Bioactivity of Endophytes from *Calliandra calothyrsus*, *Leucaena diversifolia* and *Sesbania sesban* Against *Cercospora zeae-maydis*

William Omuketi Emitaro[1*], David Mutisya Musyimi[2], George Timothy Opande[3]

[1]*Department of Biological Sciences, School of Biological and Physical Sciences, Jaramogi Oginga Odinga University of Science and Technology P.O. BOX 210 – 40601, Bondo, Kenya*

[2]*Department of Botany, School of Physical and Biological Sciences, Maseno University, Private bag, Maseno, Kenya*

[3]*Department of Biological & Agricultural Sciences, School of Science, Kaimosi friends University College, P.O BOX 385 - 50309, Kaimosi, Kenya*

**Corresponding Author*

Abstract:- **Endophytes are microorganisms that accomplish parts of their life cycle within living host tissues without causing apparent damage to the plants. Endophytes confer survival advantage to the plant as they play a role in plant resistance to diseases. *Cercospora zeae-maydis* is a fungus that causes grey leaf spot disease of maize and is responsible for over 60%yield loss. Current chemical methods for control of the disease have adverse effects on human health and environment. Little is known on the potential of endophytes of *Calliandra calothyrsus, Leucaena diversifolia* and *Sesbania sesban* as biological control of *Cercospora zeae-maydis*. The objective of this study was to investigate the antagonistic potential of bacterial and fungal endophytes of the three plants against *Cercospora zeae-maydis.* A total of 75 endophytes were isolated from the three plant species based on morphological differences on PDA and NA media. Fungal and bacterial isolates were coded based on the plant part and plant species of origin such as *FLC* - for fungi isolated from the leaf of *Calliandra calothyrsus, BLL*-bacteria isolated from leaf of *Leucaena diversifolia* and *BRS* - for bacteria isolated from the root of *Sesbania sesban*. Thirty-three fungal and forty-two bacterial isolates were tested for antagonistic activity against *Cercospora zeae-maydis* by dual culture technique. Eleven fungal and twenty-four bacterial endophytes exhibited antagonistic activity against the pathogen. There were significant ($p \leq 0.05$) antagonistic activity among fungal and bacterial isolates against the pathogen. The highest inhibitory effects among the fungal isolates included *FSC5* at 40%, *FSC1*at 37% and *FSL3* at 30% respectively. The highest bacterial isolates activity was 72% for *BLS3*, 65 % for *BRL2*, 64 % for *BRSI* and60 %for *BLC4*.It is recommended that the endophytes from the three plants could serve as potential candidates for control of *Cercospora zeae-maydis*. Future studies should investigate on the bioactive molecules produced by these microorganisms**.

Key words: **Endophytes, Antagonistic, *Calliandra calothyrsus, Leucaena diversifolia* and *Sesbania sesban***

I. INTRODUCTION

Endophytes are microorganisms that inhabit and colonize inner environment of plant organs and tissues including leaves, stems, seeds and roots, all or part of their life-cycles without causing diseases or producing visible signs and symptoms of infection [3, 17, 21], but may become pathogenic when the host senesces [11]. They are distributed in different plant parts and plant species and comprise of different communities of fungi bacteria and actinomycetes [9, 12]. Bacteria and fungi endophytes gain entry into the plant via germinating radicals, secondary roots, stomata or by secreting hydrolytic enzymes that degrades the cell wall [3, 8]. After entry, they colonize specific tissues of entry or may systemically spread and colonize different plant parts away from the point of entry establishing a mutual relationship with the plant in the intracellular, intercellular or in the vascular systems [9, 15, 25].

Endophytes confer survival advantage to the plant as they play a role in plant growth and plant resistance under stressful conditions [17, 26]. They synthesize bioactive compounds which are of great potential in agriculture, antimicrobial and anti-insect activity [13, 18, 27]. Antimicrobial potential is due to their ability to synthesize bioactive metabolites such as alkaloids, diterpenes, flavonoids, isoflavonoids and other volatile compounds [11, 15]. Some of the endophytes like *Trichoderma koningii* and *Alternaria alternate* from maize roots have antagonistic effects on *Fusarium* pathogen [22]. Banana endophytic bacteria inhibited growth of *Fusarium oxysporum* f. sp *cubense* and *Colletotrichum guaranicola* [24]. 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* [23]. Even though some research on bioactivity of endophytes has been reported, little is known on bioactivity of endophytes from *Calliandra calothyrsus*, *Leucaena diversifolia* and *Sesbania sesban* against *Cercospora zeae-maydis.*

