientific Inc, USA) at 260-280 nm and by horizontal gel electrophoresis (Thistle Scientific Ltd, USA) on a 0.8% (w/v) agarose gel at 100V for 30 minutes (Appendix VI) and visualized under UV after staining with Gel Red™ (Thermo Scientific, USA) according to Emitaro *et al*. (2017).

3.3.4.2 PCR amplification and sequencing

PCR primers 27F (AGAGTTTGATCMTGGCTCAG) and 1492R (GGTTACCTTGTTACG ACGACTT) targeting the 16S rRNA gene regions were used according to White *et al* (1990). *Escherichia coli* was used as positive control of 16S rRNA genes of bacteria. Universal primers ITS1 5' TCCGTAGGTGAACCTGCGG 3' and ITS4 5' TCCTCCGCTTATTGATATGC 3' (Sigma Aldrich) were used to amplify the fungal intergenic spacer ITS1 and ITS4 region (Chowdhary and Kaushik 2015; Ofek-Lalzar *et al*., 2016). Bioneer Accu Power® PCR Premix

(BioneerInc, USA) was used to perform PCR. To each 20µl Bioneer reaction tube, 2 µl DNA, 2 µl Taq buffer, 1.4 µl Mgcl2, dNTPs 0.4 µl, Primers 2 µl, Taq DNA Polymerase 0.4 µl, Nuclease free water 11.8 µl was added. Amplification was performed in a programmable Master thermocycler (C1000-Bio Rad, USA). The PCR conditions included denaturation, annealing, initial and final extension at temperatures of 94^{o}C for 30 sec, 55^{o}C for 1 min, 72^{o}C for 2 minutes respectively before cooling off at 15^{o}C. PCR products were separated by horizontal gel electrophoresis on 1.5% (w/v) agarose gel at 100V for 45mins and visualized under UV after staining with 2µl Gel RedTM (Thermo Scientific). The quality of amplified PCR products recovered was assessed in horizontal gel electrophoresis on 1.5% (w/v) agarose gel at 100V for 45mins and visualized under UV after staining with 2µl Gel RedTM (Thermo Scientific). The PCR products were then sent to Macrogen Europe B.V. **(**Meibergdreef 311105 AZ, Amsterdam, Netherlands) for purification and sequencing. Forward and reverse sequences were assembled and trimmed on Geneious Prime® 2020.0.4 and submitted to NCBI BLAST (www.ncbi.blast.lnm.nih.gov) to obtain the accession numbers of the isolates and similarity search.

3.3.5 Determination of species diversity of endophytic microorganisms

Shannon diversity index (H') and Simpson diversity index (1/D) was used for the evaluation of endophytic fungal and bacterial species diversity (Chowdhary and Kaushik, 2015; Meenatchi *et al.*, 2016).

Shannon wiener diversity index was calculated using the formula by Chowdhary and Kaushik (2015)

$$H' = \sum(Pi)(In\ Pi)$$

Where : Pi – is the proportion of individuals found in species I = ni/N, where ni is the number of individuals in species i and N is the total number of individuals in the community.

1n – log to base 2

Simpson's index of diversity was calculated using the formula by Meenatchi *et al.* (2016)

$$D = 1 - \sum n \frac{(n-1)}{N(N-1)}$$

Where n = the total number of organisms of a particular species while N = the total number of organisms of all species.

Bacteria and fungal percentage dominance was determined by Camargo's index ($1/D_{mn}$), where D_{mn} represents species richness (Chhipa and Kaushik, 2017).

3.4 Determination of molecular phylogenetic relationship of the endophytes

Twenty seven bacterial and twenty two fungal isolates were successfully sequenced and assembled using Geneious Prime® 2020.0.4. Assembled multiple sequences of approximately 500 bp were transferred to MEGA Version 6.0 software and aligned using Clastal W method according to Tamura *et al*. (2013). Sequences with greater than 97% similarity were retrieved for phylogenetic analysis. Evolutionary histories and diversity of the isolates were determined using the Neighbour-Joining method and distances computed using the Jukes-Cantor model (Tamura *et al.,* 2011). A bootstrap test (1000 replicates) was used to cluster associated taxa and replicate trees with above 50% likelihoods indicated on the branches.

3.5 Growth inhibition potential of endophytic bacteria and fungi against *Cercospora zeae-maydis* and *Xanthomonas campestris* pv. *musacearum*

3.5.1 Isolation of plant pathogens

Diseased banana and maize leaves were collected and processed according to Khaiyam *et al*. (2017). Diseased plant parts with specific symptoms of Grey leaf spot (GLS) of maize and *Xanthomonas* wilt of bananas based on field identification manual (CIMMYT 2004; Viljoen *et al.,* 2017) were collected using sterile scissors and polythene bags and taken to the laboratory. Samples were washed thoroughly under running tap water and surface sterilized with 4% NaOCl, rinsed several times in distilled water and blotted to dry.

3.5.1.1 Isolation of fungal pathogens

Fungal pathogens from maize leaves were isolated according to Nega *et al.* (2016). Leaf samples were cut into pieces of approximately 5 cm and placed on sterile moist blotter in a sterile petri dish. Five sections of diseased tissue were placed in each petri dish and incubated at 25^{o}C for 5 days to allow the pathogen to develop and sporulate in growth cabinets under a 12 hour fluorescent light/dark regime. The sporulating diseased sections were examined under a binocular microscope for the presence of conidia. Conidia were picked with an isolation needle and plated on PDA, allowing at least three pickings per leaf sample to obtain pure cultures. Plates were incubated at 25^{o}C for 5-7 days and hyphal tips from the advancing colony margins transferred onto PDA with isolating needle as pure culture and kept at 5^{o}C.

3.5.1.2 Isolation of bacterial pathogen

Xanthomonas campestris pv. *musacearum* was isolated according to Adriko *et al.* (2016). Approximately 1 g of the sample was crushed in 1 ml of sterile distilled water in a Petri dish. The suspension was spread on semi-selective YPGA (Yeast extract-5 g l^{-1}, Peptone-5 g /l, Glucose-4 g /1, Agar-12 g/1) medium containing antibiotics cephalexin (40 mg/1), 5-fluorouracil (10 mg/1) and cycloheximide (120 mg/1) to inhibit growth other unwanted bacteria. Inoculated plates were incubated at 28^{o}C for 48–72 hours. Mucoid yellow-pigmented colonies were picked and purified on nutrient agar (NA) medium.

3.5.2 Pathogenecity test of *Xanthomonas campestris* pv. *musacearum* and *Cercospora zeae-maydis isolate*

3.5.2.1 Pathogenicity test of *Xanthomonas campestris* pv. *musacearum* isolate

Six suckers of susceptible banana seedling were transplanted into pots measuring 22×22 cm, previously filled with sterilised forest sun-dried mixture of soil, sand and manure at a ratio of 3:1:1 in a green house according to Chala *et al*. (2016). Ports were arranged in a randomized complete block design and banana plants allowed to establish for three months to develop four to seven leaves. A suspension of the isolate was prepared and the concentration of bacterial suspension adjusted using a spectrophotometer at 0.3 optical density at 460 nm, which is equivalent to 10^{8} cfu/ml bacteria cells. Three plants were inoculated by injecting 3ml of well mixed aliquot of the bacterial suspension by inverting up and down into the petiole base of the newly expanding central leaf using a sterile 10 ml hypodermic syringe. Inoculated plants were covered with a wet plastic bag to increase humidity to maximum for 48 hours. Three plants used

as controls were inoculated with the same volume of sterile distilled water. Pathogenicity was determined by looking for yellowing of the leaf at margin side and tip, wilted leaf and blade folded upward and inward and also dry leaf 10 to 20 days after inoculation (Chala *et al*., 2016).

3.5.2.2 Pathogenicity test of *Cercospora zeae-maydis* isolate

This was done according to Lyimo *et al.* (2013). Eighteen hybrid maize were hand sown in 15 cm pots containing sterilised forest soil and kept in green house. Di-ammonium phosphate fertiliser was applied during planting at the rate of 1.5 g/pot. Calcium ammonium nitrate was applied at second leaf (V2) growth stage at the rate of 2.5 g/pot. Experimental pots were arranged in a randomized complete block design. Fungal isolate was inoculated on fresh PDA and incubated in darkness for 9 day to induce sporulation. Conidial suspension was prepared by adding 5ml of sterile distilled water onto fresh cultures, then straining suspension between two layers of cheesecloth and conidia concentration adjusted to 2×10^4 conidia/ml a in a spectrophotometer at 460 nm. Four plants were inoculated in triplicates at sixth leaf (V6) growth stage by spraying the conidia suspension of *C. zeae-maydis* using a hand sprayer until runoff. Inoculated plants were covered with transparent plastic bags for 5 day for maximum humidity. Pathogenicity was detected by observing typical lesions 13 to 19 days post inoculation.

3.5.3 Growth inhibition potential of endophytic bacterial and fungal isolates against *Xanthomonas campestris* pv. *musacearum*

3.5.3.1 Growth inhibition potential of endophytic bacterial isolates against *Xanthomonas campestris* pv. *musacearum*

A modified agar disk diffusion method was used to determine growth inhibition potential of 42 endophytic bacterial isolates against *Xanthomonas campestris* pv. *musacearum* in triplicates (Mohamad *et al.,* 2018). Endophytic bacteria and *Xc* pv. *musacearum* were separately pre-cultured in nutrient broth overnight and 5 mL of each culture centrifuged at 604 x g for 5 minutes. The supernatant was discarded and pellets suspended in sterile phosphate buffered saline (PBS) in a laminar air flow cabinet and density adjusted to 10^{-8} colony forming units (CFU) mL^{-1} using U.V spectrophotometer. Bacterial concentrate of 200 µL was evenly inoculated on nutrient agar using sterile cotton swabs and four 5-mm-diameter pieces of sterile filter paper placed on each corner of the petri dish in triplicates. A total of 10 µL concentrate of each bacterial endophyte isolate was added dropwise to the filter paper and plates were wrapped with parafilm and arranged in a completely randomized design in an oven. Culture plates were incubated at 28± 2^{o}C for 24 hours. Bacterial growth inhibition activity was assessed by measuring the diameter of the clear zone of growth inhibition.

3.5.3.2 Growth inhibition potential of endophytic bacterial isolates against *Cercospora zeae-maydis*

This was done according to Brunda *et al*. (2018). Forty two bacterial isolates were cultured on nutrient agar medium and incubated at 28^{o} C overnight while fungal pathogen was grown on

potato dextrose agar (PDA) plates and incubated for 6 days. The fungal pathogen and bacterial endophytes were inoculated at equidistant opposite sides of the PDA Petri plate. Control plates were inoculated only with the pathogen. Growth inhibition was calculated according to the formula by Brunda *et al*. (2018).

$$\text{Growth inhibition}\% = \frac{C - T}{C} \times 100$$

Where : C = mycelia growth in control (mm), T = mycelia growth in treatment (mm)

3.5.4 Growth inhibition potential of endophytic fungal isolates against *Xanthomonas campestris*pv. *musacearum* and *Cercospora zeae-maydis*

3.5.4.1 Growth inhibition potential of endophytic fungal isolates against *Xanthomonas campestris*pv. *musacearum*

Bacterial growth inhibition of fungal endophytes was assessed using agar plug diffusion (Marcellano *et al*., 2017). Thirty three isolated endophytic fungi were cultured on PDA for seven days at room temperature to obtain maximum growth. Mueller-Hinton Agar (MHA) plates were prepared and uniformly seeded with 100µl of test bacteria using sterile cotton swabs. Endophytic fungi mycelia agar plugs with diameter of approximately 8mm were cut from the PDA plate of actively growing fungi using sterile cork borer and transferred to MHA plates with the test bacteria. The plates were sealed with parafilm and then incubated at 28^{o}C for 24 hours.

Growth inhibition activity was determined by visualization and measuring the zone of growth inhibition using a transparent ruler and recorded in millimetres.

3.5.4.2 Growth inhibition potential of endophytic fungal isolates against *Cercospora zeae-maydis*

Growth inhibition activity of the 33 isolated fungi against fungal pathogens was determined using dual culture method (Katoch and Pull, 2017). Discs of isolated endophyte and pathogen measuring 0.5 mm were co-cultured at two equidistant opposite ends of PDA plates, sealed with parafilm and incubated at 25±2^{o}C for 7 days. Plates inoculated with fungal pathogen disc at the centre without endophyte served as control. The experiment was replicated three times with plates being arranged in completely randomized design. Radial growth of pathogenic fungi in the presence and absence of the endophyte was measured after 7 days, and growth inhibition percentage calculated using the formula (Katoch and Pull, 2017):

$$\text{Growth inhibition } \% = \frac{\text{CDC} - \text{CDT}}{\text{CDC}} \times 100$$

Where CDC – represents the colony radial growth of pathogen in mm on the control plate

CDT- represents the colony radial growth of pathogen in mm on the test plate

3.6 Determination of phytochemical compounds of *Calliandra calothyrsus, Leucaena diversifolia* and *Sesbania sesban* leaf extracts used for antimicrobial activity

3.6.1 Test for Steroids

2ml of chloroform and 2ml of concentrated H_2SO_4 were added with 5ml aqueous plant crude extract and then heated. Formation of a brown ring indicated the presence of steroids (Setyawati *et al.,* 2019).

3.6.2 Test for Terpenoids

Two milliliters of chloroform was mixed with 5 ml aqueous plant extract and heated in water bath and then boiled with 3 ml of concentrated H_2SO_4. Formation of grey colour indicated presence of terpenoids (Bhandary *et al*., 2012).

3.6.3 Test for Saponins

Five millilitres of test solution was mixed with 5ml of water, shaken vigorously and observed for the formation of foam, which is stable for 15 minutes for a positive result (Gul *et al.*, 2017).

3.6.4 Test for Alkaloids

Presence of alkaloids was determined according to Sheel *et al.* (2014). Five milliliters of plant extract was warmed with 2ml of 2% H_2SO_4 for two minutes, filtered and 3 drops Mayer's reagent added. Appearance of a creamy- white colour precipitate indicated a positive result

3.6.5 Test for Flavonoids

Alkaline reagent test was carried out where 2 ml of 2.0% NaOH was mixed with 5ml aqueous plant crude extract. A concentrated yellow colour was produced, which became colourless when

2 drops of diluted H_2SO_4 acid was added. Colourless appearance indicated presence of flavonoids. (Gul *et al.*, 2017).

3.6.6 Test for Tannins

Presence of tannins in the plant extract was determined by Ferric Chloride Test method as described by Sheel *et al.* (2014). 50mg of the extract was boiled with 5 ml of 45% solution of ethanol for 5 minutes, cooled and filtered. 1ml of filtrate was diluted with distilled water in a ration of 1:1 and two drops of ferric chloride added. A transient greenish to black colour indicated the presence of Tannins.

3.8 Growth inhibition activity of ethanol and aqueous extracts of leaves of *Calliandra calothyrsus, Leucaena diversifolia* and *Sesbania sesban* against *Xc.* pv *musacearum* and *Cercospora zeae-maydis*

The ethanol extracts were reconstituted using Dimethyl sulfoxide (DMSO) to make concentrations of 12.5mg/ml, 25mg/ml/ 50mg/ml and 75mg/ml while aqueous extracts were reconstituted into concentrations 12.5%, 25%, 50% and 75%. To prepare 12.5%, 12.5ml of the extract was measured and transferred into 100ml volumetric flask then toped up to 100ml using distilled water. Same procedure was repeated with other concentrations. The growth inhibition activity of leaf extracts of *Calliandra calothyrsus*, *Leucaena diversifolia* and *Sesbania sesban* against *Xc*. pv *musacearum* was determined using disc agar diffusion sensitivity test method (Bauer *et al*., 1966). Colonies from pure culture were lawn spread on Mueller Hinton agar (MHA) plates and discs impregnated with 10µl of each test extract placed on the surface aseptically. Discs impregnated with pure water and DMSO served as negative controls.

Antifungal activity of the extracts against *Cercospora zeae-maydis* was determined using poisoned food technique (Durgeshlal *et al.,* 2019) by dispensing 4 ml of each extract in petriplates and adding 16 ml of PDA them mixing and allowing them to set. A 5mm mycelia plug from 7 days old mycelia was inoculated at the centre of the plate then incubated for 7 day. PDA plated without extract was inoculated to serve as control. The treatments were done in triplicates. Petri plates were arranged in a completely randomized design (CRD) and incubated for 48 hours at 30^{o}C. Zones of growth inhibition were measured in millimeters using a transparent ruler for bacteria while for fungi radial growth of mycelia was measured. Growth inhibition percentage was determined using the formula of Durgeshlal *et al.* (2019).

$$\text{Growth inhibition } \% = \frac{\text{DC} - \text{DT}}{\text{DC}} \times 100$$

DC - colony diameters of the control

DT - colony diameters of the treated.

3.9 Data analysis

Morphological and cultural characteristics of endophytic bacterial and fungal colonies were subjected to a hierarchical cluster analysis using the squared Euclidean distance similarity and between groups linkage procedures using SPSS software version 20. Diversity indices were calculated using Simpson and Shannon Diversity indices. The percentage growth inhibition was calculated based on the ratio between the average inhibition and the average growth of the control. Phylogenetic analysis was performed by comparing sequences of the isolates deposited in the National Centre for Biotechnology Information database (http://www.ncbi.nlm.nih.gov).

The multiple sequences comparison analysis was performed with neighbough joining using MEGA software. The stability of the tree clades resulting from analysis was assessed by bootstrap analysis with 1000 replicates. Triplicate data from the antimicrobial activity of the endophytes and plant extracts was subjected to analysis of variance (ANOVA) and means separated by least significant difference at $P \leq 0.05$.

CHAPTER FOUR: RESULTS

4.1 Morphological and molecular profiling of endophytes isolated from *Calliandra calothyrsus, Leucaena diversifolia* and *Sesbania sesban*

4.1.1 Isolated endophytes

A total of 75 different colonies of both bacteria and fungi were isolated from leaves, stems and roots of the three agroforestry trees. This comprised of 42 bacterial and 33 fungal endophytes. Generally, there were more bacterial (56%) than fungal (44%) isolate in all the three plants (Table 4.1.1). Leaves of the three plants species had more bacterial isolates compared to the stem and roots while there were more fungal isolates in roots compared to stem and leaves of *C. calothyrsus, S. sesban* and *L. diversifolia* (Table 4.1.1).

