MANAGEMENT OF SORGHUM ANTHRACNOSE USING BIOCONTROL AGENTS PRODUCED BY SORGHUM RHIZOBACTERIA IN WESTERN KENYA

By

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DECLARATION

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DEDICATION

Dedicated to my beloved mother, Joan Atsembo Makumba for her foresight to educate me. To my treasured wife; Dorothy Musavi Amendi, our beloved children; Trevor and Taffie and cherished sisters for their continued support, encouragement and prayers during the study period.

ABSTRACT

The use of sorghum rhizobacteria antifungal agents in the management of sorghum anthracnose was made possible by isolating a total of 294 rhizobacterial isolates from sorghum rhizosphere soil of different agro-ecological zones of western Kenya and screening them *in vitro* against isolated sorghum foliar fungal pathogens, viz: *Alternaria alternata* (leaf spot), *Aspergillus candidus* (seed rot), *Alternaria longissima* (Grain storage mould), *Botrytis cinerea* (grey mould), *Colletotrichum gloeosporioides* (anthracnose), *Colletotrichum sublineolum* (anthracnose, red stalk rot), *Exserohilum turcicum* (leaf blight), *Fusarium equiseti* (associated with *Fusarium moniliforme*; pathogenicity still questionable), *Fusarium moniliforme* (stalk rot) and *Nigrospora oryzae* (ear rot). Screening yielded 101 sorghum rhizobacterial isolates that showed antagonism against the sorghum test fungal pathogens. Ninety five of them inhibited the pathogens by $\geq 30\%$ whilst those that inhibited at least one of the test pathogens by $\geq 70\%$ were 35. The acrisol soil type consistently produced antagonistic rhizobacterial isolates regardless of prevailing soil conditions. Of the 35, two rhizobacterial isolates labeled KaI245 and MaI254 inhibited fungal growth of all the pathogens tested, an indication that the two isolates produce potent broad-spectrum antifungal agents. Fungal growth inhibition produced by isolate KaI245 were greater and honestly significantly different from those of isolate MaI254 at $p < 0.05$ hence the former was considered to be the best antimicrobial agent producer. Biochemical tests and the API system identified isolate KaI245 as *Aeromonas hydrophila* while isolate MaI254 was identified as *Bacillus megaterium*. *Aeromonas hydrophila* KaI245 produced appreciable amounts of antifungal agents in liquid media containing potato infusion and glucose. Its culture-filtrate exhibited antibiotic activity against all the test sorghum pathogenic microorganisms. Purification of the antifungal agent-culture-filtrate by column chromatography using both polar and non-polar solvents enhanced the activity of the filtrate. This was reflected by a 65.47% increase in size of the clear zones of inhibition produced against *C. sublineolum* when the purified culture-filtrate was used. Paper chromatography combined with bioautography of the isolate's culture-filtrate produced one continuous zone of inhibition against *C. sublineolum,* an indication that the culture filtrate contained one active compound. Optimal antifungal agents production in synthetic media include: fructose as the carbon source, an incubation period of 6 days with shaker flasks agitated at a speed of 180 rpm, an initial synthetic medium pH of 7, and an incubation temperature of 28°C. *In vivo* tests in the greenhouse showed that *Aeromonas hydrophila* KaI245 antifungal agent-culture-filtrate concentrated twice significantly delayed and suppressed ($p < 0.05$) the development of sorghum anthracnose caused by *C. sublineolum* on sorghum leaves. This compared favourably with Folicur®430SC, a systemic fungicide used in the control of a range of fungal diseases including sorghum anthracnose. The results of this research provide a novel, sustainable and environmentally friendly method of controlling cereals diseases using sorghum rhizobacteria.

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LIST OF ABBREVIATIONS

µl	Microlitre
µM	Micromolar
2,4-DAPG	2, 4-diacetyl phloroglucinol
ACC deaminase	1-amino cyclopropane 1-caeboxylic acid deaminase
AFMs	Anti-fungal metabolites
ANOVA	Analysis of Variance
API	Analytical profile index
BCA	Biological control agent(s)
CABI	Centre for Agriculture and Biosciences International
CCAB	The Arab-Brazil chamber of commerce
CIMMYT	International Maize and Wheat Improvement Centre
cfu	colony forming units
CRBD	Completely randomised block design
CRD	Completely randomised design
DNA	Deoxyribonucleic acid
ELISA	Enzyme linked immunosorbent assay
EPZA	Export Processing Zones Authority
et al.	and others
FAO	Food and Agriculture Organization of the United Nations
FAOSTAT	Food and Agriculture Organization of the United Nations, Statistics Division
GFP	Green flourescent protein
GHN	The Global Health Network
GPS	Global positioning system
HPLC	High performance liquid chromatography
HSD	Honest significant difference
IAA	Indole 3-acetic acid
ICRISAT	International Crops Research Institute for Semi-Arid Tropics
ISR	Induced systemic resistance
KALRO	Kenya Agricultural and Livestock Research Organization
KARI	Kenya Agricultural Research Institute
KB medium	King's B Medium

KEPHIS	Kenya Plant Health Inspectorate Service
MIC	Minimum Inhibitory Concentration
N	Normal (Unit of concentration)
NA	Nutrient agar
NACOSTI	National Commission for Science, Technology and Innovation
NB	Nutrient broth
OMA	Oat meal agar
'P'	Phosphorous
PCA	Phenazine carboxylic acid
PCA	Potato carrot agar
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PGPB	Plant growth promoting bacteria
PGPR	Plant growth promoting rhizobacteria
pH	Potential of hydrogen
Po	Organic phosphate
R_f	Retention factor
SAR	Systemic acquired resistance
SC	Suspension concentrate
SDW	Sterile distilled water
sp.	Species (singular)
spp.	Species (plural)
UoE	University of Eldoret
UoH	University of Hawai'i
USDA	United States Department of Agriculture
USEPA	United States Environmental Protection Agency
V-P	Voges-Proskauer
WP	Wetable powder

APPENDICES

ACKNOWLEDGEMENTS

CHAPTER ONE

INTRODUCTION

1.1. Importance of sorghum

Kenya's food security is dependent on staple food crops that are mainly cereals. Among them is sorghum (*Sorghum bicolor* (L.) Moench) which is a plant native to Africa. It is ranked second to fifth among the five most important cereal crops in East Africa that include maize, wheat, rice and barley (Hulluka and Esele, 1992; FAO, 1999; Opole *et al.,* 2007; Tesfamichael *et al.,* 2015). The plant is drought resistant and performs well on a range of poor soils often out-yielding most cereals in a range of agro-ecological zones of Kenya. This includes the semi-arid areas of eastern Kenya, the coast, the water-logged and *striga* stricken areas around Lake Victoria, and the cold highlands of the Rift Valley (Rana *et al.,* 1980; Mburu, 1994; KARI, 1997; Kameri-Mbote, 2005). The crop is therefore an important commodity, which alongside millets, provides necessary food hence food security, raw materials for malted beverages, feed for livestock, and a source of income for millions of people living in arid and semi-arid environments (Maunder, 2002; Omamo *et al.,* 2006; Tesfamichael *et al.,* 2015).

1.2. Sorghum production and its constraints

The general sorghum production trend in Kenya has over the years been fluctuating (FAOSTAT, 2012; USDA, 2016). The highest production ever in recorded history was in 1976 where 223, 000 metric tonnes were realized whilst the poorest production of 54,316 metric tonnes was recorded just under a decade in 2008. Increase in production from lower yields in previous years may be attributed to changes in maize fortunes brought about by climatic changes and increased sorghum acreage (EPZA, 2005). Decline in production (yield and quality) however, is attributed to several production constraints, key among them being biotic stresses (diseases and insect pests) (Guiragossian, 1986; M'Ragwa and Kanyenji, 1987; Hulluka and Esele, 1992; Ngugi, 1998; ICRISAT, 2004) that cause an estimated total yield loss to the tune of US\$ 3,032 million (www.agbiotechnet.com/pdfs/0851995640). Abiotic stresses which predominantly are related to problematic soils (saline and acidic soils) also have a significant bearing on sorghum yields (ICRISAT, 2004).

Sorghum anthracnose caused by *Colletotrichum sublineolum* P. Henn., Kabát & Bubák and *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc stands out as one of the diseases that significantly reduces grain yield in sorghum (Prom *et al.,* 2012; Costa *et al.,* 2015). The disease has been reported to be prevalent and severe in Western Kenya fields characterized by warm and humid weather. The disease causes both direct and indirect yield losses. The premature drying of leaves and defoliation due to foliar anthracnose has been reported to reduce the yield of sorghum grain and fodder by 30—50% in susceptible cultivars during severe epiphytotics (Ali and Warren, 1992). Stalk infection (stalk rot or red rot) also results in lodging and considerable reduction in grain and fodder yields (Mishra and Siradhana, 1979; Fredericksen, 1984). Secondary infections of the stalk by red rot may cause yield losses proportional to the severity of anthracnose and stalk rot (Neya and Kabore, 1987). Grain yield has also been reported to be reduced by as much as 70% because of incomplete grain fill as shown by a decrease in seed weight and seed density. It has been reported that grain production of susceptible sorghums is severely limited when the disease develops during heading or early grain filling (Ali *et al.,* 1987). Indirect losses results due to reduced seed germination and the transmission of the disease to new geographical regions (Basu-Choudhary and Mathur, 1979).

The bulk of Kenya's total sorghum production is harvested in the western region mostly by small holder farmers. Grain yields, currently estimated at 925 kg/ha per annum are low relative to those in other parts of the world in spite attainable yields of 5,000 kg/ha (Ngugi *et al.,* 2002). Disease control has conventionally been practiced using fungicides which have over time raised environmental concerns. These include: metalaxyl, benomyl, carbendazim and lately triazoles. These fungicides, do not only oftenly produce phytotoxic effects on plants, but also generate a lot of residues that may potentially lead to substantial environmental contamination (USEPA, 2006). Besides, they are unaffordable to most of the farmers growing the crop. Furthermore, the continued use of these fungicides results in imbalances within the microbial community creating unfavourable conditions for the activity of beneficial organisms (Villajuan-Abgona *et al.,* 1996).

Phytopathogenic diseases have been controlled in the last two and a half decades by means of certain beneficial bacteria that are indigenous to the rhizosphere of plants (Thomashaw, 1996; Földes *et al.,* 2000). The rhizosphere which represents the thin layer of soil surrounding plant roots and the soil occupied by the roots supports large and metabolically active groups of bacteria (Villacieros *et al.,* 2003) known as plant growth promoting rhizobacteria (PGPR) (Kloepper *et al.,* 1980). PGPR are known to rapidly colonise the rhizosphere and suppress deleterious microorganisms as well as soil-borne pathogens at the root surface (Rangajaran *et al.,* 2003). These organisms can also be beneficial to the plant by stimulating growth (Bloemberg and Lugtenberg, 2001).

Biological suppression of sorghum anthracnose by the application of PGPR is seldom practiced in Kenya. This study was therefore designed to provide alternative environmentally friendly means of controlling sorghum anthracnose by using biological control agents produced by rhizobacteria isolated from the sorghum rhizosphere. The study will contribute to the introduction of PGPR systems in the biological control of pathogenic fungi of sorghum and other crops in Kenya.

1.3. Statement of the Problem

Food insecurity and environmental challenges posed by sorghum anthracnose are a major and chronic threat to food production and ecosystem stability, not only in Kenya but the world over. In a quest to achieve food security, there has been agricultural production intensification over the past few decades, which has consequently resulted in producers becoming more dependent on agrochemicals as a relatively reliable method of crop protection in order to stabilise their economic operations. However, the increasing use of chemical inputs has caused several undesirable effects such as development of pathogen resistance to the applied products, environment pollution and increased awareness of the detrimental effect of fungicides on man's health (Burkhead *et al.,* 1995; De Weger *et al.,* 1995; Gerhardson, 2002). Furthermore, the growing cost of pesticides, particularly in less-affluent regions of the world, and consumer demand for pesticide-free food has necessitated a search for substitutes to these products. In fact, the application of chemicals to the growing crop has not been found to be effective and remains neither

practical nor economic where sorghum is grown by small holder farmers in developing countries (Gowily, 1995).

Biological control is thus being considered as an alternative or a supplemental way of reducing the use of chemicals in agriculture (De Weger *et al.,* 1995; Gerhardson, 2002; Postma *et al.* 2003; Welbaum *et al.,* 2004). The development of biological control agents from sorghum rhizobacteria in the control of sorghum anthracnose will herald novel methods of protecting one of the staple foods in Kenya hence contribute in achieving millennium development goal No. 1 that addresses eradication of extreme poverty and hunger. This would also drive one of the economic pillars Kenya's attainment of vision 2030.

1.4. Justification

Whereas sorghum is a most reliable cereal crop for food production in the sub Saharan Africa due to its inherent ability to resist drought, the threat posed by anthracnose leading to reduced yields is quite realistic. Sustainable methods of disease control such as biological control and development of resistant cultivars offer the best alternative to anthracnose control in sorghum.

Development of resistant plant cultivars is often slow and economic pressure on land use limits its application (Wahome, 1998). Furthermore, the virulence diversity exhibited by *Colletotrichum sublineolum*, the causal agent of sorghum anthracnose, has made it difficult to develop resistant varieties (CABI, 2007). Biocontrol therefore remains the most appropriate alternative to chemical control. The suitability of this method is pegged on the fact that the biocontrol agents, which mostly are antibiotics, possess high specific activity, are target specific, and are generally biodegradable and therefore safe to use (Sharga, 1997). Currently, there is very limited knowledge regarding the biological suppression of sorghum anthracnose by the application of PGPR in Kenya. This study aimed at producing a sustainable sorghum anthracnose management method that will contribute to the introduction of PGPR systems in biological control of phytopathogenic fungi in sorghum and other crops in Kenya.

It should be appreciated that there is still a long way to go before a sound system is developed to protect plants from their predators without altering the ecological balance among species. The use of bioantagonists is certainly a very promising route.

1.5. Objectives of the study

1.5.1. Overall objective

The overall objective of the study was to manage sorghum anthracnose using biocontrol agents produced by sorghum rhizobacteria in Western Kenya.

1.5.2. Specific objectives

1) To isolate sorghum rhizobacteria from rhizosphere soil samples and sorghum foliar pathogens from diseased plant materials collected from different agro-ecological zones of the sorghum growing belt in Western Kenya.

2) To screen the isolated rhizobacteria against sorghum foliar fungal pathogens, including *Colletotrichum sublineolum* Henn. Kab & Bubak and *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc *in vitro* for antagonistic activity.

3) To characterize and identify isolated rhizobacteria that are antagonistic to sorghum foliar fungal pathogens and determine the relationship they have with different soil types isolated from.

4) To optimize the production of antibiotic(s) by the best antagonistic rhizobacteria in fermentation-shaker-flasks under different environmental conditions.

5) To determine the efficacy of the fermentation culture-filtrate produced by the best antagonistic rhizobacteria against sorghum anthracnose under greenhouse conditions.

1.6. Hypothesis of the study

1.6.1. Null hypothesis (H$_0$)

Sorghum rhizobacteria isolated from western Kenya do not produce biological control agents that are active against sorghum anthracnose

1.6.2. Alternative hypothesis (H$_1$)

Sorghum rhizobacteria isolated from western Kenya produce biological control agents that are active against sorghum anthracnose

CHAPTER TWO

LITERATURE REVIEW

2.1. Sorghum

2.1.1. The origin and distribution of sorghum

The origin and early domestication of sorghum is hypothesized to have taken place around 5000–8000 years ago in north-eastern Africa or at the Egyptian-Sudanese border (Mann *et al.,* 1983; Wendorf *et al.,* 1992; Smith and Frederiksen, 2000) with the largest diversity of cultivated and wild sorghum also found in this part of Africa (deWet, 1977; Doggett, 1988; Kimber 2000). The last wild relatives of commercial sorghum are currently confined to Africa south of the Sahara, an indication that its domestication took place there. However, the archaeological exploration of sub-Saharan Africa is in its early stages, and critical information for determining where and when sorghum could have been taken into cultivation is still lacking (Zohary and Hopf, 2000). Although rich finds of *Sorghum bicolor* have been recovered from Qasr Ibrim in Egyptian Nubia, the wild examples have been dated to *circa* 800–600 BCE, and the domesticated ones no earlier than CE 100.

The earliest archaeological evidence comes from sites dated to the second millennium BCE in India and Pakistan—where *S. bicolor* is not native. These incongruous finds have been interpreted, according to Zohary and Hopf, (2000) as indicating: (i) an even earlier domestication in Africa, and (ii) an early migration of domestic sorghum from East Africa into the Indian subcontinent. This interpretation got further support because several other African grain crops, namely: *Pennisetum glaucum* (L.) R. Br. (pearl millet), *Vigna unguiculata* (L.) Walp. (Cow pea), and *Lablab purpureus* (L.) Sweet (hyacinth bean) show similar patterns. Their wild progenitors are restricted to Africa (Zohary and Hopf, 2000).

Most cultivated varieties of sorghum can be traced back to Africa, where they grow on savannah lands. During the Muslim Agricultural Revolution, sorghum was planted extensively in parts of the Middle East, North Africa and Europe (Watson, 1974). In fact the name "sorghum" comes from Italian "sorgo", in turn from Latin "Syricum (granum)"

meaning "grain of Syria". Despite the antiquity of sorghum, it arrived late to the Near East. It was unknown in the Mediterranean area into Roman times. Tenth century records indicate it was widely grown in Iraq, and became the principal food of Kirman in Persia. In addition to the eastern parts of the Muslim world, the crop was also grown in Egypt and later in Islamic Spain. From Islamic Spain, it was introduced to Christian Spain and then France (by the 12th century). In the Muslim world, sorghum was grown usually in areas where the soil was poor or the weather too hot and dry to grow other crops (Watson, 1974).

The cultivated sorghum is a C_4 annual plant adapted to hot, semi-arid tropical and dry temperate areas of the world. It grows to a height of 50 cm to 6 m. It is cultivated both for its grain and fodder (ICRISAT, 2004). The many subspecies are divided into four groups — grain sorghums (such as milo), grass sorghums (for pasture and hay), sweet sorghums (formerly called "Guinea corn", used to produce sorghum syrups), and broom corn (for brooms and brushes). The name "sweet sorghum" is used to identify varieties of *S. bicolor* that are sweet and juicy (Wikipedia, 2011). The types used for grains have a large, erect and single culm terminating in a semi-compact or compact head or panicle. The types used for fodder purpose are generally profusely tillering with succulent stems. Many of these fodder types have high ratoonability. The plants have a fibrous root system that may penetrate 5 to 8 ft into the soil that makes sorghum one of the hardiest cereals (ICRISAT, 2004).

Sorghum bicolor is widely distributed throughout the world. Different cultivars are found in different regions depending on the climate. It is adapted to a wider range of ecological conditions. It is mostly a plant of hot, dry regions; still survive in a cool weather as well as waterlogged habitat (ICRISAT, 2004). The most common cultivar in South Africa has compact elongated heads and was previously known as *S. cafferorum* (Wyk and Gericke, 2000). A form (previously known as *S. dochna*) with more sparse open heads is often grown for its sweet canes which are chewed like sugar cane.

2.1.2. Production and uses of sorghum

Sorghum is a cereal crop whose domestication has its origins in Ethiopia and surrounding countries (Dillon *et al.,* 2007), but is mostly grown in Africa, Asia and Central America, primarily to ease food insecurity (Taylor, 2003). It is the world's fifth major grain crop and Africa's second most important in terms of acreage (FAOSTAT, 2012). Sorghum can survive very high temperatures and water-logged conditions, making it suitable to be grown in a tropical climate such as in Africa. For the reason that most cereal grain crops cannot withstand extreme harsh weather or climatic conditions, sorghum has acted as a great substitute crop. Cultivated in a large part of the world, sorghum is mainly planted to be used by people living in semiarid or subtropical areas as a substitute source of starch (Taylor, 2003).

Estimated production shows that sorghum is continually being grown throughout the world, and its uses are also on the rise. One such use is in beer production. Sorghum was formerly ignored in lager and stout beer production due to the absence of the hull, which often acted as a filter bed in lautering (Krottenthaler *et al.,* 2009). However, technology has advanced, and now sorghum is fully embraced in beer manufacturing. This is important because it reduces dependency on barley and wheat, which cannot withstand harsh weather conditions (http://www.ehow.com/facts_7200495_importance-sorghum.html).

The quantity of sorghum produced by countries is measured each year. This is then tallied against the food security situation in the country. If it is determined that sorghum would be beneficial to the country's population, the grain's uses and nutritional value are then explained and the people are taught how to consume it. In this case, sorghum aids in the control of scarce food supplies around the world (Taylor, 2003).

Research indicates that some species of sorghum are safe from pests, especially birds. This is because their tannins are bitter compared to those of maize, wheat, rice and barley. These tannins protect the sorghum grain from fungal and insect attacks (Taylor, 2003).

Research on the economic benefits of the different cereal grain crops has found that sorghum has proved to be a major income earner for some countries, including Nigeria, Sudan and Ethiopia. This crop is only being grown in specified parts of the world; other countries like the United States of America and Great Britain import the grain, which benefits the exporting countries (CCAB, 2007).

2.1.3. Diseases of sorghum

Sorghum has a number of diseases of continuing importance. Many of the important diseases are caused by fungi including grain moulds, anthracnose, stalk rots, ergots, smuts and downy mildews (Leslie, 2002). A good number of attacks come from parasitic nematodes. Bacterial and viral diseases form a small part of sorghum diseases but are of economic importance. A detailed list of sorghum diseases has been reviewed by Horne and Frederiksen (1993).

2.2. Sorghum anthracnose

2.2.1. History and geographical distribution of sorghum anthracnose

Anthracnose on sorghum was first reported in 1902 from Togo (Sutton, 1980) and has since been observed in most regions of the world where sorghum can be grown successfully (Tarr, 1962; Pastor-Corrales and Frederiksen, 1980). It is most prevalent in warm humid environments. The disease occurs widely and is considered of primary importance in Asia: China and India, Central America and the Caribbean basin: Argentina, Brazil, El Salvador, Guatemala, Honduras, Hawaii, Mexico, Panama, Puerto Rico, and Venezuela; and Africa: Botswana, Ethiopia, Kenya, Mali, Niger, Mozambique, Somalia, South Africa, Sudan, Tanzania, Uganda, Zambia and Zimbabwe (Frederiksen and Duncan, 1992; Thomas, 1995; Neya and Normad, 1998).

2.2.2. Economic importance of sorghum anthracnose

Sorghum anthracnose is prevalent whenever sorghum is grown in a warm, humid environment (Mathur *et al.,* 2002). Under optimal conditions for pathogen development, large yield losses can occur in susceptible cultivars. The extent of damage or yield loss due to anthracnose is usually related to: (a) the degree of host susceptibility to

anthracnose, (b) the environment, (c) the aggressiveness of the pathogen, and (d) the physiological status of the host (Wharton and Julian, 1996). Up to 88.7% grain loses caused by anthracnose have been reported (Ferreira and Warren, 1982). The disease causes both direct and indirect yield losses. The extent of direct losses varies with region, cultivar and prevalent climatic conditions. The reduction in 1000-seed mass, 1000-seed density and early abortion of seeds are the most important factors in yield reduction. The premature drying of leaves and defoliation due to foliar anthracnose can reduce the yield of sorghum grain and fodder by 30 to 50% in susceptible cultivars during severe epidemics (Ali and Warren, 1992). Stalk infection (stalk rot or red rot) also results in lodging and considerable reduction in grain and fodder yield (Mishra and Siradhana, 1979; Fredericksen, 1984). Secondary infection of the stalk by red rot may cause yield losses proportional to the severity of anthracnose and stalk rot (Neya and Kabore, 1987). Indirect losses also result from grain anthracnose due to reduced seed germination and transmission of the disease to new geographic regions (Basu Choudhary and Mathur, 1979).

2.2.3. Sorghum anthracnose symptomatology

The pathogen can infect all above-ground plant parts, including stem, leaves, peduncle, panicles, inflorescence, and grain (Tarr, 1962; Esela, 1995); the most common form being leaf and foliar anthracnose (Mathur *et al.,* 2002). The first signs of foliar infection are circular to elliptical dark spots, sometimes with a red pigmentation, of 0.5-5.0 mm in diameter. The diameter of some lesions can reach 2 cm. These lesions may coalesce to occupy most or all of an infected leaf and may even kill the infected leaf (Mathur *et al.,* 2002). The centre of mature lesions is straw-coloured and contains numerous acervuli containing black seta. Under humid conditions on the plant or following incubation of excised tissue, grey/cream/salmon-coloured spore masses are produced. In many instances, leaves can be entirely blighted. When the symptoms on stems are strongly coloured, the disease is often known as 'stalk red rot' (Duncan, 1984; Frederiksen 1984). Symptoms on the affected plant parts include lesions; abnormal colours; wilting; yellowed or dead leaves; discolourations on seeds and external discolourations on stems.

2.2.4. Detection and diagnosis of sorghum anthracnose

Identification of sorghum anthracnose relies almost exclusively on the observation of typical anthracnose symptoms (Holiday, 1980; Ali and Warren, 1992). The occurrence of stalk red rot due to *C. sublineolum* is much more difficult to identify because other pathogens, for example, *Fusarium moniliforme* (*Gibberella fujikuroi*) and *Macrophomina phaseolina* produce identical symptoms. *Colletotrichum sublineolum* produces symptoms on sorghum that are identical to those produced by *C. graminicola* on maize. It is important that the distinction between the two species is fully appreciated. Further research must be done to clarify the status of these species and especially to establish the nature of the pathogen causing anthracnose on other grasses and cereals (CABI, 2007). Incubation of excised leaf tissue bearing lesions under conditions of high humidity should stimulate the production of conidiomata with large quantities of slime. The conidia within this slime can be examined directly under the microscope. Alternatively, they can be transferred to an agar medium containing antibiotics. Sporulation should occur within 7-10 days allowing further analysis as required (CABI, 2007).

2.3. *Colletotrichum sublineolum* P. Henn., Kabát & Bubák.

2.3.1. Cultural and Morphological characteristics

Colletotrichum sublineolum causes anthracnose in sorghum (Crouch *et al.,* 2006). The fungus is highly variable in culture (Mordue, 1967; Saifulla and Ranganathaiah, 1990). Colonies are usually shades of grey interspersed with moist patches when sporulation occurs. Conidiomata (acervuli) contain many setae, but sclerotia are normally absent. Conidia are falcate (18.5-27.5 x 3-4.5 µm), tapering at both ends. In liquid culture ovoid spores can be produced (Thomas and Frederiksen, 1995a). Appressoria are subglobose with a slightly irregular surface (11.5-15 x 8.5-9 µm) (Sutton 1968; 1980). Ascomata are not formed (Thomas and Frederiksen, 1995b).

2.3.2. Biology and ecology

2.3.2.1. Life Cycle

As for most species of *Colletotrichum*, free water plays a major role in the development of this pathogen. Initially, conidia are prevented from germinating by the presence of self

inhibitors around spores in acervuli (Leite and Nicholson, 1992; 1993). Once these are removed by such factors like: germination stimulators especially volatile compounds, optimal germination temperatures, absence of inhibitory ions, especially those of heavy metals in air and water or a combination of any or all (Staples and Hoch, 1997), the conidia germinate to produce appressoria and penetrate the host epidermis. The pathogen produces intracellular infection vesicles within the epidermal cells of the leaf. These exist as a biotrophic phase for 1-2 days, during which time the infected cells retain their normal ultrastructure. Unlike biotrophic infection vesicles of other species of *Colletotrichum* (Bailey *et al.,* 1995), the vesicles of *C. sublineolum* are not separated from the host plasma membrane by an interfacial matrix (Wharton and Julian, 1996). Subsequently, the pathogen switches to necrotrophic growth which results in death of infected cells, destruction of host tissues and production of typical anthracnose lesions.

2.3.2.2. Transmission

The teleomorphic state is not known and thus the anthracnose disease is presumed to be spread exclusively by conidia. Conidia can be dispersed over longer distances as dry spore masses, but within smaller regions, splash dispersal of moist conidia is largely responsible for the development of epidemics. The pathogen is seed borne and this plays a major part in establishing the pathogen within the crop (Cardwell *et al.,* 1989). Transmission of the pathogen on introduced seed will also contribute to the dissemination of different strains to different regions.

2.3.2.3. Pathogen Diversity

Colletotrichum sublineolum is highly variable, both in its appearance in culture (Mordue, 1967; Casela and Frederiksen, 1994) and as a pathogen. Many different pathotypes have been described in many regions (Casela *et al.,* 1993; Casela *et al.,* 1995; 1996). Nurseries that aim to establish the extent of pathogen virulence have been set up throughout sorghum-growing regions (King and Frederiksen, 1976; Thakur, 1995). The large diversity of virulence renders the development of effective resistant varieties very difficult (CABI, 2007).

2.3.2.4. Epidemiology

The primary sources of inoculum are infected seeds (Rajasab and Ramalingam, 1989), stalk residues (Casela and Frederiksen, 1993; Misra and Sinha, 1996) and/or adjacent growing crops or weeds (Julian *et al.,* 1995). Anthracnose is most severe on mature plants, especially during the formation of the panicle (Ashok *et al.,* 1992). As with other species of *Colletotrichum*, extensive periods of rainfall are essential for the development of epidemics. The sequential senescence of sorghum leaves plays an important role in the development of the disease.

2.4. Control of sorghum anthracnose

Resistant germplasm has been regarded as the most effective method for the control of anthracnose (Harris *et al.,* 1964; Harris and Sowell, 1970; Frederiksen and Rosenow, 1971; Rosenow and Frederiksen, 1982; Abebe *et al.,* 1986; Anahosur and Laksman, 1986; de Milliano *et al.,* 1988; Evangalista and Tangonen, 1990; Bhaskar *et al.,* 1993; Casela and Frederiksen, 1993; Pande *et al.,* 1994; Anaso, 1995; Gupta *et al.,* 1996). The pathogen is highly variable and different isolates (races) show different abilities to attack different sorghum germplasm/varieties. Unfortunately, resistant varieties that are effective in one region have been found to be completely ineffective when grown in other regions (Reyes *et al.,* 1969; Pastor-Corrales and Frederiksen, 1979). Many resistant varieties continue to be produced, but the evolution, and possible introduction, of new pathogen strains means that effective resistance is not maintained. Long-term control requires that host resistance is carefully managed alongside efforts to optimise the host environment (possible by adjusting host densities and rotating crops) and to reduce inoculum arising from seed infections.

The treatment of seeds with chemicals has been shown to reduce the level of seed infection (Valarina *et al.,* 1988; Vitti *et al.,* 1993). The application of a diversity of chemicals (fungicides) to the growing crop (Gowily, 1995) has caused several negative effects such as development of pathogen resistance to the applied agents and their non-target environmental impacts (De Weger *et al.,* 1995; Gerhardson, 2002). Furthermore, the growing cost of pesticides, particularly in less-affluent regions of the world, and

consumer demand for pesticide-free food has led to a search for substitutes to these products. In the light of global chemophobia, alternative disease control strategies such as biological control have become important (Michereff *et al.,* 1993; 1994; Idris *et al.,* 2007)

2.5. Plant growth promoting rhizobacteria (PGPR)

Bacteria that can improve plant growth through various mechanisms have been known for decades and have been introduced into soil, on seeds or roots to improve plant growth and health (Raaijmakers *et al.,* 2002). The genus *Rhizobium* is the most widely known group. It has been successfully commercialized with many practical applications in agriculture by developing symbiosis with plants. Early in the last century, many bacterial species associated with plants but without symbiotic associations were discovered (Bashan and Holguin, 1998). Although many of these bacteria were able to promote plant growth, they were not widely recognized until the mid 1970's, with the discovery that some bacteria, mainly Pseudomonads, are capable of controlling soil borne pathogens and indirectly enhance plant growth (Kloepper *et al.,* 1980). The discovery of *Azospirillum* species, a diazotrophic free living bacterium that proliferates in the rhizosphere of many tropical grasses (Maria *et al.,* 2002), was the other breakthrough in the study of plant-microbe-interaction in the rhizosphere. The term plant growth promoting rhizobacteria (PGPR) was originally used to describe this unique biocontrol group (Kloepper *et al.,* 1980). As this term does not encompass all the beneficial bacteria associated with plants in the rhizosphere, generally the plant growth promoting rhizobacteria are classified into two major groups viz: Biocontrol Plant Growth Promoting Bacteria (Biocontrol PGPB) and Plant Growth Promoting Bacteria (PGPB) (Bashan and Holguin, 1998).

2.5.1. Biocontrol Plant Growth Promoting Bacteria (PGPB)

The application of chemical inputs such as fertilizers and pesticides has long been used to improve productivity in conventional agriculture. However, there is now a growing desire for alternatives to this system (Mark *et al.,* 2006). The use of bacteria as biocontrol agents of phytopathogens has been investigated for several decades (Földes *et al.,* 2000; Landa

et al., 2004). Suppression of phytopathogens has drawn considerable attention only recently as alternative farming methods to maintain productivity in agro ecosystems (Hu *et al.,* 1997; Sharga, 1997). Rhizobacteria designated as biocontrol PGPB are those that suppress plant pathogens by producing various types of inhibitory substances, or by increasing the natural resistance of the plant (Gardener *et al.,* 2001; Jetiyanun and Kloepper, 2002; Bashan and de Bashan, 2002) or by displacing (out competing) the pathogen (O'sullivan and O'Gara, 1992). Such biocontrol PGPB have the capacity to rapidly colonize the rhizosphere, and compete with deleterious microorganisms, including soil pathogens as well as phyllosphere pathogens (Rangarajan *et al.,* 2003). Some of the modes of action are discussed below.

2.5.1.1. Antibiosis

Recent advances in the understanding of genetics and the regulation of synthesis of bacterial metabolites especially antibiotics have contributed significantly to the advancement of plant protection. Biocontrol PGPBs are mainly endowed with the capacity to produce antibiotics against a number of phytopathogenic fungi and bacteria. Such biocontrol PGPB produce one or more of the antibiotics 2,4-diacetylphloroglucinol (2,4-DAPG), phenazine compounds (*Phz*), pyrrolnitrin (*Prn*), and pyoluteorin (*Plt*) (Mazzola *et al.,* 1992). These antibiotics are currently the major focus of research in biological control in soil ecosystems (Raaijmakers *et al.,* 1997). Hydrogen cyanide (HCN) is also reported to be one of the anti-fungal secondary metabolites belonging to the class of cyclic lipopeptides such as visconsinamide and tensin (Bloemberg and Lugtenberg, 2001).

Screening of microorganisms to identify biocontrol agents, which are active against many phytopathogenic fungi and bacteria, has been carried out in the past. The mode of action of many bacteria has been ascribed to the antibiotics they produce. *Pseudomonas* and *Bacillus* spp. which are active in the rhizosphere have widely been investigated and are among the potential biocontrol agents (Williams and Asher, 1996). Several strains of *Pseudomonas* spp. are used to control diseases in a variety of crops and other non-crop plants (Commare *et al.,* 2002). During their stationary growth phase, biocontrol strains of

Pseudomonas synthesize the antibiotics phenazine carboxylic acid (PCA), 2,4-DAPG, pyoluteorin and pyrrolnitrin (Schnider *et al.,* 1995). Many of these antibiotics produced by *Pseudomonas* spp. *in situ* contribute to the suppression of many plant diseases. Such antibiotic producing *Pseudomonas* spp. have been isolated from the rhizosphere soils that are naturally suppressive to diseases (Keel *et al.,* 1996). Plant diseases caused by fungal pathogens *Pythium ultimum* and *Rhizoctonia solani* are, for instance, suppressed by different strains of *Pseudomonas flourescens* (Cheryl *et al.,* 1998). The growth of *P. ultimum* mediating damping off in sugar beet has been inhibited due to the production of 2,4-DAPG by *P. flourescens* F111 biocontrol strain. This product is also produced by other *P. flourescens* strains and has been found effective against *Fusarium oxysporum* attacking tomatoes. Recently, it has been demonstrated that fluorescent *Pseudomonas* spp. producing the antibiotic 2,4-DAPG play a key role in the suppressiveness of take-all decline (TAD) in soils (de Souza *et al.,* 2003a) and the amount of 2,4-DAPG produced *in vitro* correlated with disease inhibition.