In western Kenya, maize is regarded as a staple food but its production is compromised by reduced land size, low soil fertility, pests and diseases. *Cercospora zeae-maydis* is a fungus that causes grey leaf spot (GLS) disease of maize which greatly lowers maize yield as it interacts with other environmental factors [5, 10].GLS is responsible for over 60%

loss of maize yield [2, 5]. Control of this disease is by use of synthetic chemicals, cultural practices and genetic breeding to obtain resistant varieties but farmers still experience heavy losses [5, 10]. Considering limitations of the different strategies for the management of this disease and adverse effects of synthetic chemicals on human health and environment, biological method is preferred for management of diseases but inadequate information is available on the use of endophytes as biological control. Endophytes therefore are a great choice in solving not only plant diseases but also human and animal health problems as they are chemical synthesizers inside plant. Chemicals synthesized are pharmacologically active substances with low toxicity toward mammals and environment [6]. This study aimed at isolating bacterial and fungal endophytes from leaves, stems and roots of *C. calothyrsus, L. diversifolia* and *S. sesban* and determining their antagonistic activity against *Cercospora zeae-maydis* the causal agent of grey leaf spot disease of maize.

II. MATERIALS AND METHODS

Collection of plant materials

Leaves, stems and roots of *C. calothyrsus, L. diversifolia* and *S. sesban* were collected separately in zip lock bags from Maseno university farm located 0° 10' 0" South, 34° 36' 0" East along Kisumu Busia road. Samples were collected randomly from demonstration plots in triplicate and pulled together. Fresh samples were transported to the Jaramogi Oginga Odinga University of Science and technology laboratory for isolation of the endophytes.

Isolation of bacterial endophytes

Isolation was carried out according to the procedure developed by Thanh and Diep [27]. Leaves, stems and roots were washed separately with tap water to remove attached soil dirt from the field. They were cut separately into small pieces and immersed in 70% ethanol for 3 min. They were then washed with 4% fresh sodium hypochlorite solution for 5 min and finally rinsed five times with sterile distilled water. To confirm that the sterilization process was successful, the aliquots of the sterile distilled water used in the final rinse was inoculated on nutrient agar (NA) medium plates. The plates were examined for presence or absence of bacterial growth after incubation at 28^0 C for 3 days. Samples were then 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 were serially diluted in aqueous solution (0.9 % NaCl) and plated in triplicate on NA to recover any bacterial endophytes present in the plant tissue. Plates were wrapped with parafilm then incubated at 28^oC for 1-7 days or until growth was observed. Colonies were identified and isolated in pure cultures on NA based on their morphological characteristics. Bacterial endophytes were coded based on the plant part and plant species of origin such as: (*BLC*-bacteria isolated from the leaf of calliandra, *BSC*-bacteria isolated from the stem of calliandra, *BRC*- bacteria isolated from the root of calliandra, *BLS*- bacteria isolated from the leaf of sesbania, *BSS*- bacteria isolated from the stem of sesbania, *BRS*- bacteria isolated from the root of sesbana, *BLL*- bacteria isolated from leaf of leucaena, *BSL*- bacteria isolated from stem of leucaena and *BRL*- bacteria isolated from the root of leucaena.

Isolation of fungal endophytes

Fungal endophytes were isolated according to Mahadevamurthy *et al.* [18]. Leaves, stems and roots were cut into small pieces of 5mm each and 3-5 pieces of each plant part separately. They were then plated on PDA plates incorporated with streptomycin (1.0g/l) to inhibit bacterial growth. The plates were sealed with parafilm and were incubated at 25 ± 2^oC for 7 days. The endophytic fungal colonies which emerged from plant parts were picked with sterile fine tip needle based on color appearance, and sub cultured on fresh PDA plates devoid of antibiotic to obtain pure cultures and were identified based on their morphological characteristics. Fungal endophytes were coded based on the plant part and plant species of origin such as: (*FLC*-fungi isolated from the leaf of calliandra, *FSC*-fungi isolated from the stem of calliandra, *FRC*- fungi isolated from the root of calliandra, *FLS*- fungi isolated from the leaf of sesbania, *FSS*-fungi isolated from the stem of sesbania, *FRS*- fungi isolated from the root of sesbana, *FLL*- fungi isolated from leaf of leucaena, *FSL*- fungi isolated from the stem of leucaena and *FRL*- fungi isolated from the root of leucaena.