Table 4.1.1 Percentage recovery of bacterial and fungal endophytes from leaves, stems and roots of *C. calothyrsus, S. sesban* and *L. Diversifolia*

% Recovery of endophytic bacteria and fungi from leaf, stem and roots						
Plant species /	**Bacteria**			**Fungi**		
% endopyyte	**leaf**	**stem**	**root**	**leaf**	**stem**	**root**
C. calothyrsus	37.5	31.3	31.3	9.1	45.5	45.5
L. diversifolia	42.9	35.7	21.4	21.4	28.6	50.0
S. sesban	50.0	25.0	25.0	25.0	37.5	37.5
%***Bacterial isolates***	**56**					
%***Fungal isolates***	**44**					

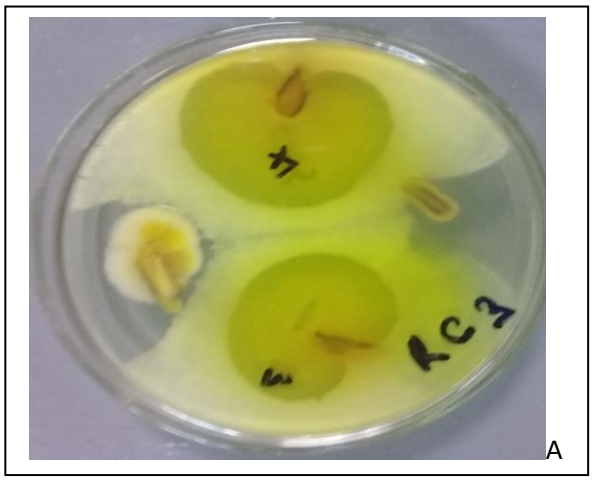
A total of 33 fungi endophytes were isolated on potato dextrose agar (PDA) based on colour of the mycelia and fruiting bodied and coded based on the plant part and plant species of origin (*FLC*-fungi leaf *Calliandra*, *FSC*-fungi stem *Calliandra*, *FRC*- fungi root *Calliandra*, *FLS*- fungi leaf *Sesbania*, *FSS*- fungi stem Sesbania, *FRS*- fungi root *Sesbania*, *FLL*- fungi leaf *Leucaena*, *FSL*- fungi stem *Leucaena* and *FRL*- fungi root *Leucaena*) (Table 4.1.2 and Plate 4.1.1A and B). On staining, most had septate mycelia while a few were aseptate (Plate 4.1.2A and B).

Using colony characteristics, 42 pure bacterial isolates were recovered on nutrient agar, identified and corded based on the part and plant species of origin (*BLC*- bacteria leaf *Calliandra*, *BSC*- bacteria stem *Calliandra*, *BRC*- bacteria root *Calliandra*, *BLS*- bacteria leaf *Sesbania*, *BSS*- bacteria stem *Sesbania*, *BRS*- bacteria root *Sesbania*, *BLL*- bacteria leaf *Leucaena*, *BSL*- bacteria stem *Leucaena* and *BRL*- bacteria root *Leucaena* (Table 4.1.2 and Plate 4.1.3). On Gram staining the isolates were grouped into Gram positive bacilli, Gram positive cocci, Gram negative Bacilli and Gram negative cocci (Plate 4.1.4 and 4.1.5 A and B).

Table 4.1.2. Fungal and bacterial endophytes isolated from *C. calothyrsus*, *L. diversifolia* and *S. sesban* leaves, stems and roots

FUNGAL ISOLATES								BACTERIAL ISOLATES							
Isolate	**code**	**plant species and parts**		**Isolate**	**Code**	**plant species and parts**		**Isolate**	**Code**	**plant species and parts**		**Isolate**	**Code**	**plant species and parts**	
1	*FLC1*	leaf		23	*FRL5*	root		1	*BLL1*	leaf		23	*BSS3*	stem	
2	*FSC1*	stem		24	*FRL6*	root		2	*BLL2*	leaf		24	*BRS1*	root	
3	*FSC2*	stem		25	*FLS1*	leaf		3	*BLL3*	leaf		25	*BRS2*	root	
4	*FSC3*	stem		26	*FLS2*	leaf		4	*BLL4*	leaf		26	*BRS3*	root	
5	*FSC4*	stem		27	*FSS1*	stem		5	*BLL5*	leaf		27	*BLC1*	leaf	
6	*FSC5*	stem		28	*FSS2*	stem		6	*BLL6*	leaf		28	*BLC2*	leaf	
7	*FRC1*	root		29	*FSS3*	stem		7	*BSL1*	stem		29	*BLC3*	leaf	
8	*FRC2*	root		30	*FSS4*	stem		8	*BSL2*	stem		30	*BLC4*	leaf	
9	*FRC3*	root		31	*FRS1*	root		9	*BSL3*	stem		31	*BLC5*	Leaf	
10	*FRC4*	root		32	*FRS2*	root		10	*BSL4*	stem		32	*BLC6*	leaf	
11	*FRC5*	root		33	*FRS3*	root		11	*BSL5*	stem		33	*BSC1*	stem	
12	*FLL1*	leaf						12	*BRL1*	root		34	*BSC2*	stem	
13	*FLL2*	leaf						13	*BRL2*	root		35	*BSC3*	stem	
14	*FLL3*	leaf						14	*BRL3*	root		36	*BSC4*	stem	
15	*FSL1*	stem						15	*BLS1*	leaf		37	*BSC5*	stem	
16	*FSL2*	stem						16	*BLS2*	leaf		38	*BRC1*	root	
17	*FSL3*	stem						17	*BLS3*	leaf		39	*BRC2*	root	
18	*FSL4*	stem						18	*BLS4*	leaf		40	*BRC3*	root	
19	*FRL1*	root						19	*BLS5*	leaf		41	*BRC4*	root	
20	*FRL2*	root						20	*BLS6*	leaf		42	*BRC5*	root	
21	*FRL3*	root						21	*BSS1*	stem					
22	*FRL4*	root						22	*BSS2*	stem					

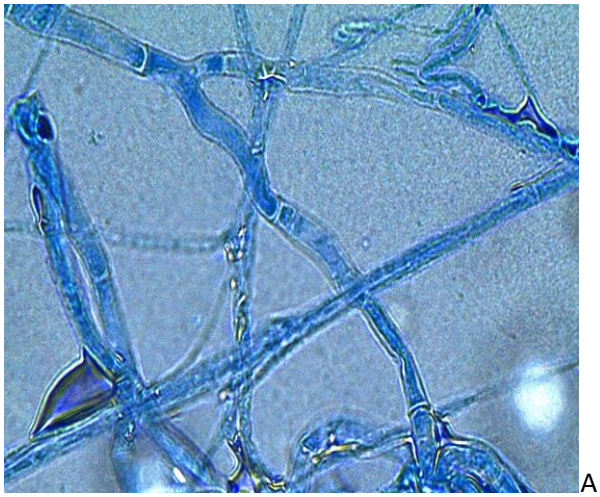
C. calothyrsus — ***S. sesban*** — ***L. diversifolia*** — ***L. diversifolia*** — ***S. sesban*** — ***C. calothyrsus***



A

B

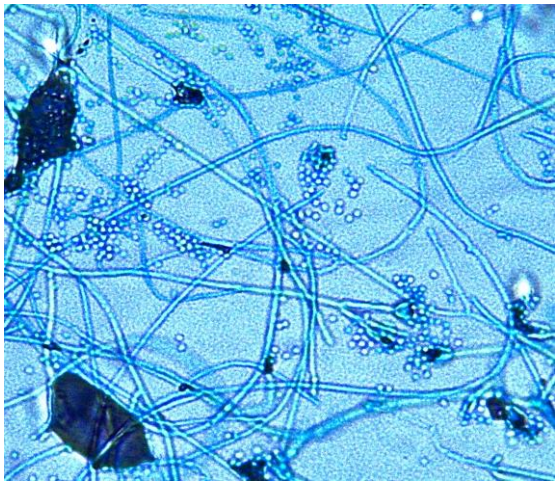
Plate 4.1.1A and 4.1.1B: Fungal isolates from roots of *C. calothyrsus* and *L. diversifolia*



A

Septas ×40

Plate 4.1.2A: - Septate mycelia,



B

Plate 4.1.2B-Aseptate mycelia (×40)

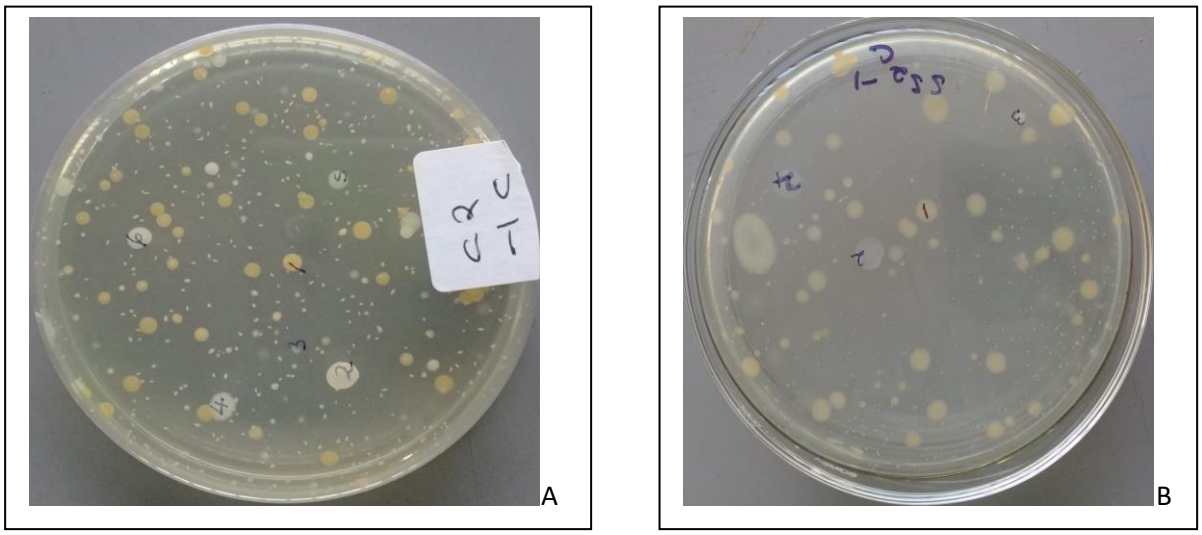


Plate 4.1.3: Bacterial isolates. A- *Calliandra* isolates, B- *Sesbania* isolat

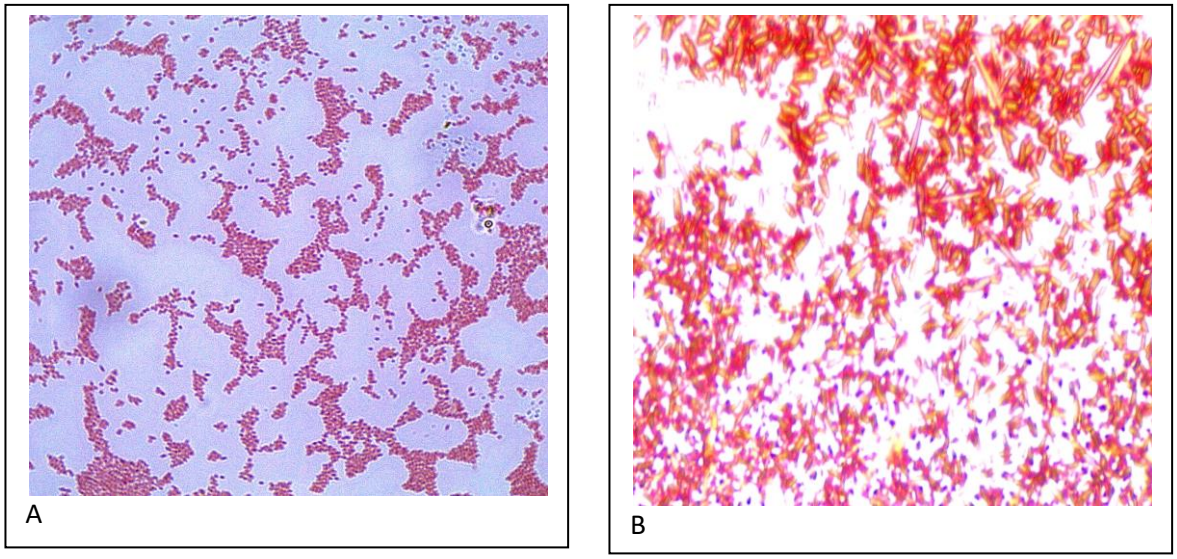


Plate 4.1.4: Gram's stained cells (×100). A- Gram negative cocci, B- Gram negative bacilli

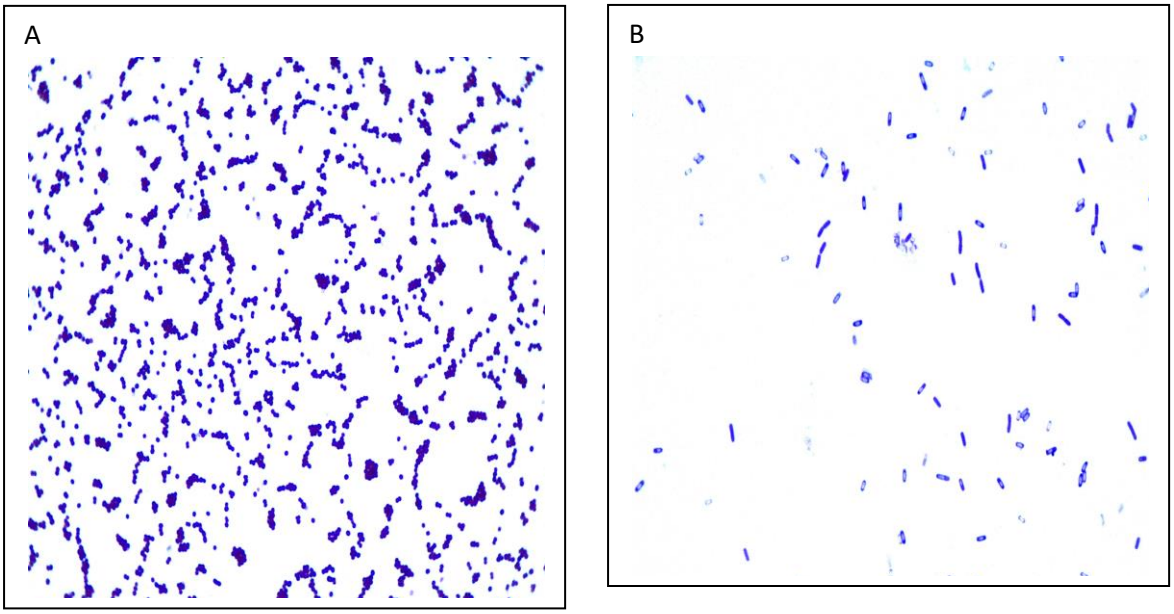


Plate 4.1.5: Gram's stained cells (×100). A- Gram positive cocci, B- Gram positive bacilli

4.1.2. Morphological characterization of endophytes

4.1.2.1 Morphological characterization of fungi isolates

Fungal isolates were grouped based on the appearance on PDA and type of septation after staining and observation using compound microscope (Table 4.1.3). White septate (*FLC1, FSC5, FRS2, FLL3, FSL1*), grey aseptate (*FRC1, FRL1, FRS1*) and green aseptate (*FRC4, FSS3, FLL2, FSL2, FRL4*) fungi were isolated from all the three plants i.e *C. calothyrsus, S. sesban* and *L. diversifolia.* White septate fungi were isolated from leaf and stem of *C. calothyrsus,* root of *S. sesban,* leaf and stem of *L. diversifolia.* Grey aseptate fungi were isolated from roots of the three plants. Green aseptate fungi were isolated from roots of *C. calothyrsus,* stem of *S. sesban,* leaf, stem and root of *L. diversifolia*. Green septate (*FRL6, FRS3*) fungi were isolated from roots of *L. diversifolia* and *S. sesban,* blue septate (*FRC5, FRL5*) and cream septate (*FRC3,FSL3*) from roots *C. calothyrsus* and roots and leaves of *L. diversifolia,* yellow aseptate (*FLS2, FSS2,FLL1,*

FSL4), green/pink septate (*FRL2*), cream aseptate (*FRL3*) and black aseptate (*FSS4*) from *S. sesban* and *L. diversifolia,* while yellow septate (*FSC3, FSS1*) and white aseptate (*FSC2, FRC2, FLS1*) were isolated from *C. calothyrsus* and *S. sesban*. Blue/green septate (*FSC4*) and yellow/cream (*FSC4*) fungi were isolated from *C. calothyrsus* only.

When the phenotypic characteristics of the fungal isolates were compared by hierarchical cluster analysis, the isolates separated into two main clusters A and B at 75% similarity level (Figure 4.1.1). Cluster A comprised of fungal isolates from roots and stems of all the three plants whose mycelia was septate but differed in terms of colour of the mycelia and fruiting bodied. Cluster B consisted of many isolates with two subclusters, 1and 2. Subcluster 1comprised of fungi isolated from the roots of the three plants, which were aseptate but only differed in colouration as one (FRL2) had green pink colour while the rest appeared grey. Subcluster 2 consisted of two further groups, I and II. Cluster B, subcluster 2, group I consisted of fungi that appeared yellow in colour but differed in septation as some (*FSC3, FSS1*) were septate while the rest were aseptate. Cluster B, subcluster 2, group II comprised of many mixed isolates. Some had septate mycelia while some had aseptate mycelia and they also varied in terms of colouration.

Table 4.1.3. Morphological characteristics of endophytic fungi isolated from *C. calothyrsus*, *L. diversifolia* and *S. sesban*

	Mycelia Characteristics		***C. calothyrsus***			***S. sesban***			***L. diversifolia***		
Fungal Isolates	**Appearance**	**septation**	**leaf**	**stem**	**root**	**leaf**	**stem**	**root**	**leaf**	**stem**	**root**
FSC3, FSS1	Yellow	septate	-	√	-	-	√	-	-	-	-
FRC4, FSS3, FLL2, FSL2, FRL4	Green	aseptate	-	-	√	-	√	-	√	√	√
FLC1, FSC5, FRS2,FLL3, FSL1	White	septate	√	√	-	-	-	√	√	√	-
FSC2, FRC2, FLS1	White	aseptate	-	√	√	√	-	-	-	-	-
FRC3,FSL3	Cream	septate	-	-	√	-	-	-	-	√	-
FLS2, FSS2,FLL1, FSL4	Yellow	aseptate	-	-	-	√	√		√	√	-
FRC1, FRL1,FRS1	Grey	aseptate	-	-	√	-	-	√	-	-	√
FRL2	Green/Pink	septate	-	-	-	-	-	-	-	-	√
FRL3	Cream	aseptate	-	-	-	-	-	-	-	-	√
FRL6, FRS3	Green	septate	-	-	-	-	-	√	-	-	√
FSC1	Blue/green	septate	-	√	-	-	-	-	-	-	-
FRC5, FRL5	Blue	septate	-	-	√	-	-	-	-	-	√
FSS4	Black	septate	-	-	-	-	√	-	-	-	-
FSC4	Yellow/cream	septate	-	√	-	-	-	-	-	-	-

Key: √ fungi present – fungi absent. Cording: 1st letter F- Fungi, 2nd- part of the plant (L-Leaf, S- Stem, R- root), 3rd- plant species (C- *Calliandra*, L- *Leucaena*, S- *Sesbania*)