Phenazine antibiotics are another group of secondary metabolites effective against phytopathogenic fungi. *Pseudomonas* strains which produce phenazine antibiotics are reported for their suppression of take-all disease of wheat caused by *Gaeumanomyces graminis* var. *tritici* (Mazzola *et al.,* 1992). To determine the importance of this antibiotic in the suppression of take-all, an experiment with phenazine deficient mutants (*Phz*⁻) generated by Tn5 mutagenesis failed to inhibit *G. graminis* var. *tritici* on media supportive of antibiotic production (Thomashow and Weller, 1990).

Antibiotics of the cyclic lipopeptides group such as visconsinamide produced by *P. flourescens* have been shown to have an impact on the control of *Pythium* spp. and *R. solani* (de Souza *et al.,* 2003b). These cyclic lipopeptides induce encystment of *Pythium* zoospores and adversely affect the mycelia of *P. ultimum* and *R. solani* by causing reduced growth and intracellular activity, hyphal swelling and increased branching (Thrane *et al.,* 2000; de Souza *et al.,* 2003b). Nielsen and Sorensen (2003) screened *P. flourescens* strains capable of antagonizing *P. ultimum* and *R. solani* on agar plates. Further investigation during the early seed germination and root development of sugar

beet revealed that the cyclic lipopeptide antibiotics were responsible for antagonistic activity *in vitro* (Nielsen *et al.,* 1999; 2000; 2002).

The next most widely researched and commercialized bacteria for biocontrol activity in soil ecosystems are the endospores forming genus *Bacillus*. Most of the antibiotics produced by *Bacillus* spp. *in vitro* were found to be peptide antibiotics and are responsible for biocontrol *in vivo* (Leifert *et al.,* 1995). *Bacillus cereus* UW85 that produce the antibiotics zwittermycin A and antibiotic B tend to suppress damping off disease more effectively than do *Bacillus* strains that do not produce antibiotics (Stabb *et al.,* 1994). This strain was initially identified from a collection of rhizosphere isolates by its ability to suppress alfalfa damping off consistently (Handelsman *et al.,* 1990). Since then, *B. cereus* UW85 has proven an effective biocontrol agent against *Phytophthora* damping off and root rot of soy beans (Emmeret and Handelsman, 1999). In general, the antibiotic zwittermycin A produced by this strain has been reported to adversely affect the growth and activity of a wide range of plant pathogenic fungi (Silo-Suh *et al.,* 1998).

Several other members of the genus have been shown to produce antibiotics of which the most important species is *Bacillus subtilis* (Földes *et al.,* 2000). *B subtilis* is one of the most widely distributed bacterial species in agricultural systems. The most commercially successful strain among this group is *B subtilis* GBO3. This strain which effectively colonizes plant roots and produce antifungal compounds is the active ingredient in one of the widely distributed biofungicide (Kodiac, Guftafson LLC) (McSpadden and Fravel, 2002). Another best known biocontrol strain of this species isolated 25 years ago in Australia is *B. subtilis* A13 (Kim *et al.,* 1997). This strain, in addition to inhibiting all the nine pathogens tested in an *in vitro* test, subsequently promoted the growth of cereals, sweet corn and carrots when applied as seed inoculants (Kim *et al.,* 1997). *Bacillus* spp. are therefore considered ideal candidates for use as biocontrol agents in seed treatment programs against soil borne pathogens (Walker *et al.,* 1998).

Based on the production of antibiotics, BALLAD[®] PLUS (a formulation of *Bacillus pumilis* strain QST 2808 antibiotics) is a registered fungicide currently used for control of

rust, powdery mildew, sheath spot, blight, rust, brown spot, leaf spots and smuts on cereals. It was discovered and developed by AgraQuest, Inc, the leading manufacturer and marketer of biological, agricultural fungicides (www.agraquest.com).

2.5.1.2. Siderophore production

Iron is one of the most abundant minerals on earth, yet in the soil, it is unavailable for direct assimilation by plants or microorganisms. This is because ferric iron (Fe^{+3}), the most common form of iron in nature, is only sparingly soluble (10^{-18} M at pH 7). Therefore, the amount of soluble iron in the soil barely supports microbial growth (Glick and Bashan, 1997). To overcome this problem, soil microorganisms secrete siderophores, iron-binding proteins of low molecular mass (400-1000 daltons) which bind Fe^{+3} with a very high affinity ($KD=10^{-20}$ to 10^{-15}). Most aerobic and facultative anaerobic microorganisms produce Fe^{+3} chelating siderophores which bind and transport ferric iron back to the microbial cells, where it is taken up by means of cellular receptors (Brait, 1992; Glick and Bashan,1997; Bultreys et al., 2001). Biocontrol PGPBs prevent the proliferation of soil borne pathogens and facilitate plant growth through the production and secretion of such siderophores. The siderophores bind most of the Fe^{+3} available in the rhizosphere thereby effectively preventing any fungal pathogen in the immediate vicinity from proliferating due to lack of iron (O'Sullivan and O'Gara, 1992). Siderophores produced by fungal pathogens have a much lower affinity for iron than those of biocontrol PGPB. Thus, biocontrol PGPB out-compete fungal pathogens for the available iron in the rhizosphere (Glick and Bashan, 1997). Siderophores also indirectly stimulate the biosynthesis of other anti-microbial compounds by making these minerals easily available to the bacteria (Duffy and Defago, 1999.)

The major types of siderophores produced by biocontrol PGPB include pyoverdin, pyochelin and salicylic acid (Lemanceau et al., 1992; Duffy and Defago, 1999; Bultreys and Gheysen, 2000). Numerous studies indicate that among the biocontrol PGPB in the rhizosphere, the fluorescent Pseudomonad species are efficient competitors for ferric iron (Fe^{+3}). The most commonly detected siderophores in these species are called pyoverdins or pseudobactins (Lemanceau et al., 1993). Many potential biocontrol strains of this

species produce pyoverdins. They are generally peptide siderophores all containing the same quinoline chromophore which is responsible for the colour of the molecule, a peptide chain and a dicarboxylic acid connected to the chromophore (Bultreys *et al.,* 2001; Bultreys *et al.,* 2003). The characteristic fluorescent pigments of fluorescent Pseudomonads are due to the pyoverdins (Budzikiwicz, 1993). Apart from this taxonomic importance and most importantly, pyoverdins produced *in-situ* chelate iron and make iron unavailable to pathogens in the rhizosphere (Loper and Henkels, 1999). Some fluorescent Pseudomonad species also produce a non-fluorescent siderophore called pyochelin, a salicylic substitute cysteine peptide (Leeman *et al.,* 1996).

Many workers have reported the suppression of disease development of several soil borne pathogens by different strains of biocontrol *Pseudomonas* spp. producing siderophores. A pyoverdin siderophore called pseudobactin 358, for instance, produced by a strain of *Pseudomonas putida* was reported as an effective biocontrol agent against *Fusarium* wilt (Lemanceau *et al.,* 1993). *Fusarium* wilt diseases are currently responsible for important yield loses on a variety of crops (de-Boer *et al.,* 1999). Many strains belonging to the *Fusarium* genus often cause severe diseases such as vascular wilt, root rot and abnormal growth in various agricultural crops (Kurek and Jaroszuk-Scisel, 2003). Although many inputs of agrochemicals are used to protect the crops against this pathogen, they are adversely affecting the quality of the food products and that of the environment (Lemanceau *et al.,* 1992).

More sophisticated techniques are currently being used to evaluate the importance of siderophore mediated competition for iron by biocontrol rhizobacteria. Studies using a well defined mutant (Pvd) have indicated the involvement of pyoverdin siderophores in the control of *Fusarium* wilt of radish and carnations (Lemanceau *et al.* 1992; Lemanceau *et al.,* 1993; Raaijmakers *et al.* 1995; Thomashow, 1996). *Pythium* induced post emergence damping off has also been suppressed in hydroponically grown tomato using strains which produce pyoverdins and pyochelins (Buysens *et al.,* 1996). In many other studies, the efficacy of siderophores of biocontrol PGPB strains of *Pseudomonas* spp. has been proven to be very promising. In this respect, for instance, a mutant strain of

Pseudomonas aureginosa that lacks the ability to produce siderophore no longer had the ability to protect tomato plants from damping off (Glick and Bashan, 1997). Normally, siderphores are produced by bacteria under iron limiting conditions in the rhizosphere. In an effort to prove this, researchers (Elsherif and Grosmen, 1994) conducted an experiment in which the amount of iron present in the soil was increased to 40 µmol Fe^{+3}/lit. The result obtained indicated a concomitant decrease in both the amount of siderophores produced and the inhibitory effect against the wheat pathogen *G. graminis* var *tritici*. Siderophore synthesis in the rhizosphere by biocontrol PGPB in response to iron limiting conditions can be detected by means of a more advanced technique, namely an ELISA assay using monoclonal antibodies. With this method, it is possible to quantify the amount of siderophores produced in an ecosystem (Buyer *et al.,* 1993).

2.5.1.3. Induction of Systemic Resistance

Under normal conditions all plants possess active defence mechanisms against pathogens' attack, which sometimes fails upon infection by a virulent pathogen. This happens as a result of the pathogen suppressing the resistance reactions (Van Loon *et al.,* 1998). If, however, defence mechanisms are triggered by a stimulus before infection by the pathogen, the disease can be minimized, thus, the plants will have enhanced defensive capacity. This systemic protection of a plant by an inducing agent when applied to a single part of the plant is known as Induced Systemic Resistance (ISR) (Liu *et al.,* 1995; Nandakumar *et al.,* 2001; Ramamoorthy *et al.,* 2002). In nature induced resistance occurs as a result of limited infection by a pathogen and the subsequent development of a hypersensitive reaction (Van Loon *et al.,* 1998). Induced resistance brought about by the inducing agent is systemic as the defensive capacity is increased not only in the primary infected tissue, but also in the non-infected tissue. According to Sticher *et al.* (1997), induced resistance is commonly referred to as Systemic Acquired Resistance *(*SAR) due to its systemic character. In some cases, however, localised acquired resistance occur only when those tissues exposed to the primary invader become more resistant (Van Loon *et al.,* 1998).

Different biotic and abiotic inducers are involved in induction of systemic induced resistance in plants against various pathogens. These include pathogens, chemical plant products and PGPB (Liu *et al.,* 1995; Leeman *et al.,* 1995; Nandakumar *et al.,* 2001). The mechanism by which these inducing agents stimulate resistance is that they activate defence genes encoding chitinases, peroxidases, β-1, 4-glucanase and enzymes involved in the synthesis of phytoalexins (Van Peer *et al.,* 1991; Maurhoef *et al.,* 1994).

Induced systemic resistance against plant pathogens by biocontrol PGPB is a relatively new topic in disease suppression. It is mediated by effective biocontrol agents such as *Pseudomonas* spp. (Leeman *et al.,* 1996). In most of the investigations so far conducted, several strains of *P. fluorescens* are rendering promising results by ISR in many crops. Biocontrol PGPBs elicit ISR in plants through fortifying the physical and mechanical strength of the cell wall as well as changing the physiological and biochemical reaction of the host plant. This leads to the synthesis of the defence chemicals against the challenge pathogen (Ramamoorthy *et al.,* 2001). Physical and mechanical strength of the cell wall was induced for instance by a biocontrol PGPB in peas (Benhamou *et al.,* 1996b). According to these researchers, treatment of pea plants with a strain of *P. fluorescens* resulted in the formation of structural barriers, that is, cell wall papillae and deposition of phenolic compounds at the site of penetration of the invading hyphae of *P. ultimum* and *F. oxysporum.* Similar experiments in potato resulted in the deposition of phenolic compounds, which inhibited the growth of *F. oxysporum* f. sp. *radicis-lycopersici* in the epidermal cell wall and outer cortex of the root system (Ramamoorthy *et al.,* 2001).

The other mechanism of ISR mediated by biocontrol PGPB is through development of biochemical or physiological changes in the plant. These include the production of PR-proteins (Pathogenesis related proteins) such as chitinases, peroxidases, synthesis of phytoalexins and other secondary metabolites (Van Peer *et al.,* 1991; Zdor and Anderson, 1992). Increased expression of plant peroxidases and chitinases enzymes in rice using strains of *P. fluorescens* was efficient enough to inhibit mycelia growth of the sheath blight fungus *R. solani* (Nandakumar *et al.,* 2001). In another experiment, seed treatment of pea by one strain of *P. fluorescens* resulted in the production of hydrolytic enzymes

accumulating at the site of penetration of the fungus. Induction of systemic resistance by biocontrol PGPB is not confined to the aforementioned plant species. *Pseudomonas* spp. mediated ISR was also observed in carnation against *F. oxysporum* f. sp. *dianthii* (Van Peer *et al.,* 1991), in cucumber against *Colletotrichum orbiculare* Berk. & Mont. (Wei *et al.,* 1996) and *Pythium aphanidermatum* (Edson) Fitzp (Chen *et al.,* 2000).

Elicitation of ISR in plants has been reported to be mediated by specific strains of *Bacillus* spp. such as *B. amyloliquifaciens, B. subtilis, B. cereus, B. mycoides,* and *B. pumilis* with significant reductions in the incidence or severity of various diseases (Kloepper *et al.,* 2004). Certain strains of *Bacillus pumilus* have been reported to be involved in ISR in plants by inducing the accumulation of phenolic compounds in the newly formed wall appositions in pea roots in response to attack by *F. oxysporum* f. sp. *pisi* (Benhamou *et al.,* 1996a; Jetiyanun and Kloepper, 2002). The phenolic compounds contribute to enhance mechanical strength of the host cell wall and may also inhibit fungal growth as phenolics are toxic to fungi in nature (Ramamoorthy *et al.,* 2002). This is an induction for the potential of *Bacillus* spp. to be used as bio-control PGPB similar to *Pseudomonas* spp. in the soil rhizosphere.

The major bacterial determinants that are claimed to produce ISR in plants by the aforementioned mechanisms are the O' antigen of cell wall lipopolysacharides, siderophores and salicylic acid (Leeman *et al.,* 1996; Bloemberg and Lugtenberg, 2001). For instance, the development of ISR in carnation against *Fusarium* wilts by *F. oxysporum* f. sp. *dianthii* is associated with lipopolysacharide present in the outer membrane of PGPB *P. flurescenes* strain (Van Peer and Schippers, 1992). Similarly in rice, the increased activity of chitinases and peroxidases has been reported to be due to the release of these signal molecules by *P. fluorescenes* (Ramamoorthy *et al.,* 2001).

The fact that bacterial cell wall lipopolysacharides are involved in ISR was proved in one experiment conducted using a mutant strain of *P. fluorescenes* lacking the O' antigen side chain of lippolysacharides (Leeman *et al.,* 1995). This mutant, unlike the wild strain, failed to induce resistance in radish, showing that the O' antigen side chain of the

lipopolysaccharides serves as a signal in the induction of systemic resistance. Lipopolysacharide is not the only trait in determining the ISR, because in another study a mutant strain lacking the O' antigen side chain has been reported to elicit defence mechanisms in Arabidopsis (Van Wees *et al.,* 1997). In this respect, while lipopolysaccharides of some *P. fluorescenes* strains are the major determinants of ISR under iron replete conditions, siderophores of the same bacterial strain are responsible for ISR in radish against Fusarium wilt under iron limited conditions. However, the mechanism of how these siderophores trigger ISR is unclear (Van Loon *et al.* 1998).

As mentioned before, salicylic acid is also involved in the induction of ISR in plants. Treatment of plants with salicylic acid decreased disease development in tobacco due to tobacco mosaic virus (Kessmann *et al.,* 1994). According to these researchers, certain PGPB strains are endowed with the capacity to produce this compound and induce systemic resistance in plants. Mutant strains lacking the ability to produce salicylic acid production lost their ability to induce systemic resistance in bean as opposed to the wild strain (De Meyer and Hofte, 1997). Various experiments indicated that ISR by bacterial determinants varies with many factors. These factors include iron limiting conditions, bacterial strains, host plants and their cultivars (Leeman *et al.,* 1996; Van Loon *et al.,* 1998). Although ISR has been studied mainly under laboratory and greenhouse conditions, reports indicate that ISR can protect plants under field conditions (Tuzun *et al.,* 1992; Zhang *et al.,* 2002). Advantages of ISR over other mechanisms of biological control systems include, once expressed, ISR activate multiple potential defence mechanisms such as increasing the activities of chitinases, β-1, 3-glucanases, peroxidases, pathogenesis related proteins and accumulation of phytoalexins (Wei *et al.,* 1996). Another important aspect of ISR is that, apart from protecting plants against a wide spectrum of pathogens once induced, it also protects plants systematically following an application of an inducing agent. Contrary to this, other mechanisms of biological control are generally not systemic (Wei *et al.,* 1996; Zhang *et al.,* 2002).

Although plants have their own defence genes, they are quiescent in normal healthy plants, thus, they are inducible genes (Nandakumar *et al.,* 2001). When these endogenous

defence mechanisms are induced by appropriate stimuli or signals, the plants' own defence mechanisms will be activated (Nandakumar *et al.,* 2001). The use of biocontrol plant growth promoting *Pseudomonas* spp. and more recently the application of *Bacillus* spp. to develop ISR in plants is now becoming a novel plant protection strategy (Bargabus *et al.,* 2002; Ryu *et al.,* 2004; Meziane *et al.,* 2005; Choudhary and Johri, 2009).

2.5.1.4. Competition in the rhizosphere

Biocontrol plant growth promoting bacteria also inhibit phytopathogens by other mechanisms other than those mentioned in sections 2.5.1.1 to 2.5.1.3 above. Competition for nutrients and a suitable niche on the root and foliar surfaces is yet another mechanism (O'Sullivan and O'Gara, 1992). The ability to compete for nutrients with indigenous microbial populations within the rhizosphere is an important trait for effective bio-control of soil-borne pathogens (Walsh *et al.,* 2001). Strains of *Pseudomonas* spp. have been reported to have the ability to metabolize the constituents of seed exudates in order to produce compounds inhibitory to *Pythium ultimum* (Glick and Bashan, 1997). There is no relationship observed between the ability of these bacteria to inhibit the fungal pathogen by the production of siderophores or antibiotics (Stephens *et al.,* 1993). This was detected by growing the bacterium on a medium that favoured the production of either antibiotics or siderophores. In a similar study, high inoculum levels of a saprophytic *Pseudomonas syringae* were reported to protect pears against *Botrytis cinerea* (gray mold) and *Penicillium expansum* (blue mold) (Bashan and de-Bashan, 2005).

Due to competition, biocontrol agents have the ability to displace some bacterial plant pathogens. The pathogenic *Pseudomonas syringae,* which increases frost susceptibility in tomato and soybean and causes ice nucleation, is reported to have been out-competed by an antagonistic ice nucleation deficient medium (Wilson and Lindow, 1994). In one greenhouse experiment, a non-pathogenic copper resistant Tn 5 mutant of *P.syringae* pv tomato, the causal agent of bacterial speck of tomato, was co-inoculated with a pathogenic strain (Cooksey, 1990). The result was that the non-pathogenic strain

decreased the disease incidence significantly by competing with the pathogen for the same niche.

On leaves there are a limited number of sites where a pathogen can attack the plant. Bacteria capable of multiplying on the leaf surface to form a large population can compete successfully with pathogens for these sites and often reduce disease. These agents can be saprophytic strains, PGPB, or nonvirulent strains of the pathogen. This has been demonstrated by the plant growth promoting bacterium *Azospirillum brasilense* which was able to displace the causal agent of bacterial speck disease of tomato, *P. syringae* pv. tomato, on tomato leaves, and consequently decreased disease development (Bashan and de-Bashan, 2002). *Azospirillum* spp. are not known as typical biocontrol PGPBs as they lack the ability to produce significant amounts of antimicrobial substances, nor do they induce systemic resistance in plants (Shah *et al.,* 1992). However, because of their rhizo-competent ability and the capacity to form large populations on leaves, *A. brasilense* displaces leaf pathogens and in the process reduces disease severity. The displacement of *P. syringae* pv. tomato by *A. brasilense* was demonstrated by the reduced colonization of the pathogen in the rhizosphere and on the leaf surfaces in the presence of *A. brasilense* (Shah *et al.,* 1992).

2.5.2 Plant Growth Promoting Bacteria (PGPB)

The second division of beneficial bacteria in the rhizosphere are those referred to as plant growth promoting bacteria (PGPB), which promote growth via production of phytohormones and improvement of plant nutritional status (Bai *et al.,* 2002). Because of these properties, the co-inoculation of these PGPB with the symbiotic rhizobia is currently becoming a valuable technique in the development of sustainable agriculture. Among the major groups of PGPB, the most widely studied and efficient group include *Azospirillum* spp. (Bertrand *et al.,* 2001), *Pseudomonas* spp. (Amy and Germida, 2002) and *Bacillus* spp. (Bai *et al.,* 2002).

2.5.2.1 Synthesis of phytohormones

The ability of rhizobacteria, particularly PGPB to synthesize various metabolites, influences plants as well as the availability of mineral nutrients for plants and soil structure. A great proportion of microorganisms capable of producing phytohormones *in vitro* are found to survive in the rhizosphere (Vancura and Jander, 1986). According to this finding, 20% of the bacteria produced phytohormones. Moreover, out of the 50 bacterial strains isolated from the rhizosphere of agriculturally important plants, 43 strains produced auxins (IAA), 29 gibberilins, 45 kinetin-like substances and 20 strains produced all three types of phytohormones. All of these strains were able to solubilize poorly soluble phosphates and thus enable phosphorous up-take (Vancura and Jander, 1986). The auxin type phytohormone known as indole-3-acetic acid (IAA) is the main type of phytohormone produced by PGPB (Patten and Glick, 1996; Gonzalez and Bashan, 2000; Patten and Glick, 2002).

One mechanism by which PGPB affect plant growth in the rhizosphere is by contributing to the host plant endogenous pool of phytohormones such as IAA (Patten and Glick, 1996). Beneficial bacteria synthesize IAA through the indole-pyruvic acid pathway. In this pathway, the amino acid tryptophan is first transformed into indole-3-pyruvic acid by oxidative deamination, which is then decarboxylated to indole-3-acetaldehyde. Indole-3-acetaldehyde is finally oxidized to IAA (Vancure and Jander, 1986; Patten and Glick, 2002).

Among the first efficient PGPB studied for their capacity to produce phytohormones are *Pseudomonas putida, P. florescens, Azospirillum* spp. and *Bacillus* spp. In all these bacteria the formation of IAA and other auxins has been proved using HPLC and mass spectroscopy (Vancure and Jander, 1986). The role of many such rhizobacterial IAA in the development of the host plant root system has been studied. In one experiment, canola seeds treated with a wild type of *P. putida* strain that produces IAA and another IAA deficient mutant constructed by insertional mutagenesis responded differently (Patten and Glick, 2002). The canola seeds primary roots treated with wild type strain were on the average longer than the roots from seeds treated with the mutant strain and the roots from un-inoculated seeds. It was previously indicated by other studies that, while low levels of

IAA stimulate primary root elongation, high levels of IAA stimulates the formation of lateral and adventitious roots (Sawar and Kremer, 1995; Xie *et al.,* 1996).

Bacterial IAA promotes root growth either directly by stimulating plant cell elongation or cell division or indirectly by its influence on 1-Amino cyclopropane-1-carboxylic acid deaminase (ACC deaminase) activity. ACC deaminase is an enzyme produced by many plant growth promoting bacteria (Glick and Bashan, 1997; Lie *et al.,* 2000). The ACC deaminase hydrolyses plant ACC, the immediate precursor of the phytohormone ethylene. Ethylene in plants acts as a secondary messenger stimulating leaf or fruit abscission, disease development and inhibition of growth (Glick and Bashan, 1997). Mutants of PGPB that do not produce ACC deaminase, for instance, have lost the ability to stimulate root elongation (Lie *et al.,* 2000). There are several other reports of the role of IAA produced by PGPB in enhancement of growth and yield of many crops (Ayyadurai *et al.,* 2006).

Another key member of the PGPB, *Azospirillum brasilense* promotes the growth of many terrestrial plants upon seed or root inoculation (Bloemberg and Lugtenberg, 2001; Bashan and De Bashan, 2002). All known *Azospirillum* species produce IAA (Gonzalez and Bashan, 2000) and it is reported that this is the most abundant phytohormone secreted by *Azospirillum*. It is generally agreed that in most *Azospirillum* species, it is the production of IAA rather than nitrogen fixation that contributes to stimulation of rooting and enhancement of plant growth (Bloemberg and Lugtenberg, 2001). The auxin type phytohormone produced by *Azospirillum* spp. affect root morphology and thereby improve nutrient uptake from soil (Barea *et al.* 2005). Apart from increasing density and length of legume root hairs, IAA secreted by *Azospirillum* increases the amount of flavonoids that are exuded and act as signals for initiations of root nodulation by rhizobial strains (Glick *et al.,* 2001). Although IAA does not apparently function as a hormone in the bacterial cells, it is important in the microbial-plant relationship, particularly when it comes to stimulating the development of the host plant root system.

2.5.2.2 Asymbiotic nitrogen fixation

In order to sustain sufficient crop production, a reliable source of nitrogen is vital. Microbial oxidation of soil organic matter may thus provide plants with potentially available nitrogen. However, in soils with poor soil organic matter, biological fixation of nitrogen is that which fills the deficiency in the soil organic nitrogen pool (Chotte *et al.,* 2002). In the rhizosphere, free living nitrogen-fixing PGPB affect plant growth directly by non-symbiotic nitrogen fixation.

Many non-legume plants have been shown to be associated with the free living diazotrophic nitrogen-fixing bacteria. With the advent and the application of the acetylene reduction assay, it has now become common practice to screen plants and microorganisms for the presence of the nitrogenase activity (Malik *et al.,* 1997). In ecosystems where legumes are sparse or absent, nitrogen fixation by free living diazotrophic bacteria is the mechanism to meet part of the nitrogen requirement of the plants (Brejda *et al.,* 1994). Most of this nitrogen fixation by free living diazotrophic bacteria in the rhizosphere is associated with the roots of grasses and is regarded as an important component of the nitrogen cycle in many ecosystems. Rhizosphere bacteria commonly known for such non-symbiotic nitrogen fixation include *Azospirillum, Herbasprillum* and *Beijerinkia* (Anonymous, 2003; Baldani and Baldani, 2005). These bacteria are commonly microaerophilic and can be best recovered from tissues by growth in semisolid media with malate as the energy source. These free living nitrogen fixers in the rhizosphere are nowadays given attention (Chotte *et al.,* 2002) as they are known for the utilization of plant exudes as a source of energy to support the fixation process.

Azospirillum spp. proliferates in the rhizosphere of many tropical grasses, fixing nitrogen and transferring it to the plant (Maria *et al.,* 2002). Field inoculation with *Azospirillum* in many investigations revealed that these bacteria are capable of promoting the yield of many important agricultural crops (Okon and Gonzalez, 1994) In wheat, for example, a non-tropical cereal, *Azospirillum* has been assayed widely for field inoculation and resulted in significant yield increase (Maria *et al.,* 2002). *Azospirillum* are also involved in pronounced nitrogen fixation in several other crops such as rice (Malik *et al.,* 1997), corn (*Zea mays*)*,* Sorghum (*Sorghum bicolor*) and switch grass (*Pinatum virgutum*)

(Bredjda *et al.* 1994). Although the main emphasis in the search for nitrogen fixing plant growth promoting rhizobacteria in the soil rhizosphere focused on the isolation of *Azospirillum* (Berge *et al.,* 1991), other nitrogen fixing strains such as *Bacillus* spp. have also been found in association with grass roots.

2.5.2.3 Solubilization and mineralization of organic and inorganic phosphates

Phosphate is the second most critical plant nutrient after nitrogen. In the soil rhizosphere, although the total phosphorous is high, only a part of this is available to plants. Thus, it can be inferred that many soils throughout the world are P-deficient as the free P-concentration even in fertile soils is not higher than 10μM at pH 6.5 (Rodriguez and Fraga, 1999; Gyanshewar *et al.,* 2002). Soluble phosphorous has a high level of reactivity with calcium, iron or aluminium. This leads to phosphorous precipitation resulting in low levels of `P´ (Gyanshewar *et al.,* 2002). The type of the soil and the pH affects the fixation and precipitation of `P´ in soil. Thus, in acidic soils, P is fixed by free oxides and hydroxides of `Al´ and `Fe´, while in alkaline soils it is fixed by `Ca´ (Jones *et al.,* 1991). To overcome the problem of P-deficiency, chemical fertilizers are added. However, the production of chemical phosphatic fertilizers is such an energy intensive process that it requires energy worth $4 billion per annum so as to meet the global need (Goldstain *et al.,* 1993). Despite the fact that most agricultural soils contain large reserves of `P´ due to regular application of phosphorous, a large part of this applied inorganic `P´ is rapidly immobilized and become unavailable to plants (Rodriguez and Fraga, 1999).

Because of the aforementioned problems of `P´ availability to plants, there is now a growing need in the selection and manipulation of biofertilizers in plant nutrition. In terms of phosphate solubilization, the arbuscular mycorrhizae belong to the former category. In the last decade the ability of different bacterial species to solubilize inorganic phosphate compounds has been detected and proved to be beneficial in agriculture (Rodriguez and Fraga, 1999; Gyanshewar *et al.,* 2002). The higher proportion of these phosphate-solubilizing bacteria is commonly found in the rhizosphere (Baya *et al.,* 1981). The mechanism by which these microorganisms solubilize Ca-P complexes is by their ability to reduce the pH of their surroundings either by the release of organic acids or

protons (Gyanshewar *et al.,* 2002). Once the organic acids are secreted, they dissolve the mineral phosphate as a result of anion exchange of PO_4^{3-} or they chelate both Fe and Al ions associated with phosphates.

Pseudomonas and *Bacillus* spp. are reported as the most important phosphate solublizers among the PGPB (Baya *et al.,* 1981). At first the production of antibiotics, siderophores and phytohormones has created confusion about the specific role of phosphate solubilization in plant growth and yield stimulation (Kloepper *et al.,* 1989). However, at present there is evidence supporting the role of this mechanism in plant growth enhancement. A strain of *P. putida* for example, stimulated the growth of roots and shoots and increased `P´ labelled phosphate uptake in canola (Lifshitz *et al.,* 1987). Inoculation of crops with *Bacillus firmus* (Datta *et al.,* 1982) and *Bacillus polymyxa* also resulted in phosphate uptake and yield increase. Rice seeds inoculated with *Azospirillum lipoferum* strain 34H, a known rhizobacteria, increased phosphate ion content and resulted in significant improvement in root length and shoot weights (Murty and Ladha, 1988).

A second major source of plant available phosphorous is that it is derived from the mineralization of organic matter. Soil contains a wide range of organic substances. Particularly in tropical soils a large part of `P´ is found in organic forms (Rodriguez and Fraga, 1999; Kwabiah *et al.,* 2003). This organic phosphate (Po) is so complex that plants can not directly utilize it but only utilize `P´ in its inorganic form. It is therefore necessary that to make the organic phosphate available to plants, it must first be hydrolyzed to inorganic `P´. This is called mineralization of organic phosphorous and it is achieved by the activity of phosphatase enzyme, which hydrolyses Po to inorganic forms (George *et al.,* 2002). Plant growth promoting bacteria in the rhizosphere show a significant phosphatase activity (Dinkelager and Marshner, 1992). Plant inoculated with phosphate-solubilizing microorganisms (PSMs) showed growth enhancement and increased `P´ content as a result of mineralization of organic phosphates. Among these, *Bacillus megaterium* is regarded as the most effective PSM in many field experiments

releasing `P´ from organic phosphate, but does not solubilize mineral phosphate (Gyanshewar, 2002).

Phosphate solubilizing bacteria are also reported to function as mycorrhizal helper bacteria (Kraus and Loper, 1995). When such bacteria are associated with mycorrhizal fungi, they promote root colonization. The principle is that, their association with mycorrhizal fungi contributes to the biogeochemical cycle of nutrients by more than just providing a greater surface area for scavenging nutrients that may be relatively immobile in soil (Toro et al., 1997). Obviously, the role of microorganisms, especially of the growth promoting rhizobacteria in `P´ solubilization and mineralization is very crucial to make `P´ easily available to plants.

2.6 Bio-formulations and application of rhizobacteria as biocontrol agents

The economic feasibility of any given biocontrol agent is affected by many factors of which formulations of these agents and their delivery systems are very critical. It has been a common practice to use seed treatment with cell suspensions of many PGPR to control several diseases. This method is however becoming impractical due to difficulty in handling, transport and storage of bacterial suspensions (Trapero-Cascas et al., 1990; Parke et al., 1991). It is very difficult to use bacterial cell suspension for large scale field use. Therefore the need arises to device techniques for the development of formulations in which the biocontrol agents can survive in a carrier material for longer periods of time (Rabindran and Vidhyasekaran, 1996). Formulations can be considered as the industrial art of converting a promising laboratory proven bacterium into a commercial field product (Bashan, 1998). Such microbial inoculum formulations not only overcome loss of viability during storage in the growers' warehouse, they also have longer shelf-life and stability over a range of temperatures between -5°C to 30°C while in the marketing distribution chain (Bashan, 1998). Formulations are generally composed of the active ingredient, that is, microorganisms, metabolites or spores which are carried in an inert material used to support delivery of the active ingredients to the target (Hynes and Boyetchko, 2005).

Bacterial formulations can be prepared either in liquid or dry forms. Liquid formulations may be oil-based, aqueous based, polymer based or combinations while dry formulations include an inert carrier such as fine clay, peat, vermiculite alginate or polyacrilamide beads (Boyetchko *et al.,* 1999). Among the dry formulations, peat based formulations have been widely investigated and used giving significant result in yield increase and bio-control efficiency. Bacteria can survive well in peat-based formulations for longer periods and PGPR have been reported to survive in such types of dry formulations (Vidhyasekeran and Muthamilan, 1995). Peat-based or talc-based dry formulations allow the antagonists to be supplied to the farmers for seed treatment or to the seed producers to supply treated seeds to the farmers.

Formulations of PGPR are generally used to promote growth and health of crop plants. Treatment with rhizobacterial formulations for instance enhanced the growth of pearl millet plants and reduced the percentage of downy mildew incidence (Nirajan-Raj *et al.,* 2003). Control of rice sheath blight caused by *R. solani* has been achieved using peat-based formulation of *P. fluorescens* (Rabindran and Vidhyasekaran, 1996). In another experiment, field emergence of chickpea plants was improved by seed treatment with talc-based *P. fluorescens* formulation (Vidhyasekaran and Muthamilan, 1995). These results were obtained as the formulated products suppressed pre-emergence damping off caused by various pathogens. Powdered formulations of PGPR in an organic carrier mixed into soilless media provide seeding growth promotion and induce systemic disease protection (Reddy *et al.,* 1999). The practical applications of these PGPR formulations were supported due to the fact that the growth promotion detected was highly significant in comparison with the non-treated controls in various experiments.