Isolation of fungal pathogen Cercospora zeae-maydis

Fungal pathogens from maize leaves were isolated according to the protocol of Nega *et al.* [20]. Leaf samples with characteristic symptoms were cut into pieces of approximately 5cm and placed on sterile moist blotter in a sterile petridish. Five sections of diseased tissue were placed in each petridish and incubated at 25^oC for 5 days to allow the pathogen to develop and sporulate in growth cabinets under a 12h fluorescent light/dark regime. The sporulating diseased sections were examined under a binocular microscope for the presence of conidia, which were then picked with an isolation needle, and plated on PDA, allowing at least three pickings per leaf sample. Plates were incubated at 25^oC for 5-7 days and hyphal tips from the advancing colony margins were transferred onto PDA with isolating needle as pure culture and kept at 5^oC. Morphological characteristics of the fungi were used to identify the pathogen [20]

Evaluation of antagonistic activity of bacterial isolates against Cercospora zeae-maydis

This was done using the methodology of Mohamad *et al.* [19]. Bacterial isolates were cultured on nutrient agar medium and incubated at 28^o C overnight. The fungal pathogens were grown on potato dextrose agar (PDA) plates and incubated for 6 days. The fungal pathogens and bacterial endophytes were inoculated at the opposite sides of the PDA Petri plate. Control

plates were inoculated only with the pathogen. Percentage antagonism was calculated according to the formula of Brunda *et al*. [7];

Antagonistic (%) =C – T/C x 100

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

Antagonistic activity of fungal isolates against Cercospora zeae-maydis

Antagonistic activity of the isolated fungi against fungal pathogens was determined according to Katoch and Pull [14] protocol. Discs of isolated endophyte and pathogen measuring 0.5 mm were co-cultured at two opposite ends of PDA plates, sealed with parafilm and incubated at 25±2^{o}C for 7 days. Plates containing the pathogens alone without endophyte served as control. Radial growth of pathogenic fungi in the presence and absence of the endophyte was measured after 7 days, and antagonistic percentage calculated using the formula of Abdennabi *et al.* [1];

Antagonistic (%) = CDC-CDT / CDCx100

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

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

Data analysis

Triplicate data of antagonistic activity of the endophytes was subjected to analysis of variance (ANOVA) and means separated by least significant difference at P = 0.05 using SAS version 2.1 software.

III. RESULTS

Isolation of endophytes

A total of 75 different colonies of both bacteria and fungi were isolated from the three agroforestry trees (Table 1) of which 42 were bacterial while 33 were fungal endophytes. The antagonistic activity of fungal endophytes from leaves, stems and roots of *Calliandra calothyrsus*, *Leucaena diversifolia* and *Sesbania sesban* against *Cercospora zeae-maydis* was exhibited by eleven out of thirty-three fungal isolates (Table 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 antagonistic percentages between the fungal isolates were significantly (p≤ 0.05) different with the highest inhibitory percentage of 40% produced by *FSC5* followed by *FSC1* and *FSL3* at 37 % and 30% respectively. The smallest inhibition was produced by *FSL1* at 4%.

Out of 42 bacterial isolates, twenty-four exhibited antagonistic activities against the pathogenic fungi with varying degrees of antagonism (Table 2). Of the 24 isolates, 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 antagonistic bacteria were from roots and leaves at nine bacterial isolates each and six were from stems. The antagonistic potential of endophytic bacteria against *Cercospora zeae-maydis* was significantly (p≤ 0.05) different with the highest inhibition percentage being produced by *BLS3* at 71.6% followed by *BRL2*, *BRSI* and *BLC4* at 65.3%, 63.5% and 60.4% respectively. The lowest antagonistic percentage was produced by *BSC4* at 1.8%.