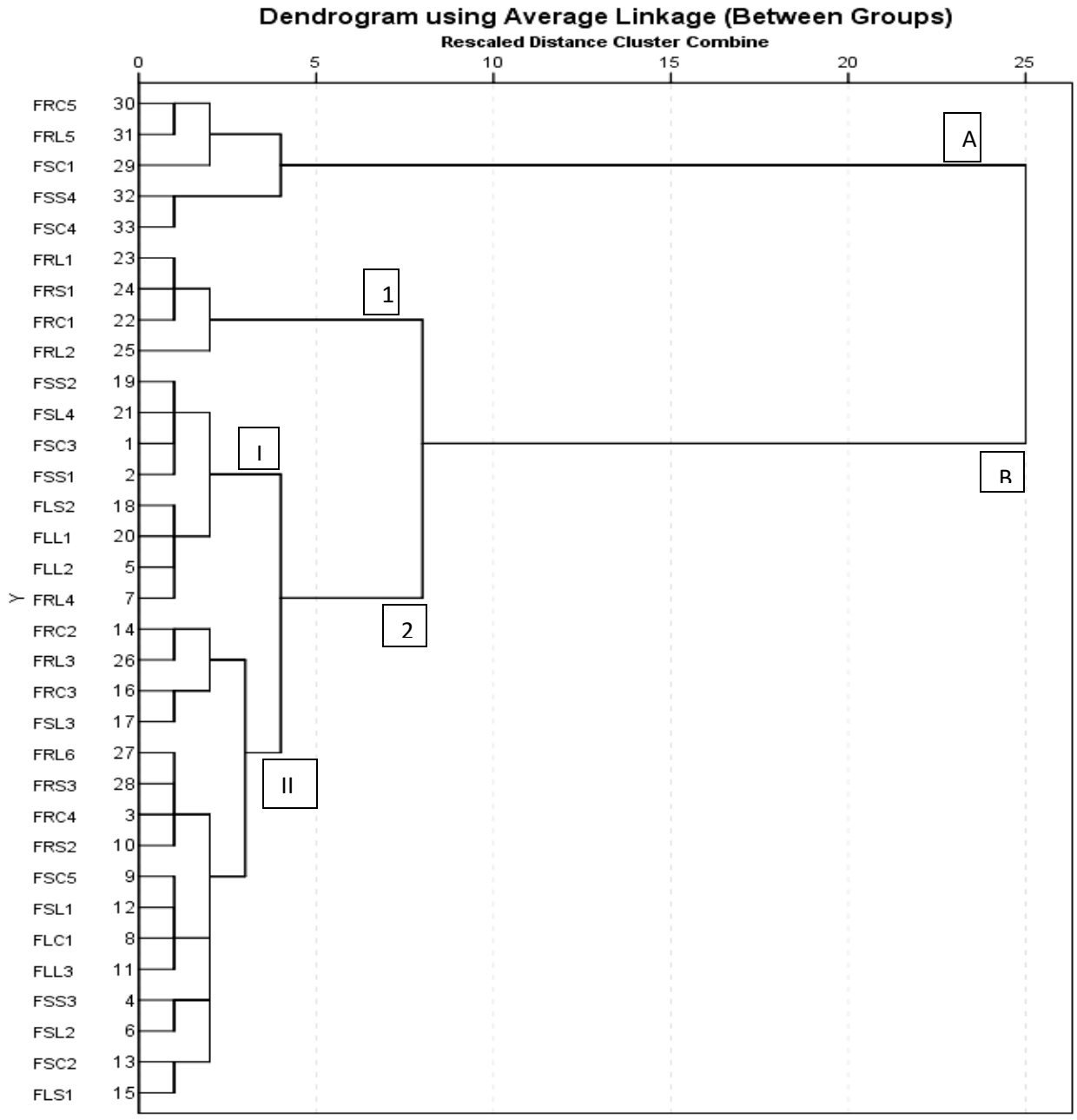


Figure 4.1.1. Dendrogram obtained by hierarchical cluster analysis of bacterial morphological characteristics. Key: *FLC*-fungi leaf *Calliandra*, *FSC*-fungi stem *Calliandra*, *FRC*- fungi root *Calliandra*, *FLS*- fungi leaf *Sesbania*, *FSS*- fungi stem Sesbania, *FRS*- fungi root *Sesbania*, *FLL*- fungi leaf *Leucaena*, *FSL*- fungi stem *Leucaena* and *FRL*- fungi root *Leucaena*

4.1.2.2 Morphological characterization of bacterial endophytes

Forty two bacterial endophytes were recovered and characterized using colony characteristics, Gram's reaction and shape of the cells (Table 4.1.4).Bacterial colonies which were yellow, raised, entire and opaque (*BLL1, BSL1, BRL1, BLS1, BSS1, BRS1, BLC1, BLL5, BSC1, BRC1*) and those exhibiting white colour, raised, entire margins and opaque (*BLL3, BSL3, BRL2, BSS2, BRC2,BLS6*) were isolated from all the three plant parts i.e leaves, stems and roots of *L. diversifolia*, *C. calothyrsus* and *S. sesban*. These two groups of colonies contained Gram negative cocci cells of bacteria. Colonies that appeared white, flat, entire, translucent and Gram negative bacilli on staining (*BSL2, BRL3, BLS2,BLC3, BRC4*) were isolated from leaves stem and roots of *L. diversifolia*, leaves and roots of *C. calothyrsus* and leaves of *S. sesban*. White, filamentous, irregular margins and opaque colonies (*BLL2, BLS4, BLC4*) which stained Gram negative and bacilli in shape were isolated from leaves of the three plants. Colonies that were cream in colour, raised, entire on margins and opaque (*BSL4, BLS3, BSS3, BRS3, BLC5, BSC2, BRC3*) were isolated from leaves, stem and roots of *C. calothyrsus, S. sesban* and stem of *L. diversifolia.* White, raised, undulated and opaque colonies (*BLC6, BSC5, BRC5*) which stained Gram positive bacilli were isolated from leaves, stems and roots of *C. calothyrsus,* light yellow raised entire opaque and Gram negative (*BLL6, BSC4*) from stem and leaves of *C. calothyrsus* and *L. diversifolia* respectively while colonies with white appearance, flat, entire margins, translucent and stained Gram positive with cocci cell (*BLL4, BLS5*) were isolated from *S. sesban* stems and *L. diversifolia* leaves.

Table 4.1.4. Morphological characteristics of endophytic bacterial isolates from *C. calothyrsus*, *L. diversifolia* and *S. sesban*

Bacterial Isolates	**Colony Characteristics**				***C. calothyrsus***			***S. sesban***			***L. diversifolia***				
	colour	**elevation**	**margin**	**opacity**	**L**	**S**	**R**	**L**	**S**	**R**	**L**	**S**	**R**	**G. stain**	**shape**
BLL4, BLS5	White	flat	entire	translucent	-	-	-	-	+		+			+ve	cocci
BLL1, BSL1, BRL1, BLS1, BSS1, BRS1, BLC1, BLL5, BSC1, BRC1	yellow	raised	entire	opaque	+	+	-	+	+	+	+	+	+	-ve	cocci
BLL3, BSL3, BRL2, BSS2, BRC2,BLS6	White	raised	entire	opaque	+	+	+	+	+	+	+	+	+	-ve	cocci
BSL2, BRL3, BLS2,BLC3, BRC4	White	flat	entire	Translucent	+	-	+	+	-	-	+	+	+	-ve	bacilli
BLL2, BLS4, BLC4	White	filamentous	irregular	opaque	+	-	-	+	-	-	+	-	-	-ve	bacilli
BSL4, BLS3, BSS3, BRS3, BLC5, BSC2, BRC3	Cream	raised	entire	opaque	+	+	+	+	+	+	-	+	-	-ve	bacilli
BLC6, BSC5,BRC5	White	raised	undulated	opaque	+	+	-	-	-	-	-	-	-	-ve	cocci
BLL6, BSC4	Light yellow	raised	entire	opaque	-	+	-	-	-	-	+	-	-	-ve	bacilli

4 Key: + Bacteria present – Bacteria absent. Cording: 1st letter B- Bacteria, 2nd- part of the plant (L-Leaf, S- Stem, R- root), 3rd- plant species (C- *Calliandra*, L- *Leucaena*, S- *Sesbania*)

Based on hierarchical cluster analysis, endophytic bacterial isolates clustered into two main clusters A and B at 75% similarity level (Figure 4.1.2). Clusters B comprised of few isolate colonies which were white in colour, opaque, Gram negative cocci and bacilli. Cluster A consisted of subcluster 1 and 2 of which subcluster 1 comprised of colonies that were raised, entire, opaque and had Gram negative bacilli. Subcluster 2 further separated into group I and II at 82.5% similarity level. Cluster A, subclaster 2, group II, consisted of colonies that were white in colour, entire, flat and translucent. The colonies varied in shape and Gram stain as some were Grarm positive cocci (*BLL4, BLS5*) while the rest were Gram negative bacilli. Cluster A, subcluster 2, group I, comprised of many isolates whose colonies were raised, entire, opaque but varied in colouration as some were white while some were yellow. All the isolates in this group were Gram negative cocci.

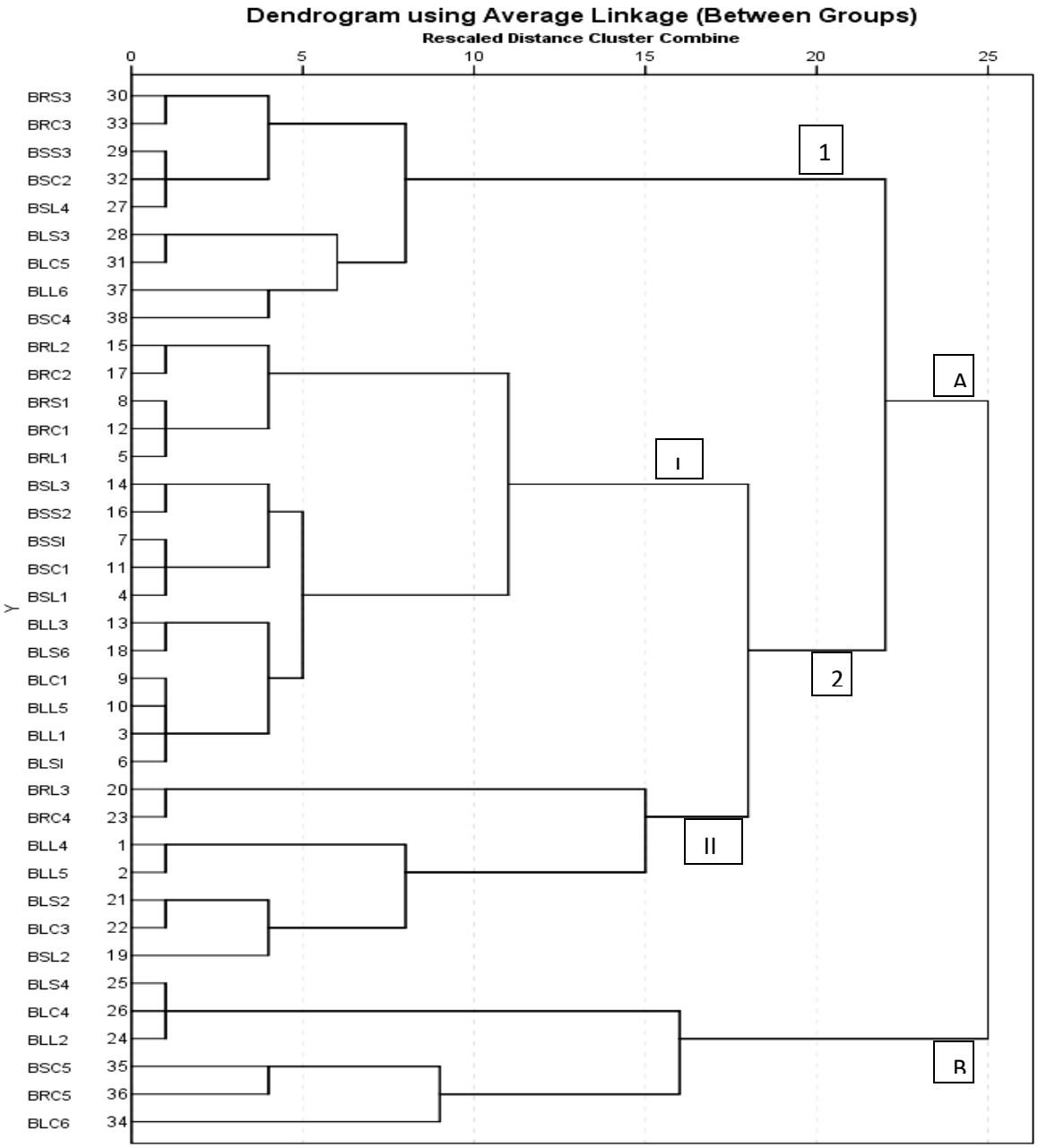


Figure 4.1.2. Dendrogram obtained by hierarchical cluster analysis of bacterial morphological characteristics.

Key: *BLC*- bacteria leaf *Calliandra*, *BSC*- bacteria stem *Calliandra*, *BRC*- bacteria root *Calliandra*, *BLS*- bacteria leaf *Sesbania*, *BSS*- bacteria stem *Sesbania*, *BRS*- bacteria root *Sesbania*, *BLL*- bacteria leaf *Leucaena*, *BSL*- bacteria stem *Leucaena* and *BRL*- bacteria root *Leucaena*

4.1.3 Molecular characterization

The molecular weight of the genomic DNA of bacterial and fungal isolates was confirmed on 0.8% agarose stained with SYBR green. Intense bands of the bacterial DNA isolate (Plate 4.16) and fungal DNA (Plate 4.17) had equal molecular weight of 1400bp and 700bp respectively along with the 100bp DNA ladder.

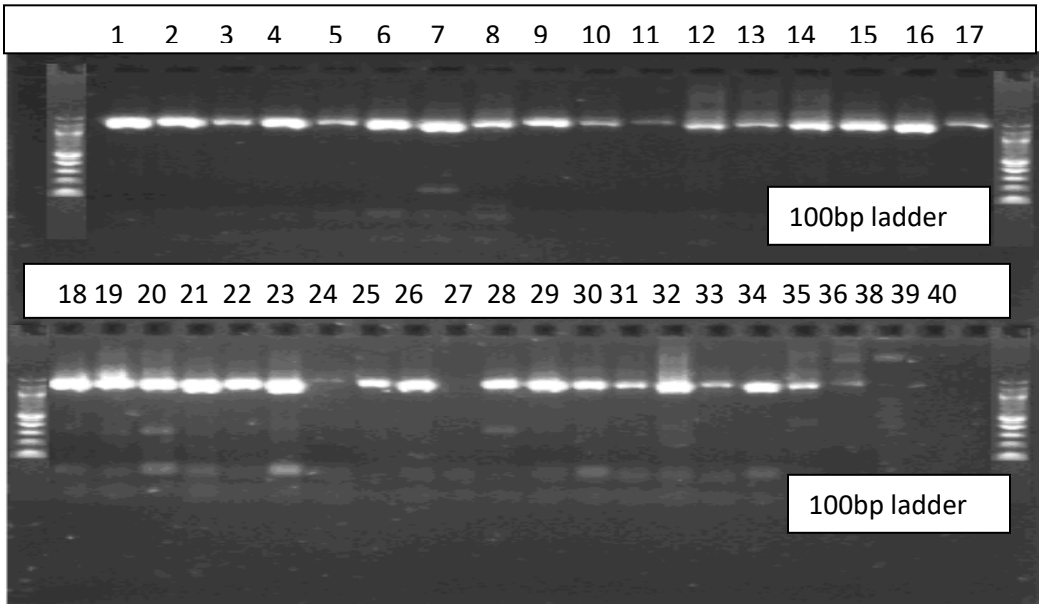


Plate 4.1.6. Gel image of 16S rRNA amplicon. 1- 40 are endophytic bacterial isolates DNA amplified at 16S rRNA region and run on agarose gel. Amplified DNA from all the bacterial isolates had same molecular weight of 1400 bp and aligned at 1400 bp of the DNA ladder as clear bands.

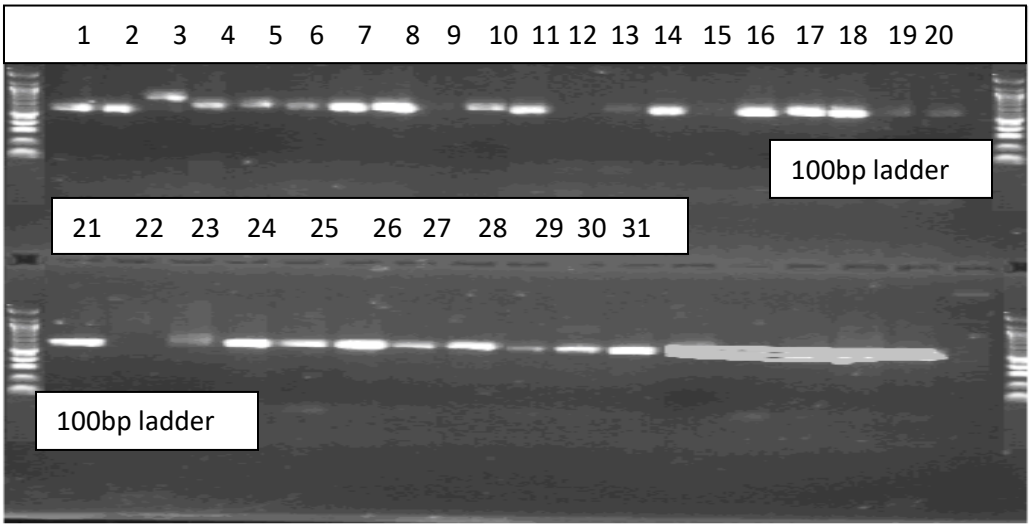


Plate 4.1.7. Gel image of ITS rRNA amplified amplicon. 1- 31 are fungal isolate rDNA amplified at ITS rRNA region and run on agarose gel. Amplified rDNA from all the fungal isolates had same molecular weight of 700 bp and aligned at 700 bp portion of the DNA ladder as clear bands.

On sequencing 16S rRNA gene for bacteria and ITS rDNA gene for fungi, sequences of the isolates were different in the arrangement of the nucleotide base pairs. Fungal sequences submitted to National Centre of Biotechnology Information (NCBI) for similarity search showed >97.00% match identity to those already deposited in the Genebank database except for isolate *FLL2* whose highest identity match was 92.00% and therefore regarded as a new species. The isolate sequences were deposited to NCBI GeneBank and given accession numbers ranging from MW262927.1 to MW262948.1 (Table 4.1.5). Based on Basic Alignment search Tool (BLAST) searches, fungal isolates were found to belong to four genera with the dominant genus being *Trichoderma* (68.2%) followed by *Mucor* (13.6%), *Aspergilus* (13.6%) and *Penicillium* (4.6%)(Table 4.1.3A).

Table 4.1.5. Maximum nucleotide identity matches of fungal isolates based on ITS sequences using BLAST analysis and accession numbers

Isolate identity	Match identity (%)	Species	Accession no	Genus % dominance
FSC4	100.00	*Trichoderma longibrachiatum*	MW262929.1	*Trichoderma*
FSC5	100.00	*Trichoderma harzianum*	MW262930.1	68.2%
FRL6	99.52	*Trichoderma harzianum*	MW262942.1	
FLS1	100.00	*Trichoderma harzianum*	MW262943.1	
FLS2	100.00	*Trichoderma longibrachiatum*	MW262944.1	
FSS1	100.00	*Trichoderma harzianum*	MW262945.1	
FSS2	97.97	*Trichoderma longibrachiatum*	MW262946.1	
FRC2	99.68	*Trichoderma harzianum*	MW262932.1	
FRC4	100.00	*Trichoderma harzianum*	MW262933.1	
FLL2	92.00	*Trichoderma harzianum*	MW262935.1	
FSL1	100.00	*Trichoderma harzianum*	MW262936.1	
FSL2	100.00	*Trichoderma harzianum*	MW262937.1	
FSL4	99.68	*Trichoderma harzianum*	MW262938.1	
FRL2	99.84	*Trichoderma harzianum*	MW262939.1	
FRS3	99.68	*Trichoderma harzianum*	MW262948.1	
FSCI	99.79	*Penicillium citrinum*	MW262927.1	*Penicillium* 4.6%
FSC3	97.00	*Mucor circinelloides*	MW262928.1	*Mucor* 13.6%
FRC1	100.00	*Mucor circinelloides*	MW262931.1	
FRL3	99.06	*Mucor fragilis*	MW262940.1	
FRL5	99.62	*Aspergillus fumigatus*	MW262941.1	*Aspergillus*
FRC5	100.00	*Aspergillus fumigatus*	MW262934.1	13.6%
FSS3	99.90	*Aspergillus niger*	MW262947.1	

Sequences with <97% identity to the closest known relative in database is considered new species (Elijah *et al.*, 2014).