Peat formulation has been the carrier of choice and the most commonly used in the rhizobia inoculation industry (Bashan, 1998). It has been common to use peat-based formulations to introduce *Azospirillum,* a biofertilizer, into the rhizosphere. There are however, some drawbacks of the peat-based formulations. Peat, as it is an undefined complex organic material, affects the final product and causes difficulties in inoculants dosage and storage condition (Bashan, 1998). Moreover, peat formulations are

susceptible to contaminations reducing the shelf life of the inoculants. Due to such problems in peat-based formulations, new trends to use unconventional synthetic materials as PGPR formulations are now becoming more practical and proved more advantageous than peat based formulations. These formulations are based on polymers, which encapsulate the living cells thereby protecting the microorganisms against many environmental stresses. Alginate is the material most commonly used for encapsulation of microorganisms and the resulting inocula are used as biological control agents and in bacterial chemotaxis research (Bashan and Holguin, 1994). It is a naturally occurring polymer composed of β-1, 4 linked D-mannuronic acid and L-glucuronic acid and it is extracted from different microalgae as well as several bacteria (Smidsrod and Skjac-Break, 1990). Compared with peat-based formulations, alginate-based PGPR formulations have such advantages as being non-toxic, biodegradable and slow release of microorganisms into the soil (Kitamikado *et al.,* 1990).

In general, the selection of appropriate formulations not only improves product stability and viability, but also reduces inconsistency of field performance of many potential biocontrol and growth promoting agents (Boyetchko *et al.,* 1999). Moreover, irrespective of the type of formulation used, effective control and yield increases also depends on the method of treatment and the concentration of the microbial inoculum used (Ribandran and Vidhyasekaran, 1996). As formulation of microorganisms or their spores determines efficient delivery, shelf-life and stability of its effectiveness against plant pathogens, it can generally be regarded as a key to bio-product success (EL-Hassan and Gowen, 2006).

2.7 Current status and future prospects of using rhizobacteria as biocontrol and growth promoting agents

Plant growth promoting bacteria interact with their biotic environments in a complex pattern. Due to this, substantial advance is being made in understanding the genetic basis of the beneficial effects of these PGPRs on plants (Thomashow, 1996; Bloemberg and Lugtenberg, 2001).

2.7.1 Current status and future prospects of Biocontrol PGPB

It has previously been mentioned that most of the biocontrol PGPB such as *Pseudomonas* produce various anti-fungal metabolites (AFMs). The genetic basis of the biosynthesis of the more frequently detected AFMs such as pyoluteorin in *P. fluorescens* Pf5 (Nowak *et al.,* 1999) and 2,4-DAPG in *P. fluorescens* Q-2-87 (Bangera and Thomashow, 1999; Delany *et al.,* 2000) has been elucidated. Various such great advances in the molecular basis of biocontrol agents have been achieved. Recently for instance, the biocontrol efficacy of *P. fluorescens* F113 has been enhanced by altering the regulation and production of 2,4-DAPG (Delany *et al.,* 2001).

In many studies, it has been demonstrated that antibiotic negative mutants of *Pseudomonas* strains have reduced ability to suppress root diseases compared with the wild strains (Schnider *et al.,* 1995). A phenazine negative mutant of *P. fluorescens* for instance was shown to lack part of its ability to suppress take-all disease of wheat (Pierson, 1994). Similarly, pyrrolnitrin defective mutant of *P. fluorescens* BL915 failed to suppress *R. solani* induced damping off in cotton (Hill *et al.,* 1994). But when antibiotic production is restored in these mutants by complementation or recombination, their biocontrol efficiency is also reduced.

Promising results are being obtained in improving the biocontrol performance of soil borne *Pseudomonas* by the introduction of antibiotic biosynthetic genes (Dowling and O'Gara, 1994). Vincent *et al.* (1991) transferred a recombinant cosmid expressing the *Phl* structural genes of *P. aurofacience* Q 2-87 to *P. fluorescens* that naturally produces phenazine carboxylic acid. The resulting recombinant strain had increased anti-fungal activity *in vitro* against *Gaeumannomyces graminis* (Sacc.) var. *tritici*, *P. ultimum* and *R. solani.* In a related experiment, by introducing the PCA biosynthetic genes of *P. fluorescens* 2-79 into different PCA non-producing strains, it was possible to develop recombinant strains. The recombinant strains proved to be more inhibitory to *G. graminis* var. *tritici in vitro* and *in vivo* than the wild types (Schnider *et al.,* 1995). It is thus reasonable to speculate that these and a number of other related advances will lead to

more efficient use of these biocontrol strains through their improvement by genetic modifications.

While all these advances of PGPB have been undertaken, it begs the question as to why biological control of soil-borne and other plant diseases achieved by most biocontrol agents including *Pseudomonas* and *Bacillus* spp. is still so inconsistent. One factor particularly associated with this inconsistency is insufficient root colonization by the introduced bacteria (Latour *et al.,* 1996; Bloemberg and Lugtenberg, 2001). Lack of knowledge about the bacterial traits that promote root colonization is another contributing factor. For a biocontrol inoculant to perform effectively, its root colonization ability and hence its rhizosphere competence is very important. In this regard the inoculant bacteria must be able to establish itself in the rhizosphere at a threshold population density (10^5 cfu/gm) sufficient to produce a beneficial effect (Raaijmarkers and Weller, 2001). Rhizobacteria have a superior ability to establish and maintain high rhizosphere population densities over an extended period of time. Saravanan *et al.* (2004) also reported that *P. fluorescens* strains that inhibited the growth of *Fusarium oxysporum* f.sp. *cubensis* causing wilt in banana do so by aggressively colonizing roots and establishing themselves at the root environment.

During the last two decades however, the population densities of many *Pseudomonas* strains has declined substantially (Mazzola and Cook, 1991). If a biocontrol agent cannot compete within the rhizosphere and colonize the root surface, it will not have an efficient biocontrol activity (Walsh *et al.,* 2001). An efficient inoculum strain must be capable of competing with the indigenous soil bacteria. Among many approaches used to overcome this problem include inoculation at higher concentration than the indigenous population, repeated inoculation and the use of genetically engineered strain with enhanced competitiveness (Nautiyal, 1997). Thus the selection of strains that are rhizosphere competent will contribute to improve the efficacy of biocontrol agents. It is important therefore to investigate bacterial colonization and gene expression *in-situ* in the rhizosphere. In recent years, the green fluorescent protein (GFP) and bioluminescence techniques have been employed to study bacterial root colonization and rhizosphere

competence (Walsh *et al.,* 2001; Bloemberg and Lugtenberg, 2001). The GFP technique together with the co focal laser scanning microscopy has facilitated the detection of a single bacterial cell. The technique revealed that *Pseudomonas* biocontrol agent often form micro colonies on the roots of crop plants (Normander *et al.,* 1999; Tombolini *et al.,* 1999).

The identification of genes and traits involved in the process of inoculation and root colonization is therefore an important strategy to improve the inadequate biocontrol activity and inconsistency in field experiments. In this regard, *P. fluorescens* genes that are specifically expressed in the rhizosphere (*rhi* genes) have been identified using the *in vivo* expression technology (Bloemberg and Lugtenberg, 2001). Many other root colonization genes and traits have been identified from *Pseudomonas* biocontrol species (Lugtenberg *et al.,* 2001).

There are, however, certain instances in which root colonization may play a less significant or even minimal role in determining the level of disease control obtained in response to rhizobacteria that suppress disease via induced systemic resistance mechanisms. Liu *et al.* (1995), for instance demonstrated that ISR activity mediated by PGPR strains did not depend on high root colonization ability and high populations. The study, conducted to determine ISR activities of *Pseudomonas putida* strain 89B-27 and *Serratia marcescens* strain 90-166 on cucumber revealed that ISR increased over time whereas the bacterial populations decreased. There was hence no relationship between ISR activity and populations of the two strains on roots.

2.7.2 Current status and future prospects of PGPB

Although inoculation with PGPB especially with non-symbiotic associative rhizosphere bacteria is not a new technology, many of the attempts failed. Inoculation trials with *Azotobacter* on a large scale in Russia in the late 1930's and an attempt to use *Bacillus megaterium* for phosphate solubilization in the 1930's also failed (Bashan, 1998). It is only in the late 1990's that a major breakthrough in plant inoculation technology was made. One of the major breakthroughs is the finding of the plant growth promoting, free

living *Azospirillum* spp. These bacteria enhance the growth of non-legume plants by directly affecting the metabolism of the plants (Bashan and Holguin, 1997). In later years after the discovery of *Azospirillum* as PGPB, many other bacteria such as *Bacillus, Flavobacterium* and *Acetobacter* have been evaluated for their potential in plant growth promotion (Tang, 1994). The biocontrol agents, mainly *P. fluorescens* and *P. putida* are also regarded as agents of plant growth promotion.

An important feature of these plant growth-promoting bacteria is their ability to colonize roots and promote plant growth (Sharma *et al.,* 2003; Patten and Glick, 2002). The potential of rhizosphere colonization by PGPB is very crucial for what is known as soil biofertilization (Villacieros *et al.,* 2003). The term 'biofertilizer', though misleading, is a widely used term to describe bacterial inoculants. It refers to preparation of microorganisms that may be a partial or complete substitute for chemical fertilization like rhizobial inoculants (Bashan, 1998).

Improving plant growth by biofertilization is a crucial mechanism by which iron acquisition in most agricultural crops is achieved. Normally, the total iron in the soil is by far much higher than most crops require. However, the concentration of free Fe^{+3} in most soils is far below that required for optimum growth (10^{-9} and $10^{-4}M$ Fe^{+3}) in the soil solution (Masahla *et al.,* 2000). Earlier studies indicate that the production of siderophores by plant growth promoting bacteria, particularly by the biocontrol *Pseudomonas* spp. increases plant iron acquisition (Masahla *et al.,* 2000). The high binding affinity and specificity for iron facilitates the transport of iron into the bacterial cells. Plants make use of this ferric-siderophore complex in their systems through the action of enzymes like ferric reductase (Sharma *et al.,* 2003). According to many reports, the possible role of plant growth promoting bacteria in iron uptake by plants in the rhizosphere is indicated by the fact that, under non sterile soil system plants show no iron deficiency symptoms in contrast to plants grown in sterile system (Walter *et al.,* 1994). Another important aspect of biofertilization is that it accounts for approximately 60% of the nitrogen supply to crops worldwide. This is achieved both by the symbiotic and free-

living nitrogen fixers. To date, the genes involved in nitrogen fixation and nitrogen assimilation have been described for *Azospirillum* (Bloemberg and Lugtenberg, 2001).

A promising trend in the field of inoculation technology with plant growth promoting bacteria is, the finding that co-inoculation of growth promoting bacteria with other microorganisms increased growth and yield (Bashan, 1998). Mixed inoculations allow the bacteria to interact synergistically and provide nutrients, remove inhibitory products and enhance some beneficial aspects of their physiology such as nitrogen fixation. *Azospirillum* spp. co-inoculated with phosphate solubilizing bacteria for instance frequently increased plant growth by providing the plant with more balanced nutrition, improved absorption of nitrogen, phosphorous and iron (Bashan and Holguin, 1997).

At present the discovery of many traits and genes involved in the beneficial effects of PGPB has resulted in a better understanding of the performance of these growth promoting agents in the field. This also provided the opportunity to enhance the beneficial effects of PGPB strains by genetic modification for future use.

CHAPTER THREE
MATERIALS AND METHODS

3.1. Study Area

The study area lied between 1.5^{o}N, 34°E and 0.25^{o}S, 35.5^{o}E which covers high potential, medium potential and semi-arid agro-ecological zones in western Kenya that produces the bulk of Kenya's sorghum. The zones were represented by West-Pokot, Trans-Nzoia, Bungoma, Busia, Siaya and Kisumu counties (Figure 1). A circular route covering these counties was navigated. Selected farms, both large and small scale that had a consistent record of cultivating the sorghum crop were identified for soil and plant sampling.
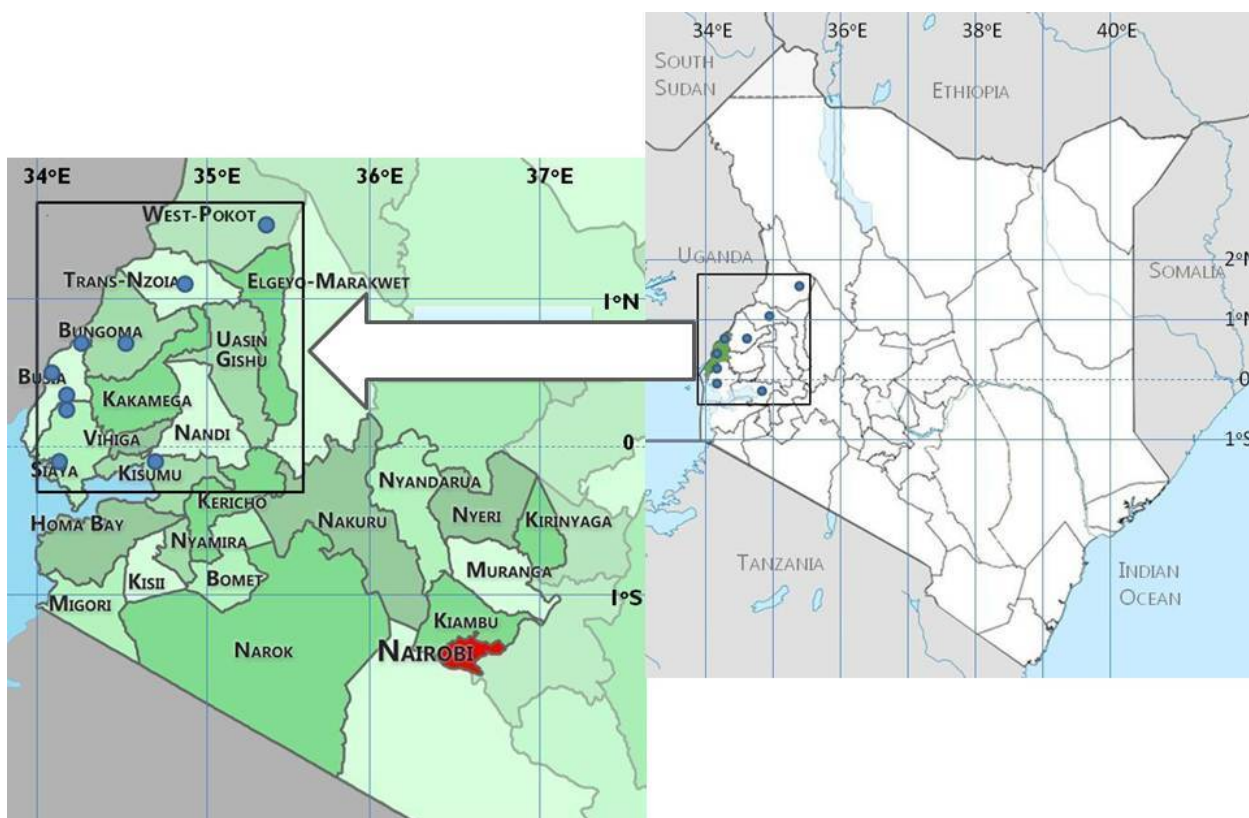
Figure 1: Right image; Map of Kenya showing specific locations within counties where soil and diseased plant samples were collected (boxed). Left image; Magnified view of the specific locations in counties of interest where sampling was carried out. The sampling locations represented different agro-ecological zones and are indicated with blue dots. Locations are approximate.
(Source: http://kenya.usaid.gov/kenya-map-west)

3.2. Collection of materials

3.2.1. Soil samples

Sampling fields were selected systematically along the route by stopping at 5 to 10 km intervals except where there were no suitable fields available (Marley *et al.,* 2001). A total of 15 farms per county were visited. Sampling within farms was done at intervals of 20-25m. For large scale sorghum plantations, fields were divided into a minimum of 2½ acre areas for sample collection (Reetz, Web article; Harold, Web article). Randomly distributed plots out of the large field were selected for sampling from which representative samples were taken. In the case of small holder fields, random collection of soil samples was done (US EPA, 1992). At each sampling site, a W-pattern of sampling was used to cover whole fields on foot. Soil from five plants along each straight line constituted a single sample. Sorghum roots were uprooted and 0.5 kg rhizosphere soil taken from each of the five plants. The soil was mixed thoroughly, out of which 1 kg was taken as a final sample (Idris *et al.,* 2007).

3.2.2. Diseased sorghum plant parts

In a study conducted by Ngugi *et al.* (2002), a number of fungal pathogens of economic importance to the production of sorghum in western Kenya with regard to their prevalence, incidence and severity were found. These included *Colletotrichum sublineolum,* the causal agent of anthracnose; *Ramulispora sorghicola*, the causal agent of oval leaf spot; *Gloeocercospora sorghi*, the causal agent of zonate leaf spot; *Cercospora sorghi*, the causal agent of grey leaf spot and *Exserohilum turcicum*, the causal agent of leaf blight. The findings by Ngugi *et al.* (2002) were used as a guideline of which phytopathogens to be expected in the present study.

Plant disease symptomatology in the present study was the basis of sample collection. While collecting diseased sorghum plant materials, emphasis was laid on symptoms of the diseases aforementioned. Nonetheless, diseased sorghum materials showing development of diseases other than those mentioned were also collected. Stops were made every 5 to 10 km and farmers sorghum fields examined for symptoms of the diseases. In areas where high cultivar diversity existed, stops were made every 25 to 50 km. At each stop, between one to five farmers fields were inspected and diseased leaf and

stem pieces cut, placed between blotter papers, labelled, packaged, and then taken to the laboratory for isolation purposes (Marley *et al.,* 2001). Disease symptom guidelines for field identification used in this study are provided by Williams *et al.* (1978) and CIMMYT (2004).

3.3. Sorghum rhizosphere soil analysis

Soil samples from the field were physically and chemically analyzed for the content of inorganic elements (Carbon, Nitrogen and Phosphorous) and textural classes in the soil analysis laboratories at the School of Agriculture and Biotechnology, University of Eldoret and Kenya Agricultural and Livestock Research Organization (KALRO) Kakamega. Another important parameter measured was soil pH. The Okalebo *et al.* (2002) methods of parameter measurements were adopted. The analysis was used to relate the soil type and composition with rhizobacteria prevailing.

3.4. Isolation of sorghum rhizobacteria

Each soil sample was mixed well before transferring 1 g to 9 ml quarter strength sterile Ringer's solution and serially diluted up to the 10^{-10} dilution (Idris *et al.,* 2007). A 0.1 ml aliquot of the 10^{-10} dilution suspension was spread-plated on King's B medium (King *et al.,* 1954) and nutrient agar (NA) medium in triplicates. The spread-plate cultures were incubated for 24 h at 28°C. Representative colonies, with different morphological appearances were selected from the countable plates and re-streaked on a new plate but of the same media to obtain pure colonies. Colonies were maintained on NA slants. Preliminary characterization of cultures was done using the following tests: Gregorson's KOH (Gregorson, 1978), cytochrome oxidase (Kovacs, 1956; Shields and Cathcart 2010), oxidation fermentation (Hugh and Leifson, 1953; Hanson, 2008), catalase (Reiner, 2010) and motility (Shields and Cathcart, 2011) tests.

3.5. Isolation of sorghum fungal phytopathogens

The test fungal pathogens were isolated using basic techniques. Fresh diseased sorghum plant samples collected from the field were washed free of soil with running tap water. Diseased plant parts were cut into 3 mm pieces and then surface sterilized by immersing

in 1% sodium hypochlorite for 1 minute then in 70% alcohol (ethanol) for 30 seconds (Marley *et al.,* 2001). The diseased plant pieces were aseptically rinsed in 3 changes of sterile distilled water (SDW) and blotted dry by turning them up and down on sterile filter papers to remove excess water. The surface sterilized plant pieces were then plated on Potato Dextrose Agar (PDA) and Oat Meal Agar (OMA) containing 200 ppm streptomycin to suppress bacterial growth. Incubation of inoculated plates was done under continuous fluorescent light for 7-14 days at 28°C (Souza-Paccola *et al.,* 2003a; Zanette *et al.,* 2009). Sub-culturing on PDA was done to purify the pathogen after positive identification of colonies of interest. Microscopic examination of the fungal morphology was also carried out to confirm the identity of the pathogens. PDA slants of purified fungal pathogens were prepared and preserved at 4°C for subsequent uses (Mortensen, 1994). Long term preservation was done on PDA slants bearing fungal isolates that were overlaid with mineral oil and stored at room temperature (Nakasone *et al.,* 2004).

3.6. Identification of the sorghum fungal phytopathogens

Identification was based on cultural and morphological characteristics of pure cultures of the pathogen isolates, by visual, microscopic examinations of conidiogenous structures and synoptic descriptions or keys.

3.6.1. Visual and microscopic examination

Pure cultures of 7 to 14 days old grown on PDA were used for both visual and microscopic examination. Visual observations on mycelia colour and growth form were used for preliminary identification. Microscopic slides were prepared from each fungal isolate and observed under a Chinese made compound microscope (Biological Microscope, Model XSZ-N107BIII), with cotton blue in lacto-phenol as the mounting medium at 640X and oil immersion at 1600X for the presence of conidia, conidial type, and vegetative structures such as hyphal formation, type of septa and branching.

3.6.2. Observation of conidiogenous cells

The slide culture technique used in this study adopted a method developed by Dade (1960) which is a modification of the original method described by Riddel (1950) that allows for longer incubation enabling maturity of the fungi under investigation to be reached.

Cover slips were sterilised either by dipping in alcohol and flaming or by autoclaving in a glass petri dish containing filter paper to absorb any moisture. The sterilised cover slips were then fixed to the floor of a Petri dish using small amounts of sterile agar. PDA was then gently poured over the top and allowed to set. Once set, the agar was cut away from above the cover slips using a flamed scalpel or a cork borer to remove a plug from above the cover slips. All four edges of the cover slips were then inoculated with the fungal isolates separately and the plates incubated at 28°C for 14 days. The cover slips were then removed and mounted directly on to a microscope slide with cotton blue in lacto phenol as the mounting medium and examined under the microscope at 640X and under oil immersion at 1,600X (Nugent *et al.,* 2006).

3.6.3. Use of synoptic identification descriptions and/or keys

Synoptic identification descriptions/keys described by Olive *et al.* (1946); Ellis and Holiday (1971); Chidambaram *et al.* (1973); Al-Doory and Domson (1984); Sivanesan (1987); Ahmed and Ravinder (1993); Navi *et al.* (1999); Munaut *et al.* (2001); Mims and Vaillancourt (2002); Timmer *et al.* (2003); Souza-Paccola *et al.* (2003a); Crouch *et al.* (2006); Zakaria *et al.* (2009); Chowdappa *et al.* (2012); Gautam (2013); Gautam (2014) and Abass and Mohammed (2014) were used for characterizing intact morphological features of fungal isolates obtained from the Dade slides. The keys were used to determine: conidia types, production, colour and their shapes; colours and shapes of conidiophores; lengths of conidia and conidiophores; colour of hyphae; formation of appressoria, sporodochia and stroma. Identification of phytopathogens was done by screening each isolate through the parameters given and grouping them into their specific species as identified.

3.7. Fungal inoculum preparation

Fungal conidia/spore suspensions were prepared by flooding 7 to 21-day-old (depending on the species) pure plate cultures of each test fungal pathogen with 10 ml of SDW. Fungal growth was gently scrapped with the edge of a sterile glass slide (sterilized by dipping in 70% alcohol and flaming) to dislodge the conidia/spores. The conidia/spore suspension of each test fungus was then separately filtered using sterile cheese cloth (sterilized by autoclaving at $121^{o}C$ and 1 bar for 15 minutes) to remove mycelia clumps (Wahome, 1998). Conidia/spore counts were done by placing a drop of suspension on an improved Neubauer haemocytometer and examining under a microscope. Total conidia/spore counts were used to calculate the conidia/spore densities that were then adjusted to an appropriate concentration using SDW.

3.8. Pathogenicity test of isolated *Colletotrichum sublineolum*

Pathogenicity of the isolated *C. sublineolum* was tested using the sorghum "Serena" variety seeds obtained from the Kenya Seed Company that is relatively susceptible and are commonly cultivated by Kenyan farmers. Seven seeds were planted in each of the 20cm by 10.5cm diameter plastic pots containing sterile soil mixture (planting medium). The planting medium was prepared from soil, manure (cow dung) and sand in the ratio of 2:1:1 by volume respectively (Isanda, 1995). The soil mixture was sterilized by subjecting to $121^{o}C$ and 1 bar for 2 hours in an autoclave, allowed to stand for 7 days to release toxic gases such as ammonia that are capable of inhibiting germination of seeds, then ¾-filled into 30 cm diameter polythene sleeves. Brigade 25EC (2.5%v/v), an insecticide/miticide, whose active ingredient/molecule is Bifenthrin, was sprinkled on the surface of each pot to control insect pests. These pots were kept in the greenhouse at room temperature ($22 \pm 5^{o}C$) and watered regularly. After seed germination, thinning was done to leave 3-5 seedlings per pot.

Conidia suspension of the isolated *C. sublineolum* was prepared as described in section 3.7 above. The test was conducted by spraying 14-day-old plants with a conidial suspension at a concentration of 4×10^{4} conidia per millilitre using an atomizer, until all the leaves were wet. The plants were then covered using a transparent polythene bag for

72 h to induce high relative humidity conditions of about 100% and $28 \pm 5^{\circ}C$, for proper germination of conidia and high plant infection. Control plants were sprayed with SDW. After 72 h, the plants were removed from the polythene chamber and placed on greenhouse benches. Disease reactions were recorded 8 days after inoculation (Borges, 1983). After symptom development, re-isolation of *C. sublineolum* was carried out from disease lesions onto artificial medium, and then examined under the microscope to check the morphology conformity.

3.9. Bacterial inoculum preparation

Isolates were grown in Nutrient Broth (NB) or Potato Dextrose Broth (PDB) on a rotary shaker at $28^{\circ}C$ and 180 rpm for 24 h. The suspensions were centrifuged in 50 ml capacity sterile falcon tubes at 5000 rpm for 10 min. The pellets were re-suspended in quarter strength sterile Ringer's solution to give a final concentration of 10^8 cfu/ml (OD = 0.5) at 550 nm using the viable plate count method and optical density measurement (Idris *et al.*, 2007).

3.10. *In vitro* screening of the sorghum rhizobacteria isolates for antagonistic activity

Given the large number of sorghum rhizobacteria isolated, *in vitro* inhibition of mycelia growth of the isolated fungal phytopathogens was tested using the technique described by Loeffler *et al.* (1986). Three 6 mm diameter NA agar discs, each originating from different pure rhizobacterial isolates were equidistantly spot inoculated 2cm from the margins of Potato Dextrose Agar (PDA) plates and incubated at $28^{\circ}C$ for 24 h. A similar diameter agar disc from fresh PDA cultures of the test phytopathogenic microorganisms were placed at the centre of the rhizobacteria inoculated PDA plates and incubated at $27 \pm 1^{\circ}C$ for seven days. The radii of the fungal colony towards and away from the bacterial colony were measured using an outside vernier calliper and percentage growth inhibition calculated using the following formula:

$$\% \text{ Inhibition} = \left[\frac{(R - r) \times 100}{R} \right]$$

Where, *r* is the radius of the fungal colony opposite the bacterial colony and, *R* is the maximum radius of the fungal colony away from the bacterial colony.

This Loeffler *et al.* (1986) culture technique formed preliminary screening tests of the rhizobacterial isolates for antagonism against the test sorghum fungal foliar pathogens.

To establish consistency of results for those rhizobacterial isolates that showed a degree of antagonism, repeat screening PDA plates using the dual culture technique method described by Paulitz *et al.* (1992) and Landa *et al.* (1997) with modifications were set up. In this case, one 10 µl drop from 10^8 cfu/ ml suspension of each bacterial isolate was placed 2 cm from the margins of Potato Dextrose Agar (PDA) plates and incubated at 28^oC for 24 h. A 6 mm diameter agar disc from fresh PDA cultures of the phytopathogenic microorganisms were then placed at the centre of the PDA plates for each bacterial isolate and incubated at 27 ± 1^oC for seven days.

All rhizobacterial isolates which resulted in more than 30% mycelia growth inhibition against the test phytopathogens were stored in screw-capped vials containing NB or PDB supplemented with an equal volume of 50% glycerol and stored at -70^oC for long-term storage (Idris *et al.,* 2007; Addgene, web article).

Results obtained from the modified dual culture technique tests were used to eliminate isolates to come up with the good ones based on the % colony reduction and diversity. Those selected were once again screened for antagonism *in vitro* against the fungal pathogens listed above, using a direct method described by Besson *et al.* (1978) with some modifications. The fungal conidia/spore suspensions were prepared as described in section 3.7 above.

Sterile PDA was used, out of which portions of 20 ml were cooled to 45^oC and inoculated with 0.5 ml of conidia/spore suspensions adjusted to 4×10^4 conidia ml^{-1} using an improved Neubauer haemocytometer. The culture media thus seeded was poured into petri dishes, mixed thoroughly and set in a horizontal position. After the agar had

solidified, each seeded medium was inoculated with the selected rhizobacterial isolate by a single streak of inoculum in the centre of the dish using a 2 mm diameter inoculation loop. The plates were then incubated first at 28°C for 72 h. Continued incubation was done for plates that did not show any tangible changes for a few more days at 30°C and analysis carried out then. The experiment was conducted in triplicate. Control plates, not inoculated with rhizobacterial isolates, were also prepared. Microbial interactions were analysed by determining the size and shape of the inhibition zone using an outside vernier caliper.

The Besson *et al.* (1978) method of screening did not necessarily guarantee equal amounts of bacterial inocula against all the test sorghum pathogens. A modification of this method with a consideration of applying the same amount of bacterial inoculum per pathogen was employed. Ten µl of rhizobacterial inocula bearing 10^8 cfu/ml in NB or PDB determined by the viable plate count method were inoculated at the centre of plates seeded with 0.5ml of conidia/spore suspensions adjusted to 4 x 10^4 conidia ml^{-1} and incubated at 28°C for 72 h. Microbial interactions were analysed by determining the size and shape of the inhibition zone using an outside vernier caliper. Experimental set-ups were conducted in triplicates. Control plates were inoculated with SDW.

3.11. Production and purification of antimicrobial agents from the best antagonistic rhizobacterial isolate

3.11.1. Batch fermentation

Antibiotic production by the best performing antagonistic rhizobacterial isolate was carried out in liquid media on shaker flasks. Synthetic media described by Farhana *et al.* (2011) with modifications consisting of: 25.0g of Glucose, 4.0g of infusion from potatoes, 5.0g of $(NH_4)_2SO_4$, 1.0g of Yeast extract, 1.0g of KH_2PO_4, 0.5g of $MgSO_4.7H_2O$, 0.5g of NaCl and 1.0g of $CaCO_3$ in 1 litre of distilled water was used as the production medium. Sterilized medium (200ml) was added to sterile 1litre Erlenmeyer flasks and pH adjusted to 7.0 using 1N NaOH and 1N HCl. The flasks were then inoculated with 20% v/v of the rhizobacterial suspension (Wang *et al.*, 2008; Farhana *et al.,* 2011) prepared as described above. The inoculated flasks were incubated

at 28°C for 7 days on a rotary mechanical shaker at a revolution speed of 180 r.p.m (Farhana *et al.,* 2011; Song *et al.,* 2012).

3.11.2. Extraction of antimicrobial agents

A cell-free filtrate was obtained by centrifuging (10,000 rpm for 20 min. at 4°C), followed by filtration of the supernatant through a 0.22 μm pore size Millipore® cellulose acetate filters. Iso–amylalcohol was then added to the supernatant in the ratio of 1:1. The mixture was then thoroughly and carefully shaken before putting in a separating funnel and left for 30 minutes for the two layers to separate. The supernatant was then run off into a clean beaker and the Iso-amylalcohol layer containing the extracted antibiotics was put in a separate beaker. Using the same supernantant but with fresh Iso-amylalcohol, the extraction process was repeated 2 more times to ensure that most of the antibiotics in the supernatant were extracted.The resulting organic phases were mixed and concentrated to dryness under vacuum by using a rotary evaporator (Atta *et al.,* 2009).

3.11.3. Purification of antimicrobial agents

A mass of 50 g of Silica Gel G was heated at 105°C for 30 min and then hydrophobized by adding 100 ml of distilled hexane and continuously heated for 20 min at 180°C in a drying oven. The prepared Silica Gel G was then packed into a 1" x 8" column (inside diameter/length) by the wet packing method (Nakanishi *et al.,* 1998). After loading 20 ml of rhizobacterial culture-filtrate (recovered from the dried organic phase obtained in the extraction process by adding distilled solvent [iso-amyl alcohol]), the column was washed with a series of 100 ml eluent (distilled hexane and distilled ethyl acetate in the ratios of; 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, and 1:9) at a rate of 2 ml/min. Eluates were gathered at 10 ml/bottle (Tang *et al.,* 2011). Thin Layer Chromatography (TLC) was performed for each bottle collected to determine number of compounds and their polarity to inform eluent addition into the column. The eluates per bottle were evaporated on a rotary vacuum evaporator and inhibitory activity performed against *C. sublineolum.* The purified antibiotic(s) were then used to determine the minimum inhibitory concentration (MIC).

3.12. Paper-disc agar-plate diffusion assay for antimicrobial agent(s) produced by the best antagonistic rhizobacterial isolate

Assay of the culture-filtrate of the best performing rhizobacterial isolate for antibiotic production and activity against the fungal phytopathogenic microorganisms was done using the paper disc agar diffusion assay as described by Loo *et al.* (1945). The method involves the use of seeded media. Sterile PDA was cooled to about 45°C in a water bath and seeded with the adjusted conidia/spore suspensions (10^4 conidia/spores ml^{-1}) of the phytopathogenic microorganisms at 10% v/v. With the help of sterile pipettes, 10ml of the seeded media were carefully dispensed into sterile 9cm diameter Petri dishes and kept on a flat surface inside a lamina flow hood to maintain a uniform medium depth.

Three autoclaved 11mm diameter Whatman No.1 filter paper discs were soaked in the rhizobacterial culture-filtrate and then placed at three equidistant points about 1cm from the periphery of the plate. The plates were incubated at room temperature (22 ± 5°C) for 72 h, after which the diameter of the clear zone around the filter paper discs was measured using an outside vernier calliper. The experiment was laid out in a completely randomized design and replicated 3 times. Similar filter paper discs soaked in SDW were used for the control treatment.

3.13. Determination of the minimum inhibitory concentration (M.I.C.) of the purified antimicrobial agent(s) produced by the best antagonistic rhizobacterial isolate against *C. sublineolum*

Minimum inhibitory concentration is the lowest antibiotic concentration that will inhibit growth of specific organisms (Andrews, 2001). Sterile PDA at 45°C was seeded with a conidia suspension (10^4 conidia ml^{-1}) of *Colletotrichum sublineolum*. Ten ml of the seeded medium were poured into Petri dishes and allowed to set. Two millilitres of SDW were dispensed into each of the ten sterile universal bottles using a sterile pipette. One millilitre of the purified antibiotics was pipetted into the 2 ml water blank in the first bottle. This were thoroughly shaken (vortexed) and successive serial dilutions carried out by pipetting 1 ml into each of the successive 2 ml water blanks. This was done up to the 10th dilution to obtain the dilution series: 3^{-1}, 3^{-2}, 3^{-3}, 3^{-4}, 3^{-5}, 3^{-6}, 3^{-7}, 3^{-8}, 3^{-9}, and 3^{-10}.

Starting with the highest dilution, 0.5ml of each dilution was pipetted and poured on separate plates containing seeded medium. The solutions were evenly spread using a flamed glass rod over the plates. The plates were then incubated at 28°C for 48 h to 72 h during which observations were made for the presence of growth of *C. sublineolum* (Wahome, 1998; Muiru, 2000).