IV. DISCUSSION

This study has confirmed that plants harbour diverse endophytes. Bacterial endophytes from the three plants exhibited antifungal activity when tested against maize fungal pathogen *Cercospora zeae-maydis* the causative agent of grey leaf spot. Majority of the antagonistic bacteria were isolates from roots and leaves of the three agroforestry trees. Roots are in constant interaction with soil pathogenic bacteria and fungi exposing endophytic bacteria to hydrolytic enzymes secreted for penetration. Bacterial endophytes protect themselves and host plant against these harsh conditions by synthesizing antifungal chemicals that are thought to have been secreted to inhibit the growth of *Cercospora zeae-maydis.* Bacterial endophytes in leaves prevent germination of fungal spores and growth of bacterial by synthesizing and secreting antifungal chemicals that are thought to have antagonized growth of *Cercospora zeae-maydis.* These results are in agreement with those of Yuliar *et al.* [28] who reported antagonistic activity of endophytic bacteria from different plants against *Rhizoctonia solani* and *Fusarium oxysporum* plant fungal pathogens. The antifungal activities of endophytic bacteria is attributed to their ability to secrete toxins and surface-active compounds and extracellular digestive enzymes that outcompetes fungal phytopathogen, thus inhibiting their growth [7, 19).

Thirteen fungal endophytes exhibited antagonistic activity against maize fungal pathogen *Cercospora zeae-maydis* with varying inhibition percentages, five from *L. diversifolia* while *L. calothyrsus*,and *S. sesban* had three isolates each. The difference in the number of fungal endophytes from the three plants showing antagonistic activity could be as a result of different endophytes colonizing different species of plants with ability to synthesize different chemical compounds with antifungal activity. High numbers of antagonistic fungi were from stems, probably because of high levels of alkaloids and antifungal proteins they synthesize to protect the host plant against pathogens. These results are similar to those reported by Latz *et al.* [16] that above ground endophytic fungi *Epichloe festucae* secretes proteins that inhibits the development of *Sclerotinia homoeocarpa*. The bioactive compounds synthesized by the endophytes may have the

ability to antagonize the proliferation of pathogenic microorganisms [4], hence their ability to antagonize fungal pathogens of maize and bananas.

V. CONCLUSION

From this study, it is evident that *Calliandra calothyrsus*, *Leucaena diversifolia* and *Sesbania sesban* have diverse endophytes which can be used as an alternative to synthetic chemicals in controlling grey leaf spot of maize caused by *Cercospora zeae-maydis*, hence minimizing the environmental degradation. Future studies should investigate on the bioactive molecules produced by these microorganisms.

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Table 1. Endophytic Bacteria and Fungi Isolated from Parts of *C. calothyrsus*, *L. diversifolia* and *S. sesban*

	Bacterial isolates per plant part				**Fungal isolates per plant part**			
Plant species	**Leaf**	**Stem**	**Root**	**Total**	**Leaf**	**Stem**	**Root**	**Total**
C. calothyrsus	6	5	5	**16**	1	5	5	**11**
L. diversifolia	6	5	3	**14**	3	4	7	**14**
S. sesban	6	3	3	**12**	2	3	3	**8**
Total				**42**				**33**

Table 2. Antagonistic Potential of Bacterial and Fungal Endophytes against *Cercospora zeae-maydis*

Fungi isolates against *Cercospora zeae-maydis*				**Bacteria isolate against *Cercospora zeae-maydis***		
S/NO	**Fungi isolate**	**Mean zone of inhibition (%)**		**S/NO**	**Bacterial isolate**	**Mean percentage inhibition (%)**
1	*FSC1*	37.0^{a}		1	*BLC3*	13.7^{ghk}
2	*FSC4*	27.5^{ab}		2	*BLC4*	60.4^{abc}
3	*FSC5*	40.7^{a}		3	*BLC5*	6.6^{jk}
4	*FSS2*	26.4^{abc}		4	*BLC6*	26.7^{ij}
5	*FRS3*	16.4^{bcd}		5	*BRC1*	44.2^{bc}
6	*FRS2*	13.2^{bcd}		6	*BRC2*	16.4^{ijk}
7	*FSL1*	3.7^{d}		7	*BRC3*	30.6^{fhj}
8	*FSL3*	29.6^{ab}		8	*BSC1*	21.6^{hij}
9	*FRL6*	6.3^{cd}		9	*BSC4*	1.8^{k}
10	*FLL1*	27.4^{ab}		10	*BSC5*	7.4^{jk}
11	*FSL4*	22.7^{abc}		11	*BLS3*	71.6^{a}
	P Value	0.0177		12	*BRS1*	63.5^{abc}
	LSD	20.3		13	*BRS2*	39.5^{cd}
	COV	52.5		14	*BRS3*	36.5^{dfh}
				15	*BSS2*	59.8^{bc}
				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 along the column are not significantly different at $p \leq 0.05$

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