Bacterial sequences submitted to National Centre of Biotechnology Information (NCBI) for similarity search showed >97.00% match identity to those already deposited in the Genebank database except for isolate *BLS1 and BLS2* whose highest identity match was 91.07% and 87.08 respectively and were regarded as a new species. The isolate sequences were deposited to NCBI Bankit and given accession numbers ranging from MW251519.1 to MW251545.1 (Table 4.1.6). Basic Alignment search Tool (BLAST) search revealed ten bacterial genera dominated by genus *Bacilli* (33.3%), followed by *Staphylococcus* (22.2%), *Alcaligens* (11.2%), *Xanthomonas* and *Sphingomonas* at (7.4%) each. Other genera were *Pantoea, Pseudomonas, Acinetobacte, Bacterium* and *Enterobacteria* at 3.7% each (Table 4.1.3B).

Table 4.1.6. Maximum nucleotide identity match of bacterial isolates based on 16S rRNA sequences using BLAST analysis and accession numbers.

Isolate identity	**Match identity (%)**	**species**	**Accession no**	**Genus dominance**	**%**
BLL4	99.86	*Staphylococcus pasteuri*	MW251519.1	*Staphylococcus*	
BLL6	99.41	*Staphylococcus epidermidis*	MW251521.1	22.2%	
BSL1	99.93	*Staphylococcus warneri*	MW251522.1		
BLC4	100.00	*Staphylococcus epidermidis*	MW251536.1		
BLC5	99.49	*Staphylococcus sp*	MW251537.1		
BLC6	100.00	*Staphylococcus pasteuri*	MW251538.1		
BSS1	99.78	*Bacillus tequilensis*	MW251529.1	*Bacillus* 33.3%	
BSS2	99.93	*Bacillus sp.*	MW251530.1		
BRS3	99.29	*Bacillus toyonensis*	MW251533.1		
BLC1	100.00	*Bacillus altitudinis*	MW251534.1		

BLC3	99.48	*Bacillus toyonensis*	MW251535.1	
BLL5	99.35	*Bacillus toyonensis*	MW251520.1	
BSC1	99.08	*Bacillus toyonensis*	MW251539.1	
BSC3	100.00	*Bacillus cereus*	MW251541.1	
BRC1	99.33	*Bacillus toyonensis*	MW251543.1	
BSL3	99.93	*Xanthomonas campestris pv. campestris*	MW251523.1	*Xanthomonas* 7.4%
BRL3	99.85	*Xanthomonas campestris pv. campestris*	MW251524.1	
BLS1	91.07	*Alcaligenes aquatilis*	MW251525.1	*Alcaligenes* 11.2%
BLS2	87.08	*Alcaligenes faecalis*	MW251526.1	
BLS3	98.91	*Alcaligenes faecalis*	MW251527.1	
BLS5	97.81	*Enterobacteriaceae bacterium*	MW251528.1	*Enterobacteriaceae* 3.7%
BRC3	99.37	*Sphingomonas echinoides*	MW251544.1	*Sphingomonas*
BRC5	99.22	*Sphingomonas echinoides*	MW251545.1	7.4%
BSS3	98.19	*Acinetobacter johnsonii*	MW251531.1	*Acinetobacter* 3.7%
BRS1	94.84	*Bacterium strain*	MW251532.1	*Bacterium* 3.7%
BSC2	99.90	*Pantoea agglomerans*	MW251540.1	*Pantoea* 3.7%
BSC5	99.03	*Pseudomonas plecoglossicida*	MW251542.1	*Pseudomonas* 3.7%

Sequences with <97% identity to the closest known relative in database is considered new species (Elijah *et al.*, 2014).

4.1.4 Diversity of fungal and bacterial isolates

Following Shannon-Wiener index (H´) and Simpson diversity index (D) calculation, it was noted that *C. calothyrsus* (H'=0.95, D=0.75) had higher diversity of fungal endophytes as compared to *L. diversifolia* (H'=0.74, D=0.46) and *S. sesban* (H'=0.45, D=0.33) (Table 4.1.7). For bacterial isolates, higher diversity indices were recorded in *S. Sesban* followed by *C. calothyrsus* then *L. diversifolia* Table 4.1.8. Diversity of bacterial endophytes in the three plants was compared to fungal endophytes in both Shannon-Wiener index (H´) and Simpson diversity index (D). Diversity indices were, Shannon diversity index (H'=0.9) and Simpson diversity (D= 0.8) for fungal endophytes, while bacterial isolates had Shannon diversity index of (H'=1.9) and Simpson diversity index of (H=0.5) Table 4.1.9.

Table 4.1.7. Diversity indices of fungal isolates of *C. calothyrsus, L. diversifolia* and *S. sesban*

C. calothyrsus				***L. diversifolia***				***S. sesban***			
Genus	**no**	**H cal.**	**D cal.**	**Genus**	**no**	**H cal**	**D cal**	**Genus**	**no**	**H cal**	**D cal**
Penicillium	1	-0.259	0	*Trichoderma*	6	-0.215	0.535	*Trichoderma*	5	-0.151	0.666
Mucor	2	-0.346	0.035	*Mucor* spp	1	-0.259	0	*Aspergillus*	1	-0.298	0
***Trichoderm*a**	4	-0.346	0.214	*Rhizopus*	1	-0.259	0		5	0.450	0.333
Aspergillus	1	-0.259	0		**8**	0.735	0.464				
	8	0.953	0.75								
H'	**0.95**				**0.74**				**0.45**		
D	**0.75**				**0.46**				**0.33**		

Key; H- Shannon diversity Index, D- Simpson diversity Index

Table 4.1.8. Diversity indices of bacterial isolates of *C. calothyrsus, L. diversifolia* and *S. sesban*

C. calothyrsus				***L. diversifollia***				***S. sesban***			
Genus	**no**	**H Calc.**	**D Calc.**	**Genus**	**no**	**H Calc.**	**D Calc.**	**Species**	**no**	**H Calc.**	**D Calc.**
Bacillus	5	-0.36478	0.151515	*Staphylococcus*	3	-0.3631	0.1429	*Alcaligenes*	2	-0.3466	0.0357
Staphylococcus	3	-0.34657	0.25	*Bacillus*	1	-0.2780	0.00	*Bacterium*	1	-0.2599	0.00
Pantoea	1	-0.20708	0.0	*Alcaligenes*	1	-0.2780	0.00	*Bacillus*	3	-0.3678	0.1071
Pseudomonas	1	-0.20708	0.0	*Xanthomonas*	2	-0.3579	0.0476	*Acinetobacter*	1	-0.2599	0.00
Sphingomonas	2	-0.29863	0.015152		7	1.2770	0.8095	Enterobacteriaceae	1	-0.2599	0.00
	12	1.42413	0.583333						8	1.4942	0.8571
H'		**1.4**				**1.3**				**1.5**	
D		**0.6**				**0.8**				**0.9**	

Key; H- Shannon diversity Index, D- Simpson diversity Index

Table 4.1.9. Diversity of bacterial and fungal isolates of the three plants

Bacterial isolates				**Fungal isolates**			
Genus		**H cal.**	**D cal.**	**Genus**		**H calc.**	**D calc**
Staphylococcus	6	-0.3342	0.0427	*Penicillium*	1	-0.1405	0
Bacillus	9	-0.3662	0.1026	*Mucor*	3	-0.2717	0.012987
Sphingomona	2	-0.1928	0.0028	*Trichoderma*	15	-0.26113	0.454545
Xanthomonas	2	-0.1928	0.0028	*Aspergillus*	3	-0.2717	0.012987
Pseudomona	1	-0.1221	0.0000		22	0.945023	0.519481
Pantoea	1	-0.1221	0.0000				
Alcaligenes	3	-0.2441	0.0085	**H'**	**0.9**		
Enterobacteriaceae	1	-0.1221	0.0000	**D**	**0.5**		
Acinetobacter	1	-0.1221	0.0000				
Bacterium	1	-0.1221	0.0000				
		1.9405	0.8405				
H'		**1.9**					
D		**0.8**					

Key; H'- Shannon diversity Index, D- Simpson diversity Index

4.2 Phylogenetic relationship of endophytes of *Calliandra calothyrsus, Leucaena diversifolia* and *Sesbania sesban*

4.2.1 Evolutionary relationships of fungal isolates

The evolutionary profile of the fungal endophytes isolated from *C. calothyrsus, L. diversifolia* and *S. sesban* conducted in Molecular Evolutionary Genetic Analysis (MEGA 6.0) software clustered the isolates into three clades (Figure 4.2.1) representing three orders which included hypocreales, eurotiales and mucorales. Out of 22 sequences analyzed, 15 clustered in Clade I (order hypocreales) with 100% bootstrap support and the clade comprised of fungi in the genus *Trichoderma* isolated from the three plants. Clade II had isolates in the order eurotiales divided into genus *Penicillium* and *Aspegilus* with 100% bootstrap support. *Aspagillus* were isolates from all the three plants while *Penicillium* was isolated from *C. calothyrsus* only. Clade III had three isolates from *L. diversifolia* and *C. calothyrsus* all belonging to order mucorales and genus *Mucor* with 100% bootstrap support (Table 4.2.1). Evolutionary, members of order hypocreales and order eurotiales belong to the phylum Ascomycota while members of the order mucorales belong to the phlum Zygomycota. Majority of the isolates were from phylum Ascomycota (86.4%) while a few were from phylum Zygomycoata (13.6%) Table 4.2.1.

Figure 4.2.1. Neighbour joining phylogenetic tree of fungal isolates of *C. calothyrsus L. diversifolia* and *S. sesban* based on ITS rDNA gene sequence. Clade I-V shows how the sequences of the fungal isolate clustered in relation their similarity. Fungi clustering in the same clade have highly similar sequences and are closely related

Key: ●- *C. calothyrsus* isolates, ■- *L. diversifolia* isolates, ▲- *S. sesban* isolates.

Table 4.2.1. Evolutionary relationship of endophytic fungal isolates

Accession no	Species	Genus	Family	Order	Class	Phylum % dominance
MW262929.1	*Trichoderma longibrachiatum*					
MW262930.1	*Trichoderma harzianum*					
MW262942.1	*Trichoderma harzianum*					
MW262943.1	*Trichoderma harzianum*	*Trichoderma*	Hypocreaceae	Hypocreales	Sordariomycetes	
MW262944.1	*Trichoderma longibrachiatum*					
MW262945.1	*Trichoderma harzianum*					
MW262946.1	*Trichoderma longibrachiatum*					**Ascomycota 86.4%**
MW262932.1	*Trichoderma harzianum*					
MW262933.1	*Trichoderma harzianum*					
MW262935.1	*Trichoderma* sp					
MW262936.1	*Trichoderma harzianum*					
MW262937.1	*Trichoderma harzianum*					
MW262938.1	*Trichoderma harzianum*					
MW262939.1	*Trichoderma harzianum*					

MW262948.1	*Trichoderma harzianum*					
MW262927.1	*Penicillium citrinum*	*Penicillium*	Trichocomaceae	Eurotiales	Eurotiomycetes	
MW262941.1	*Aspergillus fumigatus*					
MW262934.1	*Aspergillus fumigatus*		Trichocomaceae	Eurotiales	Eurotiomycetes	
MW262947.1	*Aspergillus niger*	*Aspergillus*				
MW262928.1	*Mucor circinelloides*	*Mucor*	Mucoraceae	Mucorales	Mucoromycetes	**Zygomycota**
MW262931.1	*Mucor circinelloides*					13.6%
MW262940.1	*Mucor fragilis*					

4.2.2 Evolutionary relationship of endophytic bacterial isolates

The phylogenetic tree of endophytic bacterial isolates of the three plants showed six clades (I-VI) representing different orders as follows; bacillales, sphingomonadeles, xanthomonadales, , enterobacterales, pseudomonodales and burkholderiales (Figure 4.2.2). The orde bacillales had two genus; *Bacilli* with nine sequences and *Staphylococci* with six sequences clustered at 100% bootstrap support each. Bacterial endophytes in the order bacilllales were isolated from all the three plants i.e *C. calothyrsus L. diversifolia* and *S. sesban.* Order sphingomonadeles and xanthomonadales had two isolates each supported by 100% bootstrap. Order enterobacterales had three genera; *Enterobacteriaceae, Pantoea* and *Bacterium* supported by 100% bootstrap. Order pseudomonodales had two genera *Acinetobacter* and *Pseudomonas* while order burkholderiales had one genus Alcalgenes. Isolates in the order pseudomanodales and those in the order burkholderiales were supported by 65% and 100% bootstraps respectively. Endophytic bacteria in the order pseudomonodales were from *C. calothyrsus* and *S. sesban* while those in the order burkholderiales were from *C. calothyrsus* and *L. diversifolia.* Most of the isolates belonged to phylum proteobacteria (66%) while a few were in the phylum firmicutes (44%) (Table 4.2.2).

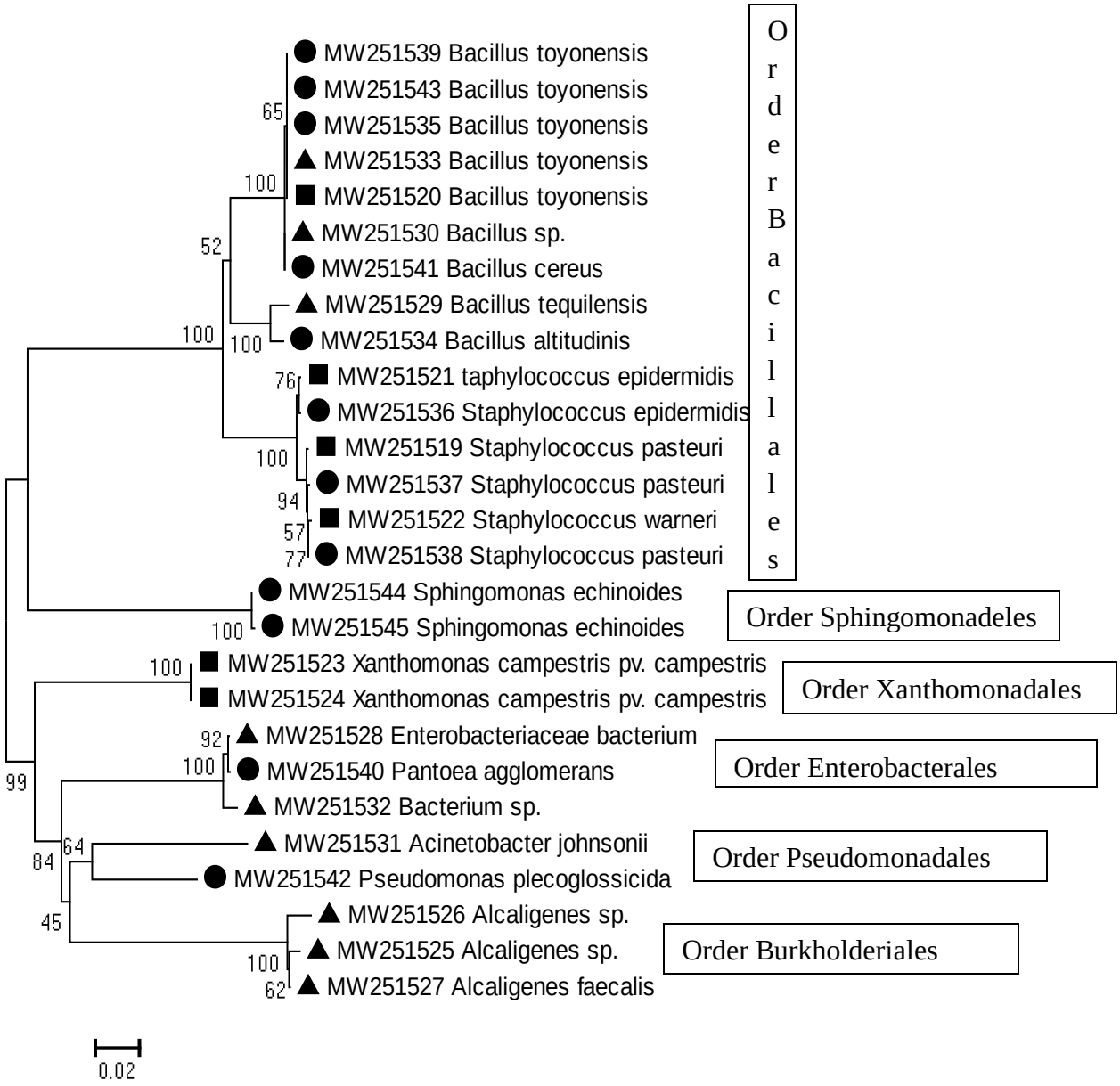


Figure 4.2.2. Neighbour joining phylogenetic tree of bacterial isolates of *C. calothyrsus* with gene bank isolates based on 16S rRNA gene sequence. Clade I-VI shows how the sequences of the bacterial isolate clustered in relation to their similarity. Bacteria clustering in the same clade have highly similar sequences and are closely related.

Key: ●- *C. calothyrsus* isolates, ■- *L. diversifolia* isolates, ▲- *S. sesban* isolates.

Table 4.2.2. Evolutionary relationship of endophytic fungal isolates

Accession no	Species	Genus	Family	Order	class	Phylum % dominance
MW251519	*Staphylococcus pasteuri*	*Staphylococci*				
MW251521	*Staphylococcus epidermidis*		Staphylococcaceae			
MW251522	*Staphylococcus warneri*					
MW251536	*Staphylococcus epidermidis*					
MW251537.1	*Staphylococcus sp*					
MW251538.1	*Staphylococcus pasteuri*			Bacillales	Bacilli	
MW251529.1	*Bacillus tequilensis*	*Bacilli*	Bacillaceae			
MW251530.1	*Bacillus sp.*					
MW251533.1	*Bacillus toyonensis*					
MW251534.1	*Bacillus altitudinis*					
MW251535.1	*Bacillus toyonensis*					Firmicutes
MW251520.1	*Bacillus toyonensis*					44%
MW251539.1	*Bacillus toyonensis*					
MW251541.1	*Bacillus cereus*					
MW251543.1	*Bacillus toyonensis*					
MW251523.1	*Xanthomonas campestris pv. campestris*	*Xanthomonas*	Xanthomonadaceae	Xanthomonadales	Gamma proteobacteria	

MW251524.1	*Xanthomonas campestris pv. campestris*					
MW251528.1	*Enterobacteriaceae bacterium*	*Enterobacteria*	Enterobacteriaceae	Enterobacterales	Gamma Proteobacteria	
MW251532.1	*Bacterium strain*	*Bacterium*				
MW251540.1	*Pantoea agglomerans*	*Pantoea*	Erwiniaceae			
MW251531.1	*Acinetobacter johnsonii*	*Acinetobacter*	Moraxellaceae			Proteobacteria 66%
MW251542.1	*Pseudomonas plecoglossicida*	*Pseudomonas*	Pseudomonadaceae	Pseudomonadales		
MW251544.1	*Sphingomonas echinoides*	*Sphingomonas*	Sphingomonadaceae	Sphingomonadales	Alpha proteobacteria	
MW251545.1	*Sphingomonas echinoides*					
MW251525.1	*Alcaligenes aquatilis*	*Alcaligenes*	Alcaligenaceae	Burkholderiales	Betaproteobacteria	
MW251526.1	*Alcaligenes faecalis*					
MW251527.1	*Alcaligenes faecalis*					

4.3 Growth inhibition potential of endophytes from *Calliandra calothyrsus*, *Leucaena diversifolia* and *Sesbania sesban* against fungal and bacterial plant pathogens

4.3.1 Growth inhibition potential of bacterial endophytes against *Xc*. pv *musacearum* and *Cercospora zeae-maydis*

Out of 42 pure bacterial endophytes isolated from *Calliandra calothyrsus, Leucaena diversifolia* and *Sesbania sesban,* thirteen exhibited growth inhibition activity against banana pathogenic bacteria *Xanthomonas campestris* pv. *musacearum* (Table 4.3.1). Six of the bacterial endophytes that inhibited growth of *Xanthomonas campestris* pv. *musacearum* were from *C. calothyrsus* (*BLC2, BLC6, BRC1, BRC2, BRC4* and *BSC1*), two from *S. sesban* (*BLS3* and *BSS2*) and five from *L. diversifolia* (*BRL1, BRL2, BRL3, BSL2* and *BSL4*). Majority of bacteria with growth inhibition potential were from roots at six, followed by stem at four then leaves at three. The growth inhibition activities of the bacterial endophytes against *Xc*. pv *musacearum* were significantly ($p \leq 0.05$) different with the largest zone of inhibition being produced by *BRL3* (*Xanthomonas campestris pv. campestris*) at 21.3±0.9mm followed by *BRL1*and *BRC4* at 20.6±1.5mm and 20.0±1.2mm respectively. The smallest zone of growth inhibition was produced by *BLC6* at 10.0±0.6mm.