3.14. Determination of the number of antimicrobial agent(s) in culture-filtrate of the best antagonistic rhizobacterial isolate using paper chromatography

3.14.1. Development of chromatograms

Sterilized Whatman No. 1 Chromatography paper strips (5 mm wide) were applied with 5 μl of antibiotic-culture-filtrate of the best antagonistic rhizobacterial isolate 3cm from the lower edge of the chromatography paper strip. The paper strips were then dried. A solvent system of 1-butano1/acetic acid/water (4:3:3 v/v) was placed in chromatography tank(s) and lined with filter papers to ensure saturation. The upper ends of the chromatography paper strips were attached onto the inner surface of the tank lid(s) using adhesive tape. The paper strips were then immersed in the solvent system to a depth of 1cm from the lower end. Ascending development of chromatograms were allowed for about 15-30 minutes after which the chromatograms were removed, solvent front marked and then air-dried (Wahome, 1998; Muiru, 2000).

3.14.2. Making of the assay medium and bioautography

Bioautography was carried out in chromatography boxes. Approximately 30 ml of sterilised PDA were poured using sterile pipette into three sterilized chromatography boxes placed on a flat surface to make a uniform basal layer. After the media had set, 20 ml of PDA seeded with (10^4 conidia ml^{-1}) of *Colletotrichum sublineolum* was evenly spread on the unseeded layer. This was allowed to set before placing the developed chromatogram strips centrally over the seeded medium surface. The set ups were then incubated at 28°C for not less than 72 h after which the position of the inhibition shown by clear zones on the medium determined (Wahome, 1998; Muiru, 2000).

3.15. Optimization of environmental conditions for maximum antimicrobial agent(s) production by the best rhizobacterial isolate

3.15.1. Effect of different carbon sources

The effect of different carbon sources to optimize the maximum antibiotic production by the best rhizobacterial isolate was tested by inoculating liquid media with 20% v/v of 48 h rhizobacterial suspension having an equal amount (25g/l) of mannitol, fructose, glucose and glycerol. Samples were drawn after 7 days, centrifuged and supernatants analysed for antimicrobial activity against *C. sublineolum* (Farhana *et al.,* 2011).

3.15.2. Effect of incubation period

This was done to determine the optimum time at which the best antagonistic rhizobacterial isolate produced maximum antibiotics in liquid medium containing 25g/l of fructose and 4g/l potato infusion. The antagonistic rhizobacterial isolate was incubated in the liquid medium at 28°C in an orbital shaker at 180 rpm and samples taken 24 hourly from 24 to 216 h. The cell free supernatant of samples taken at different times were used against *Colletotrichum sublineolum* as the test pathogen and activity measured in terms of zone of inhibition (Song *et al.,* 2012).

3.15.3. Effect of initial pH

Effect of initial pH for optimum antibiotic production was studied by inoculating the organism in synthetic media containing 25g/l of fructose and 4g/l potato infusion adjusted to different pH values (1, 3, 5, 7, 9 and 11) using 0.1 N NaOH and 0.1 N HCl. Samples were drawn after 6 days, centrifuged and supernatants used for analysis of antimicrobial activity against *C. sublineolum* as the test pathogen using 3 sterile paper discs per plate. Triplicate plates were prepared for each treatment. The plates were incubated for 72 h for the inhibition zones to develop (Muhammad *et al.* 2009; Song *et al.,* 2012).

3.15.4. Effect of incubation temperatures

Effect of temperature on growth of the best rhizobacterial isolate in synthetic media was checked for the optimum production of antimicrobials by incubating the production media inoculated with bacterial strain at various temperatures: 20°C, 24°C, 28°C, 32°C,

36°C and 40°C. Samples were collected and processed for antifungal assay (Delrio *et al.,* 1972; Awais *et al.,* 2008; Song *et al.,* 2012). Samples that were not subjected to any specific temperature conditions (room temperature) were deemed as the control treatment. *Colletotrichum sublineolum* was used as the test pathogen.

3.15.5. Effect of storage of antimicrobial agent culture filtrate

Effect of storage on the best rhizobacterial isolate antimicrobial agent(s) culture-filtrate was checked at different storage conditions. The active antimicrobial agent(s) culture-filtrate (10 ml) from the best antagonistic rhizobacterial isolate were placed in sterile universal bottles and properly closed with a screw cap to prevent contamination during storage. Half of the samples were placed at room temperature while the others were placed in a refrigerator at $+4^{\circ}$C. The antimicrobial activity of the stored antimicrobial agent(s) against *Colletotrichum sublineolum* was determined every 10 days for a period of eight months (Wahome, 1998; Muiru, 2000)

3.16. Identification of the isolated sorghum rhizobacterial isolates
3.16.1. Identification using biochemical tests

Based on the results of the preliminary characterization described in section 3.4 above, the most promising rhizobacterial isolate(s) were selected and further identified to the species level by means of further biochemical testing (Norris *et al.,* 1981; Smirbert and Krieg, 1981; Sneath, 1984; Lehman, 2005; Hanson, 2008; MacWilliams, 2009a & b; Brink, 2010; McDevitt, 2010; Edison and dela-Cruz 2012; Lal and Cheeptham, 2012; Reiner, 2012).

3.16.2. Identification using the API system

Isolates identified by performing further biochemical testing were also identified to the species level by means of the API identification system assisted by analytical profile index (API) Plus computer software (bioMérieux®SA, Marcy-l'Etoile, France). Gram positive, endospore forming rods were identified to the species level using API®50 CH test strips in combination with API 50CHB/E (bioMérieux) medium. Gram negative rod isolates with fermentative reaction in the Hugh and Leifsons O/F test were identified

using the API® 20 E test strip while those with oxidative reaction were identified by the API® 20 NE test strip (bioMérieux®SA, Marcy-l'Etoile, France; Idris *et al.* 2007; GHN, 2013).

3.17. Greenhouse evaluation of the best antagonistic rhizobacterial isolate(s) in the control of sorghum anthracnose

3.17.1. Planting medium and Inoculation

The planting medium that was used for greenhouse evaluation of the best rhizobacterial isolate(s) in the control of sorghum anthracnose is as described in section 3.8 above. Inoculum preparation of *C. sublineolum* is also described in section 3.7 above. A conidia suspension at a concentration of 4×10^4 conidia per millilitre was sprayed on 14-day-old plants using an atomizer, until all the leaves were wet. Inoculation was done in the evening. The plants were then covered using a transparent polythene bag for 72 h to induce high relative humidity conditions of about 100% and 28 ± 5^oC, for proper germination of conidia and high plant infection. After 72 h, the plants were removed from the polythene chamber and placed on greenhouse benches.

3.17.2. Determination of the effectiveness of the best rhizobacterial antimicrobial agent(s) culture-filtrate in controlling sorghum anthracnose

Cell-free antimicrobial agent culture-filtrate from rhizobacteria that produced the greatest inhibition zones against the fungal phytopathogens was selected for *in vivo* evaluation in the greenhouse (Idris *et al.,* 2007). Inoculated sorghum plants were treated with the active antimicrobial agent culture filtrate. A conventional fungicide; Folicur®430SC whose active molecule is Tebuconazol (http://www.bayercropscience.ca/) and a commonly used biofungicide; Ballad®Plus (http://www.agraquest.com) were used as standards. Inoculated plants were treated after a period of 48 h with the following culture-filtrate concentrations: Original culture-filtrate concentration, double concentration culture-filtrate, half concentration culture-filtrate and quarter concentration culture-filtrate. Two control treatments and a non treated one were set up as described in Table 1 below. The experiments were arranged in the greenhouse in a completely randomized design layout with each treatment bearing three potted plants. In each of the sorghum pots, two leaves

were tagged for scoring purposes. Observations were made every week for 5 weeks (Wahome, 1998). An ICRISAT scale of 1-9 was used to evaluate anthracnose on leaves as outlined in Table 2. In this scale 1 = no disease and 9 = more than 75% anthracnose severity (Thakur *et al.,* 1998). The treatments which plants were subjected to are described in Table 1 below:

Table 1: Plant treatments in pot studies on the effectiveness of rhizobacterial antimicrobial agents

Plants	Treatments
A	Plants sprayed with original culture filtrate of the antagonistic rhizobacteria.
B	Plants sprayed with half concentrated culture filtrate from antagonistic rhizobacteria
C	Plants sprayed with quarter concentrated culture filtrate from the antagonistic rhizobacteria.
D	Plants sprayed with double concentrated culture filtrate from the antagonistic rhizobacteria.
E	Plants sprayed with Biofungicide Ballad®Plus (0.2% v/v)
F	Plants sprayed with conventional fungicide Folicur®430SC (0.1% v/v)
G	Plants sprayed with *C. sublineolum* spore suspension only (Done as +ve control)
H	Plant sprayed with SDW only (Done as –ve control treatment)
I	Non-treated plants (Done to determine the phytotoxicity of rhizobacterial culture filtrates).

Note:

Double concentration culture filtrate- The antimicrobial agent culture-filtrate was concentrated using a rotary vacuum evaporator by removing half the amount of initial volume of culture filtrate.
Original concentration culture filtrate- The antimicrobial agent culture-filtrate was used as it was after harvesting and centrifuging.
Half concentration culture filtrate- The original culture-filtrate was diluted by adding an equal volume of SDW.
Quarter concentration culture filtrate- The original culture-filtrate was diluted by adding a volume of SDW three times that of the original culture-filtrate.

The dose response curve to determine the appropriate concentration of most effective sorghum rhizobacteria was determined by plotting % leaf relief (% disease suppression) against dose concentration (treatments A to D). The concentration at which the highest % leaf relief occurred was deemed as the optimal concentration. Based on the disease severity index, % disease suppression of anthracnose as described by Villajuan-Abgona *et al.* (1996) was calculated as follows:

$$\text{\% Anthracnose Suppression} = \left[\frac{(A - B)}{A}\right] \times 100$$

Where;

A = % disease severity exhibited on the leaves due to *Colletotrichum sublineolum* alone,

B = % disease severity exhibited on the leaves after inoculation with both the pathogen and the various culture-filtrate concentrations of the best bacterial antagonist and standard controls (Folicur®430SC.and Ballad®Plus).

Table 2: **Severity scale for evaluation of anthracnose severity on sorghum**

Scale	Description
1	No disease
2	1-5% anthracnose severity
3	6-10% anthracnose severity
4	11-20% anthracnose severity
5	21-30% anthracnose severity
6	31-40% anthracnose severity
7	41-50% anthracnose severity
8	51-75% anthracnose severity
9	> 75% anthracnose severity

Note: The scale is used for evaluation of disease severity on the: leaf surface; rachis; peduncle; panicle (including rachis, rachis branches, glumes and grain); and grains (damage resulting in reduction in grain size or grain abortion).

3.18. Insect pests and weed control

Red spider mites, leaf miner, aphid, sciarid fly, and white fly damage were controlled using the miticide Romectin® 1.8EC and insecticide Duduthrin® 1.75EC. The miticide was applied at a rate of 10ml/20liters while the insecticide was applied at a rate of 50ml/30litres of water. Pots were kept weed free by regular hand weeding.

3.19. Statistical data analysis

The experiment on optimization of antimicrobial production, inhibitory concentration of antimicrobial substances against pathogenic fungi and greenhouse trials were conducted in a completely randomized design (CRD). Recorded data was analyzed with Statgraphics® Centurion 16.1 Software (Statpoint Technologies, Inc., 2010). Treatment effects were tested by ANOVA and the means compared using the Tukey-Kramer Multiple-Comparison Test at a 5% probability level (Gomez & Gomez, 1984).

CHAPTER FOUR
RESULTS

4.1. Collection of materials

Soil and diseased plant materials where sorghum had been grown consistently for at least the past seven years were collected in three seasons. The first season collection was done between March and October 2012. The second season collection was done between March and October 2013 whereas the third and final season collection was done between March and November 2014. Global Positioning System (GPS) locations where samples were collected were recorded and agro-ecological zones identified. The agro-ecological zones are listed and described in appendix 3. Samples were logged at the laboratory as shown in Appendix 1. Soil samples were obtained from a total of sixteen sampling sites as shown in Figure 2. Samples' laboratory logs were used to assign strain numbers to both the rhizobacterial and phytopathogenic isolates. Sorghum plants were sampled at a stage when they were maturing towards the end of the third month or early in the third month after planting. Sorghum rhizosphere soil samples were collected from six different agro-ecological zones that collectively yielded a total of 101 antagonistic rhizobacterial isolates from which 35 were capable of inhibiting test sorghum fungal pathogens *in vitro* by ≥ 70%. Descriptions of the agro-ecological zones are given in Appendix 3.

4.2. Soil particle size analysis and chemical composition

Soil samples had varied particle sizes, composition and organic contents. Generally, the soil from the LM1 agro-ecological zone had a pH ranging from very strongly acidic (4.66) to slightly acidic (6.06) (USDA, 1993). The organic carbon content varied from 1.07% – 2.3% whilst the nitrogen content varied from 0.10% – 0.75% to effectively give a C:N range of 2.83 – 14.97. Soil particle sizes varied from 56% – 81% coarse sand, 10% – 13% silt and 5% – 15% clay to indicate that the soil samples textural classes ranged from sandy-clay-loam to sandy-loam. The phosphorous content ranged from 0.69 mg/kg to 8.05 mg/kg (Appendix 5).

Soil from this agro-ecological zone produced thirty nine (39) antagonistic rhizobacterial isolates, 20 of which originated from the Sega site, Siaya County (Appendix 2) whose

soil type is an orthic and ferralo-orthic acrisol (Figure 2, Appendix 4). This site had a total of 12 soil samples (Appendix 1).

Alupe's six soil samples produced fifteen rhizobacterial isolates whilst Burumba's two soil samples yielded one antagonistic rhizobacteria. Bugeng'i's two soil samples gave three antagonistic rhizobacteria isolates (Appendices 1 and 2). The three sites are within Busia County. The soil types in Alupe and Burumba are orthic acrisols with orthic ferralsols while Bugeng'i's soil type is a dystric planosol, dystric and vertic gleysols and pellic vertisol (Jaetzold *et al.,* 2005).

The LM2 agro-ecological zone as well had a pH that ranged from very strongly acidic to moderately acidic (4.76 – 5.74) (USDA, 1993). The organic carbon content varied from 0.83% – 2.37% whilst the nitrogen content varied from 0.07% – 0.35% to effectively give a C:N range of 4.91 – 12.94. The soil particle size varied from 55% - 81% coarse sand, 3% – 13% silt and 13% – 36% clay to give textural classes of sandy clay loam, sandy loam and loamy sand. The phosphorous content ranged from 0.27 mg/kg to 8.05 mg/kg (Appendix 5).

This agro-ecological zone produced a total of 16 rhizobacterial isolates. Eight soil samples from the Kibos site in Kisumu County yielded two antagonistic isolates whose soil type is a veto-eutric planosol with chromic vertisol and solodic planosol. This soil is also commonly referred to as the "black cotton soil".

Busende sampling site yielded two antagonistic rhizobacterial isolates from two soil samples that are classified as eutric gleysols and pellic vertisols. The one soil sample from Busire—Siriwo produced five antagonistic isolates (Appendices 1 and 2). The site's soil is a chromic and orthic acrisol, rhodic ferralsols and dystric nitisols.

The Akiriamet site yielded five rhizobacterial isolates from 2 soil samples (Appendice 1 and 2) whose soil classification is humic acrisol (Figure 2, Appendix 4).

Kanduyi site produced two antagonistic rhizobacteria from two soil samples, one of which was among the best antagonists yielded in the entire study (Appendices 1 and 2). The site's soil type is an orthic acrisol with humic acrisol and ferralic arenosol.

The LM3 agro-ecological zone had a strong acidic to moderate acidic pH (5.14 – 5.9) (USDA, 1993). The organic carbon content varied from 0.71% – 0.93% whilst the nitrogen content varied from 0.08% – 0.12% to effectively give a C:N range of 7.68 – 9.75. The soil particle size varied from 79% - 88% coarse sand, 5% – 6% silt and 7% – 15% clay to give textural classes of sandy-clay-loam, sandy-loam and loamy-sand. The phosphorous content ranged from 0.66 mg/kg to 0.9 mg/kg (Appendix 5).

This agro-ecological zone yielded a total of twenty antagonistic rhizobacterial isolates. A combined four soil samples from Angurai and Awaat sampling sites within Busia County produced nineteen (19) antagonistic isolates while the Mayanja site yielded one antagonistic rhizobacterial isolate from 2 soil samples (Appendices 1 and 2). The soil class found in Angurai and Awaat sampling sites are ferralo-orthic acrisols (Figure 2, Appendix 4) while that found in Mayanja is an orthic acrisol. Angurai and Awaat sites are found within Busia County while Mayanja is found in Bungoma County.

The LM4 agro-ecological zone also had a strong acidic to moderate acidic pH (5.09 – 5.55) (USDA, 1993). The organic carbon content varied from 1.01% – 1.3% whilst the nitrogen content varied from 0.12% – 0.15% to effectively give a C:N range of 8.42 – 8.67. The soil particle size varied from 56% - 58% coarse sand, 9% – 10% silt and 32% – 34% clay to give a textural class of sandy clay loam. The phosphorous content ranged from 1.51 mg/kg to 1.53 mg/kg (Appendix 5). The soil type of this agro-ecological zone's site at Ureje in Siaya County is described as chromic luvisols and ferralo-chromic acrisols. (Appendices 2 and 4). The site yielded seven rhizobacterial isolates from four soil samples (Appendix 1).

The LM5 agro-ecological zone had a slightly acidic pH (6.19 – 6.5) (USDA, 1993). The organic carbon content varied from 0.72% – 1.28% whilst the nitrogen content varied from 0.05% – 0.48% to effectively give a C:N range of 1.91 – 16.47. The soil particle size varied from 67% - 75% coarse sand, 8% – 12% silt and 17% – 23% clay to give textural classes of sandy-clay-loam and loamy-sand. The phosphorous content ranged from 1.31 mg/kg to 8.92 mg/kg (Appendix 5).

This agro-ecological zone's site at Sigor within West Pokot County yielded eighteen antagonistic rhizobacterial isolates from eleven soil samples (Appendices 1 and 2). The soil class is described as chromic luvisols, rhodic ferralsols and luvic to ferralic arenosols.

The UM4 agro-ecological zone had a very strong acidic pH (4.96 – 5.11) (USDA, 1993). The organic carbon content varied from 1.5% – 1.74% whilst the nitrogen content varied from 0.13% – 0.27% to effectively give a C:N range of 6.42 – 11.88. The soil particle size varied from 62% - 68% coarse sand, 8% silt and 24% – 30% clay to give textural classes of sandy-clay-loam, sandy-loam and loamy-sand. The phosphorous content ranged from 0.80 mg/kg to 1.27 mg/kg (Appendix 5). The five soil samples collected in this zone at Miti Majambazi and Misemwa sites of Trans Nzoia County yielded only one antagonic rhizobacterial isolate (Appendices 1 and 2). Soil from these sampling sites are rhodic ferralsols (Jaetzold *et al.,* 2011).

Figure 2: **Soil types of sampled sites. Locations are approximate.(Source:** *http://kenya.usaid.gov/kenya-map-west*)**. Soil types of each sampling site is given in the key below:**

Site No.	Site Name	Soil type	Mean rhizobacteria/ soil sample
1:	Kibos	Veto-eutric Planosols with chromic Vertisols and solodic Planosols	<1
2:	Ureje/Usigu	Chromic Luvisols and ferralo-chromic Acrisols	2
3:	Sega	Orthic and ferralo-orthic Acrisols	2
4:	Busire-Siriwo	Chromic and orthic Acrisols and rhodic Ferralsols	5
5:	Busende	Eutric Gleysols and solodic Planosols & mollic Gleysols & dystric Histosols	1
6:	Bugeng'i	Dystric Planosols, dystric and vertic Gleysols and pellic Vertisols	2
7:	Burumba	Orthic Acrisols with orthic Ferralsols	<1
8:	Alupe	Orthic Acrisols with orthic Ferralsols	3
9:	Awaat	Ferralo-orthic Acrisols	5
10:	Angurai	Ferralo-orthic Acrisols	5
11:	Akiriamet	Humic Acrisols with humic Cambisols	3
12:	Kanduyi-Makutano	Orthic Acrisols with humic Acrisols	1
13:	Mayanja	Orthic Acrisols	1
14:	Misemwa	Rhodic Ferralsols	1
15:	Miti-Majambazi	Rhodic Ferralsols	0
16:	Sigor	Chromic Luvisols, rhodic Ferralsols and luvic to ferralic Arenosols	2

4.3. Isolation of sorghum rhizobacteria

Mixtures of distinct colonies were obtained from primary King's B medium and NA isolation plates. Representative colonies with different morphological appearances were selected from the countable plates and re-streaked on new plates, but of the same media to obtain pure colonies. The study generated a total of 294 rhizobacterial isolates (Appendix 2). Antagonistic isolates against test sorghum foliar fungal pathogens numbered 101 with those that were able to inhibit growth of sorghum test fungal pathogens by $\geq$ 30% being 95 whilst those that inhibited at least one of the test pathogens by $\geq$ 70% were 35. There were 193 non-antagonistic isolates.

4.4. Isolation and Identification of sorghum fungal phytopathogens

The procedure undertaken while isolating the fungal pathogens experienced little contamination by common laboratory contaminants such as *Penicillium* spp. and *Aspergillus* spp. except for a few cases. Microscopic examinations included determination of conidia types, production, colour and their shapes; colours and shapes of conidiophores; lengths of conidia and conidiophores; colour of hyphae; formation of appressoria, sporodochia and stroma and measurements of conidia and appresoria using a stage micrometer. Synoptic descriptions and keys were then employed to identify the phytopathogens. Images of pure culture plates are given in the subtitles below (Plates 1—10). Observations and inferences made for every fungal pathogen examined are outlined in Appendix 6.

4.4.1. *Alternaria alternata* (Fr.) Keissler
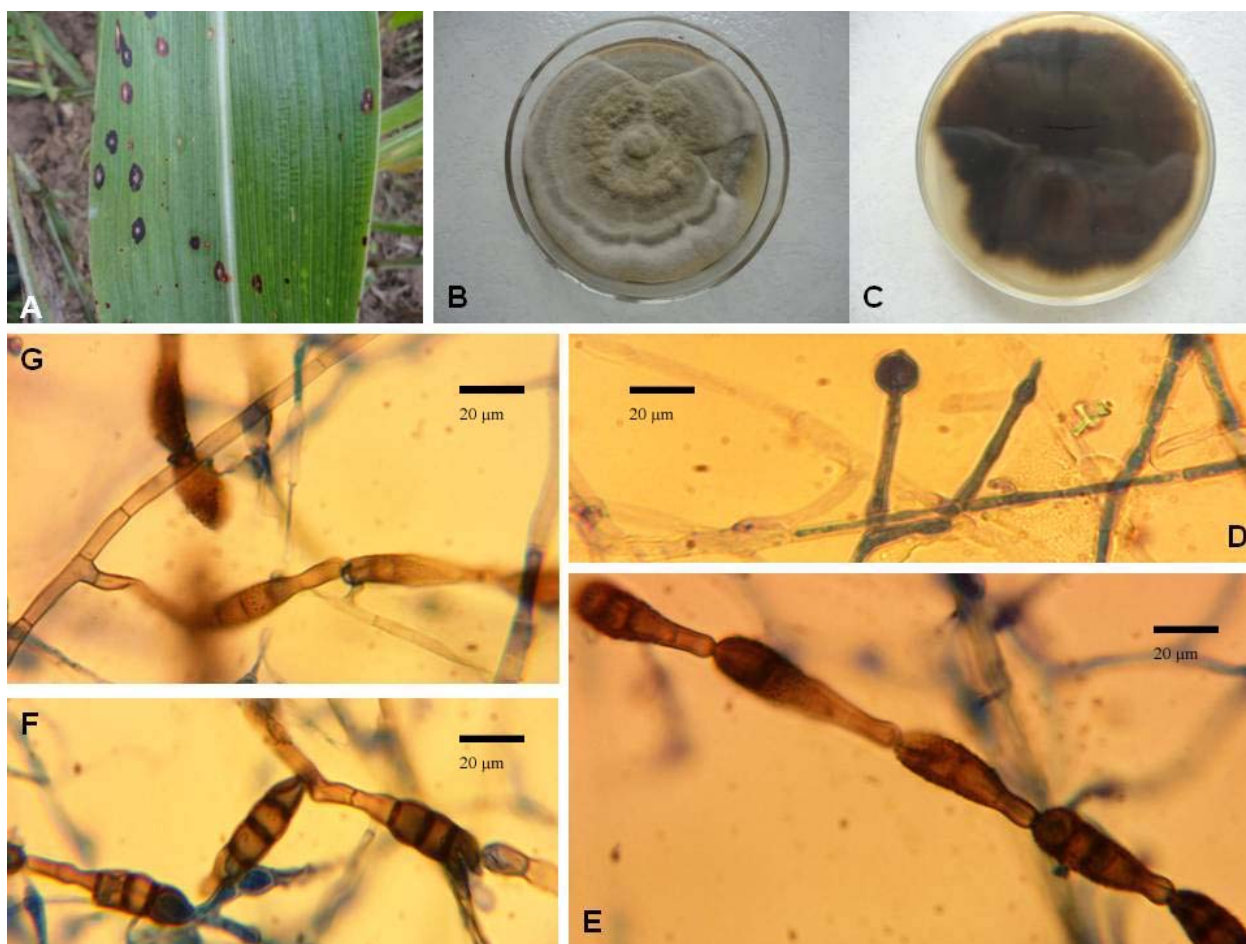
Plate 1: **Micrographs of isolated *Alternaria alternata* Kb06. A; Sorghum leaf where the pathogen was isolated; B; Top petri dish view of isolated *Alternaria alternata* Kb06 growing on PDA. C; Reverse (Bottom) petri dish view of B. D–G; Conidiophores and characteristic conidia as observed at 1600X. Scale bars D, E, F and G = 20µm. Source: (Author) 2016.**

4.4.2. *Aspergillus candidus* Link.
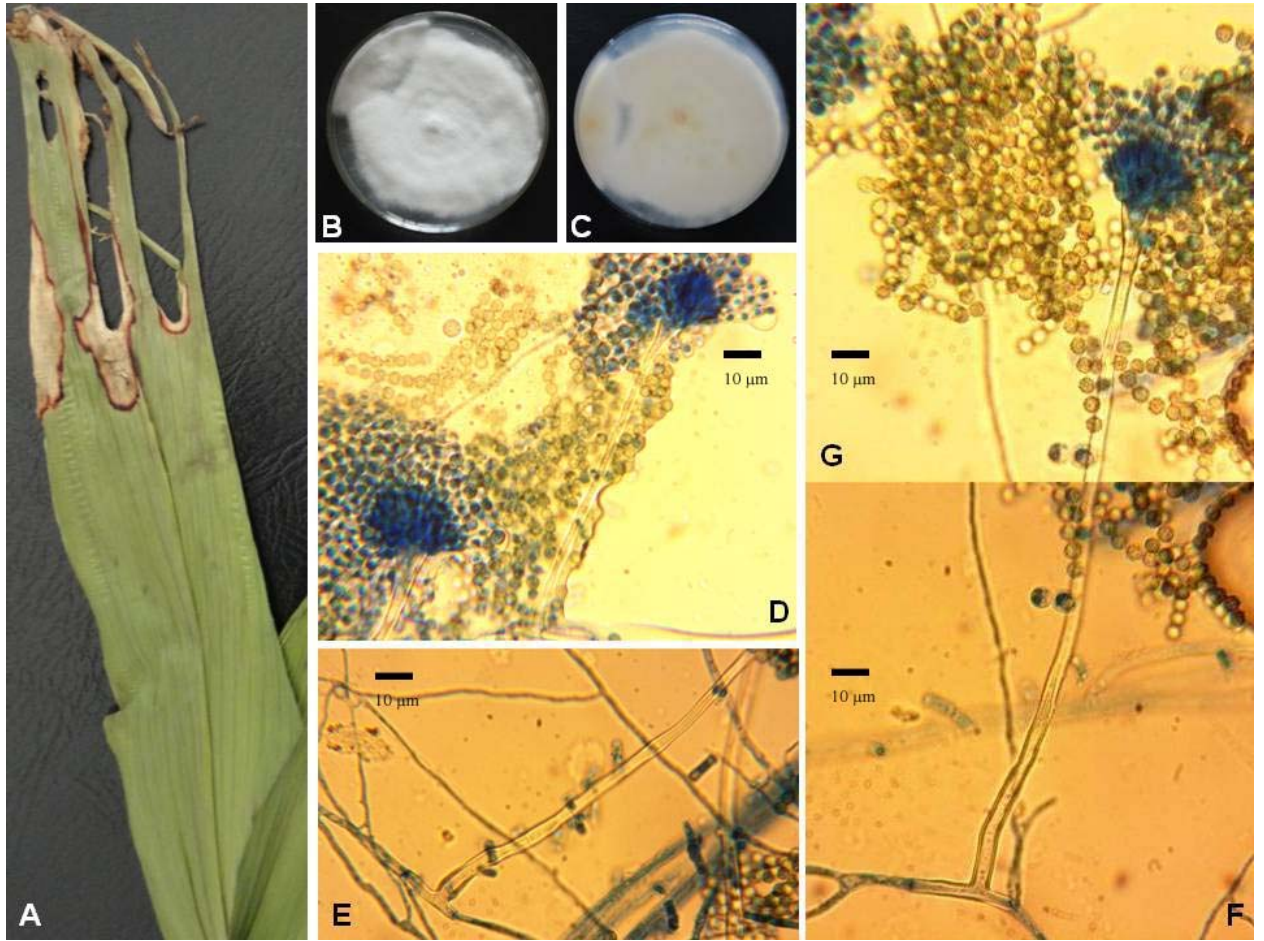
Plate 2: **Micrographs of isolated *Aspergillus candidus* Sr03. A; Sorghum leaves where the pathogen was isolated; symptoms not necessarily caused by isolate. B; Top petri dish view of isolated *Aspergillus candidus* Sr03 growing on PDA. C; Reverse (Bottom) petri dish view of B. D–G; Conidiophores bearing vesicles that have sterigmata on which globose/sub-globose conidia are attached in chains as observed at 1600X. Scale bars D, E, F and G = 10µm. Source: (Author) 2016.**

4.4.3. *Alternaria longissima* Deighton & MacGarvie
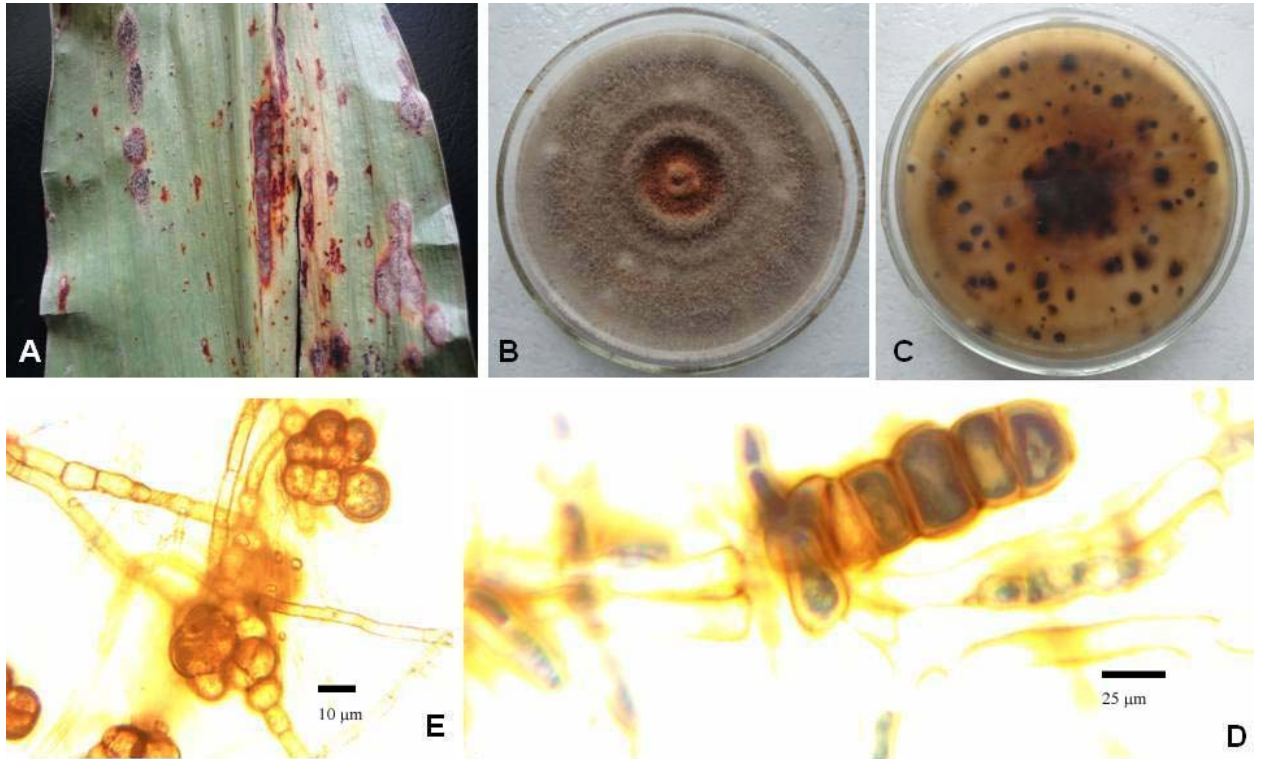
Plate 3: **Micrographs of isolated *Alternaria longissima* Kb08. A; A sorghum leaf where the pathogen was isolated; symptoms not necessarily caused by isolate. B; Top petri dish view of isolated *Alternaria longissima* Kb08 growing on PDA. C; Reverse (Bottom) petri dish view of B. D; A conidium as observed at 1600X. Scale bar D = 25µm. E; Multicellular chlamydospores as observed at 1600X. Scale bar E = 10µm. Source: (Author) 2016.**

4.4.4. *Botrytis cinerea* Pers. Ex Pers.
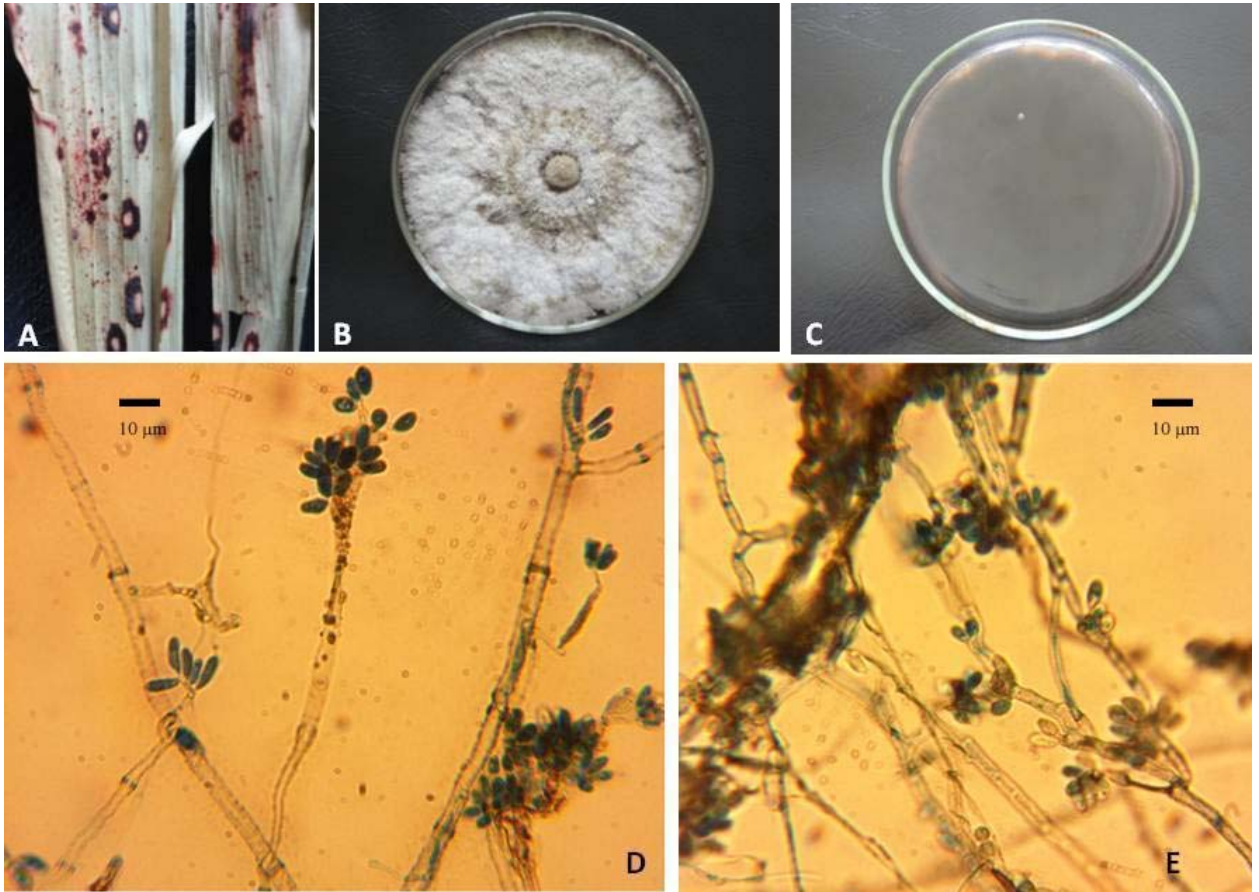
Plate 4: Micrographs of isolated *Botrytis cinerea* Sg01. A; Sorghum leaves where the pathogen was isolated; symptoms not necessarily caused by isolate, B; Top petri dish view of isolated *Botrytis cinerea* Sg01 growing on PDA. C; Reverse (Bottom) petri dish view of B. D–E; Conidiophores and conidia as observed at 1600X. Scale bars D and E = 10μm. Source: (Author) 2016.