The bacterial endophytes also showed growth inhibition activity against maize pathogen *Cercospora zeae-maydis*. Out of 42 isolates, twenty four exhibited growth inhibitory activity against the pathogenic fungi with varying degrees of growth inhibition potential (Table 4.3.1). Ten were from *C. calothyrsus* (*BLC3, BLC4, BLC5, BLC6, BRC1, BRC2, BRC3, BSC1, BSC4 and BSC5*), six from *S. sesban* (*BLS3, BRS1, BRS2, BRS3, BSS2 and BSS3*) and eight from *L. diversifolia* (*BLL2, BLL4, BLL5, BLL6, BRL1, BRL2, BRL3 and BEL4*). Majority of the bacteria

with growth inhibition potential were from roots and leaves at nine bacterial isolates each and six were from stems of *C. calothyrsus, L. diversifolia* and *S. sesban*. Growth inhibition potential of endophytic bacteria against *Cercospora zeae-maydis* was significantly ($p \leq 0.05$) different with the highest growth inhibition percentage being produced by *BLS3* (*Alcaligenes faecalis*) at 71.6% followed by *BRL2, BRSI* and *BLC4* at 65.3%, 63.5% and 60.4% respectively. The lowest growth inhibition percentage was produced by *BSC4* at 1.8%

Table 4.3.1 Growth inhibition potential of bacterial endophytes against *Xc*. pv *musacearum* and *Cercospora zeae-maydis*

Bacteria against *Xc*. pv *musacearum*			**Bacteria against *Cercospora zeae-maydis***		
S/NO	**Bacterial isolate**	**Mean zone of growth inhibition (mm)**	**S/NO**	**Bacterial isolate**	**Mean growth inhibition percentage (%)**
1	*BLC2*	14.0 ± 0.6^{df}	1	*BLC3*	13.7^{ghk}
2	*BLC6*	10.0 ± 0.6^{g}	2	*BLC4*	60.4^{abc}
3	*BRC1*	18.0 ± 2.6^{abc}	3	*BLC5*	6.6^{jk}
4	*BRC2*	16.0 ± 0.6^{cd}	4	*BLC6*	26.7^{ij}
5	*BRC4*	20.0 ± 1.2^{ab}	5	*BRC1*	44.2^{bc}
6	*BSC1*	11.6 ± 0.8^{fg}	6	*BRC2*	16.4^{ijk}
7	*BLS3*	16.6 ± 1.3^{bcd}	7	*BRC3*	30.6^{fhj}
8	*BSS2*	12.3 ± 0.9^{fg}	8	*BSC1*	21.6^{hij}
9	*BRL1*	20.6 ± 1.5^{a}	9	*BSC4*	1.8^{k}
10	*BRL2*	15.0 ± 0.5^{cd}	10	*BSC5*	7.4^{jk}
11	*BRL3*	21.3 ± 0.9^{a}	11	*BLS3*	71.6^{a}
12	*BSL2*	11.3 ± 1.2^{fg}	12	*BRS1*	63.5^{abc}
13	*BSL4*	11.6 ± 0.3^{fg}	13	*BRS2*	39.5^{cd}
	P Value	<.0001	14	*BRS3*	36.5^{dfh}
	LSD	3.3	15	*BSS2*	59.8^{bc}
	COV	13.1	16	*BSS3*	26.3^{ij}
			17	*BLL2*	32.6^{fh}
			18	*BLL4*	14.3^{hij}
			19	*BLL5*	14.5^{hij}
			20	*BLL6*	9.2^{jk}
			21	*BRL1*	35.4^{fh}
			22	*BRL2*	65.3^{ab}
			23	*BRL3*	46.8^{cd}
			24	*BSL4*	59.6^{abc}
				P Value	<.0001
				LSD	25
				COV	45.4

Means followed by the same super script letters down the column are not significantly different at $p<0.05$

Key: BR-Bacteria root, BS- Bacteria stem, BL- bacteria leaf, the last later is plant species

L- *Leucaena,* C- *Calliandra,* S- *Sesbania*

4.3.2 Growth inhibition potential of fungal endophytes against *Xc*. pv *musacearum* and *Cercospora zeae-maydis*

Thirty three fungal endophytes were isolated from leaves, stems and roots of *Calliandra calothyrsus*, *Leucaena diversifolia* and *Sesbania sesban.* Nineteen of the fungal isolates produced growth inhibition activity against maize pathogen *Xc*. pv *musacearum* (Table 4.3.2 and Plate 7). Six of the fungal endophytes were from *C. calothyrsus* (*FLC1, FSC3, FSC4, FSC5, FRC3, FRC4*), eight from *L. diversifolia* (*FLL1, FLL2, FLL3, FSL2, FSL3, FSL4, FRL6, FRL7*) and five from *S. sesban* (*FLS2, FSS2, FRS1, FRS2, and FRS3*). Majority of the fungi with growth inhibition activity were from roots and stems at seven each while leaves had five. There was significant ($p \leq 0.05$) difference in the antagonistic activities of endophytic fungi against *Xc*. pv *musacearum.* The largest zone of growth inhibition was produced by *FLL2* (*Trichoderma harzianum*) at 21.3±1.3mm followed by *FLL1, FLL3* and *FRL3* at 19.0±0.6mm, 18.3±0.3mm respectively. The smallest zone of growth inhibition was 2.3±1.4mm produced by FRL6.

Growth inhibition potential of fungal endophytes of *Calliandra calothyrsus, Leucaena diversifolia* and *Sesbania sesban* against *Cercospora zeae-maydis* was exhibited by eleven out of thirty three fungal isolates (Table 4.3.2). Three of the fungal endophytes were from *C. calothyrsus* (*FSC1, FSC4, FSC5*), three from *S. sesban* (*FSS2, FRS3, FRS2*) and five from *L. diversifolia* (*FSL1, FSL3, FSL4, FLL1, FRL6*). Of the eleven fungal isolates, seven (*FSC1, FSC4, FSC5, FSS2, FSL1, FSL3* and *FSL4*) were from stems, three (*FRS3, FRS2 and FRL6*) from roots and one (*FLL1*) from leaves. The growth inhibition percentages between the fungal isolates were significantly ($p \leq 0.05$) different with the highest inhibitory percentage of 40% produced by *FSC5* (*Trichoderma harzianum*) followed by *FSC1* and *FSL3* at 37.0% and 29.6% respectively. The lowest growth inhibition percentage was produced by *FSL1* at 3.7%.

Table 4.3.2 Growth inhibition potential of fungal endophytes against *Xc*. pv *musacearum* and *Cercospora zeae-maydis*

Fungal endophytes against *Xc*. pv *musacearum*			**Fungal endophytes against *Cercospora zeae-maydis***		
S/NO	**Bacteria isolate**	**Mean zone of growth inhibition (mm)**	**S/NO**	**Fungi isolate**	**Mean zone of growth inhibition (%)**
1	*FLC1*	12.6 ± 2.3^{eg}	1	*FSC1*	37.0^{a}
2	*FRC3*	15.3 ± 0.3^{cde}	2	*FSC4*	27.5^{ab}
3	*FRC4*	16.3 ± 2.2^{c}	3	*FSC5*	40.7^{a}
4	*FSC3*	16.3 ± 0.3^{c}	4	*FSS2*	26.4^{abc}
5	*FSC4*	15.3 ± 3.2^{cde}	5	*FRS3*	16.4^{bcd}
6	*FSC5*	14.0 ± 2.6^{cde}	6	*FRS2*	13.2^{bcd}
7	*FLL1*	19.0 ± 0.6^{ab}	7	*FSL1*	3.7^{d}
8	*FLL2*	21.3 ± 1.3^{a}	8	*FSL3*	29.6^{ab}
9	*FLL3*	18.3 ± 0.3^{ac}	9	*FRL6*	6.3^{cd}
10	*FRL6*	2.3 ± 1.4^{h}	10	*FLL1*	27.4^{ab}
11	*FRL7*	18.3 ± 0.3^{ac}	11	*FSL4*	22.7^{abc}
12	*FSL2*	8.3 ± 0.3^{g}		P Value	0.0177
13	*FSL3*	16.3 ± 2.3^{c}		LSD	20.3
14	*FSL4*	14.6 ± 0.3^{cde}		COV	52.5
15	*FLS2*	11.6 ± 1.5^{eg}			
16	*FRS1*	17.0 ± 1.2^{ac}			
17	*FRS2*	10.0 ± 1.2^{g}			
18	*FRS3*	17.3 ± 0.9^{ac}			
19	*FSS2*	16.6 ± 1.2^{c}			
	P Value	<.0001			
	LSD	4.3			
	COV	17.9			

Means followed by the same super script letters along the column are not significantly different at $p<0.05$.

Key: FR-Fungi root, FS- Fungi stem, FL- Fungi a leaf, the last later is plant species

L- *Leucaena,* C- *Calliandra,* S- *Sesbania*

Amongst the isolates, bacterial endophytes from roots had higher percentage (46.2%) of growth inhibition against *Xc*. pv *musacearum* while those from leaves had higher growth inhibition percentage (42.1%) against *C. zeae-maydis.* With fungal endophytes, higher percentage of growth inhibition against *Xc*. pv *musacearum* was produced by root (36.8%) endophytes while against *C. zeae-maydis* was produced by stem endophytes (63.6%) (Table 4.3.3).

Table 4.3.3 Percentage growth inhibition of bacterial and fungal endophytes against *Xc*. pv *musacearum* and *C. zeae-maydis*

Plant parts	**% growth inhibition of Bacterial endophytes**		**% growth inhibition of Fungal endophytes**	
	Xc. pv *musacearum*	*C. zeae-maydis*	*Xc*. pv *musacearum*	*C. zeae-maydis*
Leaf	23.1	42.1	31.6	9.1
Stem	30.8	26.3	31.6	63.6
Root	46.2	31.6	36.8	27.3

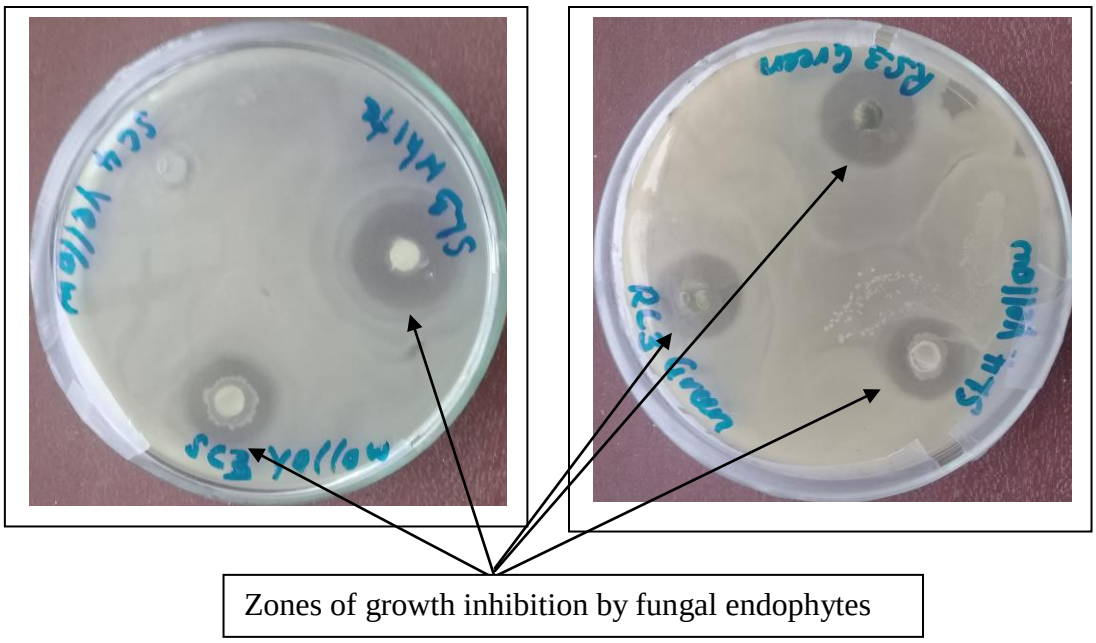


Plate 4.3.1: Growth inhibition of fungal endophytes against *Xc*. pv *musacearum*

4.4 Phytochemical screening of *C. calothyrsus, L. diversifolia* and *S. sesban* leaves extracts

Phytochemical screening revealed that terpenoids, flavonoids and saponins were present in the leaf extracts of the three plant species (Table 4.4). Tannins were present in *C. calothyrsus* and *S. sesban* but absent in *L. diversifolia*. Steroids were present in *L. diversifolia* and *S. sesban* but absent in *C. calothyrsus*. Alkaloids were present only in the leaf extracts of *S. sesban*. *Calliandra calothyrsus* had higher concentrations of tannins, terpenoids, saponins and flavonoids while *S. sesban* contained higher amounts of steroids and alkaloids. Flavonoids were also in high concentration in *L. diversifolia.*

Table 4.4 Phytochemical compounds of *C. calothyrsus, L. diversifolia* and *S. sesban*

Plant species	**Tannins**	**Terpenoids**	**steroids**	**Saponins**	**Flavonoids**	**Alkaloids**
C. calothyrsus	++	++	-	++	++	-
L. diversifolia	-	+	+	+	++	-
S. sesban	+	+	++	++	+	++

Key. - = Absent, += Present in low concentration, ++= Present in high concentration.

4.5 Growth inhibition activity of leaf extracts of *Sesbania sesban, Calliandra calothyrsus* and *Leucaena diversifolia* against *Xanthomnas campestris pv. musacearum* and *Cercospora zeae-maydis*

4.5.1. Growth inhibition activity of *S. sesban, C. calothyrsus* and *L. diversifolia* leaf ethanol extract against *Xc.* pv. *musacearum* and *Cercospora zeae-maydis*

The growth inhibition activity of *S. sesban* , *C. calothyrsus* and *L. diversifolia* leaf ethanol extracts against *Xc*. pv *musacearum* and *Cercospora zeae-maydis* was significantly ($p \leq 0.05$) different with *S. sesban* producing large mean zone of growth inhibition of 13.9±0.7 mm for bacteria and 72.2% growth inhibition for fungi compared to *C. calothyrsus* and *L. diversifolia* (Table 4.5.1). Based on treatments, there was no significant ($p \geq 0.05$) difference in the mean zone of growth inhibition at different treatments against *Xc*. pv *musacearum.* There was significant difference in the growth inhibition percentage of treatments against *Cercospora zeae-maydis* with treatment 25% giving highest growth inhibition percentage of 71.7%. Different concentrations of each plant extract inhibited the growth of *Xc*. pv. *Musacearum.* There was no significant difference in the growth inhibition of different concentrations of extracts from *C.*

calothyrsus and *L. diversifolia* against *Xc*. pv. *musacearum* and *Cercospora zeae-maydis* while there was significant difference in the growth inhibition of different concentrations of *L. diversifolia* against the two pathogens. Treatment 25mg/ml produced the largest growth inhibition percentage of 71.1% followed by 50mg/ml and 12.5mg/ml at 65.3% and 65% respectively (Table 4.5.1).

Table 4.5.1 Effect of *S. sesban*, *C. calothyrsus* and *L. diversifolia* leaf ethanol extract on *Xc*. pv *musacearum* and *Cercospora zeae-maydis*

Xc*. pv *musacearum				***Cercospora zeae-maydis***			
Plant species	**Mean growth inhibition (mm)**	**Treatments (mg/ml)**	**mean growth inhibition (mm)**	**Plant species**	**Mean growth inhibition (mm)**	**Treatments (mg/ml)**	**% growth inhibition**
S. sesban	13.9±0.7[a]	**12.5**	11.0±1.0[a]	***S. sesban***	72.2[a]	12.5	65[ab]
C. calothyrsus	11.0±0.6[b]	**25.0**	10.8±0.9[a]	***C. calothyrsus***	68[a]	25.0	71.7[a]
L. diversifolia	10.0±0.8[b]	**50.0**	12.2±0.8[a]	***L. diversifolia***	55.2[b]	50.0	65.3[ab]
P value	0.0014	**75.0**	12.5±0.9[a]	**P value**	0.0001	75.0	58.4[b]
LSD	1.98	**P value**	0.4727	**LSD**	7.0789	P value	0.0251
CV	20.15	**LSD**	2.2875	**CV**	12.9032	LSD	8.174
		CV	20.15			CV	12.90316

Effect of different concentrations on pathogens growth

***Xc*. pv *musacearum* (mean growth inhibition in mm)**				***Cercospora zeae-maydis* (mean growth inhibition in %)**		
Treatments (mg/ml)	***S. sesban***	***C. calothyrsus***	***L. diversifolia***	***S. sesban***	***C. calothyrsus***	***L. diversifolia***
12.5	14.3±1.8[a]	10.3±0.8[a]	8.3±0.3[b]	74[a]	57[a]	64[a]
25.0	13.3±0.9[a]	11.0±1.5[a]	8.3±1.2[b]	75[a]	78.3[a]	61.6[a]
50.0	13.6±2.0[a]	10.3±0.3[a]	12.6±1.2[a]	64.6[a]	62.6[a]	68.6[a]
75.0	14.3±1.3[a]	12.3±1.8[a]	11.0±1.7[ab]	75[a]	74[a]	23.3[b]
P value	0.95	0.65	0.09	0.2	0.17	<.0001
LSD	5.09	4.1	3.99	11.73	21.95	11.45
CV	19.45	19.81	21.03	8.63	17.14	11.02

Means followed by the same letters down the column are not significantly different at p p≤ 0.05

4.5.2. Growth inhibition activity of *S. sesban, C. calothyrsus* and *L. diversifolia* leaf aqueous extract against *Xc.* pv. *musacearum* and *Cercospora zeae-maydis*

In aqueous extracts, there was significant ($p \leq 0.05$) difference in the growth inhibition activity of *S. sesban, C. calothyrsus* and *L. diversifolia* against *Xc.* pv. *musacearum* and *Cercospora zeae-maydis. Sesbania sesban* produced largest zone of growth inhibition and growth inhibition percentage of 13.0±0.9mm and 78.3% respectively (Table 4.5.2 and plate 8). *Calliandra calothyrsus* produced the least mean zone of growth inhibition (9.4±0.6mm) and inhibition percentage (59.1%) compared to *S. sesban* and *L. diversifolia.* In different treatments, there was no significant ($p \geq 0.05$) difference in the growth inhibition against *Xc.* pv. *musacearum* while there was significant difference against *Cercospora zeae-maydis.* The highest treatment of 75% had the highest mean growth inhibition percentage of 75.3% followed by 50%, 12% and 25% respectively. At different concentration of individual plant, there was significant ($p \leq 0.05$) difference in the growth inhibition of extracts from *C. calothyrsus* and *L. diversifolia* against *Xc.* pv. *Musacearum, S. sesban* against *Cercospora zeae-maydis* (Table 4.5.2). The highest concentration (75%) for both *C. calothyrsus* and *L. diversifolia* had the highest growth inhibition percentage of 72% and 74% respectively.

Table 4.5.2. Effect of *S. sesban*, *C. calothyrsus* and *L. diversifolia* leaf aqueous extract on *Xc*. pv *musacearum* and *Cercospora zeae-maydis*

Xc*. pv *musacearum				***Cercospora zeae-maydis***			
Plant species	**Mean growth inhibition (mm)**	**Treatments (%)**	**Mean growth inhibition (mm)**	**Plant species**	**(%)growth inhibition**	**Treatments (%)**	**% growth inhibition**
S. sesban	13.0±0.9[a]	**12.5**	10.0±0.5[b]	***S. sesban***	78.3[a]	12.5	68[b]
C. calothyrsus	9.4±0.6[b]	**25.0**	12.2±1.0[a]	***C. calothyrsus***	59.1[c]	25.0	67.7[b]
L. diversifolia	12±0.3[a]	**50.0**	11.7±1.2[ab]	***L. diversifolia***	71[b]	50.0	68[b]
P value	0.0016	**75.0**	12.1±1.8	**P value**	<.0001	75.0	75.3[a]
LSD	1.87	**P value**	0.15	**LSD**	2.7	P value	<.0001
CV	19.38	**LSD**	2.16	**CV**	4.54	LSD	3.0852
		CV	19.38			CV	4.54

Effect of different concentrations on pathogens

***Xc*. pv *musacearum* (growth inhibition in mm)**				***Cercospora zeae-maydis* (%growth inhibition)**		
Treatments (%)	***S. sesban***	***C. calothyrsus***	***L. diversifolia***	***S. sesban***	***C. calothyrsus***	***L. diversifolia***
12.5	9.7±0.3[b]	8.7±0.3[ab]	11.6±0.8[a]	80[a]	55.3[b]	68.6[b]
25.0	15.0±1.2[a]	10.3±0.9[a]	11.3±1.8[a]	78[a]	53.3[b]	72a[b]
50.0	14.3±2.6[ab]	8.3±0.3[b]	12.3±1.5[a]	79[a]	55.6[b]	69.3[b]
75.0	13.3±1.4[ab]	10.3±0.7[a]	12.6±1.5[a]	80[a]	72[a]	74[a]
P value	0.16	0.08	0.91	0.71	0.0028	0.05
LSD	5.24	1.95	4.64	4.61	8.31	4.06
CV	21.27	11.05	20.55	3.09	7.47	3.04

Means followed by the same letter along the column are not significantly different at p p≤ 0.05.

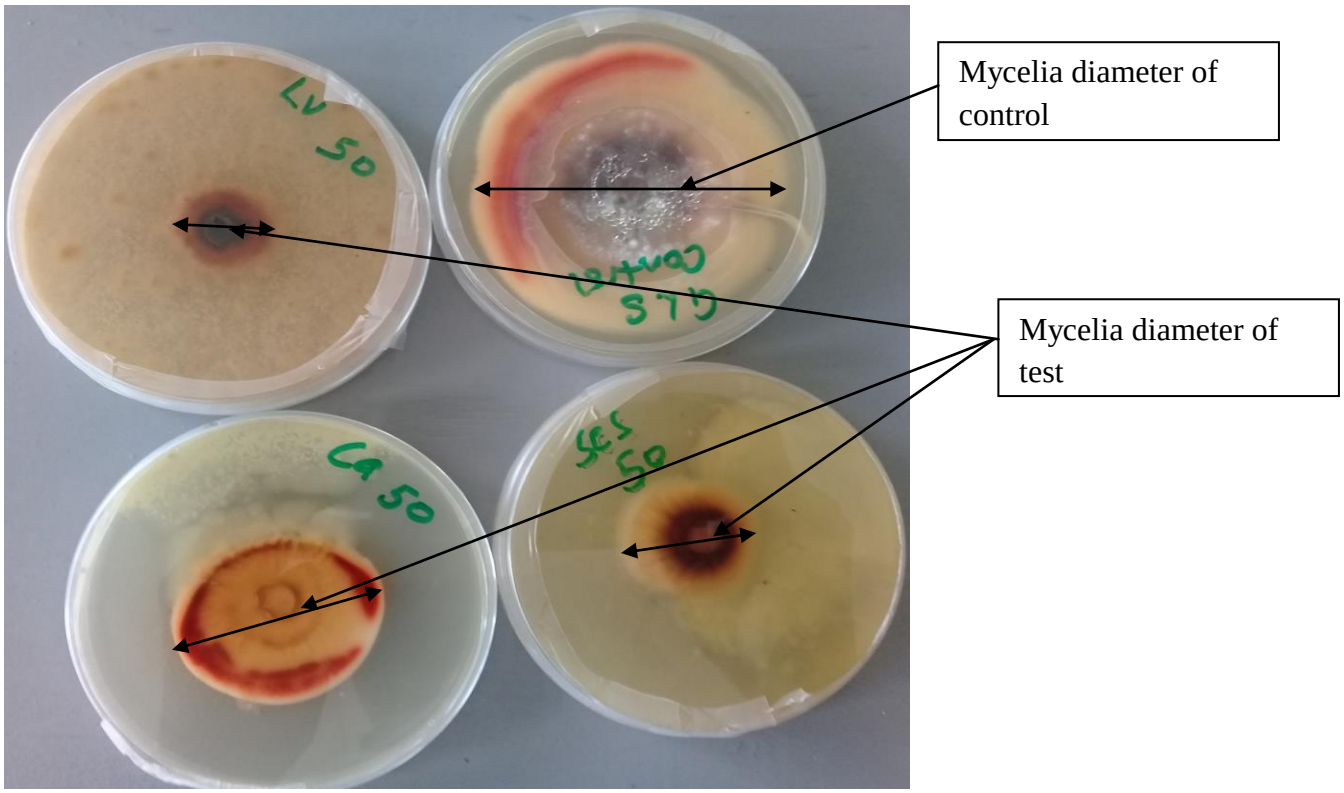


Plate 4.5: Growth inhibition activity of plant extracts against *Cercospora zeae-maydis*

CHAPTER FIVE: DISCUSSION

5.1 Morphological and molecular profiling of endophytes isolated from *Callindra calothyrsus, Leucaena diversifolia* and *Sesbania sesban*.

5.1.1 Morphological characterization of bacterial and fungal endophytes

Endophytic bacterial communities were high in the three plant species compared to fungal isolates (Table 4.1.1). This is probably because of their small size that enabled them to occupy both intracellular and extracellular spaces and multiply faster than fungi. Furthermore, there were more bacterial isolates from leaves than any other part of the plants probably because they are attracted to the leaves due to readily available food as a result of photosynthesis. This results are in agreement with the report that there is high diversity of endophytes in leaves than any other organ in plants (Chowdhary and Kaushik, 2015; Katoch and Pull, 2017). Similarly El-Deeb *et al*. (2013) reported that bacteria endophytes are the predomonant endophytes followed by fungi which concure with our findings.

Based on hierarchical cluster analysis, dendogram of bacterial endophytes revealed clustering of bacteria from different plant species and plant parts in the same clusters. The clustering of isolates in the same cluster implies that they are closely related as it was our case where colonies of different isolates from different plant species and parts shared the same characteristics. This result agrees with the report by Ondieki *et al*. (2017) that bacteria isolates from different sites could have similarity in colony characteristics. For fungal isolates, they also clustered in different clusters with fungi sharing similar phenotypic characteristics clustering together. Similar results were reported by Riley *et al*. (2001) and Tomsone *et al.* (2012) when determining morphological diversity of soil bacteria. Endophytic bacteria isolated from leaves, stems, and

roots of *C. calothyrsus*, *S. sesban* and *L. diversifolia* were morphologically different based on colony characteristics such as colour, elevation, shape, opacity and Gram staining results (Table 4.1.4 and Plate 4.1.3, 4.1.4, 4.1.5). Similar results were reported by Nhu and Diep (2017) after isolating different bacteria based on morphological characteristics from Soybean (*Glycine max*). The variation in the morphological characteristics of bacterial colonies of isolates could be due to difference in the metabolism of different components of the culture media. Based on morphological characteristics, there is a wide variation in colony morphology of isolates from the same plant species, plant organ and plant tissue (Sondang *et al.*, 2019; Padder *et al.*, 2017). Variations between bacterial endophytes are based on colony colour, shape, opacity, elevation, size and appearance after Gram staining (Padder *et al.*, 2017). According to Sinha *et al.* (2017), variation in colour of the colonies is as a result of the bacteria synthesising pigments as secondary metabolites while metabolising different nutritional components of the media. Pigments are synthesized by microorganisms during their growth in culture media to protect the cells from injurious effect of light rays of visible and near ultraviolet range which could be the reason of different pigmentation amongst the bacterial isolates.

Based on Gram's reaction, the endophytic bacterial isoltes distinguished into Gram negative bacilli, Gram negative cocci, Gram positive bacilli and Gram positive cocci (Plate 4.1.4 and 4.1.5). These results concur with the report of Bhagya *et al.* (2019) where they isolated both Gram negative and Gram positive bacterial endophytes from Green gram (*Vigna radiata L.*). The difference in appearance of the cells after Gram staining is due to variation in the structure of the cell walls of different bacteria. Gram negative bacteria cell wall contain lipopolysaccharide layer which dissolves in alcohol during decolourisation making the cells to lose crystal violet used as

primary stain therefore appearing red. Gram positive bacteria have peptidoglycan and teichoic acid layers in their cell wall which forms matrices that enables them to retain crystal violet stain thus appearing purple (Padder *et al*., 2017).

Fungal endophytes isolated from *C. calothyrsus, S. sesban* and *L. diversifolia* differed in mycelia structure, upper and bottom surface appearance (Table 4.1.3). The difference in colour on growth media could be attributed to the different chemical compounds they synthesise during their growth period and the type of spores they produce. Similar results were reported by Akutse *et al.* (2017) while isolating endophytic fungi from *Phaseolus vulgaris* and *Visia faba.* Al-Jaradi *et al.* (2018) also used morphological characteristics such as colony appearance, pigmentation and growth rate to characterise endophytic fungi of *Phaseolus vulgaris, Vigna unguiculata, V. radiata* and *Vicia faba.* Morphological characteristics have been used to describe fungi isolate based on features such as ascospore and peridium morphology, odour, hyphae form, conidia shape, spore size, colonies upper surface, reverse surface and type of concentric (Akutse *et al.,* 2017; Dashyal *et al.,* 2019).

Morphological features have been used by different researches (Hanin and Fitriasari 2019; Rabha *et al.,* 2016; Akutse *et al.,* 2017) to characterise fungal and bacterial endophytes but these features are not adequate for conclusive identification of endophytes due to existence of intermediate forms. Therefore in this study, conclusive characterisation of the endophytes could only be achieved by combining morphological and molecular methods.

5.1.2 Molecular characterisation and diversity of endophytic fungi and bacteria of *C. calothyrsus, S. sesban* and *L. diversifolia.*

Molecular identification of endophytic fungi based on ITS rDNA gene revealed 22 species belonging to four genera with genus *Trichoderma* being most dominant followed by genus *Mucor* and *Aspergillus* (Table 4.1.5). Endophytic fungi isolated from the three plants were dominated by genus *Trichoderma* especially *Trichoderma harzianum* which is reported to be an important species in synthesizing secondary metabolites with antimicrobial properties (Katoch *et al.,* 2019; Harman *et al.* 2019). The dominance of genus *Trichoderma* could therefore be because of their role in protecting *C. calothyrsus, L. diversifolia* and *S. sesban* against microbial and insect pathogens enabling them to survive in the environment. Similarly, genus *Mucor* enhances plant tolerance to metal toxicity (Domka *et al.,* 2019; Rozpądek *et al.,* 2018) and their presence in the three leguminous plants guarantees healthy environment for other microorganisms such as *Rhizobium* for nitrogen fixation.

Members of the genus Aspergillus synthesis growth stimulating hormones and other secondary metabolites with antimicrobial properties (El-hawary *et al*., 2020; Lubna *et al*., 2018). Their high number as endophytes could be due to the role they play in growth promotion and protection of these agroforestry trees. Among the identified fungal isolates, isolate *FLL2* (MW262935) sequence, had the lowest match identity of 92.00% with *Trichoderma harzianum* isolate Mc2151 (MK738146) isolated from citrus probably because it was isolated from a leguminous plant while the later was isolated from citrus plants. Endophytes are specific to the specie of plant they colonize (Li *et al.,* 2020) hence the variation in fungal species isolated from the three plants.

Endophytic bacteria also form part of inner microbial community of plants colonizing every plant organ and tissue. Of the 27 bacterial endophytes identified by 16S rRNA gene, majority were found to belong to genus *Bacilli* and *Staphylococci*. This could be because they play vital role in plant growth such as protection against plant pathogen and synthesis of plant growth promoting hormones. According to Ek-Ramos *et al.* (2019), endophytes of genus *Bacilli* enables the plant to withstand biotic and abiotic stress. Similar results were reported by Brígido *et al*. (2019) when they isolated endophytic bacteria of Chickpea (*Cicer arietinum* L.) plants.

Generally in both leguminous and non leguminous plants, the predominant genera are *Bacillus, Pseudomonas, Burkholderia, Rhizobium,* and *Klebsiella* (Webster *et al.,* 2020 ; Brígido *et al*., 2019). Members of genus *Bacilli* and *Pseudomonas* are predominantly found in leguminous plants because of their mutualistic association with plants. Some species of genus *Bacilli* such as *Bacillus amy-loliquefaciens* are responsible for nitrogen fixation, phosphorous, potassium, and zinc solubilisation, production of phytohormones (IAA) and act as bio-control agent (Rana *et al*., 2020) hence their dominance as endophytic bacteria of *C. calothyrsus, S. sesban* and *L. diversifolia.*

Isolates *BLS1, BLS2* and *BRS1* had match identities of 91.07%, 87.08% and 94.84% with genebank bacteria *Alcaligenes aquatilis, Alcaligenes faecalis* and *Bacterium strain* respectively. The isolates were therefore named and given accession numbers as *Alcalgenes* sp. (MW251526), *Alcalgenes* sp. (MW251527) and *Bacterium* sp. (MW251532). Low similarity match with genebank isolates could be because they were isolated from different ecological conditions and from different plant species as endophytic communities are influenced by soil type, plant species and organ or tissue (Katoch and pull, 2017; Correa-galeote *et al.,* 2018). Molecular

characterisation of microorganisms using 16S rRNA and ITS rDNA gene nucleotide sequences provides bacteria and fungi species specific signature and hence is considered a precise method of microbial identification (Bind and Nema, 2019). This method is therefore suitable for rapid and accurate identification of both culture dependent and culture independent microorganisms (Ikeda *et al*., 2013).

Within the endosphere of the plant, bacteria are the most abundant and diverse endophytes followed by fungi in both leguminous and non leguminous plants (Rna *et al.,* 2020; Vasileva *et al.,* 2019). In this study, Shannon diversity indices and Simpson diversity indices for fungal endophytes from *C. calothyrsus, L. diversifolia* and *S. sesban* ranged between 0.45 to 0.95 and 0.33 to 0.75 respectively while for bacteria indices ranged between 1.3 to1.5 and 0.l to 0.9. High diversity of both fungal and bacterial endophytes could be due to large number of culture depended species of endophytes which were distributed evenly in the plant species under study. High number of type of species of endophytes of *C. calothyrsus, L. diversifolia* and *S. sesban* could be because they are legumes and are endowed with high nutritional contents which influence colonisation of the plants by endophytes. Shannon-Weaver and Simpson diversity indices takes into account species richness and evenness and therefore increase in species richness and evenness, results in high diversity indices (Kim *et al.,* 2017) which is in agreement with this study that *C. calothyrsus, L. diversifolia* and *S. sesban* had high diversity of fungi and bacteria endophytes. Characterisation and diversity of bacterial and fungal endophytes was carried out on culture dependent which limits the identification of endophytes colonising these three plants.

5.2 Phylogenetic analysis of fungal and bacterial endophytes of *C. calothyrsus, L. diversifolia* and *S. sesban*

The clustering of the fungal endophytes isolated from *C. calothyrsus, L. diversifolia* and *S. sesban* into different clades supported by 100% bootstrap implies that each clade arose from a common ancestor. Organisms clustering in the same clade could be having similar sequences with significant functional or structural likeliness, therefore being close to each other in a phylogenetic tree. According to Munjal *et al.* (2018), similarity in the sequences signifies lack or low levels of mutation that could cause rearrangement in the nucleotide sequences resulting into divergence in the sequences.

Majority of operational taxonomic units in this study belonged to phylum Ascomycota. Members of phylum Ascomycota predominantly colonize the internal organs of the plant (Hamzah *et al*., 2018; Chowdhary and Kaushik, 2015) probably because they form mutualistic association with plant. The association could be by synthesis of protective and growth enhancement compounds. The phylum has genera such as *Tichoderma* and *Aspergillus* that synthesise compounds with antifungal and antibacterial activity (Mishra and Nautiya, 2018) and also growth promoting hormones such as gibberellins and Indole acetic acid (Lubna *et al*., 2018). The identification of most fungi from phylum Ascomycota could also be because, most members of this group are culture dependent and are able to metabolise nutritional components of standard isolating media for their growth and withstands constant changes in laboratory environment. Majority of fungi from other phylum such as Basidiomycota and Zygomycota are Culture-independent (Hamzah *et al*., 2018) and only a few can grow in synthetic culture media hence their low recovery in this study. Phylum Ascomycota is the largest and most diverse phyla of

eukaryotes that has been found to dominate plants as endophytes due to their interaction in enhancing carbon and nitrogen cycles (Challacombe *et al.,* 2019; González-teuber and Bascuñán-godoy, 2017).

Bacterial endophytes also clustered into different orders each having >98% bootstrap support with the majority belonging to phylum Proteobacteria. Clustering into orders implies that members of the clade have high similarities in the structure of the nucleotide arrangement and the sequences align close to each other during analysis (Horiike, 2016; Munjal, 2018). The results are consistent with studies by Chimwamurombe *et al.* (2016) that members of phylum Proteobacteria are the most abundant followed by those of Firmicutes. The high number of endophytic bacterial species belonging to the phylum proteobacteria could be as a result of their ability to overcome plant defence mechanism to penetrate and systemically colonize different organs and tissues of the host plant. Bacterial endophytes benefit the plant directly by improving nutrient uptake and stimulating synthesis of growth and stress related phytohormones (Zhang *et al.,* 2019). Similarly, these endophytic bacteria improve plant health by synthesizing secondary metabolites that targets pests and microbial pathogens of the plant hence improving plant defences (Elmagzob *et al.,* 2019; Zhang *et al.,* 2019).