4.4.5. *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc.
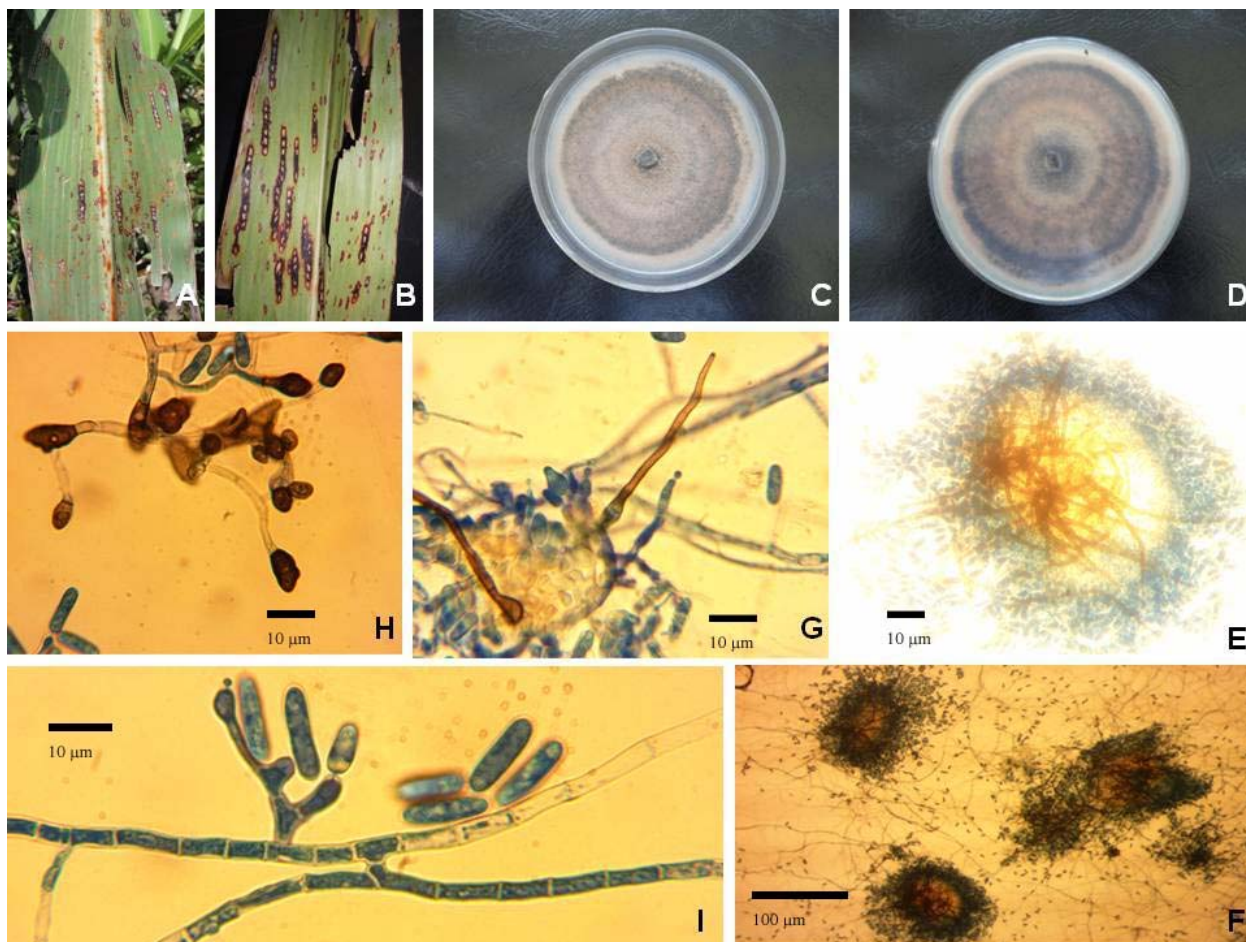
Plate 5: Micrographs of isolated *Colletotrichum gloeosporioides* **Kb08. A–B; Sorghum leaves showing anthracnose symptoms, C; Top petri dish view of isolated *Botrytis cinerea* Sg01 growing on PDA. D; Reverse (Bottom) petri dish view of C. E; An acervulus as seen at 640X, F; Three acervuli as seen at 160X, G; Characteristic septate setae emerging from an acervulus as observed at 1600X, H; Appressoria as seen at 1600X, I; Conidia and conidiogenous cells as seen at 1600X. Scale bars E, G, H and I = 10µm. Scale bar F = 100 µm. Source: (Author) 2016.**

4.4.6. *Colletotrichum sublineolum* P. Henn., Kabát & Bubák
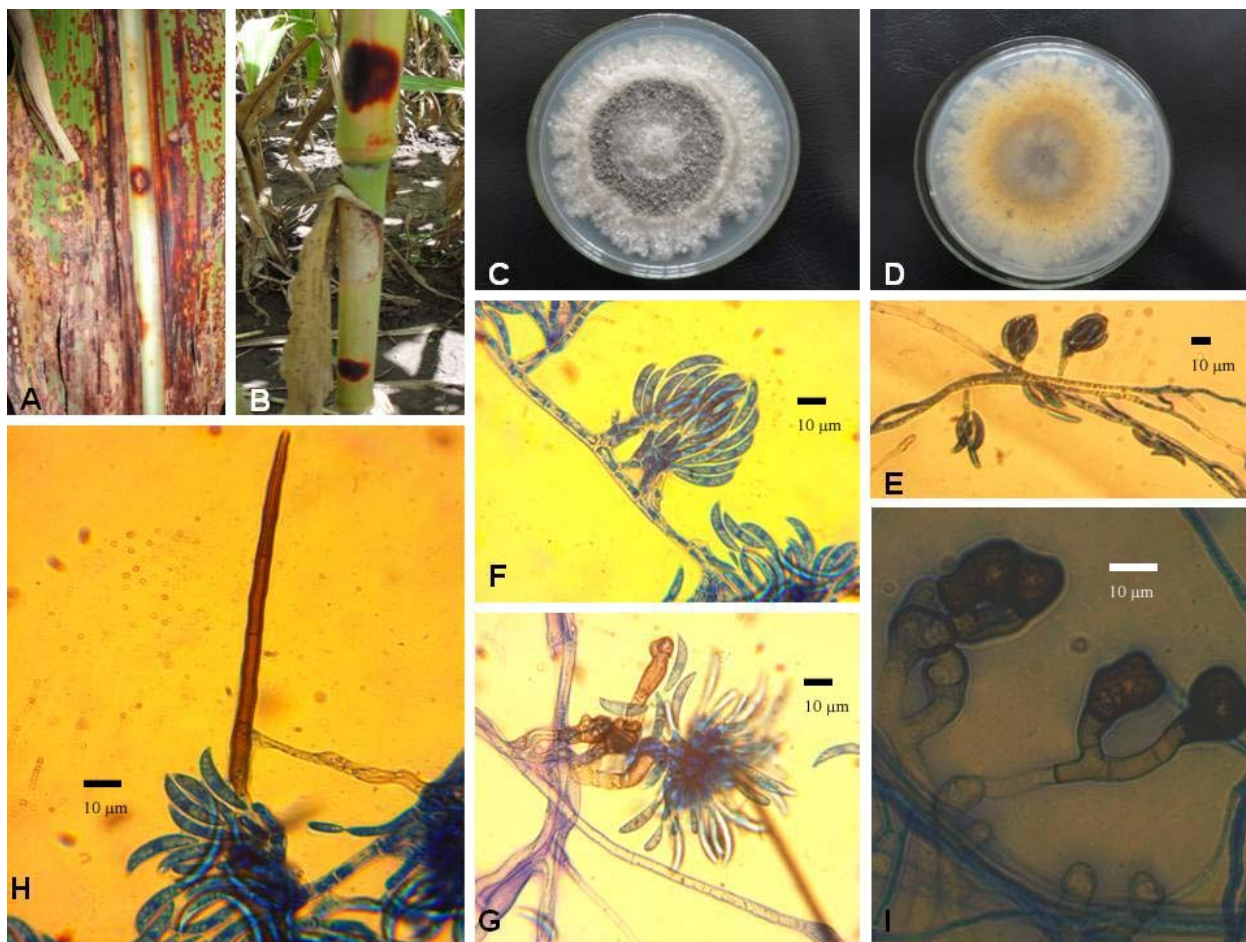
Plate 6: Micrographs of isolated *Colletotrichum sublineolum* Kb04. A–B; Symptoms of anthracnose (necrotic lesions with a straw colour at the centre and red margins on the leaf and its midrib; red/tan necrosis on leaf sheaths. C; Top petri dish view of isolated *Colletotrichum sublineolum* Kb04 growing on PDA. D; Reverse (Bottom) petri dish view of C showing the production of the characteristic yellow exudates. E–F; Conidiophores and conidia at 640X and 1600X respectively. G–H; Acervuli observed at 1600X showing the characteristic septate setae. H; Conidia at 1600X positioned at the base of spines. I; Appressoria as seen at 1600X. Scale bars E, F, G, H and I = 10µm. Source: (Author) 2016.

4.4.7. *Exserohilum turcicum* **(Pass.) Leonard & Suggs**
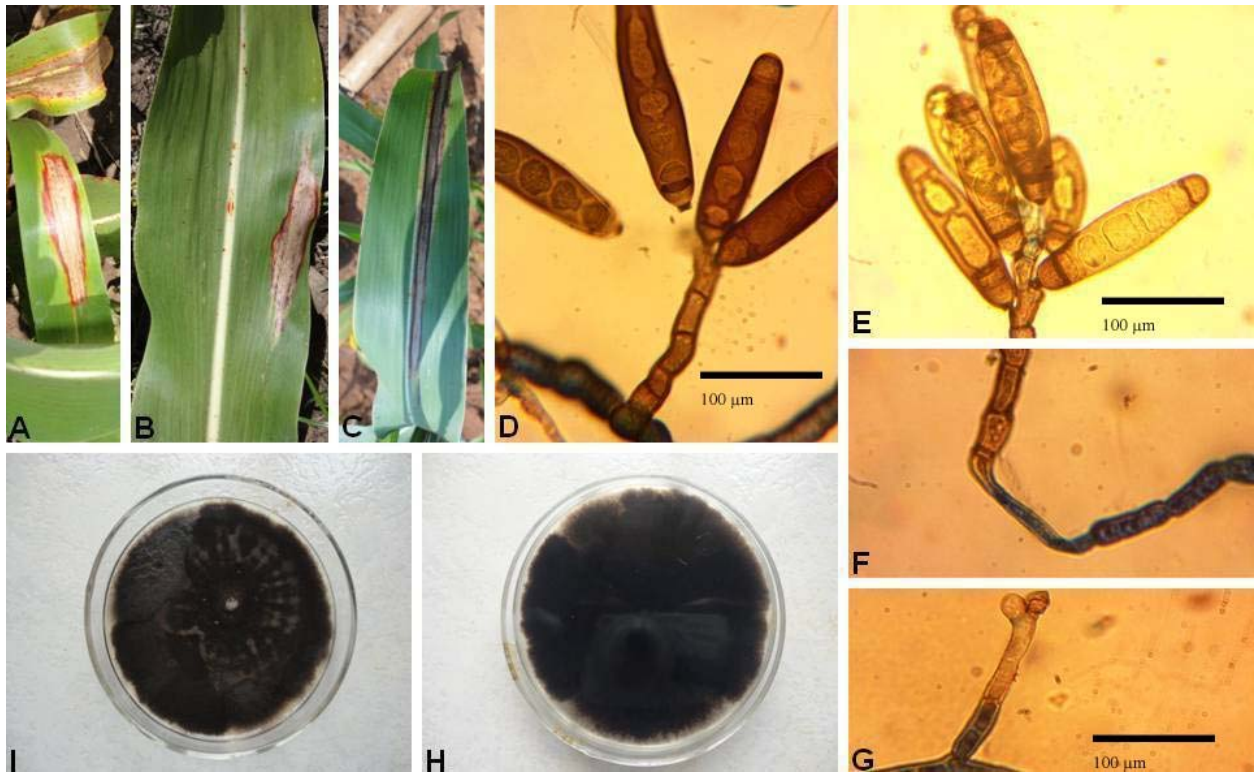
Plate 7: **Micrographs of isolated** *Exserohilum turcicum* **Sr02. A–C; Sorghum leaves showing leaf blight symptoms of long elliptical necrotic lesions , straw colored in the centers with dark margins. D–G; Conidiophores and conidia as observed at 1600X. H; Reverse (Bottom) view of isolated** *Exserohilum turcicum* **Sr02 growing on PDA giving black colonies. I; Top petri dish view of petri dish view of H. Scale bars D, E, F and G = 100µm. Source: (Author) 2016.**

4.4.8. *Fusarium equiseti* **(Corda) Sacc.**
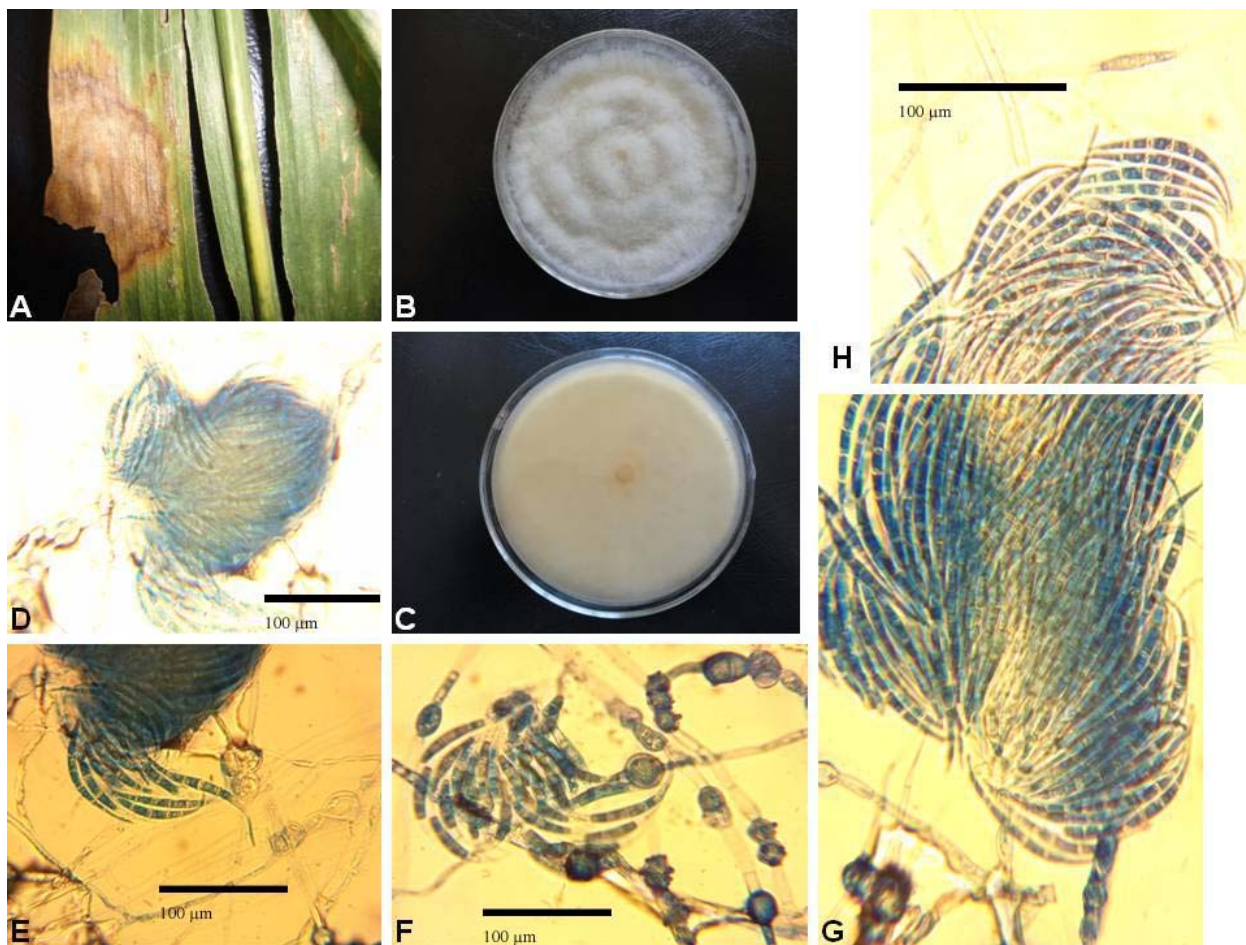
Plate 8: **Micrographs of isolated** *Fusarium equiseti* **Sr01. A; Sorghum leaf showing symptoms of blight/wilting. B; Top petri dish view of isolated** *Fusarium equiseti* **Sr01 growing on PDA. C; Reverse (Bottom) petri dish view of B showing the production of the characteristic yellowish-brown pigment. D–E, G– H; Sporodochia bearing macroconidia. No microconidia present. Macroconidia tapering towards both ends and strongly bent mainly in the central part with a more or less distinctly elongated, straight or whip-like bent apical cell and a very distinctly pedicellate basal cell, mostly 5 to 7 septa as observed at 1600X. F; Chlamydospores abundant in hyphae, intercalary, solitary, in pairs, frequently forming chains or clusters, globose to subglobose, smooth- or rough-walled, becoming ochraceous. Scale bars D, E, F, G and H = 100µm. Source: (Author) 2016.**

4.4.9. *Fusarium moniliforme* J. Sheld. Lisea fujikuroi Sawada
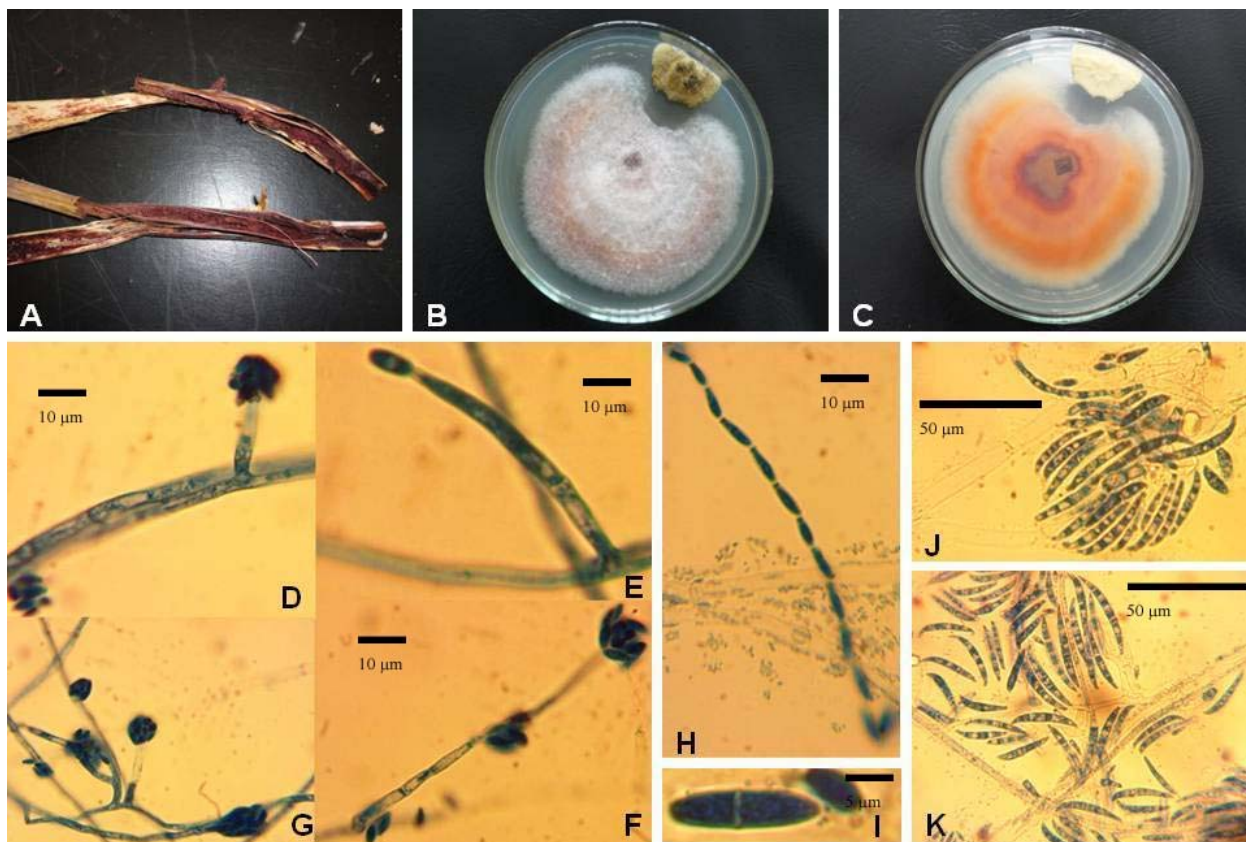
Plate 9: Micrographs of isolated *Fusarium moniliforme* Kb04. A; A sorghum stem showing symptoms of stalk rot disease: discoloured pith/internal matter. B; Top petri dish view of isolated *Fusarium moniliforme* Kb04 growing on PDA. C; Reverse (Bottom) petri dish view of B showing the production of the characteristic pink pigment. D–G; False heads of microconidia/microconidium on monophialides as observed at 1600X. H–I; Egg/oval/club-shaped microconidia that have flat bases either in beaded-chains or aggregated, catenulate and two-celled as observed at 1600X. J–K; Macroconidia curved to almost straight, 3-7 septate, with a foot-shaped basal cell as observed at 1600X. Scale bar I = 5µm. Scale bars E, D, F and H = 10µm. Scale bar G = 20 µm. Scale bars J and K = 50µm. Source: (Author) 2016.

4.4.10. *Nigrospora oryzae* **(Berk. & Br.) Petch**
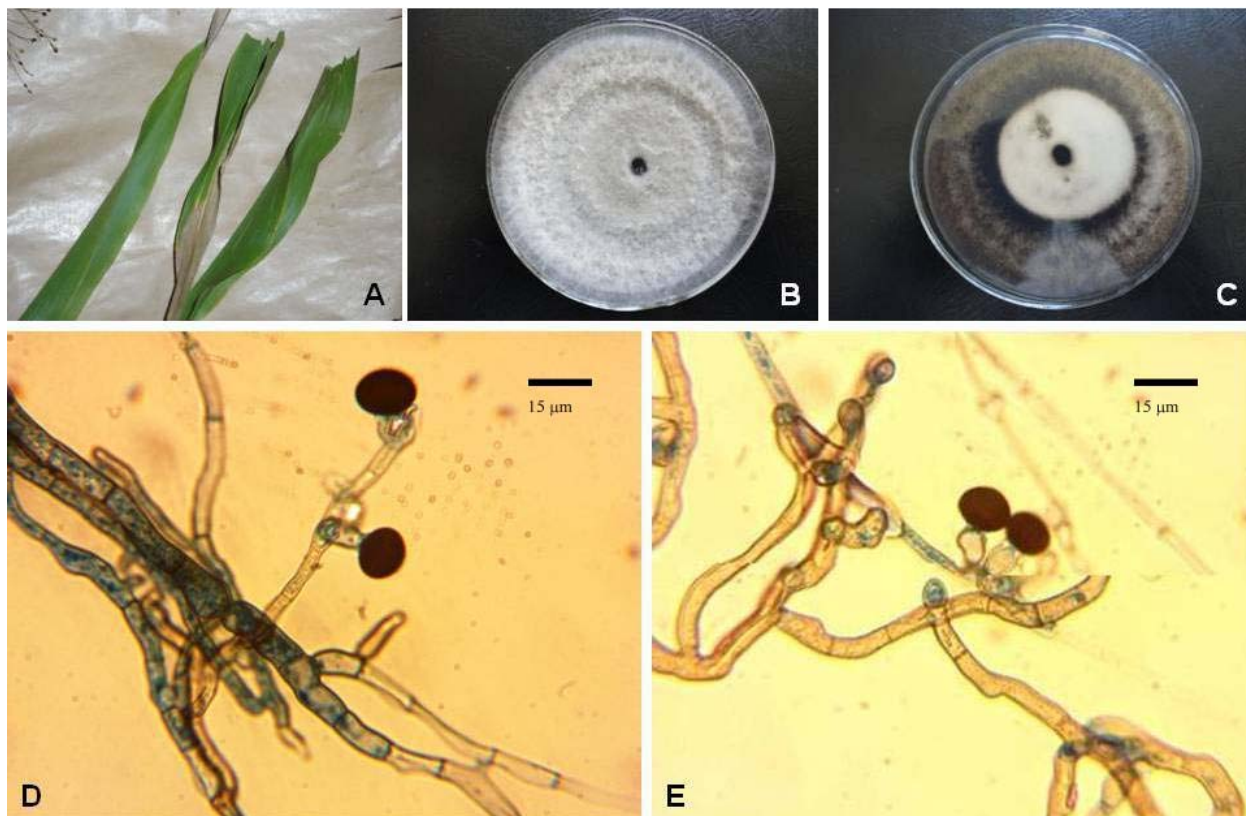
Plate 10: **Micrographs of isolated** *Nigrospora oryzae* **Sr01. A; Sorghum leaves where the pathogen was isolated; symptoms not necessarily caused by isolate, B; Top petri dish view of isolated** *Nigrospora oryzae* **Sr01 growing on PDA. C; Reverse (Bottom) petri dish view of B. D–E; Conidiophores and conidia as observed at 1600X. Scale bars D and E = 15µm. Source: (Author) 2016.**

4.5. Pathogenicity test of *Colletotrichum sublineolum* **Henn. Kab & Bubak**

All the seedlings inoculated with *Colletotrichum sublineolum* produced characteristic anthracnose symptoms after 6 days. The symptoms began as minute specks, which enlarged lengthwise on the leaves and leaf sheaths. The infection occurred along the veins as brick to rust brown lesions, which later turned to black (Plate 11a & b). These symptoms resembled those of plant materials from which the pathogen was first isolated. Reisolation and plating produced fungal cultures with similar characteristics as those of the cultures used to provide inoculum for pathogenicity. Observation of the morphological characteristics of *C. sublineolum* after preparation of Dade slide (under a microscope: 1600X objective) showed hyaline conidial cells that are lunate shaped with acute apices measuring an average of 23 x 3.5 µm. Acervuli bore dark-brown septate setae while appressoria were globose to perprolate, ovoid or obovoid or clavate, smooth

or lobate or multi-lobate, apice cylindrical or obtuse, edges irregular with an average height of 16.5µm, width 14.8 µm (Plate 6). This confirmed that the pathogen in question was indeed *Colletotrichum sublineolum.*
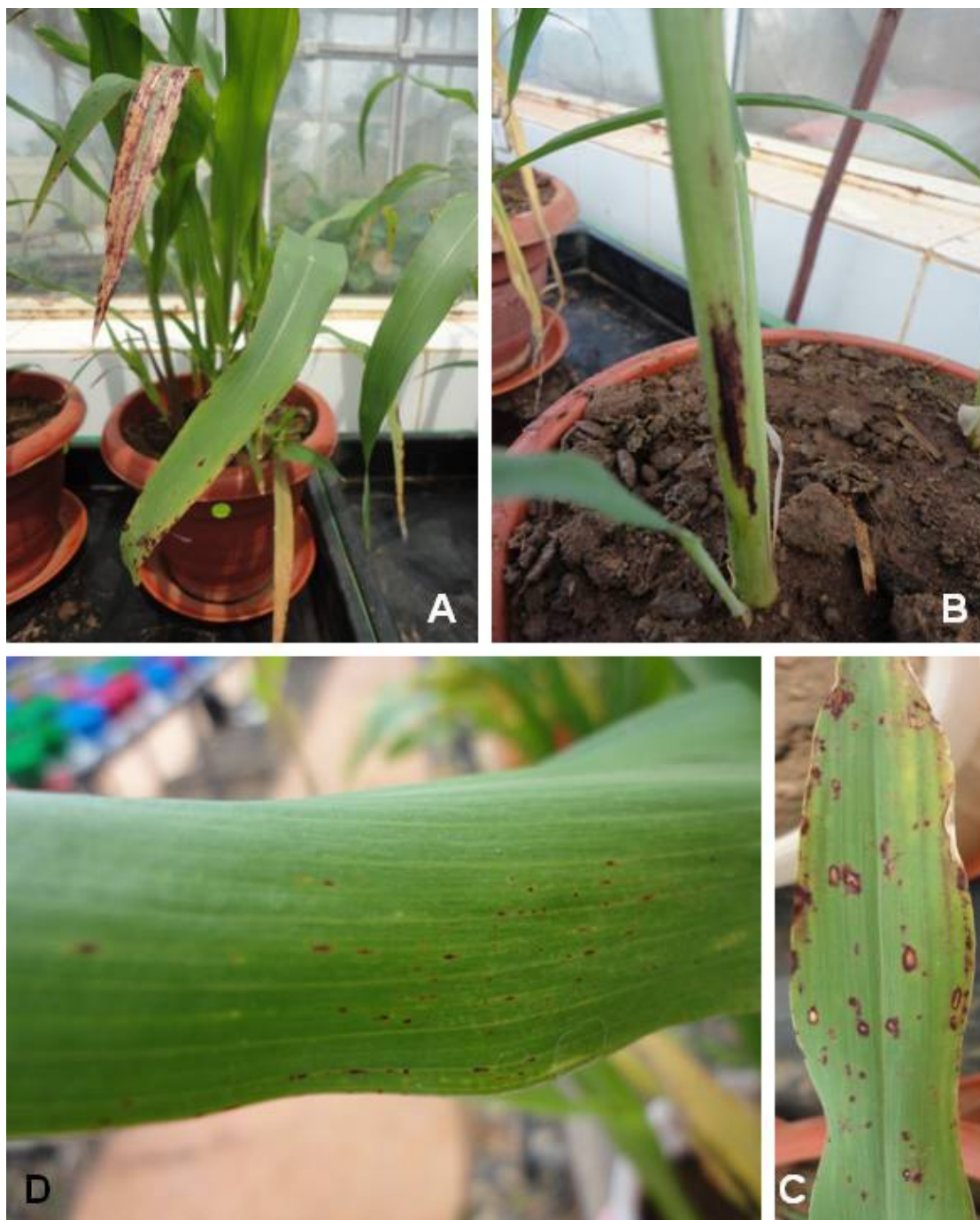
Plate 11a): A; Sorghum plants infected with *C. sublineolum* showing characteristic anthracnose symptoms on leaves. B; Leaf sheath showing anthracnose symptoms. C—D; Close-up images of infected leaves. Source: (Author) 2016.

Plate 11b): **E; Control sorghum plants uninfected with** *C. sublineolum*. **F—G; Close-up images of a clean leaf and leaf sheaths. Source: (Author) 2016.**

4.6. *In vitro* screening of the sorghum rhizobacterial isolates for antagonistic activity

Out of the 294 sorghum rhizobacteria isolated; ninety five exhibited at least up to 30% mycelia growth inhibition of the test fungal phytopathogens while thirty five gave mycelia growth inhibition of up to 70% for at least one of the pathogens when the Loeffler *et al.* (1986) method of screening was used. Results of this method of screening are given in Appendix 7 for rhizobacterial isolates that inhibited mycelia growth by ≥ 70% for at least one of the economically important test fungal pathogens. From the 35 rhizobacterial isolates two, viz: KaI245 and MaI254, exhibited clear zones of inhibition against the test sorghum fungal pathogens. As a result, no visible growth of the pathogens was observed within the clear zones of inhibition, an indication that they produced potent antifungal agents suitable for biocontrol. Images of screening using the Loeffler *et al.* (1986) method are shown in plates 12 and 13.
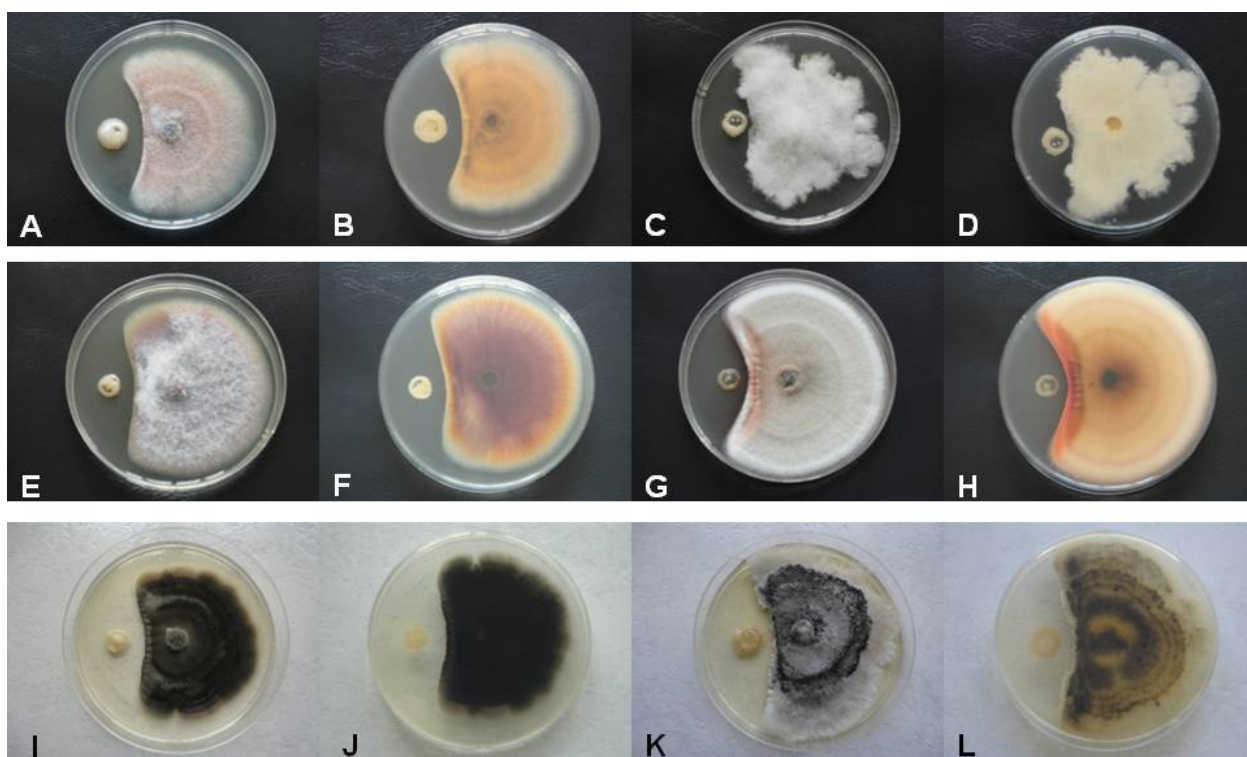
Plate 12: Top and bottom views of the inhibitory growth effects of sorghum rhizobacterial isolate KaI245 on sorghum foliar fungal pathogens 6 days after inoculation unless stated otherwise using a modified Loeffler *et al.* (1986) method. A–B; *Colletotrichum gloeosporioides* Kb08. C–D; *Fusarium equiseti* Sr01. E–F; *Fusarium moniliforme* Kb04. G–H; *Alternaria longissima* Kb08. I–J; *Exserohilum turcicum* Sr02, (10 days after inoculation) and K–L; *Colletotrichum sublineolum* Kb04, (14 days after inoculation). Notice the clear zones of inhibition between the fungal pathogens and the rhizobacterial isolates. Source: (Author) 2016.
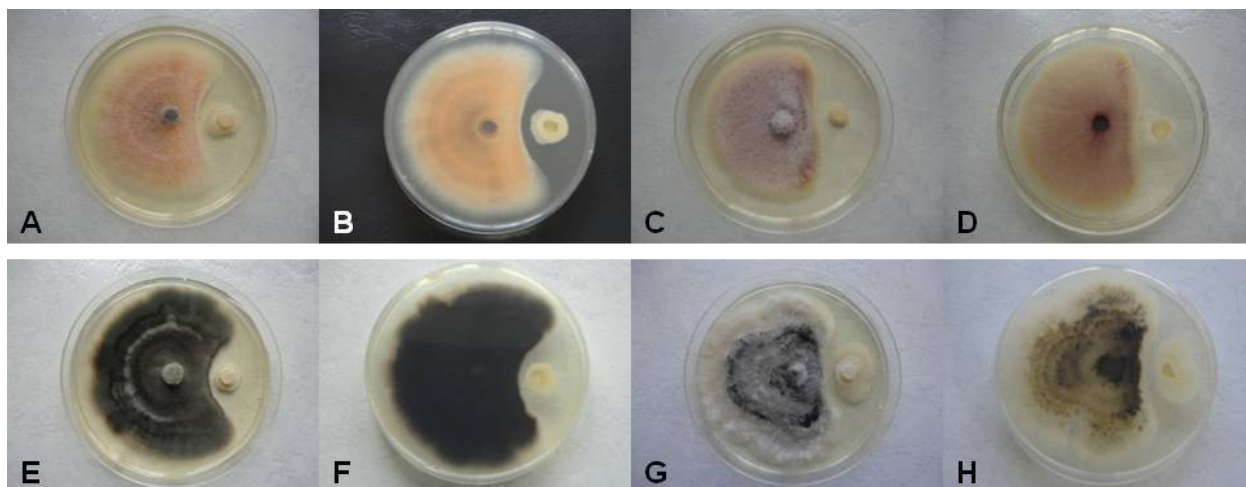
Plate 13: Top and bottom views of the inhibitory growth effects of sorghum rhizobacterial isolate MaI254 on sorghum foliar pathogens 6 days after inoculation unless stated otherwise using a modified Loeffler *et al.* (1986) method. A–B; *Colletotrichum gloeosporioides* Kb08. C–D; *Fusarium moniliforme* Kb04. E–F;*Exserohilum turcicum* Sr02 (10 days after inoculation) and G–H; *Colletotrichum sublineolum* Kb04 (14 days after inoculation). Source: (Author) 2016.

Appendix 9 gives a summary of the screening results obtained by the Paulitz *et al.* (1992) and Landa *et al.* (1997) methodology for rhizobacterial isolates KaI245 and MaI254 against all the test fungal pathogens isolated in this study. Images of the screening are shown in Plates 14 and 15.
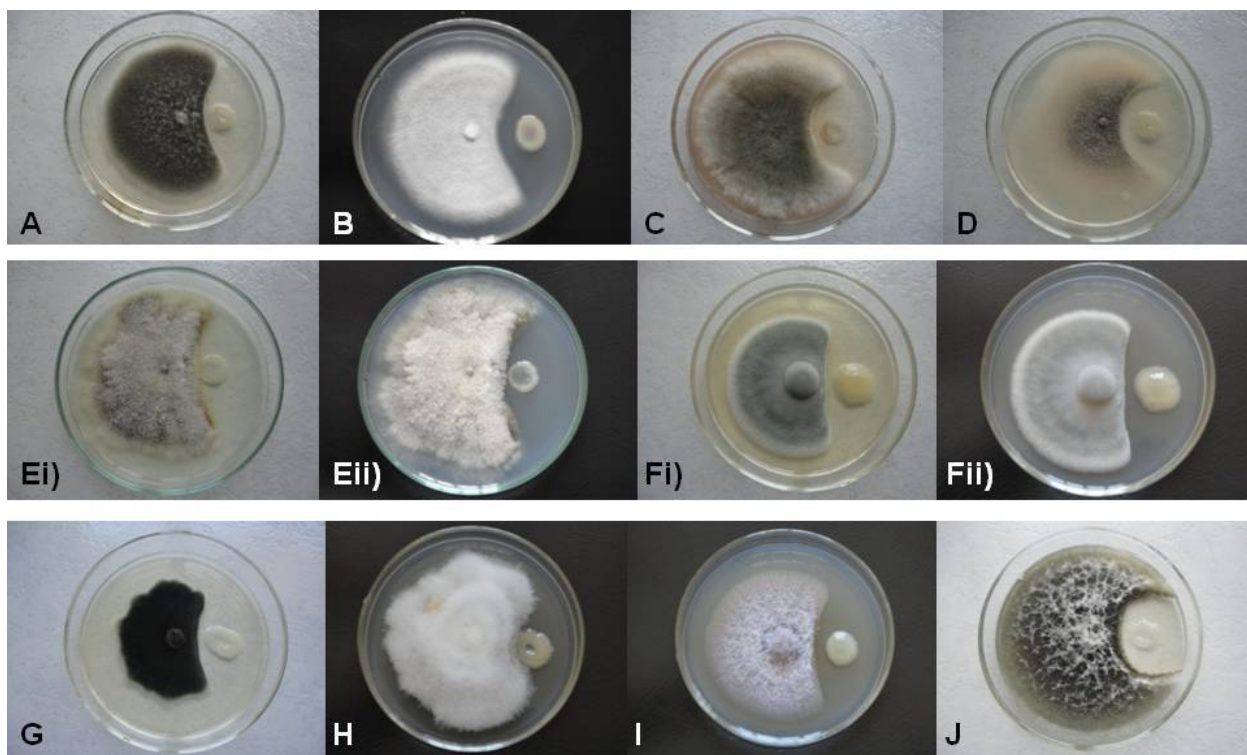
Plate 14: Inhibitory growth effects of sorghum rhizobacterial isolate KaI245 on sorghum foliar fungal pathogens 7 days after inoculation. A; *Alternaria alternata* Kb06. B; *Aspergillus candidus* Sr03. C; *Alternaria longissima* Kb08. D; *Colletotrichum gloeosporioides* Kb08. Ei—ii; *Botrytis cinerea* Sg01, Fi—ii; *Colletotrichum sublineolum* Kb04, G; *Exserohilum turcicum* Sr02, H; *Fusarium equiseti* Sr01, I; *Fusarium moniliforme* Kb04, J; *Nigrospora oryzae* Sr01. Source: (Author) 2016.
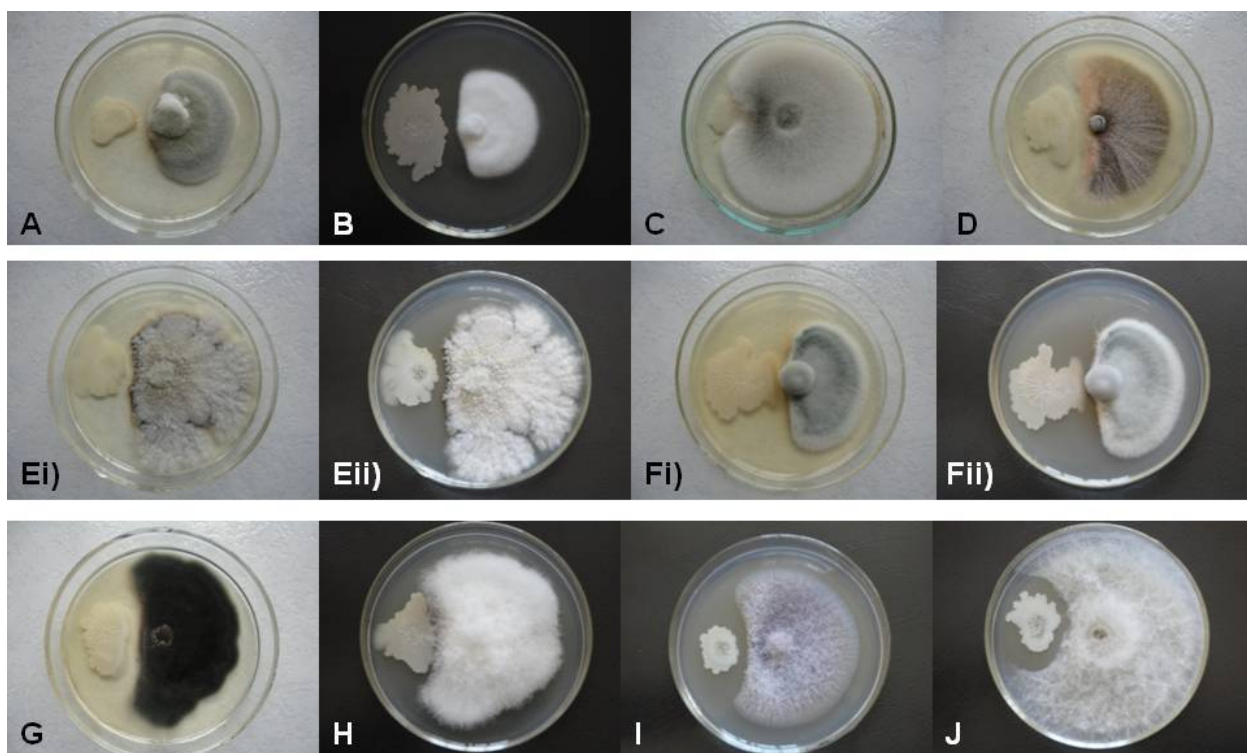
Plate 15: **Inhibitory growth effects of sorghum rhizobacterial isolate MaI254 on sorghum foliar fungal pathogens 7 days after inoculation. A;** *Alternaria alternata* **Kb06. B;** *Aspergillus candidus* **Sr03. C;** *Alternaria longissima* **Kb08. D;** *Colletotrichum gloeosporioides* **Kb08. Ei—ii;** *Botrytis cinerea* **Sg01, Fi—ii;** *Colletotrichum sublineolum* **Kb04, G;** *Exserohilum turcicum* **Sr02, H;** *Fusarium equiseti* **Sr01, I;** *Fusarium moniliforme* **Kb04, J;** *Nigrospora oryzae* **Sr01. Source: (Author) 2016.**

The screening results given in Table 3 using the Paulitz *et al.* (1992) and Landa *et al.* (1997) methodology indicates that growth inhibition by the two rhizobacterial isolates was not significantly different for the sorghum test fungal pathogens save for *Alternaria longissima* Kb08, *Colletotrichum sublineolum* Kb04 and *Nigrospora oryzae* Sr01. Thus, anyone of the two sorghum rhizobacteria could be used for biocontrol of phytopathogens tested in this study. This observation was further put under scrutiny using the Besson *et al.* (1978) method of screening that produces growth inhibition results 360° around the antagonist.

Table 3: **Mean % growth inhibition produced by rhizobacterial isolates KaI245 and MaI254 tested against sorghum pathogens using the Paulitz *et al.* (1992) and Landa *et al.* (1997) methodology**

Phytopathogen	Mean % growth inhibition by:	
	Rhizobacterial isolate KaI245	**Rhizobacterial isolate MaI254**
Alternaria alternata Kb06	70.44[c]	71.47[c]
Aspergillus candidus Sr03	71.24[c]	70.80[c]
Alternaria longissima Kb08	64.41[e]	63.88[b]
Botrytis cinerea Sg01	74.19[c]	72.28[c]
Colletotrichum gloeosporioides Kb08	71.63[c]	71.22[c]
Colletotrichum sublineolum Kb04	70.55[c]	75.60[d]
Exserohilum turcicum Sr02	64.10[b]	64.11[b]
Fusarium equeseti Sr01	54.27[a]	56.97[a]
Fusarium moniliforme Kb04	63.28[b]	63.77[b]
Nigrospora oryzae Sr01	75.80[d]	74.06[c]

Results are means of nine replicate dual cultures. Means followed by different letters for the effect of rhizobacterial isolates KaI245 and MaI254 on the same test pathogens are honestly significantly different (HSD) at $p < 0.05$. Significant differences obtained by Tukey-Kramer Multiple-Comparison Test.

Mycelia growth inhibition was ambiguous at first when the Besson *et al.* (1978) method was used, but became more and more pronounced as incubation continued. Inhibition of growth by the isolate KaI245 was greater by comparison to isolate MaI254 for all the test pathogens (Plates 16 and 17, Table 4, Appendix 10). Clear zones were measured in millimeters after 72 h of incubation at room temperature. The widest zone of inhibition was recorded in *B. Cinerea* Sg01 plates that recorded a mean of 30.70 mm followed by *N. oryzae* Sr01*, C. sublineolum* Kb04, *Aspergillus candidus* Sr03, *C. gloeosporioides* Kb08, *Alternaria longissima* Kb08, *Exserohilum turcicum* Sr02, *Fusarium equiseti* Sr01, *Alternaria alternata* Kb06 and *F. moniliforme* Kb04 which gave mean inhibition zones of 26.49 mm, 22.44 mm, 20.28 mm, 19.11mm, 15.99 mm, 9.08 mm, 8.90 mm, 8.31 mm and 4.41 mm respectively. The zones were clear with distinct boundaries and persisted for more than 7 days (Plates 16 and 17).
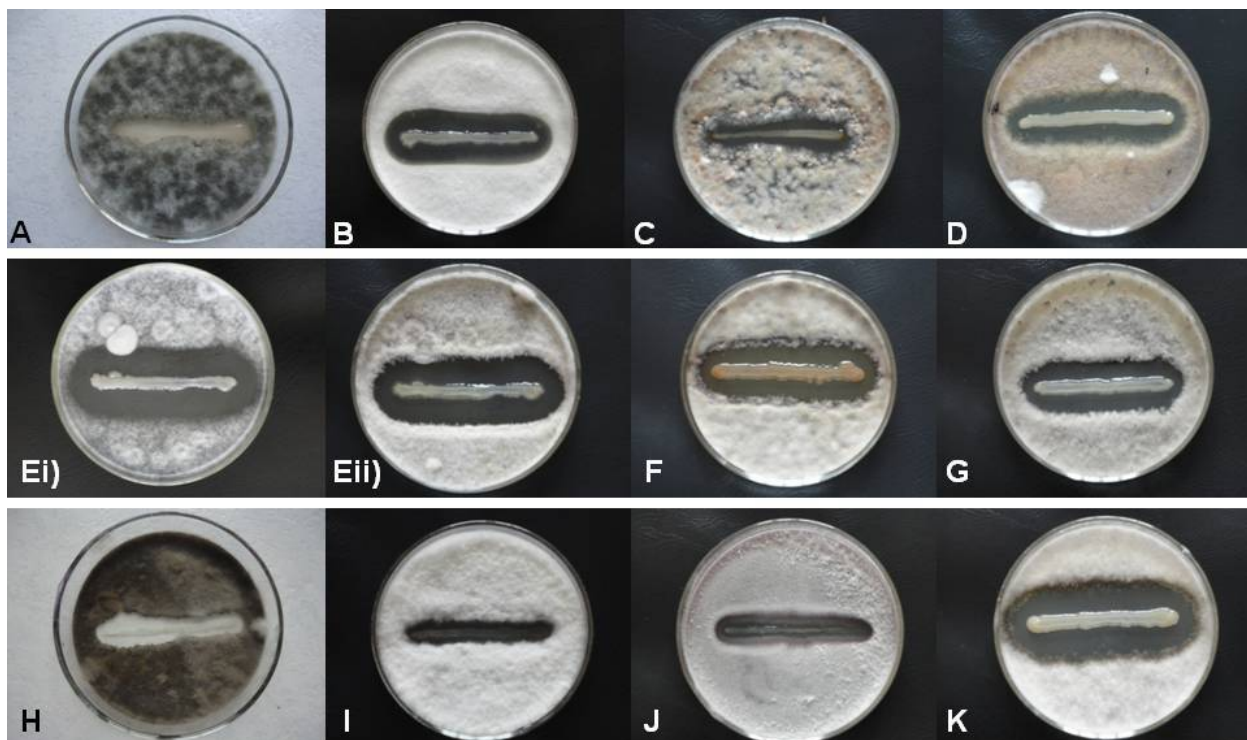
Plate 16: **Screening results of rhizobacterial isolate KaI245 tested against sorghum pathogens using the Besson *et al.* (1978) methodology, 8 days after inoculation: A; *Alternaria alternata* Kb06, B; *Aspergillus candidus* Sr03, C; *Alternaria longissima* Kb08, D; *Colletotrichum gloeosporioides* Kb08, Ei); *Botrytis cinerea* Sg01 3 days after inoculation, Eii); *Botrytis cinerea* Sg01, F; *Colletotrichum sublineolum* Kb04, G; *Colletotrichum sublineolum* Kb07, H; *Exserohilum turcicum* Sr02, I; *Fusarium equiseti* Sr01, J; *Fusarium moniliforme* Kb04, K; *Nigrospora oryzae* Sr01. Source: (Author) 2016.**
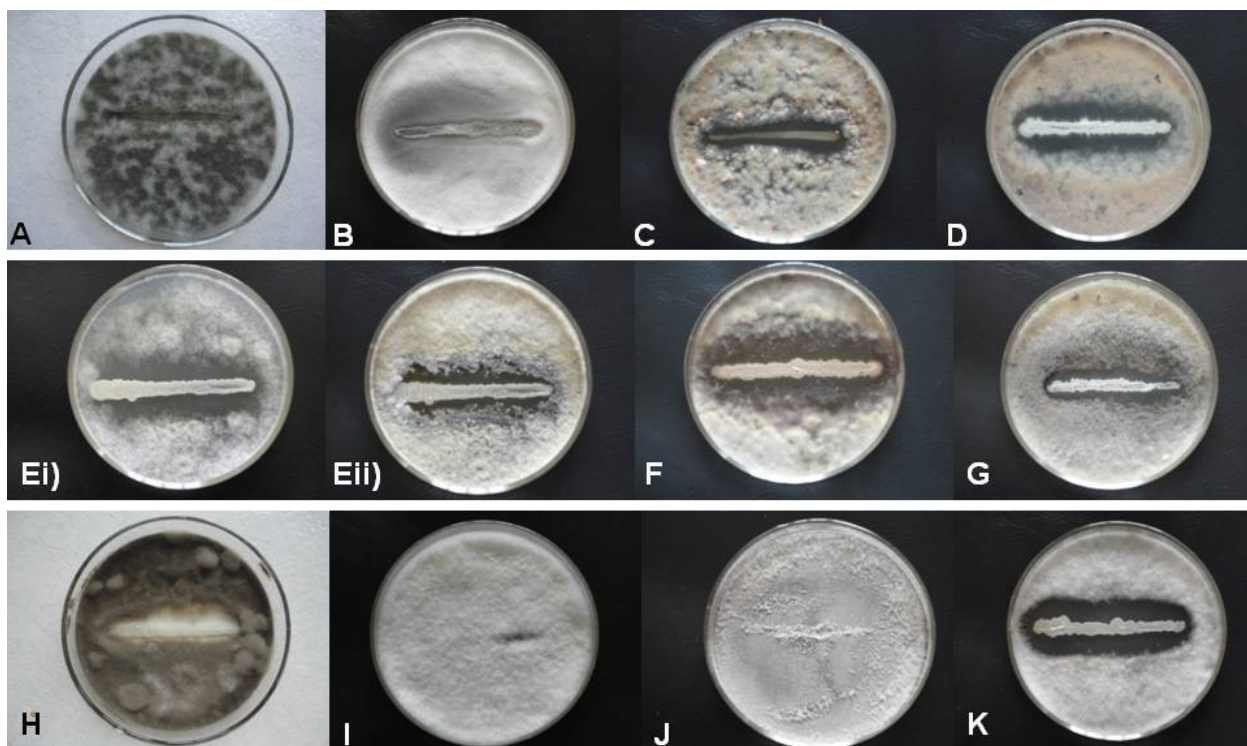
Plate 17: Screening results of rhizobacterial isolate MaI254 tested against sorghum pathogens using the Besson *et al.* (1978) methodology, 8 days after inoculation: A; *Alternaria alternata* **Kb06, B;** *Aspergillus candidus* **Sr03, C;** *Alternaria longissima* **Kb08, D;** *Colletotrichum gloeosporioides* **Kb08, Ei);** *Botrytis cinerea* **Sg01 3 days after inoculation, Eii);** *Botrytis cinerea* **Sg01, F;** *Colletotrichum sublineolum* **Kb04, G;** *Colletotrichum sublineolum* **Kb07, H;** *Exserohilum turcicum* **Sr02, I;** *Fusarium equiseti* **Sr01, J;** *Fusarium moniliforme* **Kb04, K;** *Nigrospora oryzae* **Sr01. Source: (Author) 2016.**

Table 4: **Mean clear zones of growth inhibition produced by rhizobacterial isolates KaI245 and MaI254 against sorghum fungal pathogens using the Besson *et al.* (1978) methodology**

Phytopathogen	Mean clear zone of growth inhibition (mm) by:	
	Rhizobacterial isolate KaI245	Rhizobacterial isolate MaI254
Alternaria alternata Kb06	8.31[c]	2.76[b]
Aspergillus candidus Sr03	20.28[f]	5.16[b]
Alternaria longissima Kb08	15.99[e]	5.99[b]
Botrytis cinerea Sg01	30.70[i]	21.41[g]
Colletotrichum gloeosporioides Kb08	19.11[f]	17.72[e]
Colletotrichum sublineolum Kb04	22.44[g]	12.63[d]
Exserohilum turcicum Sr02	9.08[c]	7.98[b]
Fusarium equeseti Sr01	8.90[c]	-1.85[a]
Fusarium moniliforme Kb04	4.41[b]	-2.00[a]
Nigrospora oryzae Sr01	26.49[h]	21.41[g]

Results are means of nine replicate dual cultures. Means followed by different letters for the effect of rhizobacterial isolates KaI245 and MaI254 on the same test pathogens are honestly significantly different (HSD) at p < 0.05. Significant differences obtained by Tukey-Kramer Multiple-Comparison Test. Negative values indicate that fungal growth overran bacterial growth.

Statistical analysis for this particular method of screening indicates that all the mean zones of inhibition produced by the two rhizobacterial isolates pitted against the same pathogens were significantly different from each other at p < 0.05 (Table 4, Appendix 10). This outcome is at variance with what was obtained when the Paulitz *et al.* (1992) and Landa *et al.* (1997) methodology was used.

Results of the modified Besson *et al.* (1978) method of screening confirmed observations made from the Besson *et al.* (1978) method of screening. Isolate KaI245 produced greater clear zones of mycelia growth inhibition compared to isolate MaI254 for all the test pathogens except for *C. gloeosporioides* (Plates 18 and 19, Table 5, Appendix 11). Besides, zones of inhibition for rhizobacterial isolate KaI245 were unambigous in comparizon to rhizobacterial isolate MaI254. The widest zone of inhibition was recorded in *N. oryzae* Sr01 plates that recorded a mean of 14.41 mm followed by *B. Cinerea* Sg01, *Alternaria alternata* Kb06, *Aspergillus candidus* Sr03, *C. gloeosporioides* Kb08, *C.*

sublineolum Kb04, *F. equiseti* Sr01, *Alternaria longissima* Kb08, *E. turcicum* Sr02, and *F. moniliforme* Kb04 which gave mean inhibition zones of 11.65 mm, 10.89 mm, 8.91 mm, 8.83mm, 8.61 mm, 7.17 mm, 3.90 mm, 3.34 mm and 1.76 mm respectively. The zones were clear with distinct boundaries and persisted for at least 6 days (Plates 18 and 19).
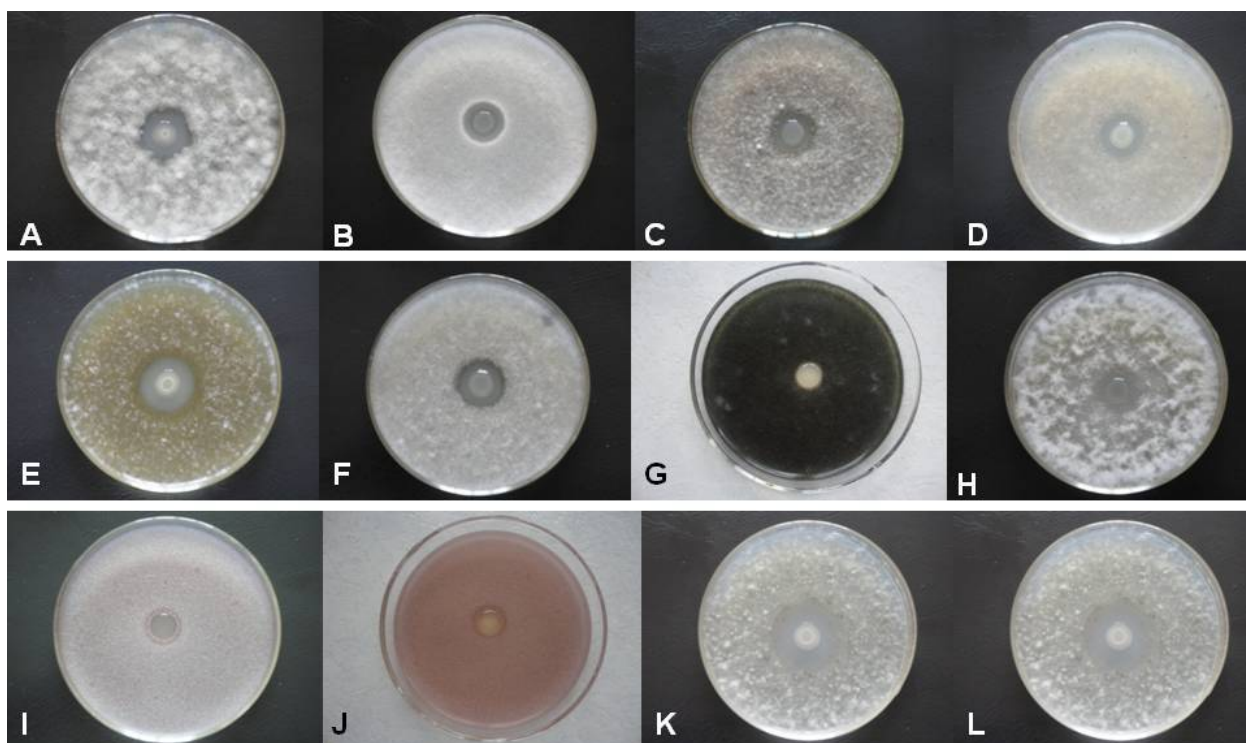
Plate 18: **Screening results of rhizobacterial isolate KaI245 tested against sorghum pathogens using the modified Besson** *et al.* **(1978) methodology, 3 days after inoculation: A;** *Alternaria alternata* **Kb06, B;** *Aspergillus candidus* **Sr03, C;** *Alternaria longissima* **Kb08, D;** *Colletotrichum gloeosporioides* **Kb08, E);** *Botrytis cinerea* **Sg01, F;** *Colletotrichum sublineolum* **Kb04, G;** *Exserohilum turcicum* **Sr02, H;** *Fusarium equiseti* **Sr01, I—J;** *Fusarium moniliforme* **Kb04, K—L;** *Nigrospora oryzae* **Sr01. Source: (Author) 2016.**
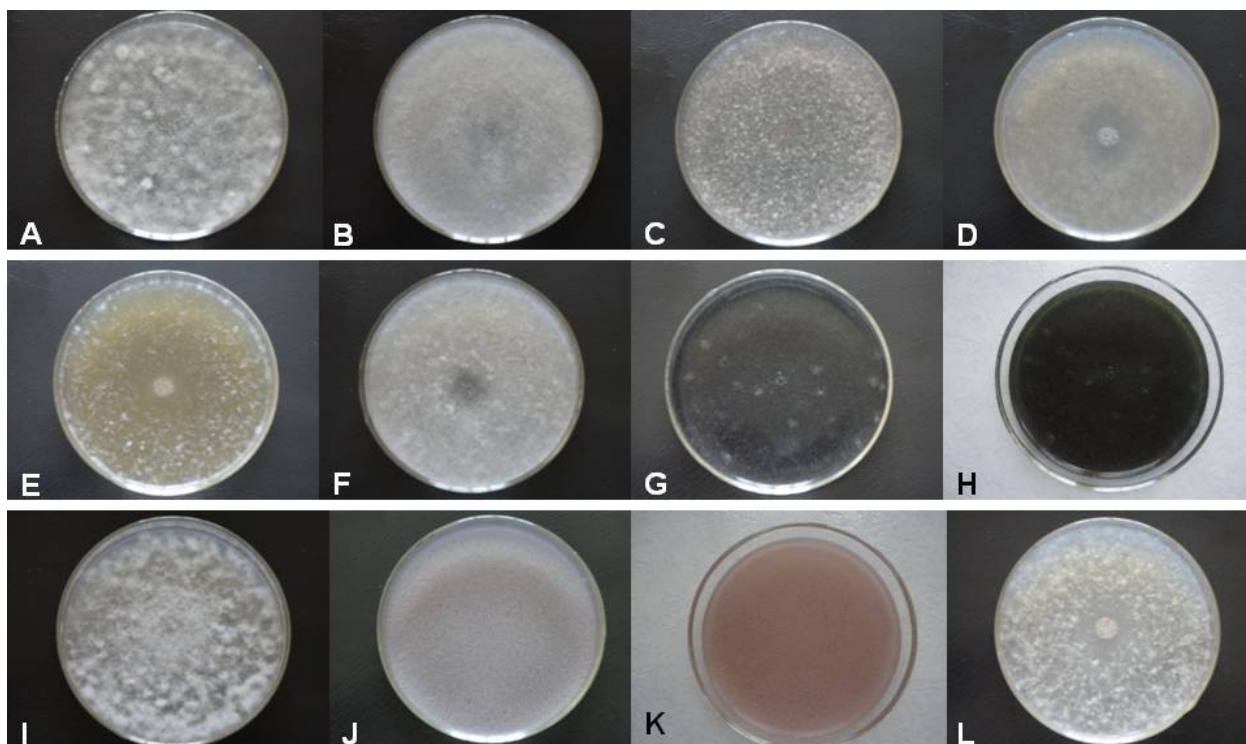
Plate 19: Screening results of rhizobacterial isolate MaI254 tested against sorghum pathogens using the modified Besson *et al.* (1978) methodology, 3 days after inoculation: A; *Alternaria alternata* Kb06, B; *Aspergillus candidus* Sr03, C; *Alternaria longissima* Kb08, D; *Colletotrichum gloeosporioides* Kb08, E); *Botrytis cinerea* Sg01, F; *Colletotrichum sublineolum* Kb04, G—H; *Exserohilum turcicum* Sr02, I; *Fusarium equiseti* Sr01, J—K; *Fusarium moniliforme* Kb04, L; *Nigrospora oryzae* Sr01. Source: (Author) 2016.

Table 5: **Mean clear zones of growth inhibition produced by rhizobacterial isolates KaI245 and MaI254 against sorghum pathogens using the modified Besson *et al.* (1978) methodology**

Phytopathogen	Mean clear zone of growth inhibition (mm) by:	
	Rhizobacterial isolate KaI245	Rhizobacterial isolate MaI254
Alternaria alternata Kb06	10.89[h]	-0.71[b]
Aspergillus candidus Sr03	8.91[g]	1.02[c]
Alternaria longissima Kb08	3.90[d]	0.19[c]
Botrytis cinerea Sg01	11.65[h]	3.87[d]
Colletotrichum gloeosporioides Kb08	8.83[f]	9.12[g]
Colletotrichum sublineolum Kb04	8.61[f]	7.13[f]
Exserohilum turcicum Sr02	3.34[d]	-8.00[a]
Fusarium equeseti Sr01	7.17[f]	-9.00[a]
Fusarium moniliforme Kb04	1.76[c]	-8.00[a]
Nigrospora oryzae Sr01	14.41[i]	6.59[e]

Results are means of nine replicate dual cultures. Means followed by different letters for the effect of rhizobacterial isolates KaI245 and MaI254 on the same test pathogens are honestly significantly different (HSD) at $p < 0.05$. Significant differences obtained by Tukey-Kramer Multiple-Comparison Test. Negative values indicate that fungal growth overran bacterial growth.

Statistical analysis for the modified Besson *et al.* (1978) method of screening indicates that all the mean zones of inhibition produced by the two rhizobacterial isolates pitted against the same pathogens were significantly different at $p < 0.05$ except for *Colletotrichum sublineolum* (Table 5, Appendix 11). This outcome is as well at variance with what was obtained when the Paulitz *et al.* (1992) and Landa *et al.* (1997) methodology was used but consistent with the Besson *et al.* (1978) method with almost negligible variations. On account of greater mycelia inhibition and persistence of results, rhizobacterial isolate KaI245 is better suited for biocontrol of pathogens tested in this study. Subsequent experimentation beyond identification of rhizobacterial isolates were therefore restricted to this isolate.

4.7. Identification of sorghum rhizobacterial isolates

4.7.1. Preliminary identification

Given that many of the primary sorghum rhizobacterial isolates that showed antagonistic potency against the test fungal pathogens were more or less morphologically similar, preliminary identification procedures including: Gregorson's KOH, cytochrome oxidase, oxidation/ fermentation, catalase and motility tests were performed. Preference was given to the 35 rhizobacterial isolates that inhibited at least one of the test phytopathogen by ≥ 70%. A summary of the results obtained is given in Table 6.