In this study, it was also noted that some similar bacterial and fungal endophytes were isolated from all the three plants and clustered together while some showed specificity in the plant species colonization. *Trichoderma harzianum* and *Bacilli toyonensis* (Figure 4.21 and 4.2.2) were found to colonise all the three plants probably because the plants are related as they belong to the family leguminosaea. The ability of similar endophytes to colonize different plant species

could be because the three plants were legumes and could secrete exudates with same chemical composition that attracted similar microbial endophytes. On the other hand, *Penicillin citrinum, Mucor fragalis, Mucor circinolloides, Alcaligens spp, Acinetobacteria johnsonii and Pantoea agglomerans* showed specificity in the plant of origin. The specificity could be attributed to difference in chemical composition of the plant or organ and genetic variability that determines the preference of colonizing endophytes. Studies by Magginii *et al.* (2019) indicated that different plant species and compartments select different endophytic microorganisms due to the presence of bioactive compounds such as alkamides, caffeic, acid derivatives, polysaccharides and alkene.

Moreover, attraction of bacterial endophytes to both leguminous and non leguminous plant roots and seeds is a function of the exudates rich in various amino acids, sugars and phenolics (Iyer and Rajkumar, 2017; Liu *et al.*, 2017; Maggini *et al.*, 2019). Members of the genus *Trichoderma, Aspergilus* and *Mucor* for fungi and genus *Bcilli, Saphylococcus* and *Alcaligenes* for bacteria were found to be colonizing all the three plants. According to Card *et al*. (2016), most endophytes are quite promiscuous and naturally associate with a number of hosts disregarding host specificity theory which is in line with findings in this study. Similarly, endopytic fungi can be transmitted horizontally among individual plants in a community through sexual or asexual spores (Sahoo *et al.*, 2017). This explains the colonization of different plant species and plant organs by similar endophytic microorganism as shown in this study. The study utilised endophytes of the plants in phylogenetic analysis which are affected by rhizospher microorganisms which this research was not able to determine.

5.3 Growth inhibition potential of endophytes from *Calliandra calothyrsus, Leucaena diversifolia* and *Sesbania sesban* against fungal and bacterial plant pathogens

5.3.1 Growth inhibition potential of bacterial endophytes against *Xc*. pv *musacearum* and *Cercospora zeae-maydis*

Endophytes isolated from *C. calothyrsus, L. diversifolia* and *S. sesban* had varying growth inhibition potential against banana bacterial pathogen *Xc*. pv *musacearum*. Most of the isolates from *C. calothyrsus* showed growth inhibition activities compared to those isolated from *L. diversifolia* and *S. Sesban* (Table 4.3.1). The ability of the endophytes to inhibit growth of the *Xc*. pv *musacearum* could be because they synthesised chemical compounds in the media that interfered with some of the metabolic function such as cell wall and protein synthesis hence arresting growth. Similarly, the difference in the growth inhibition activity between bacterial isolates could be due to difference in concentration of secondary metabolites synthesised by the endophytes that produced different growth inhibition percentages. Similar results were reported by Selim *et al.* (2016) on bacteria endophytes from *Pisum sativum* roots against *Erwinia carotovora* responsible for soft rot in potato. Endophytes from non leguminous plants have also been reported (Yousefi *et al.*, 2018) to be active against bacteria pathogens such as *Xanthomonas oryzae* pv. *oryzae* (Yousefi *et al.*, 2018) of rice, *Pectobacterium atrosepticum* and *Clavibacter michiganensis* subsp. *epedonicus* (Pageni *et al.*, 2014) causing soft rot in potatoes.

Among the isolates that exhibited growth inhibition potential, most of them were from roots followed by stem and a few from leaves (Table 4.3.3). Roots of most plats have high concentration of phytochemicals which bacterial endophytes could have mimicked and

synthesized during their growth in the media to inhibit the growth of the bacterial pathogen. Similar results of growth inhibition activity of bacterial endophytes from different plant parts against plant pathogens have been reported (Yuliar *et al.,* 2013). Roots exudates different chemical compounds in the surrounding which attracts diverse microbial communities in which some may be pathogenic to the plant while some may secrete chemicals toxic to other microbes modifying the environment. Such environment may trigger the endophytes to respond by synthesizing chemicals that could inhibit the growth of pathogenic microorganisms by interfering with the uptake of essential elements for bacterial growth. The chemicals synthesized by bacterial endophytes includes antibiotics, siderophores or bactericins (Tidke *et al.,* 2017) which could have been secreted on culturing to inhibits the growth of *Xc*. pv *musacearum*. Roots are also thought to be a site of storage of some of secondary metabolites like tannins which could be mimicked and synthesised by root endophytes thus contributing to increased growth inhibition rate.

Bacterial endophytes from stems and leaves produced growth inhibition activity probably because of their ability to synthesise secondary metabolites with growth inhibition effect against other microorganisms. According to Singh *et al.* (2017) and Tidke *et al.* (2017), endophytic bacteria synthesise chemical such as alkaloids, steroids, terpenoids, peptides, polyketones, flavonoids, quinols and phenols, and the natural insecticide azadirachtin that are active against invading microorganisms. Such chemical compounds could have been synthesised by bacterial endophytes in the growth media to interrupt some vital cellular metabolism of *Xc*. pv *musacearum*.

Bacterial endophytes also exhibited antifungal activity when tested against maize fungal pathogen *Cercospora zeae-maydis* the causative agent of grey leaf spot with varying degree of growth inhibition (Table 4.3.1). Reports show that endophytic bacteria from Soybean (Zhao *et al.,* 2018) and Faba bean (Bahroun *et al.,* 2017) have growth inhibitory potential against *Phytophthora sojae, and Fusarium solani*. As reported by Zhao *et al.* (2018), most bacteria endophytes produce siderophores which are thought to have been produced in the media chelating iron in the media making it unavailable to the pathogen hence arresting growth. Majority of the endophytic bacteria with growth inhibitory activity against fungal pathogen were from leaves. Leaves interact with foliar pathogen, insect pests and herbivores which can induce endophytes to synthesize protective compounds which they could have released in the media to inhibit the growth of the pathogen. This interaction could have triggered bacterial endophytes to synthesise antifungal chemicals that were secreted in media on culturing to inhibit the growth of *Cercospora zeae-maydis.* The results are in agreement with Brunda *et al.* (2018) report on growth inhibition of endophytic bacteria from different parts of soybean plants against soil borne pathogenic fungi *Sclerotium rolfsii*, *Rhizoctonia bataticola* and *Fusarium oxysporum* infecting soybean.

Bacterial endophytes are useful in plants for they are involved in promoting plant growth, controlling plant pathogens, or helping host plant to defeat or tolerate stress responses from environment (El-Deeb *et al.,* 2013; Nawangsih *et al.,* 2011; Yuliar *et al.,* 2013). Growth inhibitory activity of bacterial endophytes against plant pathogens is achieved either by endophytes directly inhibiting the pathogen or indirectly by strengthening the plant immune system that in turn stops the growth and development of pathogens in the plants (Liu *et al.,* 2017;

Mohamad *et al.,* 2018). In this study growth inhibition could have been achieved by direct inhibition where the endophytes synthesised and secreted growth inhibitory chemicals in the media. Even though studies reveal that endophytes synthesise antimicrobial compounds it was not possible to harness these vital compounds to test for their antimicrobial activity.

5.3.2 Growth inhibition potential of fungal endophytes against *Xc*. pv *musacearum* and *Cercospora zeae-maydis*

Endophytic fungi from *L. diversifolia, C. calothyrsus* and *S. sesban* exhibited growth inhibition potential against *Xc.* pv *musacearum* with varying degrees (Table 4.3.2). Growth inhibition of endophytic fungal isolates against *Xc.* pv *musacearum* could be attributed to their ability to synthesise extracellular enzymes in the growth media that degrades the cell wall of bacteria antagonising their growth. Fouda *et al.* (2015) reported the ability of endophytic fungi to synthesise enzymes such as pectinases, cellulases, lipases, amylases, laccases, and proteinases which may play a significant role in biodegradation and hydrolysis of bacterial processes thus arresting growth. These results support the report by Khare *et al.* (2018) on the versatility of endophytic fungi to inhibit growth of phytopathogens.

Most of the fungi with growth inhibitory potential were isolates from roots and stems and only a few were from leaves (Table 4.3.3). This could be attributed to the fact that different plant organs store different secondary metabolites which could have influenced the type of chemical compounds synthesized by the endophytic fungi. Also roots are known to have higher concentrations of phytochemicals such as taninnins which the fungal endophytes could have mimicked and synthesised into the growth media to inhibit the growth of the bacteria pathogen.

The results concur with report by Srivastava *et al*. (2015) that endophytic fungi from different parts of leguminous weed *Prosopis juliflora* are active against *Xanthomonas vesicatoria.* Roots and stems had equal number of growth inhibiting fungi probably because these parts of the plant are prone to attack by feeding insects which may inject pathogenic microorganisms hence having endophytes that can synthesize protective chemicals. Leaves had few growth inhibiting fungi probably because most of the endophytes in leaves are involved in synthesis of chemicals that prevent herbivory rather than microbial attack.

The fungal endophytes also exhibited growth inhibition activity against maize fungal pathogen *Cercospora zeae-maydis* with varying growth inhibition percentage (Table 4.3.2). Inhibition of fungal mycelia growth could be attributed to the synthesis and secretion of antifungal chemical compounds in the media that probably halted the metabolic activities of the fungal pathogen. As reported by Fadiji and Babalola (2020), endophytic fungi are capable of synthesising chemical compounds such as trihydroxycadalene, cadinane sesquiterpenes, volatile oil, n-butanol and ethyl acetate that are active against fungal pathogens of plants. Such chemical compounds may have been synthesised during growth of endophytic fungi that inhibited advancement of mycelia of fungal pathogen. Zuhria *et al.* (2016) also reported growth inhibition of fungal endophytes from soybeans against *Sclerotium rolfsii.*

Endophytic fungi from different plants have been reported (Handayani *et al.,* 2018; Pelo *et al*., 2010; Fadiji and Babalola, 2020) to have many beneficial attributes to the host plant. They have been found to be a source of different plant hormones that enhance the growth of their host plants while some produce deferent bioactive compounds, such as alkaloids, diterpenes, flavonoids, and isoflavonoids, to increase the resistance of the host plant to biotic and abiotic

stresses (Fouda *et al.,* 2015; Mohamad *et al.,* 2018). Some of the endophytic fungi also promote the accumulation of secondary metabolites (including important medicinal components or drugs) originally produced by plants (Jia *et al.,* 2016; Bamisile *et al.,* 2018). The bioactive compounds synthesized by endophytes have the ability to antagonize the proliferation of pathogenic microorganisms including nematodes and root feeding insects (Bamisile *et al.,* 2018) hence their ability to inhibit growth of both bacterial and fungal pathogens of maize and bananas.

5.4 Phytochemical compounds in *C. calothyrsus, L. diversifolia* and *S. sesban* leaf extracts used as antimicrobial agent

Phytochemical screening of *C. calothyrsus* leaf ethanol extract revealed the presence of tannins, saponins, flavonoids, and terpenoids and absence of steroids and alkaloids. The results were contrary to those reported by Setyawati *et al.* (2019) that *C. calothyrsus* contains saponins, tannins, alkaloids, flavonoids and steroids. Difference in phytochemical compounds detected could be due to the solvents used in extraction as he used hexane while in this study ethanol was used. Also, the difference may be due to the fact that these plants occupied different ecological zones.

Lucaena diversifolia leaf extract contained saponins, flavonoids, steroids and terpenoids but lacked tannins and alkaloids. Presence of steroids is indicated by appearance of brown colure due to the hydroxyl group (-OH) of cholesterol reacting with chloroform, acetic acid and sulphuric acid. Similar results were reported by Revathi (2018) during his test of presence of phytochemical compounds in *Leucaena leucocephala* a different species in the genus *Leucaena.*

Sesbania sesban on the other hand contained all the tested phytochemical compounds including alkaloids that were absent in *L. diversifolia* and *C. calothyrsus*. The presence of all the phytochemical compounds tested in *S. sesban* is in agreement with the results of Nirosha *et al.* (2019) and this explains their significance in antimicrobial activity. Alkaloids are a class of naturally occurring organic compounds that mostly contain nitrogen atoms. They are precipitated from neutral or slightly acidic solution by Mayer's reagent to give a cream colour precipitate. The results supports Gomase *et al*. (2012) report on the phytochemical compounds present in *S. Sesban*. These results differ from those reported by Mythili and Ravindhran (2012) that the plant does not contain saponins because studies were carried out in different ecological zones. Different ecological zones have different climatic factors such as light intensity and water availability which are known to affect significantly the phytochemical content and profile of a plant (Borges *et al*., 2018).

The variation in the concentration of the of phytochemical compounds in the leaf extracts of *C. calothyrsus S. sesban* and *L. diversifolia* could be attributed to the type of solvent used and response of individual plant to biotic and abiotic factors as the plants were obtained from the same ecological zone. The concentration of bioactive compounds in each plant species depends on the environmental conditions, age of the plant, relative humidity of harvested materials and method of extraction (Izah 2018; Borges *et al.*, 2018; Musyimi *et al*., 2008). Tannins, saponins, flavonoids and terpenoids were highly concentrated in *C. calothyrsus* leaf extract and less concentrated in *L. diversifolia* and *S. sesban* probably because *C. calothyrsus* is prone to attack by many pathogens and insect herbivores which induce the plant to synthesize these compounds for protection against microbial and insect pathogen. Saponins and flavonoids were present in all

the three plants but saponins were highly concentrated in *C. calothyrsus* and *S. sesban* than *L. diversifolia* may be because the availability of banana plants attracted more molluscs which also fed on the two plants stimulating it to synthesis secondary metabolites with mollucicide activity. Flavonoids were present in high concentration in *C. calothyrsus* and *L. diversifolia* probably because the two plants have a wide range of microbial pathogens by which they have to protect themselves by synthesizing the compound in high concentrations. Steroids and alkaloids were highly concentrated in *S. sesban* than the other two plants probably because *S. sesban* is easily attacked by bacterial pathogens hence the need to synthesize the two compounds for protection against the pathogens. The difference in quantity and quality of the phytochemical compounds in different plants is a function of genetic constitution of the plant and environmental factors including temperature, salinity, light wavelength, soil nutrients and signalling molecules (Borgas *et al.*, 2018; Borges *et al*., 2013). Similarly, plant growth stage, post harvest handling, altitude, soil pH, plant organ and rainfall also affect the quality and quantity of the phytochemical present in any given plant (Borgas *et al.*, 2018; Raya *et al.*, 2015). However, this condition were not considerd in this study as well qualitative analysis due to resource constrain.

5.5 Growth inhibition activity of *S. sesban, C. calothyrsus* and *L. diversifolia* leaf extracts against *Xc. pv musacearum* and *Cercospora zeae-maydis*

5.5.1. Growth inhibition activity of *S. sesban, C. calothyrsus* and *L. diversifolia* leaf ethanol extract against *Xc.* pv *musacearum* and *Cercospora zeae-maydis.*

Leaf ethanol extracts of *S. sesban, C. calothyrsus* and *L. diversifolia* significantly inhibited the growth of *Xc.* pv *musacearum* (4.5.1). Growth inhibition could be attributed to the

phytochemical compounds detected in the leaf extracts that may have interfered with normal metabolic functions for growth of the pathogen. The results are in agreement with the report of Izah *et al*. (2018) and Salhi *et al.* (2017) that extracts from plants have alkaloids, quinines, flavonoids and many other secondary metabolites with antimicrobial properties.

Sesbania sesban proved to be more effective against *Xc.* pv *musacearum* pathogen as it produced large zones of growth inhibition compared to *C. calothyrsus* and *L. diversifolia.* The difference in performance could be attributed to high concentration of saponins, steroids and alkaloids in the leaf extract of *S. sesban* (Table 4.4) which could have hindered the uptake of growth nutrients from the media hence inhibiting growth. The results are in agreement with the report by Ahmed *et al*. (2013) that *S. sesban* extracts are active against plant bacterial pathogen *Erwinia amylovora.* At different concentrations, the antimicrobial activities of the three agroforestry trees were significantly different implying that *Xc.* pv *musacearum* although all the treatments produced significant inhibition. This implies that the pathogen can be inhibited by a wide range of concentration of ethanol extracts. This property may be because the active ingredients in the extract inhibited cell wall or protein synthesis arresting the growth of the pathogen.

Ethanol extracts also inhibited radial growth of *Cercospora zeae-maydis* fungi with *Sesbania sesban* and *Calliandra calothyrsus* producing larger growth inhibition percentages compared to *Lucaena diversifolia.* This was probably because the two plant leaves contained high concentration of flavonoids with antifungal activity (Table 4.4). According to Mierziak *et al.* (2014), flavonoids have antifungal activity which may have inhibited fungal spore germination and elongation of fungal mycelia. Different concentrations of the extracts also produced

significant growth inhibition of fungal pathogen. Lower concentrations produced largest growth inhibition percentage than high concentrations. This could be because high concentrations have high amounts of the active ingredients which could antagonize each other's performance thus leading to low growth inhibition percentage. The fluctuation in percentage of inhibition across the concentration was also reported by Ahmed *et al*. (2013).

The ability of *S. sesban, C. calothyrsus* and *L. diversifolia* extracts to inhibit the growth of *Xc.* pv *musacearum* is attributed to secondary metabolites they synthesize that have antibacterial properties. The leaves of all the three plants were found to have tannins, steroids and saponins (Table 4.4) which posse's antibacterial properties (Revathi, 2018; Pizzi, 2019; Iqbal *et al.,* 2015). *Sebania sesban* has been reported to be active against bacterial plant pathogens such as *Erwinia amylovora* (Mythili and Ravindhran, 2012) and animal pathogens *Escherichia coli*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* (Nirosha *et al.,* 2019; Walekhwa *et al.,* 2020). There are no reports of the antimicrobial activity of *C. calothyrsus* and *L. diversifolia* against plant pathogens but some of the closely related species like *Calliandra haematocephala* (Tiwari and Rai, 2016) and *Leucaena leucocephala* (Abu *et al*., 2016) have great antimicrobial activity against both Gram positive and Gram negative bacteria.

5.5.2. Growth inhibition activity of *Sesbania Sesban, Calliandra calothyrsus* and *Leucaena diversifolia* leaf aqueous extract against *Xc.* pv *musacearum* and *Cercospora zeae-maydis*.

Aqueous leaf extracts of *S. sesban, C. calothyrsus* and *L. diversifolia* significant inhibited growth of *Xc.* pv *musacearum* with *S. sesban* and *L. diversifolia* being more active compared to *C.*

Calothyrsus (Table 4.5.2). This was probably because the bioactive compounds in *S. sesban*, and *L. diversifolia* were more water soluble and dissolved in aqueous solvent completely thus being able to exert their antimicrobial activity. The antimicrobial activity of these extracts could be due to the ability to inhibit activity of enzymes involved in cell division therefore arresting the growth of the bacteria. Different concentrations did not show any significant difference in the zones of inhibition but all were active against *Xc.* pv *musacearum.* This shows that this bacterial pathogen is sensitive to a wide range of concentrations which can be applied to control its growth.