Table 6: Preliminary characterization of rhizobacterial isolates producing ≥ 70% mycelia inhibition of at least one of the test sorghum foliar phytopathogens based on biochemical tests

Rhizobacterial isolate	Gram test	Endo-spore	Catalase test	Cytochrome oxidase test	Motility* test	O/F test	Inference
KbI01[NE]	+	−	+	+	−	Nd	Gram +ve
UrI15[NE]	−	−	+	+	+	O/F−	Gram −ve
SgI29[B]	+	+	+	+	−	Nd	Gram +ve
SgI31[B]	+	+	+	+	−	Nd	Gram +ve
SgI34[B]	+	+	+	+	−	Nd	Gram +ve
SgI41[B]	+	+	+	+	−	Nd	Gram +ve
BuSiI62[B]	+	+	+	+	−	Nd	Gram +ve
SgII77[B]	+	+	+	+	+	Nd	Gram +ve
SgII78[B]	+	+	+	+	+	Nd	Gram +ve
BrI125 [B]	+	+	+	+	−	Nd	Gram +ve
BrI131 [B]	+	+	+	+	−	Nd	Gram +ve
UrI132 [B]	+	+	+	+	−	Nd	Gram +ve
UrI144 [B]	+	+	+	+	−	Nd	Gram +ve
AlB$_2$I157 [NE]	+	−	+	+	−	Nd	Gram +ve
AlB$_2$I159 [B]	+	+	+	+	−	Nd	Gram +ve
AkI163 [B]	+	+	+	+	−	Nd	Gram +ve
AkI164 [B]	+	+	+	+	−	Nd	Gram +ve
AkI165 [B]	+	+	+	+	−	Nd	Gram +ve
AkI166 [E]	−	−	+	−	+	O/F+	Gram −ve
AkI167 [B]	+	+	+	+	−	Nd	Gram +ve
AwI171 [NE]	−		+	+	+	O+	Gram −ve
AngI180 [NE]	−	−	+	+	−	O/F+	Gram −ve

AngI181 [E]	–	–	+	–	+	O/F+	Gram –ve
AngI182 [NE]	–	–	+	+	–	O+	Gram –ve
SrI194 [E]	–	–	+	–	+	O/F+	Gram –ve
SrI209 [B]	+	+	+	+	+	Nd	Gram +ve
AlB$_1$I215 [B]	+	+	+	+	–	Nd	Gram +ve
SrII225 [B]	+	+	–	+	–	Nd	Gram +ve
SrII226 [B]	+	+	+	+	–	Nd	Gram +ve
SrIV236 [B]	+	+	+	+	–	Nd	Gram +ve
SrIV238 [B]	+	+	+	+	–	Nd	Gram +ve
SrIV243 [B]	+	+	+	+	–	Nd	Gram +ve
KaI244 [B]	+	+	+	+	+	Nd	Gram +ve
KaI245 [NE]	–	–	+	+	+	O/F+	Gram –ve
MaI254 [B]	+	+	+	+	+	Nd	Gram +ve

B = Bacillaceae; **E**. = Enterobacteriaceae; **NE**. = Non-Enterobacteriaceae; *****:+ = Motile, – = Non-motile; O/F+: Oxidative/Fermentative Positive; O/F– = Oxidative/Fermentative Negative; O+ = Oxidative, F+ = Fermentative; Nd = Not determined for Gram positives.

Results obtained broadly indicate that both Firmicutes (Gram +ve) and Gracilicutes (Gram –ve) antagonistic bacteria reside in the sorghum rhizosphere. Further, the preliminary identification results tentatively places the rhizobacterial isolates into three groups as follows (bioMérieux sa 69280 Marcy l'Etoile, France):

1) Bacillaceae; that included Gram +ve, spore forming rhizobacterial rods. This group constituted about 71.4% of the 35 rhizobacterial isolates.

2) Enterobacteriaceae; Gram –ve, oxidase –ve and fermentative rhizobacterial isolates from the Hugh and Leifson's oxidation-fermentative test, equivalent to 8.6% of the 35 rhizobacterial isolates.

3) Non-Enterobacteriaceae; rhizobacterial isolates that gave positive oxidase reactions and oxidative results from the Hugh and Leifson's oxidation-fermentative test representing 20% of the 35 rhizobacterial isolates.

Generally, any one of these isolates could be used in the biocontrol of sorghum foliar pathogens.

4.7.2. Identification based on further biochemical characterization

Among the 35 isolates that underwent preliminary identification, the rhizobacterial isolates KaI245 and MaI254 were further morphologically and biochemically analyzed based on their exceptional antagonistic potency. They produced visible zones of inhibition against test sorghum fungal pathogens that were not observed with the other isolates. A summary of the morphological and biochemical characteristics of the two isolates is given in Table 7 and Appendix 12.

Table 7: Morphological and biochemical characteristics of rhizobacterial isolates KaI245 and MaI254

Sr. No.	Tests	Isolate KaI245	Isolate MaI254
1.	Colonial characteristics on NA	Small, copiously mucoid, convex with entire margins	Large, non-mucoid, flat with erose margins
2.	Pigmentation	Nil	Nil
3.	Microscopic characteristics	Plump rods, from curved to straight with rounded edges	Plump rods with centrally placed endospores
4.	Acid production from glucose [a]	+ve	+ve
5.	Gas production from glucose [a]	+ve	−ve
6.	Casein hydrolysis [b]	−ve	+ve
7.	Citrate utilization [c]	+ve	+ve
8.	Gelatin liquefaction [d]	+ve	+ve
9.	Growth at 50°C [e]	Nd	−ve
10.	Growth at 65°C [e]	Nd	−ve
11.	Growth in 7% NaCl [e]	Nd	+ve
12.	Growth in anaerobic agar [e]	Nd	−ve
13.	Indole production [f]	+ve	Nd
14.	Glucose fermentation [g]	+ve	Nd
15.	Fructose fermentation [g]	−ve	Nd
16.	Lactose fermentation [g]	+ve	Nd
17.	Sucrose fermentation [g]	+ve	Nd
18.	Nitrate reduction [b]	+ve	−ve
19.	Voges-Proskaeur test [h]	+ve	−ve
20.	H$_2$S production [i]	−ve	Nd
21.	Urease production [j]	−ve	Nd

22.	Starch hydrolysis [k]	+ve	+ve

Nd = Not determined.

[a] Reiner, (2012)

[b] Norris *et al.* (1981); Smirbert and Krieg, (1981); Sneath, (1984)

[c] MacWilliams, (2009a)

[d] Edison and dela-Cruz (2012)

[e] Norris *et al.* (1981); Sneath, (1984)

[f] MacWilliams, (2009b)

[g] Huge and Leifson, (1953); Hanson, (2008)

[h] McDevitt, (2010)

[i] Lehman, (2005)

[j] Brink, (2010)

[k] Lal and Cheeptham, (2012)

From the above phenotypic characteristics, synoptic descriptions and keys tentatively identify rhizobacterial isolate KaI245 as an *Aeromonas* sp. (Breed *et al.,* 1948; Buchanan and Gibbons, 1974; Sakazaki and Ballows, 1981; Holt *et al.,* 2000; Abbott *et al.,* 2003) while rhizobacterial isolate MaI254 is identified as *Bacillus megaterium* (Norris *et al.,* 1981; Sneath, 1984; Abis Encyclopaedia, 2015).

4.7.3. Identification using the API system

The identities assigned to rhizobacterial isolates KaI245 and MaI254 by general biochemical characterization were confirmed using the API system. Identification for rhizobacterial isolate KaI245 was done using both API[®] 20 E and API[®] 20 NE test strips whilst that of rhizobacterial isolate MaI254 was done using the API[®]50 CH test strips in combination with API 50CHB/E (bioMérieux) medium. Both the API[®] 20 E and API[®] 20 NE test strips identified rhizobacterial isolate KaI245 as *Aeromonas hydrophila* while the API[®]50 CH test strips identified rhizobacterial isolate MaI254 as *Bacillus megaterium*. These results are in tandem with what general biochemical characterization produced.

4.8. Production, extraction and purification of antifungal agents from *Aeromonas hydrophila* KaI245

Fourty millilitres of a primary culture containing 1.0 x 10^8 cfu/ml of the antagonistic *Aeromonas hydrophila* strain KaI245, as determined by the plate count method, was inoculated in sterile 200 ml of modified Farhana *et al.* (2011) medium in 1 litre conical

flasks at pH 7.0 for 7 days. Initially the liquid medium bearing mannitol as the carbon source was almost clear in appearance save for the insoluble $CaCO_3$ particles at the bottom of the conical flasks. Conical flasks with glucose, fructose and glycerol as the carbon sources bore tinges of yellow-cream for glucose broth, cream for glycerol broth whilst the fructose broth bore a vanish brown colour. As bacterial growth continued on the orbital shakers, the colours remained the same albeit with a little intensification. Growth of the rhizobacterial isolate, though submerged, was also noticeable on the surface of the conical flasks as creamy-white coloured rings. A brown colouration was observed on the ring formed in the fructose flasks.

In spite of tight plugging of the conical flasks with cotton wool and aluminium foil, a distinctive awful smell was produced especially in the mannitol and glycerol broth flasks. No contamination was noticed during the antibiotic production period. The pH of the liquid medium after incubation and shaking for the 7 days was determined as 5.02, 5.62, 5.14 and 5.68 for the glucose, fructose, mannitol and glycerol broths respectively. Upon centrifugation, at 10,000 rpm for 20 minutes at 4°C, the colour of the supernatants were yellow-cream, vanish-brown, colourless and light cream for liquid media prepared with glucose, fructose, mannitol and glycerol as carbon sources respectively. The supernatants were slightly viscous, especially that obtained from glucose broth, and maintained the foul smell. There was increased viscosity observed in the supernatants when they were concentrated on a rotary vaccum evaporator. Colour intensification was also observed. These attributes were retained through the process of purification.

Thin Layer Chromatography revealed that the antimicrobial substance(s) in the ten bottle contents collected during the purification process was one and the same owing to the distance travelled on the chromatography paper. The distances were more or less the same.

4.9. Evaluation of the antifungal agent(s) from *Aeromonas hydrophila* KaI245
4.9.1. Paper-disk agar-plate diffusion assay for antifungal activity

The antagonistic culture filtrate obtained from *Aeromonas hydrophila* KaI245 showed antibiotic activity owing to the production of inhibition zones on media seeded with the

plant pathogens. Filter paper discs dipped in active culture filtrate gave clear zones of inhibition for all the test pathogens. Filter paper discs dipped in distilled water produced no clear zones. The clear zones were measured in millimeters after 72 h of incubation at room temperature. The widest zone of mycelia growth inhibition was recorded in *B. cinerea* Sg01 plates that recorded a mean of 33.19 mm followed by *N. oryzae* Sr01, *C. sublineolum* Kb04, *C. gloeosporioides* Kb08, *F. equiseti* Sr01, *F. moniliforme* Kb04, *A. longissima* Kb08, *A. candidus* Sr03, *A. alternata* Kb06 and *E. turcicum* Sr02 which gave mean inhibition zones of 32.70 mm, 29.03 mm, 22.15 mm, 18.09 mm, 16.79 mm, 15.22 mm, 8.04 mm, 2.83 mm and 0.30 mm respectively. Statistical analysis indicated that the mean zones of inhibition were honestly significantly different from each other at $p < 0.05$ except for the zones produced against *B. cinerea* Sg01 and *N. oryzae* Sr01 (Table 8, Appendix 13). The zones were clear with distinct boundaries and persisted for more than 7 days (Plate 20).
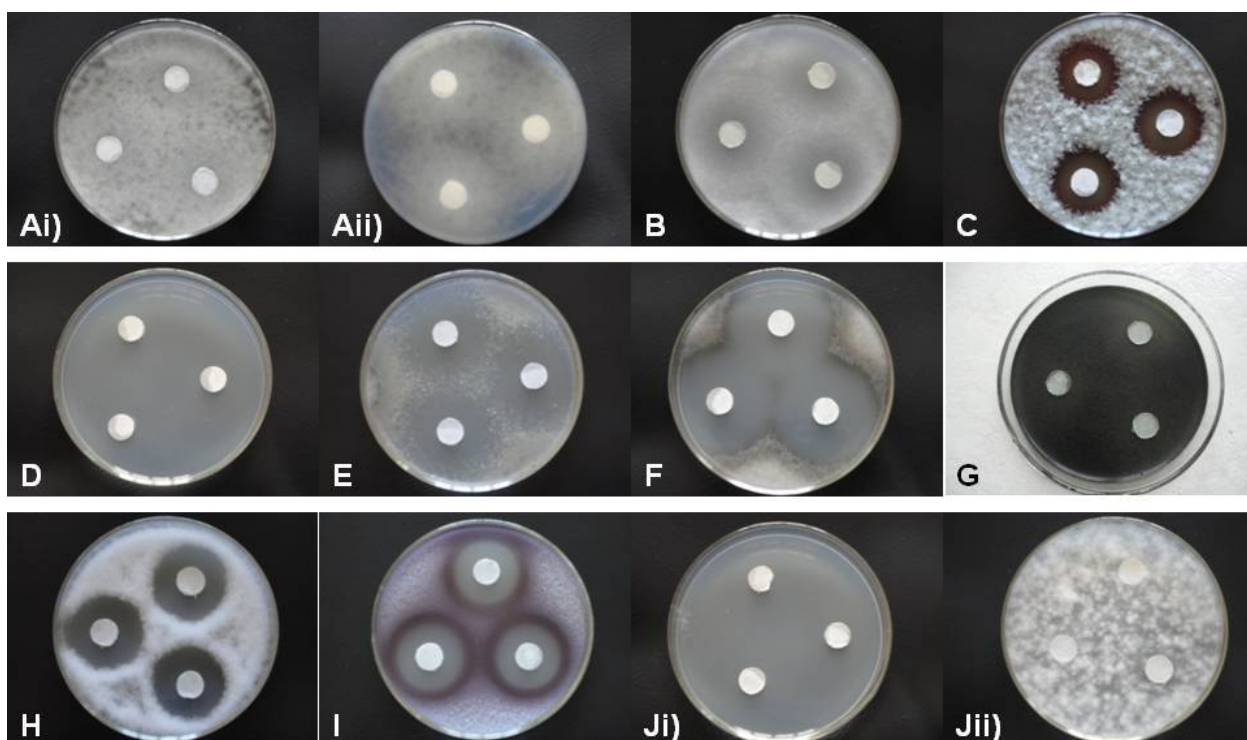
Plate 20: **Paper-disk-diffusion-assay of** *Aeromonas hydrophila* **KaI245's culture-filtrate against sorghum foliar fungal pathogens, 3 days after inoculation. Ai); Top Petri-dish view of** *Alternaria alternata* **Kb06, Aii); Bottom Petri-dish view of** *Alternaria alternata* **Kb06, B;** *Aspergillus candidus* **Sr03, C;** *Alternaria longissima* **Kb08, D;** *Botrytis cinerea* **Sg01, E;** *Colletotrichum gloeosporioides* **Kb08, F;** *Colletotrichum sublineolum* **Kb04, G;** *Exserohilum turcicum* **Sr02, H;** *Fusarium equiseti* **Sr01, I;** *Fusarium moniliforme* **Kb04, Ji);** *Nigrospora oryzae* **Sr01, Jii); Control plate of** *Nigrospora oryzae* **Sr01. Source: (Author) 2016.**

Table 8: Mean clear zones of growth inhibition produced by the antibiotic-culture-filtrate[†] of *Aeromonas hydrophila* **KaI245 tested against the sorghum fungal pathogens 3 days after inoculation**

Phytopathogen	Mean clear zone of mycelia growth inhibition (mm)
Alternaria alternata Kb06	2.83[b]
Aspergillus candidus Sr03	8.04[c]
Alternaria longissima Kb08	15.22[d]
Botrytis cinerea Sg01	33.19[i]
Colletotrichum gloeosporioides Kb08	22.15[g]
Colletotrichum sublineolum Kb04	29.03[h]
Exserohilum turcicum Sr02	0.30[a]
Fusarium equeseti Sr01	18.09[f]
Fusarium moniliforme Kb04	16.79[e]
Nigrospora oryzae Sr01	32.70[i]

† Active-culture-filtrate obtained from liquid medium bearing glucose as its carbon source
Results are means of nine replicate dual cultures. Means followed by different letters for the effect of active-culture-filtrate of *Aeromonas hydrophila* KaI245 on the test pathogens are honestly significantly different (HSD) at $p < 0.05$. Significant differences obtained by Tukey-Kramer Multiple-Comparison Test.

4.9.2. Determination of the minimum inhibitory concentration (M.I.C) of antifungal agent(s) produced by *Aeromonas hydrophila* KaI245 against *C. sublineolum* Henn

Petri dishes seeded with *C. sublineolum* and treated with the purified antibiotic culture filtrate were analyzed after an incubation period of 72 h. Plates treated with a dilution of 3^{-5} (1:243) and lower did not show any growth of *C. sublineolum* while plates treated with a dilution of 3^{-6} (1:729) and higher had fungal growth. Thus the minimum inhibitory concentration of antibiotics produced by *Aeromonas hydrophila* KaI245 falls between 3^{-5} and 3^{-6}.

4.9.3. Determination of the number of antifungal agents by paper chromatography

Antibiotics contained in the culture filtrate from *Aeromonas hydrophila* KaI245 were eluted by the solvent system containing a mixture of n-butanol, acetic acid and water in the ratio of 4:3:3 v/v (Wahome, 1998; Muiru, 2000). The elution of antibiotics on chromatography paper strips was evidenced by the formation of a clear zone of inhibition

at a distance from the original spot when using the bioautographic detection method. One zone of inhibition after bioautographic detection was noted. This implied that the culture filtrate of *Aeromonas hydrophila* KaI245 contained one antibiotic that is active against *C. sublineolum* (Plate 21).
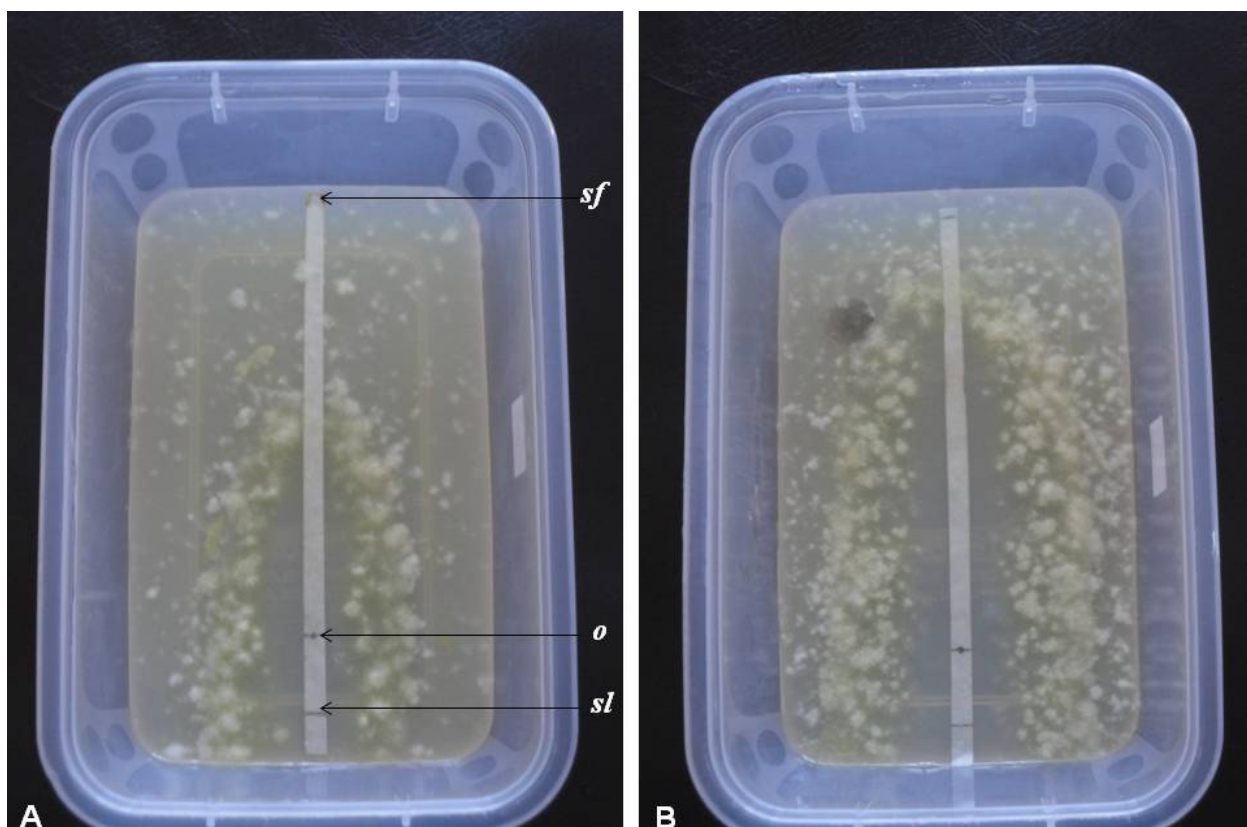
Plate 21: **Zones of inhibition produced by eluted antibiotics of *Aeromonas hydrophila* KaI245 in bioautography boxes against *C. sublineolum*. A; Chromatography strip with the antibiotic eluted from crude *Aeromonas hydrophila* KaI245 culture filtrate. B; Chromatography strip with the antibiotic eluted from purified *Aeromonas hydrophila* KaI245 culture filtrate. The lower line towards the bottom of the images (*sl*) is 1 cm from the lower edge—the portion that was immersed in the solvent system. The origin (*o*) is indicated as the upper horizontal line. The line beyond the zone of inhibition—towards the upper part of the image (*sf*)— is the solvent front. Source: (Author) 2016.**

The plate above clearly shows that the crude culture-filtrate had one antibiotic eluted. Right from the origin, a continous zone of inhibition was exhibited up to the upper edge to indicate that the antibiotic was continously eluted and remained along the chromatogram strip up to the furthest point it could be eluted. The eluted antibiotic had a strong activity of inhibiting the growth of *C. sublineolum*. Further up the chromatogram strip was a short non-inhibitory zone before the solvent front. In the crude *Aeromonas*

hydrophila KaI245 culture filtrate, the antibiotic traveled 5.22 cm from the origin whist a distance of 9.64 cm was covered by the antibiotic from the purified *Aeromonas hydrophila* KaI245 culture-filtrate. In both cases, there was a linear clear zone of inhibition along the chromatogram strip against *C. sublineolum* wherever the antibiotic was deposited.

The culture filtrate's antibiotic showed different R_f values while in the crude and purified forms. The R_f values were calculated as the ratio of the distance moved by the antibiotic and solvent from the origin to the solvent front (Table 9). The distance moved by the antibiotic was measured from the origin to the geometric center of the zone of inhibition.

Table 9: R_f values measurements of crude and purified *Aeromonas hydrophila* KaI245 antibiotic-culture-filtrate[†] obtained by paper chromatography using butanol-acetic acid-water solvent system

Culture filtrate	Measurement in (cm)				
	i	**j**	**Z**	**Y**	**R_f**
Crude	5.22	1.15	3.01	12.03	0.10
Purified	9.64	3.33	3.04	12.04	0.28

† Antibiotic-culture-filtrate obtained from liquid medium bearing glucose as its carbon source

Key:

i	= Distance from the origin to the upper end of clear zone
j	= Distance from the origin to the geometrical center of the clear zone
Z	= Distance from the origin to the lower edge of the clear zone
Y	= Distance from the origin to the solvent front

R_f value = j/Y.

The process of purification of the antibiotic enhanced the activity of the antibiotic-culture-filtrate as attested to by the greater size of inhibition zones produced by the purified antibiotic culture-filtrate. Zones of inhibition produced were 65.47% greater compared to those of the crude antibiotic culture-filtrate.

4.9.4. Optimization of environmental conditions for maximum antibiotic production from *Aeromonas hydrophila* KaI245

4.9.4.1. Effect of different carbon sources

The antifungal substance (antibiotic) produced in liquid medium amended with fructose as the carbon source yielded the greatest mean clear zone of mycelia growth inhibition of 32.58 mm against *C. sublineolum*. The values for glucose, mannitol and glycerol were 28.70, 29.73 and 27.34 mm respectively which were lower than that produced by fructose and more importantly, statistically significantly different from each other at $p < 0.05$ (Table 10).

Table 10: **Clear zones of growth inhibition (mm) produced by *Aeromonas hydrophila* KaI245 antibiotic-culture-filtrates prepared from different carbon sources and tested against *Colletotrichum sublineolum***

Paper disc replicate	Clear zones of mycelia growth inhibition (mm) produced by KaI245 culture filtrate of carbon source:			
	Glucose	**Fructose**	**Mannitol**	**Glycerol**
1	28.91	32.43	29.96	27.77
2	28.12	33.14	29.76	27.99
3	28.53	34.02	29.35	27.78
4	29.34	31.98	29.88	26.89
5	29.13	32.23	29.47	26.97
6	29.17	33.73	29.97	26.68
7	28.53	32.78	29.54	27.01
8	28.01	31.96	29.68	27.23
9	28.53	30.99	29.98	27.75
Means:	**28.70[b]**	**32.58[d]**	**29.73[c]**	**27.34[a]**

Means followed by different letters for the effect of antibiotic-culture-filtrates of *Aeromonas hydrophila* KaI245 on *C. sublineolum* are honestly significantly different (HSD) at $p < 0.05$. Significant differences obtained by Tukey-Kramer Multiple-Comparison Test.

4.9.4.2. Effect of incubation period

Culture filtrates obtained from the various incubation periods exhibited different strengths in colour and viscosity. At 36 h, the culture filtrate was very light cream—almost transparent. Subsequent incubation days produced culture filtrates with increased amount of colour and viscosity.

The antibiotic activity of the culture filtrate obtained from the *Aeromonas hydrophila* KaI245 increased with increase in incubation time (Table 11). The increase was steady up to the 6th day. Thereafter, fluctuations in inhibition zone values were noted. In the first two days of incubation, no visible zones of inhibition were seen. The 3rd to the 5th days produced zones that were faint and had diffuse boundaries compared to clear zones with distinct boundaries from culture filtrates harvested from the 6th day onwards up to the 10th day.

A maximum mycelia growth inhibition zone of 32.87mm was obtained on the 7th day, 18.28mm bigger compared to that produced in the 3rd day of incubation. Statistical analysis of clear inhibition zones showed that there were significant differences at $p \leq 0.05$ on activities of filtrates harvested at day 3 to 7. However, there were no significant differences of clear inhibition zones produced from culture filtrates harvested at day 7 and 8, whose recorded values showed a drop. Generally, clear inhibition zones produced by culture filtrates increased in size from that harvested at day 3 to 6 followed by a steady decrease from day 7 to 10.

Table 11: Clear zones of growth inhibition (mm) produced by *Aeromonas hydrophila* KaI245 antibiotic-culture-filtrate[†] harvested at different incubation times and tested against *Colletotrichum sublineolum*

Paper disc rep	Clear zone of mycelia growth inhibition (mm) produced by KaI245 culture filtrate harvested at:									
	24h	**48h**	**72h**	**96h**	**120h**	**144h**	**168h**	**192h**	**216h**	**240h**
1	0.00	0.00	14.62	20.76	27.04	32.34	32.37	31.34	30.58	29.45
2	0.00	0.00	14.98	20.23	27.11	31.59	32.12	31.10	29.23	28.44
3	0.00	0.00	15.34	21.63	28.12	32.47	32.87	30.22	28.79	27.11
4	0.00	0.00	14.89	20.02	28.24	32.59	32.41	31.12	29.34	27.14
5	0.00	0.00	14.67	19.78	27.00	32.01	32.12	31.19	28.98	27.35
6	0.00	0.00	14.92	20.03	27.57	31.78	30.27	31.43	29.56	26.54
7	0.00	0.00	15.01	20.88	28.67	31.76	30.44	30.34	28.44	26.89
8	0.00	0.00	14.76	20.01	27.45	32.14	31.54	30.32	28.45	26.78
9	0.00	0.00	14.59	19.32	26.99	31.83	31.30	31.47	29.49	28.99
Means:	**0.00[a]**	**0.00[a]**	**14.86[b]**	**20.30[c]**	**27.58[d]**	**32.06[g]**	**31.72[f]**	**30.95[f]**	**29.21[e]**	**27.63[d]**

† Antibiotic-culture-filtrate obtained from liquid media bearing fructose as its carbon source
Means followed by different letters for the effect of antibiotic-culture-filtrates of *Aeromonas hydrophila* KaI245 on *C. sublineolum* are honestly significantly different (HSD) at $p < 0.05$. Significant differences obtained by Tukey-Kramer Multiple-Comparison Test.

4.9.4.3. Effect of initial pH

The synthetic medium used to test the effect of initial pH contained fructose as the carbon source. The recommended pH of the synthetic medium was 7.0. It was befitting to test the effect of pH 6.0 that falls between pH 5.0 and pH 7.0. A control set up whose initial pH was not adjusted was also included.

Generally, the optimal initial pH range for antibiotic production from rhizobacterial isolate KaI245 was wide and fell between pH 3 and pH 11. The antibiotic activity in the culture filtrate was adversely affected at initial pH levels below 3. No observable zones of mycelia growth inhibition were noticeable at initial pH 1. The clarity of inhibition zones increased as the initial pH of the culture filtrate was adjusted towards level 7 from both the alkaline and acid pH range. Thus, production of active antibiotic-culture-filtrate of *Aeromonas hydrophila* KaI245 was possible over a very wide pH range but with optimum production at pH 7.

Statistical analysis indicated that the mean clear zones of inhibition produced by samples adjusted to initial pH 1, 3, 5, 6, 9 and 11 were significantly different at $p \leq 0.05$ from the mean clear zones of inhibition produced by the non-adjusted control sample. However, there was no significant difference between the mean clear zones of control plates and those whose pH had been adjusted to 7. The mean clear zone of mycelia growth inhibition produced by the sample adjusted to pH 7 was slightly higher (31.79) than that of non-adjusted control (31.63), an indication that this antibiotic is best produced at pH level 7 (Table 12).

Table 12: **Clear zones of inhibition (mm) produced by *Aeromonas hydrophila* KaI245 antibiotic-culture-filtrate[†] subjected to different pH levels prior to incubation and tested against *Colletotrichum sublineolum***

Paper disc replicate	Clear zones of mycelia growth inhibition (mm) produced by KaI245 culture filtrate subjected to various pH levels:							
	Control	**1**	**3**	**5**	**6**	**7**	**9**	**11**
1	32.14	0.00	19.54	23.51	27.40	32.40	16.30	15.17
2	29.67	0.00	19.45	23.33	27.14	31.24	17.29	15.76
3	30.37	0.00	19.78	24.11	27.23	32.13	17.19	15.78
4	32.90	0.00	20.28	23.78	27.65	31.59	16.37	16.39
5	31.23	0.00	20.18	23.23	28.39	31.54	17.39	16.71
6	32.98	0.00	19.59	24.54	27.98	31.94	16.89	16.49
7	32.58	0.00	19.88	26.74	27.76	30.86	17.01	16.88
8	32.78	0.00	20.34	24.87	27.45	32.96	18.49	17.43
9	29.98	0.00	19.89	24.99	27.99	31.45	18.89	16.76
Means:	**31.63[f]**	**0.00[a]**	**19.88[c]**	**24.34[d]**	**27.67[e]**	**31.79[f]**	**17.31[b]**	**16.37[b]**

† Active-culture-filtrate obtained from liquid medium bearing fructose as its carbon source
Means followed by different letters for the effect of antibiotic-culture-filtrates of *Aeromonas hydrophila* KaI245 on *C. sublineolum* are honestly significantly different (HSD) at $p < 0.05$. Significant differences obtained by Tukey-Kramer Multiple-Comparison Test.

4.9.4.4. Effect of temperature conditions

The activity of the culture-filtrate showed that temperature conditions influenced quality of antibiotic produced in liquid medium by *Aeromonas hydrophila* KaI245. There was a steady increase in the mean clear zone of *C. sublineolum* mycelia growth inhibition by

antibiotic-culture-filtrates produced at temperatures 20°C to 28°C (Table 13). A similar decline was observed for culture-filtrates at temperature values of 32°C to 40°C. From the clear zones of inhibition recorded, the optimal temperature range for maximum antibiotic production was between 28°C—32°C with 28°C being the optimal temperature. Values recorded at these temperature conditions together with those produced at room temperature (22 ± 5°C) that doubled-up as the control were not significantly different at p < 0.05. Mean inhibition zones recorded at 20°C, 24°C, 36°C and 40°C were not significantly different at p < 0.05. Values recorded at these temperature conditions were lower from the optimal value for maximum antibiotic production (Table 13).

Table 13: Clear zones of growth inhibition (mm) produced by *Aeromonas hydrophila* KaI245 antibiotic-culture-filtrate[†] obtained at different temperature levels and tested against *Colletotrichum sublineolum*

Paper disc replicate	Clear zones of mycelia growth inhibition (mm) produced by KaI245 culture filtrate obtained at different temperature levels:						
	Control	**20°C**	**24°C**	**28°C**	**32°C**	**36°C**	**40°C**
1	31.93	29.15	30.22	33.19	33.28	31.37	30.09
2	32.41	29.78	32.01	33.18	33.33	32.12	30.24
3	32.98	29.02	31.82	34.02	33.12	31.87	30.31
4	33.58	31.07	31.45	33.42	30.65	31.41	30.66
5	32.94	32.00	31.56	33.32	31.78	32.12	30.43
6	33.64	31.73	31.68	33.38	31.64	30.27	31.83
7	32.49	30.48	29.78	31.77	33.11	30.44	32.78
8	31.96	30.66	30.36	31.78	33.31	31.54	31.96
9	32.79	30.19	30.12	31.43	31.44	31.30	30.99
Means:	**32.75^{b}**	**30.45^{a}**	**31.00^{a}**	**32.83^{b}**	**32.41^{b}**	**31.38^{a}**	**31.03^{a}**

† Active-culture-filtrate obtained from liquid medium bearing fructose as its carbon source
Means followed by different letters for the effect of antibiotic-culture-filtrates of *Aeromonas hydrophila* KaI245 on *C. sublineolum* are honestly significantly different (HSD) at p < 0.05. Significant differences obtained by Tukey-Kramer Multiple-Comparison Test.

4.9.4.5. Effect of storage of antibiotic agent culture-filtrate

The antibiotic activity of the culture filtrate remained stable after storage at refrigeration (4°C) and at room temperature (22 ± 5°C) against *C. sublineolum* for a period of 8 months. The clear zones of mycelia inhibition values recorded at the two storage temperatures at different intervals were significantly not different at $p < 0.05$ (Table 14; Appendices 14 and 15) in spite of the fluctuations observed. It therefore follows that the antibiotic remained somewhat stable at the two different storage conditions for a period of eight months and can be recommended to be stored at any one of those two conditions.

Table 14: Mean clear zones of inhibition (mm) produced by *Aeromonas hydrophila* KaI245 antibiotic-culture-filtrate[†] subjected to different storage conditions for 8 months and tested against *Colletotrichum sublineolum*.

Storage period (days)	Mean clear zone of mycelia growth inhibition (mm) at:	
	Refregiration (+4°C)	Room temperature (22 ± 5°C)
0	32.19[b]	32.19[b]
40	31.44[b]	31.16[a]
80	31.53[b]	31.57[b]
120	30.77[a]	30.95[a]
160	32.83[c]	32.81[c]
200	31.70[b]	31.98[b]
240	32.85[c]	32.88[c]

† Active-culture-filtrate obtained from liquid medium bearing fructose as its carbon source

Means followed by different letters for the effect of antibiotic-culture-filtrates of *Aeromonas hydrophila* KaI245 on *C. sublineolum* are honestly significantly different (HSD) at $p < 0.05$. Significant differences obtained by Tukey-Kramer Multiple-Comparison Test.

4.10. Greenhouse evaluation of *Aeromonas hydrophila* KaI245 antibiotic-culture-filtrate in controlling sorghum anthracnose

Sorghum anthracnose was considerably controlled with the *Aeromonas hydrophila* KaI245 antibiotics-culture-filtrate. There was a delayed manifestation of the disease symptoms on the treated plants compared with the +ve control. Delayed disease development was also observed on plants sprayed with a biofungicide Ballad[®]Plus and the conventional fungicide Folicur[®]430SC.

In the first week, mean disease scores of 1.17, 1.50, 1.67, 1.67, 2.17, 2.33 and 2.5 were recorded on sorghum leaves treated with; Folicur[®]430SC, original antibiotic-culture-filtrate, culture-filtrate concentrated twice, culture-filtrate diluted to half the original concentration, Ballad[®]Plus, sterile distilled water control and culture-filtrate diluted to a quater the original concentration respectively (Table 15, Appendix 16). There was no significant difference at $p < 0.05$ between culture filtrates at double, original and half diluted concentrations. Sorghum anthracnose was well suppressed by the standard chemical fungicide—Folicur[®]430SC whose disease progress value was well below those recorded with other treatments and significantly different at $p < 0.05$ (Table 15, Appendix 14). The antibiotic-culture-filtrate diluted to quater the original, the commercial biofungicide—Ballad[®]Plus and the positive control recorded higher scores that were not significantly different.