The aqueous extract of the three plants also inhibited the radial growth of *Cercospora zeae-maydis* with *S. sesban* posting a higher percentage of growth inhibition compared to *C. calothyrsus* and *L. diversifolia* (Table 4.5.2). The ability of *S. sesban, C. calothyrsus* and *L. diversifolia* to inhibit the growth of *Cercospora zeae-maydis* is attributed to the secondary metabolites they posses like alkaloids, saponins and tannins which have antifungal activities. These results concur with the report of Ahmed *et al*. (2013) that *S. sesban* extracts have antifungal activities against plant fungal pathogens like *A. fumigatus* and *F. oxysporum.* As reported by Mythili and Ravindhran (2012) and Walekhwa *et al.* (2020), extracts from *S. sesban* have the ability to inhibit growth of *Curvularia lunata*, *Fusarium oxysporum Candida albicans and Aspergilus fumigatus*.

There are no reports on the antifungal activity *C. calothyrsus* leaf extracts but tannins from leaves have been reported to be active against *Ceratobasidium ramicola* fungi that is harmful and destructive to forest and horticultural crops (Firmansyah *et al.,* 2020). Similarly, some of the

species of *Leucaena* which have been tested for their antifungal activity did not show inhibitory effect. *Leucaena leucocephala* extract had no antifungal activity against *Aspergilus niger, Rhizopus stolon, Penicillum notatum* and *Candida albicans* (Aderibigbe *et al.,* 2011). These results therefore, could form the basis for further research that could lead to isolation and development of antimicrobial agents alternative to synthetic chemicals to control *Cercospora zeae-maydis* and *Xanthomonas campestris* pv. *Musacearum* pathogens of maize and bananas in order to improve the yield. In this study, only plant leaves were tested for their antimicrobial activity without considering other parts which could have higher antimicrobial activity but were left out as harvesting them could negatively affect the plants in the intercrop.

CHAPTER SIX: CONCLUSION, RECOMMENDATION AND SUGGESTIONS FOR FUTURE RESEARCH

6.1 CONCLUSION

i. Morphologically, most of bacterial endophytes were Gram negative cocci and bacilli while a few were Gram positive cocci and bacilli. Fungal endophytes were divided into two based on mycelia morphology as septate and aseptate. Molecularly, sequences of fungal endophytes had >97% similarity match with genebank isolates except for isolate FLL2 (MW262935.1) which was identified as *Trichoderma* sp. Fungal endophytes belonged to four genera; *Trichoderma*, *Mucor*, *Aspergilus* and *Penicillium.* Majority of bacterial endophytes had >97% similarity march with genebank isolates except isolates *BLS1* (MW251525.1), *BLS2* (MW251526.1) and *BRS*1 (MW251526.1) identified as *Alcaligenes* sp. *Alcaligenes* sp. and *Bacterium* species respectively. Bacterial endophytes belonged to ten genera; *Bacilli*, *Staphylococcus, Alcaligens, Pantoea, Sphingomonas, Pseudomonas, Acinetobacte, Xanthomonas, Bacterium and Enterobacteria.* Morphological dendogram cluster analysis indicated high similarity level between two groups at 75% similarity level. Shannon-Wiener index (H´) and Simpson diversity index (D) were high for both fungal and bacterial isolates implying that the three plants are rich in the number of culture dependent species which are evenly distributed. Furthermore, amongst the isolates, bacterial isolates showed specificity to the plant of origin as there was no single bacterium that was isolated from all the three plants i.e *L. diversifolia, C. calothyrsus* and *S. Sesban.* With the fungal isolates *Trichoderma harzianum* was isolated from the three plants and also from the three plant parts i.e leaves, stem and roots.

ii. Phylogenetically, fungal endophytes clustered in three orders; hypocreales, eurotiales, and mucorales of which majority belonged phylum Ascomycota while bacteria endophytes clustered in six orders; bacillales, sphingomonadeles, xanthomonadales, , enterobacterales, pseudomonodales and burkholderiales and majority belonged to phylum Proteobacteria. Order hypocreales and eurotiales comprised of fungi endophytes isolated from the three plants while order mucorales comprided of fungal endophytes isolated from *L. diversifolia, C. Calothyrsus*. Endophytic bacteria belonging to order bacillales were isolated from all the three plants while those belonging to the other orders were specific to the plant of origin. All the orders were supported by >90% bootstrap which shows that the isolates had high similarity in their sequences with minimum differences. This implies that most of the isolates had a common ancestor.

iii. Thirteen fungal isolates showed growth inhibition against *X.c* pv. *musacearum* and twenty four isolates inhibited growth of *C. zeae-maydis.* Nineteen bacterial isolates inhibited the growth of *X. campestris* pv. *musacearum* while eleven inhibited growth of *C. zeae-maydis*. Isolate *FLL2* (*Trichoderma* sp.) and *BRL3* (*Xc.pv campestris*) produced larger inhibitory percentage against *X.c* pv. *musacearum* while isolate *FSC5* (*Trichoderma harzianum*), and *BLS3* (*Alcaligenes faecalis*) had higher antagonistic percentages against *C. zeae-maydis.* Therefore, endophytes from the three plants could serve as potential candidates as biocontrol of *X.c* pv. *musacearum* and *Cercospora zeaemaydis*.

iv. Phytochemical analysis of *L. diversifolia*, *C. calothyrsus* and *S. sesban* leave extracts shows that tannins, steroids and saponins were detected in the three plants; terpenoids in *L. diversifolia* only, flavonoids in *L. diversifolia* and *C. calothyrsus* while alkaloids were

present in *C. calothyrsus* and *S. sesban*. The presence of phytochemical compounds explains the antimicrobial properties of the extracts.

v. Extracts from the three plant species showed growth inhibitory activity against *X.c* pv. *musacearum* and *C. zeae-maydis* pathogens for both water and ethanol extract. There was significant difference in the antimicrobial among plant species while there was no significance different between treatments. The extracts therefore can be an alternative to synthetic chemicals in controlling grey leaf spot of maize and banana bacterial wilt incited by *X.c* pv. *musacearum* and *C. zeae-maydis* respectively in order to improve yield.

6.2 Recommendations

i. Molecular tools should be used in conjunction with morphological, biochemical and physiological characteristics for precise identification of microbial endophytes to avoid incomplete identification when morphological characteristic are utilized solely.

ii. Sequences of isolates that had <97% match identity with any of the GeneBank sequences should be regarded as new strains of fungi and bacteria from *L. diversifolia*, *C. calothyrsus* and *S. sesban.*

iii. It is recommended that endophytes from the three plants serve as potential candidates for control of *X.c* pv. *musacearum* and *Cercospora zeaemaydis* in order to increase yield of maize and bananas.

iv. Qualitative and quantitative methods should be used when analysing phytochemical compounds for proper understanding of phytochemical compounds in plants.

v. *Sesbania sesban* extracts should be used while controlling grey leaf spot and banana bacteria wilt incited by *X.c* pv. *musacearum and C. zeae-maydis* at high concentrations to achieve desired results when controlling the disease to achieve better results.

6.3 Suggestions for future research

i. Characterisation and diversity of bacterial and fungal endophytes was carried out on culture dependent microorganisms only therefore future research should focus on culture independent microorganisms to expose the diversity of both culture dependent and culture independent microorganisms.

ii. Phylogenetic analysis of endophytic and rhizophytic microorganisms should be determined to understand their evolutionary relationship as the rhizosphere affects the endosphere microorganisms.

iii. Growth inhibition potential was determined of using isolates themselves without identifying the bioactive compounds they synthesise. Therefore, future studies should focus on extracting and identifying bioactive chemicals synthesized by the endophytes.

iv. Qualitative phytochemical analysis was done using ethanol extracts. Future studies should focus on quantitative analysis and use of less polar solvents, which extracts more nonpolar compounds.

v. Future studies should also focus on induced phytochemicals to assess their antimicrobial activity.

vi. Organic volatile compounds from the three plants should also be a point of future researches to ascertain their antimicrobial activities.

vii. Crude extracts were used for antimicrobial studies but future studies should focus on identifying individual compounds in the extracts with antimicrobial activity.

viii. Some other media apart from nutrient agar for bacteria and potato dextrose agar for fungi should be used in isolation to recover more culturable endophytes from the three plants.

ix. In this study only bacteria and fungi were targeted for isolation. Future studies shoud target isolation of actinomycetes which are belied to synthesie more of antimicrobial compounds.

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APPENDICES

Appendix I: Analysis of variance of growth inhibition percentage of bacterial endophytes against *Xanthomonas campestris* pv. *musacearum* and *Cercospora zeae-maydis*

PARAMETERS	Source of variation	DF	SS	MS	F	Pr>F
Growth inhibition percentage of bacterial endophytes against *Xc.* pv. *musacearum*	Model	12	533.2307692	44.4358974	11.04	<.0001
	Error	26	104.6666667	4.0256410	<.0001	
	Corrected total	38	637.8974359			
Growth inhibition percentage of bacterial endophytes against *C. zeae-maydis*	Model	23	31496.21653	1369.40072	5.89	<.0001
	Error	48	11159.84000	232.49667		
	Corrected total	71	42656.05653			

Appendix II: Analysis of variance of growth inhibition percentage of fungal endophytes against *Xanthomonas campestris* pv. *musacearum* and *Cercospora zeae-maydis*

PARAMETERS	Source of variation	DF	SS	MS	F	Pr>F
Growth inhibition percentage of fungal endophytes against *Xc.* pv. *musacearum*	Model	18	1028.877193	57.159844	8.10	<.0001
	Error	3856	268.000000	7.052632		
	Corrected total		1296.877193			
Growth inhibition percentage of fungal endophytes against *C. zeae-maydis*	Model	10	4189.460606	418.946061	2.90	0.0177
	Error	20	3172.880000	144.221818		
	Corrected total	32	7362.340606			

Appendix III: Analysis of variance of growth inhibition of aqueous extracts against *Xanthomonas campestris* pv. *musacearum* and *Cercospora zeae-maydis*

PARAMETERS	Source of variation	DF	SS	MS	F	Pr>F
Growth inhibition of *C. calothyrsus, L. diversifolia* and *S. sesban* against *Xc.* pv. *musacearum*	Model	11	149.6666667	13.6060606	2.74	0.0016
	Error	24	119.3333333	4.9722222		
	Corrected total	35	269.0000000			
Growth inhibition of different treatments against *Xc.* pv. *musacearum*	Model	11	149.6666667	13.6060606	1.91	0.1542
	Error	24	119.3333333	4.9722222		
	Corrected total	35	269.0000000			
Growth inhibition of different concentration of *S. sesban* against	Model	3	50.9166667	16.9722222	2.19	0.1670
	Error	8	62.0000000	7.7500000		
	Corrected total	11	112.9166667			

Xc.* pv. *musacearum						
Growth inhibition of different concentration of *C. calothyrsus* against *Xc.* pv. *musacearum*	Model	3	10.25000000	3.41666667	3.15	0.0862
	Error	8	8.66666667	1.08333333		
	Corrected total	11	18.91666667			
Growth inhibition of different concentration of *L. diversifolia* against *Xc.* pv. *musacearum*	Model	3	3.33333333	1.11111111	0.18	0.9052
	Error	8	48.66666667	6.08333333		
	Corrected total	11	52.00000000			
Growth inhibition of *C. calothyrsus, L. diversifolia* and *S. sesban* against *C. zeae-maydis*	Model	11	3206.888889	291.535354	122.67	<.0001
	Error	24	241.333333	10.055556		
	Corrected total	35	3448.222222			

Growth inhibition of different treatments against *C. zeae-maydis*	Model	11	3206.888889	291.535354	12.29	<.0001
	Error	24	241.333333	10.055556		
	Corrected total	35	3448.222222			
Growth inhibition of different concentration of *S. sesban* against *C. zeae-maydis*	Model	3	8.25000000	2.75000000	0.46	0.7189
	Error	8	48.00000000	6.00000000		
	Corrected total	11	56.25000000			
Growth inhibition of different concentration of *C. calothyrsus* against *C. zeae-maydis*	Model	3	676.9166667	225.6388889	11.57	0.0028
	Error	8	156.0000000	19.5000000		
	Corrected total	11	832.9166667			
Growth inhibition of different	Model	3	54.66666667	18.22222222	3.90	0.0548
	Error	8	37.33333333	4.66666667		
	Corrected	11	92.00000000			

concentration of *L. diversifolia* against *C. zeae-maydis*	total					

Appendix IV: Analysis of variance of growth inhibition of methanol extracts against *Xanthomonas campestris* pv. *musacearum* and *Cercospora zeae-maydis*

PARAMETERS	Source of variation	DF	SS	MS	F	Pr>F
Growth inhibition of *C. calothyrsus*, *L. diversifolia* and *S. sesban* against *Xc*. pv. *musacearum*	Model	11	147.3333333	13.3939394	8.70	0.0014
	Error	24	132.6666667	5.5277778		
	Corrected total	35	280.0000000			
Growth inhibition of different treatments against *Xc*. pv. *musacearum*	Model	11	147.3333333	13.3939394	1.17	0.3433
	Error	24	132.6666667	5.5277778		
	Corrected total	35	280.0000000			
Growth inhibition of different	Model	3	2.25000000	0.75000000	0.10	0.9564
	Error	8	58.66666667	7.33333333		

concentration of *S. sesban* against *Xc.* pv. *musacearum*	Corrected total	11	60.91666667			
Growth inhibition of different concentration of *C. calothyrsus* against *Xc.* pv. *musacearum*	Model	3	8.00000000	2.66666667	0.56	0.6554
	Error	8	38.00000000	4.75000000		
	Corrected total	11	46.00000000			
Growth inhibition of different concentration of *L. diversifolia* against *Xc.* pv. *musacearum*	Model	3	40.91666667	13.63888889	3.03	0.0932
	Error	8	36.00000000	4.50000000		
	Corrected total	11	76.91666667			
Growth inhibition of *C. calothyrsus,* *L. diversifolia* and *S. sesban* against *C. zeae-maydis*	Model	11	6389.555556	580.868687	13.35	0.0001
	Error	24	1694.000000	70.583333		
	Corrected total	35	8083.555556			
Growth inhibition	Model	11	6389.555556	580.868687	3.72	0.0251

of different	Error	24	1694.000000	70.583333		
treatments	Corrected	35	8083.555556			
against *Xc*. pv.	total					
musacearum						
Growth inhibition	Model	3	227.0000000	75.6666667	1.95	0.2004
of different	Error	8	310.6666667	38.8333333		
concentration of	Corrected	11	537.6666667			
***S. sesban* against**	total					
C. zeae-maydis						
Growth inhibition	Model	3	876.666667	292.222222	2.15	0.1720
of different	Error	8	1087.333333	135.916667		
concentration of	Corrected	11	1964.000000			
C. calothyrsus	total					
against *C. zeae-*						
maydis						
Growth inhibition	Model	3	3401.666667	1133.888889	30.65	<.0001
of different	Error	8	296.000000	37.000000		
concentration of	Corrected	11	3697.666667			
L. diversifolia	total					
against *C. zeae-*						
maydis						

Appendix V: ZR Fungal/Bacterial DNA MiniPrep ™ Short Protocol

The extraction of DNA was corried out according to the proctocal recommended by the manufacturer (Zymo Research cooperation)

1. Add 50 – 100 mg (wet weight) fungal or bacterial cells that have been resuspended in up to 200 µl of water or isotonic buffer (e.g., PBS) or up to 200 mg of tissue to a ZR BashingBead™ Lysis Tube (0.1 mm & 0.5 mm). . Add 750 µl Lysis Solution to the tube.
2. Secure in a bead beater fitted with a 2 ml tube holder assembly and process at maximum speed for 5 minutes.
3. Centrifuge the ZR BashingBead™ Lysis Tube (0.1 & 0.5 mm) in a microcentrifuge at 10,000 x g for 1 minute.
4. Transfer up to 400 µl supernatant to a Zymo-Spin™ IV Spin Filter (Orange Top) in a Collection Tube and centrifuge at 7,000 x g for 1 minute.
5. Add 1,200 µl of Genomic Lysis Buffer to the filtrate in the Collection Tube from Step 4.
6. Transfer 800 µl of the mixture from Step 5 to a Zymo-Spin™ IIC Column in a Collection Tube and centrifuge at 10,000 x g for 1 minute.
7. Discard the flow through from the Collection Tube and repeat Step 6.
8. Add 200 µl DNA Pre-Wash Buffer to the Zymo-Spin™ IIC Column in a new Collection Tube and centrifuge at 10,000 x g for 1 minute.
9. Add 500 µl g-DNA Wash Buffer to the Zymo-Spin™ IIC Colum n and centrifuge at 10,000 x g for 1 minute.
10. Transfer the Zymo-Spin™ IIC Column to a clean 1.5 ml microc entrifuge tube and add 100 µl (35 µl minimum) DNA Elution Buffer directly to the column matrix. Centrifuge at 10,000 x g for 30 seconds to elute the DNA

Appendix VI: Gel electrophoresis protocol

Separation of the DNA material was done according to Lee *et al.* (2012) as follows;

Preparation of the Gel

1. Weigh out the appropriate mass (0.8%) of agarose into an Erlenmeyer flask.
2. Add running buffer to the agarose-containing flask. Swirl to mix. The most common gel running buffers are TAE (40 mM Tris-acetate, 1 Mm EDTA) and TBE (45 mM Tris-borate, 1 mM EDTA).
3. Melt the agarose/buffer mixture over a Bunsen flame. At 30 s intervals, remove the flask and swirl the contents to mix well. Repeat until the agarose has completely dissolved.
4. Add ethidium bromide (EtBr) to a concentration of 0.5 μg.
5. Allow the agarose to cool either on the bench top or by incubation in a 65 °C water bath. Failure to do so will warp the gel tray
6. Place the gel tray into the casting apparatus and place an appropriate comb into the gel mold to create the wells.
7. Pour the molten agarose into the gel mold. Allow the agarose to set at room temperature. Remove the comb and place the gel in the gel box.

Setting up of Gel Apparatus and Separation of DNA Fragments

1. Add loading dye to the DNA samples to be separated. Gel loading dye is typically made at 6X concentration (0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol).

Loading dye helps to track how far your DNA sample has traveled, and also allows the sample to sink into the gel.

2. Program the power supply to desired voltage (1-5V/cm between electrodes).
3. Add enough running buffer to cover the surface of the gel. It is important to use the same running buffer as the one used to prepare the gel.
4. Attach the leads of the gel box to the power supply. Turn on the power supply and verify that both gel box and power supply are working.
5. Remove the lid slowly and carefully load the DNA sample(s) into the gel. An appropriate DNA size marker should always be loaded along with experimental samples.
6. Replace the lid to the gel box. The cathode (black leads) should be closer the wells than the anode (red leads). Double check that the electrodes are plugged into the correct slots in the power supply.
7. Turn on the power. Run the gel until the dye has migrated to an appropriate distance.

Observing Separated DNA fragments

1. When electrophoresis has completed, turn off the power supply and remove the lid of the gel box.
2. Remove gel from the gel box. Drain off excess buffer from the surface of the gel. Place the gel tray on paper towels to absorb any extra running buffer.
3. Remove the gel from the gel tray and expose the gel to uv light. This is most commonly done using a gel documentation system.