In the second week, the mean disease scores of Folicur[®]430SC, the original culture-filtrate and culture-filtrate concentrated twice were 1.67, 2.0 and 2.17. These were not significantly different at $p < 0.05$ (Table 15, Appendix 16), but were significantly different from the other treatments. During the week, the effects of culture filtrates at normal (original) strength and that concentrated twice in controlling sorghum anthracnose resulted in lower disease severity from the one whose concentration had been diluted and the biofungicide Ballad[®]Plus.

The activity of the culture filtrate concentrated twice in the third week compared favourably with the chemical check (Folicur[®]430SC) following the disease mean scores of 2.33 and 2.17 recorded by the two treatments respectively though they were significantly different at $p < 0.05$. The double concentrated-culture-filtrate, the original culture-filtrate, half diluted culture-filtrate and Ballad[®]Plus whose mean scores were 2.33, 2.67, 3.0 and 3.17 respectively were not significantly different from each other but did against the quater diluted culture-filtrate whose score was 3.5 (Table 15, Appendix 16).

The fourth week saw the culture-filtrate concentrated twice, original culture-filtrate and half diluted culture-filtrate give disease mean scores of 2.67, 3.0, and 3.5 that were significantly different at $p < 0.05$ (Table 15, Appendix 14). It is noticeable that at this stage, sorghum anthracnose development was suppressed to a greater level when the culture-filtrate concentrated twice was used compared to the other culture-filtrate treatments. This trend was also observed in the third week of disease severity assessment. The +ve control whose disease severity value increased to 6.17 was significantly different from the water control that gave 4.67. It is worth noting that all the treatments in the week except for the double concentrated culture-filtrate had significantly higher disease severity scores than the standard chemical Folicur®430SC whose score was 2.33 (Table 15, Appendix 16).

In the fifth week of scoring, most of the leaves in the +ve control had a mean disease score of 7.17. This was significantly higher than the other treatments at $p < 0.05$. However the original culture-filtrate and culture-filtrate concentrated twice had disease mean scores of 3.17 and 2.83 respectively which were significantly different. Folicur®430SC had a mean score of 2.33 that was significantly lower from those scored for original strength and double concentrated culture-filtrates (Table 15, Appendix 14). This notwithstanding, the culture filtrate concentrated twice remained consistent in suppressing the development of sorghum anthracnose on plants from the onset and compared well with Folicur®430SC from the third week to week 5.

Plates 22 to 25 show the effect of different treatments on anthracnose infected leaves over the treatment period of 5 weeks.

Plate 22: **Anthracnose disease progress on a tagged sorghum leaf after treatment with *Aeromonas hydrophila* KaI245 double concentration antibiotic-culture-filtrate. A; Sorghum leaf at the end of week 1. B; Sorghum leaf at the end of week 2. C; Sorghum leaf at the end of week 3. D; Sorghum leaf at the end of week 4. E; Sorghum leaf at the end of week 5. Source: (Author) 2016.**
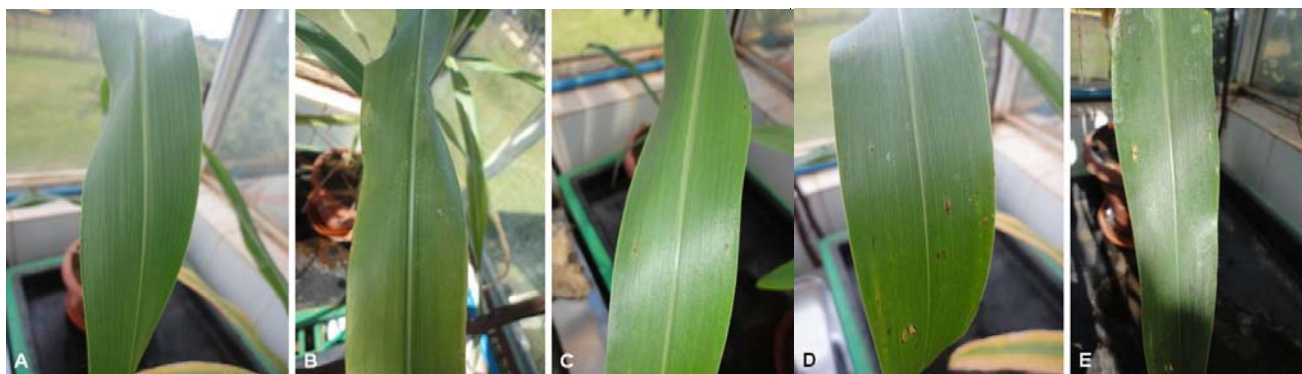
Plate 23: **Anthracnose disease progress on a tagged sorghum leaf after treatment with Folicur®430SC (0.1%)— (Chemical control). A; Sorghum leaf at the end of week 1. B; Sorghum leaf at the end of week 2. C; Sorghum leaf at the end of week 3. D; Sorghum leaf at the end of week 4. E; Sorghum leaf at the end of week 5. Source: (Author) 2016.**
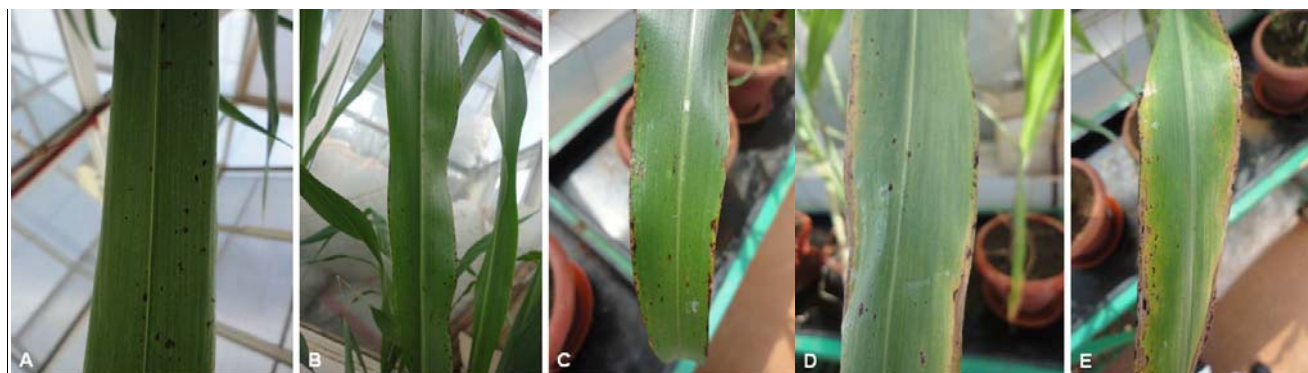
Plate 24: **Anthracnose disease progress on a tagged sorghum leaf after treatment with *Colletotrichum sublineolum* conidial suspension—(+ve control). A; Sorghum leaf at the end of week 1. B; Sorghum leaf at the end of week 2. C; Sorghum leaf at the end of week 3. D; Sorghum leaf at the end of week 4. E; Sorghum leaf at the end of week 5. Source: (Author) 2016.**

Plate 25: **Non-treated tagged sorghum leaf, A; At the end of week 1. B; At the end of week 2. C; At the end of week 3. D; At the end of week 4. E; At the end of week 5. Source: (Author) 2016.**

Table 15: **Mean sorghum anthracnose disease scores against different treatments under greenhouse conditions**

	Treatment mean anthracnose disease scores							
	A	**B**	**C**	**D**	**E**	**F**	**G**	**H**
Week 1	1.5^b	1.6667^b	2.5^c	1.6667^b	2.3333^c	1.1667^a	2.3333^c	2.1667^b
Week 2	2.0^d	2.5^f	3.3333^g	2.1667^e	2.8333^f	1.6667^d	3.6667^g	2.8333^f
Week 3	2.6667^i	3.0^i	3.5^j	2.3333^i	3.1667^i	2.1667^h	4.8333^k	3.3333^i
Week 4	3.0^n	3.5^o	4.0^o	2.6667^m	3.8333^o	2.3333^l	6.1667^q	4.6667^p
Week 5	3.1667^t	3.5^t	4.1667^u	2.8333^s	4.3333^u	2.3333^r	7.1667^w	6.1667^v

Mean sorghum anthracnose scores per week for the various treatments followed by different letters are honestly significantly different (HSD) at $p < 0.05$. Significant differences obtained by Tukey-Kramer Multiple-Comparison Test.

A: Original antibiotic-culture-filtrate.
B: Half concentrated antibiotic-culture-filtrate.
C: Quarter concentrated antibiotic-culture-filtrate.
D: Double concentrated antibiotic-culture-filtrate.
E: Biofungicide Ballad®Plus (0.2%)
F: Chemical fungicide Folicur®430SC (0.1%)
G: *C. sublineolum* spore suspension only (Done as +ve control)
H: Sterile distilled water only (Done as –ve control treatment)

The disease severity scores from Table 15 indicate that the double concentration of *Aeromonas hydrophila* KaI245 antibiotic-culture-filtrate compared very well with the chemical treatment (Folicur®430SC) in controlling sorghum anthracnose *in vivo* vis á vis the other concentrations whose performances were dismal. This is reflected in the values recorded of 2.8333 and 2.3333 respectively at the end of the fifth week, albeit they were

significantly different at $p < 0.05$. This observation renders the double concentration of *Aeromonas hydrophila* KaI245 antibiotic-culture-filtrate the optimal concentration for the control of sorghum anthracnose. The dose response curves plotted in Figure 3 below confirms this assertion where the double concentration curve is well above the other curves. However, it should be noted that there is a degree of anthracnose suppression by all the concentrations. In the first week of experimentation, there was greater manifestation of anthracnose symptoms for plants treated with quarter concentration antibiotic-culture-filtrate compared to the +ve control which was treated with *C. sublineolum* suspension only hence the negative value.
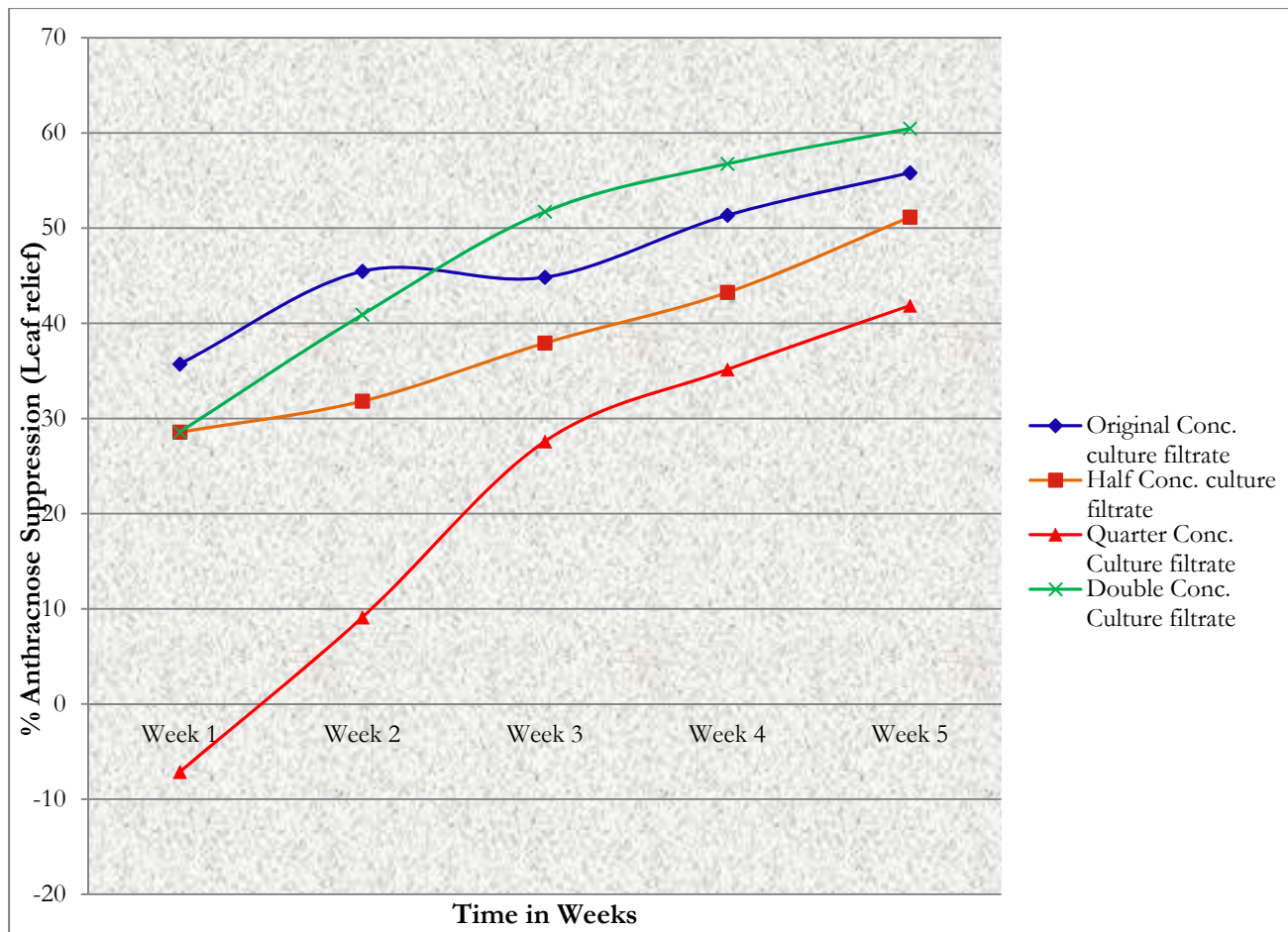
Figure 3: % Anthracnose disease suppression on sorghum leaves treated with different concentrations of *Aeromonas hydrophila* KaI245 antibiotic-culture-filtrate against time. The negative value in the quater concentration curve is attributed to greater anthracnose symptoms compared to the +ve control. Source: (Author) 2016.

Suppression of anthracnose was also dipicted when disease scores were plotted against time. The lower the disease devlopment curve, the better the treatment in the management of sorghum anthracnose. The double concentrated culture-filtrate's curve lay very close to the chemical control curve, an indication that it was the best biological control treatment vis á vis the other treatments (Appendix 17—22).

4.11 Phytotoxicity of *Aeromonas hydrophila* KaI245 antibiotic-culture-filtrate

Phyototoxicity was evident on sorghum plants treated with *Aeromonas hydrophila* KaI245 antibiotic-culture-filtrate. The degree of phytotoxicity varied with antibiotic concentration levels. The manifestation of phytotoxicity on the treated leaves was systematic and gradual. Full manifestation on the foliage was achieved on the 3rd day after antibiotic treatment. At the beginning, some of the affected leaves wilted at the tips and with time dried off but a majority recovered. Some leaves expressed phytotoxicity as cream spots of dead necrotic tissues on the edges. With time, the green surfaces of affected leaves changed into yellow (Plate 26). Some of the tagged leaves were affected but a good number were among those that recovered at least after another 13 days. Scoring for disease severity on the affected leaves was done in comparison to those that did not get affected to distinguish between phytotoxic effects and sorghum anthracnose symptoms. These manifestations were elaborate on plants treated with the culture filtrate with a double concentration. The other three concentrations (original diluted to half concentration and diluted to a quater concntration) did not show visible signs of phytotoxicity. Non-sprayed plants remained healthy (Plate 27).
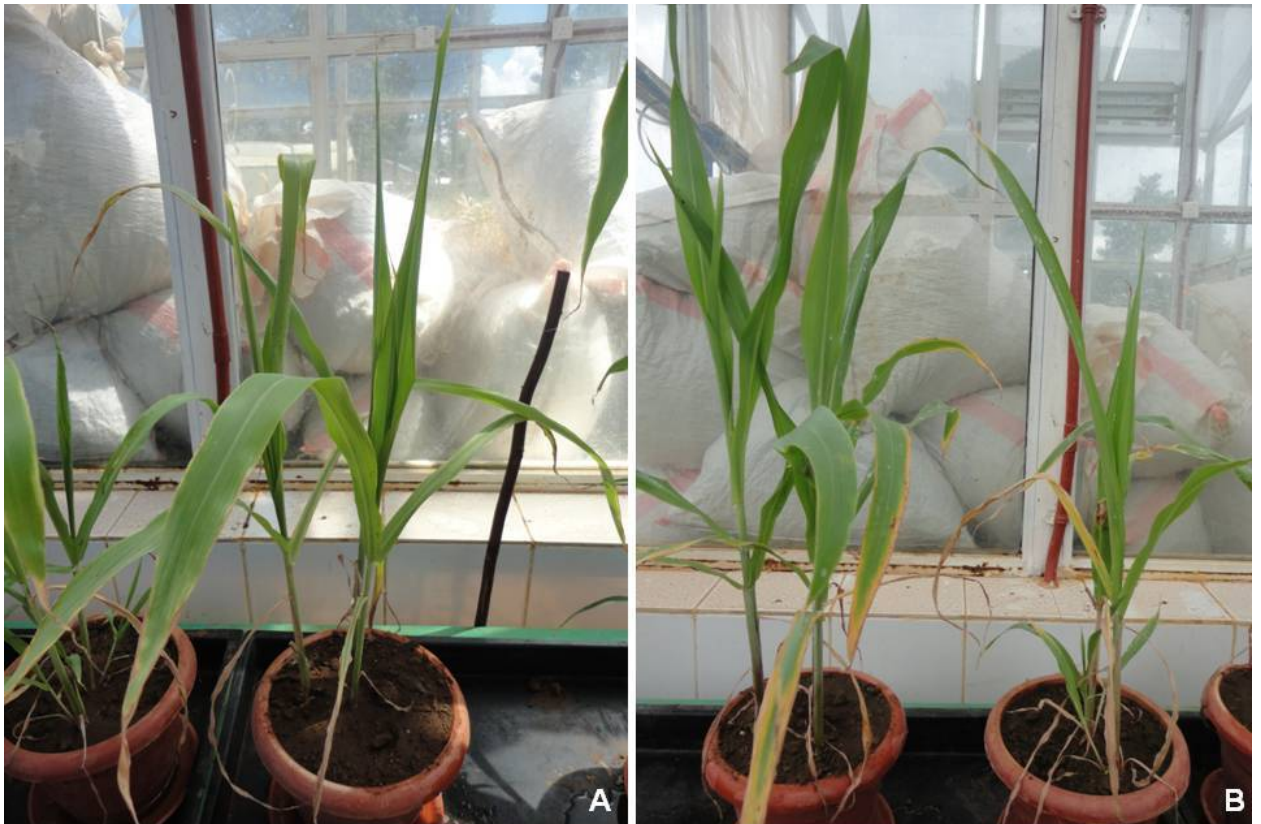
Plate 26: A; Sorghum plants exhibiting the phytotoxic effect of the double concentrated *Aeromonas hydrophila* KaI245 antibiotic-culture-filtrate. B; Recovery of the same sorghum plants 13 days later. Source: (Author) 2016.

Plate 27: Non-sprayed sorghum plants at: A; The end of week 2. B; The end of week 5. Source: (Author) 2016.

CHAPTER FIVE

DISCUSSIONS

5.1. Soil particle size analysis, chemical composition and their influence on sorghum rhizobacteria

The present study, like many other studies revealed that rhizosphere soil is a habitat of a host of bacteria, some of which are beneficial such as antimicrobial agents producers. By virtue of the fact that bacterial isolates obtained in this study were from the sorghum rhizosphere, it points to what has already been established that certain bacteria tend to congregate in the soil immediately adjacent to plant roots (the rhizosphere), where they may feed off the sugars that plant roots exude and many are able to attach to root surfaces and to Arbuscular Mycorrhizas (AM) and other fungal hyphae (Teri *et al.,* 2010).

Within this group of bacteria, there are those that are able to provide several benefits (affecting the host plant either directly or indirectly) including: nitrogen fixation, stimulation of root development (due to phytohormone production), solubilization of soil phosphates, defence of plants from soil borne pathogens and improving host tolerance to abiotic stresses. This constitutes the biocontrol PGPB (Bashan and Holguin, 1998). This group is taxonomically diverse including assorted groups of Gram negative bacteria (e.g., *Acinetobacter, Aeromonas, Alcaligenes, Azoarcus, Azospirillium, Azotobacter, Beijerinckia, Burkholderia, Enterobacter, Erwinia, Flavobacterium, Gluconacetobacter, Klebsiella, Pseudomonas, Serratia, Rhizobium*), Gram-positive bacteria (*Arthrobacter, Bacillus* and new genus *Paenibacillus, Clostridium, Streptomyces*) and even some archaea (Burdman *et al.,* 2000; Sudhakar *et al.,* 2000; Hamaoui *et al.* 2001; Bertrand *et al.* 2001; Lugtenberg *et al.* 2001; Mirza *et al.* 2001; Bonaterra *et al.,* 2003; Esitken *et al.,* 2003; Murphy *et al.,* 2003; Raj *et al.,* 2004; Joo *et al.,* 2004; Esitken *et al.,* 2006; Podile and Kishore 2006; Saleem *et al.,* 2007).

This was attested to when an *Aeromonas* sp. was isolated from Kanduyi in the LM2 agro-ecological zone and a *Bacillus* sp., isolated from Mayanja in an LM3 agro-ecological zone, all originating from Bungoma County that are very potent antagonists towards sorghum foliar pathogens tested in this study. Besides, data generated from the study also

indicates that there is an abundance of Gram-positive bacteria vis á vis Gram-negative bacteria in sorghum rhizosphere soil. This is in tandem with other studies such as the one conducted by Bossio *et al.* (2005) while analyzing soil microbial community responses to land use change in an agricultural landscape of western Kenya. Their study reported that agricultural soils have higher proportions of actinomycetes and Gram-positive bacteria than Gram-negative bacteria.

From all the sites that were sampled in this study, the Sega site of Siaya County in an LM1 agro-ecological zone produced the highest number of beneficial rhizobacteria— twenty isolates from twelve soil samples giving an approximate average of 2 rhizobacterial isolates per soil sample. This could be attributed to its condusive pH of 5.29 that supports bacterial activity. According to Teri *et al.* (2010), the soil community and its habitat are strongly influenced by soil acidity or alkalinity (pH). Bacterial activity is usually optimal within a pH range of 5–9 (Smith and Doran, 1996; Rousk *et al.,* 2009). The site's soil type was an orthic and ferralo-orthic acrisol (Figure 2, Appendix 4). According to Jaetzold *et al.* (2009), these soils are acidic with a low base status, which are strongly leached but less weathered. The base saturation percentage (BSP) of the B horizon is less than 50 %; thus indicating low fertility. This not withstanding, the site's soil fertility seems to be moderate and is reflected in the C:N ratio and phosphorous content that recorded the second highest and third highest from all sites sampled with a value of 14.97 and 7.37545mg/kg respectively (Appendix 5). A C:N ratio below 15 and near 10 is indicative that decomposition is complete and there is net mineralization with nutrients available to the plants/crops (Inbar *et al.,* 1990; UoH, 2015). Soil microorganisms themselves have a C:N ratio near 8:1. In this case, a C:N ratio of 14.97 indicates that there is still an amount of mineralized nutrients in the soil that can be utilized by the microorganisms for respiration.

Sorghum plants were sampled at a stage when they were maturing towards the end of the third month or early in the third month after planting, suggesting that a considerable amount of added organic and inorganic matter to the soil in the form of fertilizer had been consumed by the plants leaving the soil barely with any nutrient. The high C:N ratio of

14.97 and a phosphorous content of 8.05 mg/kg at Sega is an indicator of high organic content in the soil, attributed to the soil amendment practices. The site is a farm which over the years has been used for sorghum research purposes and it is possible that consistent use of inorganic fertilizers resulted in moderate fertility able to sustain a considerable number of beneficial sorghum rhizobacteria. The high number of rhizobacterial isolates could also be attributed to the number of soil samples analyzed. However, there is a consistency of number isolated with soil nutrition.

A combined 16 rhizobacterial isolates from 8 soil samples translating to 2 isolates per soil sample were obtained from Alupe and Burumba sites of Busia County whose soil type is orthic acrisol with orthic ferralsol. Orthic acrisols are acidic soils with a low base status and is usually strongly leached. The base saturation (BSP) of the B horizon of the soil is less than 50 %, indicating low fertility (Jaetzold *et al.,* 2005). Soil fertility of orthic ferralsols is low to very low due to low mineral contents, kaolinites (as clay minerals) and a low Cation Exchange Capacity (CEC) of less than 16 me/100 g of clay. The two sites had C:N values of 10.7 and 10.46 respectively (Appendix 5) indicating that minerals were about to be depleted. The dystric planosol, dystric and vertic gleysol and pellic vertisol of Bugeng'i site are imperfectly to poorly drained and do not seem to be a promising habitat of sorghum rhizobacteria according to this study. The two soil samples from this site only yielded 3 rhizobacterial isolates. This is as well reflected in the C:N value of 7.62 (Appendix 5) that could just be the C:N of the rhizobacteria themselves. There are obviously no nutrients in the soil.

The Sega soil was able to support a good number of sorghum rhizobacteria on account of its fertility compared to the soil collected in Busia County all found within the confines of the same agro-ecological zone.

The veto-eutric planosol with chromic vertisol and solodic planosol soil type of Kibos site of Kisumu County in the LM2 agro-ecological zone produced two antagonistic rhizobacterial isolates from 8 soil samples. This soil is also commonly referred to as "black cotton soil". It is described as dark montmorillonite-rich, poorly drained cracking clays of the bottom-lands with peloturbation processes. The clay content is higher than 30 % and usually contain high amounts of $CaCO_3$ and other minerals with a high CEC due

to the montmorillonitic clay minerals (Jaetzold *et al.,* 2009). The site had a C:N ratio and phosphorous content of 12.94 and 8.05195 respectively (Figure 2, Appendix 5), all indicative of available nutrition to the resident bacteria. The pH of the site was also conducive with a value of 5.16 that favours bacterial growth. All these conditions notwithstanding, the site had very low numbers of rhizobacteria.

Busende sampling site yielded two antagonistic rhizobacterial isolates from two soil samples that are classified as eutric gleysols and pellic vertisols. The one soil sample from Busire—Siriwo produced five antagonistic isolates (Appendices 1 and 2). The site's soil is a chromic and orthic acrisol, rhodic ferralsol and dystric nitisol. Orthic acrisols as described above are acid soils with a low base status and are usually strongly leached. Chromic acrisols contain high chroma. Their base saturation (BSP) of the B horizon of these soils is less than 50 %; thus indicating low fertility (Jaetzold *et al.,* 2005). The soil pH of 5.21 (Appendix 4) recorded in this site is conducive for bacterial growth (Teri *et al.,* 2010). However, the C:N ratio of 6.86 is not indicative of a soil that can sustain bacterial growth (Inbar *et al.,* 1990; UoH, 2015). It is possible that most of the fertilizer applied early in the growth of sorghum had been used up by the crop. However, this site produced an average of 5 rhizobacterial isolates per soil. The soil, inspite of the nutritional content, is a conducive habitat for sorghum rhizobacteria.

The Akiriamet site yielded five rhizobacterial isolates from 2 soil samples; approximately 3 rhizobacterial isolates per soil sample (Appendice 1 and 2) whose soil classification is humic acrisol (Figure 2, Appendix 4). This soil besides its acidic nature, has an umbric A horizon, rich in humic substances. The C:N ratio of this site was 8.99 with a phosphorous content of 0.8999; attributes of the low nutritive value of the soil. However, due to the presence of humic substances, the soil was able to support a considerable number of rhizobacteria as stated above.

Kanduyi site produced two antagonistic rhizobacteria from two soil samples, one of which was among the best antagonist yielded in the entire study (Appendices 1 and 2). The acidic orthic acrisol soil with humic acrisols and ferralic arenosols gave a C:N ratio of 12.62 with a phosphorous content of 0.2737 (Figure 2, Appendices 4 and 5). Ferralic arenosols have high sesquioxide contents. Sustainability of the rhizobacteria could be

attributted to the level of high organic matter content reflected in the C:N value that was still available in the soil.

The LM3 agro-ecological zone yielded a total of twenty antagonistic bacterial isolates. A combined four soil samples from Angurai and Awaat sampling sites within Busia County produced nineteen (19) antagonistic isolates which translates to 5 rhizobacterial isolates per soil sample (Appendices 1 and 2). The soil class found in these sampling sites are ferralo-orthic acrisols (Figure 2, Appendix 4) that are acidic with a low base status and are usually strongly leached. They also posses ferralic characteristics which imply that they have oxic horizons and their soil fertility is low to very low due to low mineral contents, kaolinites (as clay minerals) and a low Cation Exchange Capacity (CEC) of less than 16 me/100 g of clay (Jaetzold *et al.,* 2005). The C:N ratios of these sites were 8.01 and 7.68 with phosphorous contents of 0.656 and 0.896 respectively (Appendix 5), attributes of the low nutritive value of the soil. An average of five rhizobacterial isolates were obtained from each soil sample. These sites were found within Busia County.

The Mayanja site yielded one rhizobacterial isolate from 2 soil samples (Appendices 1 and 2) whose soil classification is orthic acrisol (Figure 2, Appendix 4). This soil's acidic nature (5.9), and low fertility with a C:N ratio of 9.75 and a phosphorous content of 0.8042 (Appendix 5), are indicative of the low nutritive value of the soil. However, the site produced the second best antagonistic rhizobacterial isolate.

The LM4 agro-ecological zone's site at Ureje in Siaya County (Appendix 2) yielded seven rhizobacterial isolate from four soil samples (Appendix 1). The site's soil is a chromic luvisol and ferralo-chromic acrisol. Luvisols are strongly leached soils that have argillic B horizons with a relatively high base status and BSP of more than 50 %. They also have chromic material while ferralo-chromic acrisols are acrisols that bear ferralic and chromic charcteristics described above (Jaetzold *et al.,* 2009). The C:N ratio of this site was 8.67 with a phosphorous content of 1.52915 (Appendix 5), attributes of the low nutritive value of the soil. An average of two rhizobacterial isolates were obtained from each soil sample.

The LM5 agro-ecological zone's site at Sigor within West Pokot County yielded eighteen antagonistic rhizobacterial isolates from eleven soil samples (Appendices 1 and 2). The soil class in this site is a chromic luvisol, rhodic ferralsol and luvic to ferralic arenosol (Jaetzold *et al.,* 2011). The soil pH in these sites was very conducive to bacterial growth and survival (Appendix 5). However, the C:N ratio fluctuated between the Sigor sites, the most favourable being 16.47 recorded at Sigor II. Sigor III failed to produce any antagonistic rhizobacterial isolate. It is possible that the soil type in this site does not favour the proliferation of bacterial growth. This is in spite of known conducive conditions of pH and C:N ratio for bacterial growth recorded at the site.

The five soil samples collected in the UM4 agro-ecological zone at Miti—Majambazi and Misemwa sites of Trans Nzoia County yielded only one antagonic rhizobacterial isolate (Appendices 1 and 2). Soil from the sampling sites is rhodic ferralsols (Jaetzold *et al.* 2011) that seem not to have supported beneficial rhizobacteria.

There seems to be no particular pattern of any relationship between antagonistic rhizobacterial isolates obtained from different agro-ecological zones with corresponding soil content characteristics as revealed this study. Nonetheless, soil of the acrisol type consistently produced considerable numbers of antagonistic rhizobacterial isolates regardless of prevailing soil conditions. It should be noted however that soil known to have high nutritive value can sustain high numbers of bacterial populations including beneficial ones regardless of soil type (Inbar *et al.,* 1990; UoH, 2015).

5.2. Isolation and identification of sorghum foliar pathogens

Sorghum fungal pathogens were isolated using basic techniques. Identification was based on cultural and morphological characteristics of pure cultures of the pathogen isolates, by visual, microscopic examinations of conidiogenous structures and synoptic descriptions or keys developed by Olive *et al.* (1946); Ellis and Holiday (1971); Chidambaram *et al.* (1973); Sivanesan (1987); Al-Doory and Domson (1984); Ahmed and Ravinder (1993); Navi *et al.* (1999); Manuat *et al.* (2001); Mims and Vaillancourt (2002); Timmer *et al.* (2003); Souza-Paccola *et al.* (2003a); Crouch *et al.* (2006); Zakaria *et al.* (2009);

Chowdappa *et al.* (2012); Gautam (2013), Gautam (2014) and Abass and Mohammed (2014).

Symptomatology informed what piece of the diseased sorghum plants were to be inoculated on fresh isolation medium. The resultant fungal growth on media was examined and formed the cultural characteristics of the fungal isolate. There were fungal isolates that originated from the surface of sorghum leaves that looked diseased yet the symptoms observed were not necessarily associated with them. They included *Aspergillus candidus*, *Alternaria longissima*, *Botrytis cinerea* and *Fusarium equiseti*. This observation could be attributed to the presence of the fungus on seeds prior to planting and therefore generally present on the plant (Navi *et al.,* 1999). Nonetheless, for the rest of the fungal isolates, identification was based on disease symptoms, morphological characters and dimensions of reproductive structures including conidia, conidiophores, acervuli, sporodochia and other structures such as appresoria.

An earlier study done by Ngugi *et al.* (2002) had indicated that the most prevalent sorghum fungal phytopathogens in Western Kenya in decreasing order of prevalence were oval leaf spot (*Ramulispora sorghicola*), rust (*Puccinia purpurea*), ladder leaf spot (*Cercospora fusimaculans*), zonate leaf spot (*Gloeocercospora sorghi*), gray leaf spot (*Cercospora sorghi*), leaf blight (*Exserohilum turcicum*), and anthracnose (*Colletotrichum sublineolum*). The Ngugi *et al.* (2002) study was conducted in the month of July in the years 1995 and 1996. The observation made by the current study is that sorghum anthracnose caused by *Colletotrichum sublineolum* was the most prevalent foliar disease in the Western Kenya sorghum growing belt. It is probable that the pathogen has built itself over time. A repeat of the Ngugi *et al.* (2002) study needs to be carried out to ascertain sorghum foliar pathogen prevalence in Western Kenya. The resulting information will be very important in understanding dynamics of pathogens (epiphytotics) and putting in place viable management strategies including biological control using antimicrobial agents from *Aeromonas hydrophila* KaI245.

5.3. Antimicrobial activity of sorghum rhizobacterial isolates against sorghum foliar pathogens

The use of microorganisms for biocontrol has become an effective alternative to the control of plant pathogens using agrochemicals. There are many examples of bacterial or fungal strains formulations with biocontrol applications. Among them are biocontrol PGPB which recently are increasingly and extensively being used in biological control of fungal plant diseases (Altindag *et al.,* 2006; Lourenco *et al.,* 2006; Saravanakumar *et al.,* 2007; Akgul and Mirik 2008; Sang *et al.,* 2008; Dutta *et al.,* 2008).

The aim of this study was to isolate, screen and select rhizobacteria inoquous to sorghum plants with antagonistic activity against *Colletotrichum sublineolum* associated with anthracnose. Bacterial isolates were screened *in vitro* for their biocontrol activity against *C. sublineolum* and the effectiveness of their extracted metabolites determined *in vivo* against sorghum anthracnose. The isolates were obtained from the rhizosphere of sorghum plants from major growing fields in Western Kenya. This approach provided an opportunity to select effective biocontrol strains capable of antagonizing foliar sorghum pathogens that can be used commercially (Landa *et al.,* 1997).

In vitro assays are known to have certain limitations in that the biocontrol efficiencies may not be equally expressed under gnotobiotic (axenic) and *in vivo* conditions (Inam-ul-Haq *et al.,* 2003). However, the *in vitro* assays conducted in this study were used to screen and select potential biocontrol agents and subsequently test their ability to suppress anthracnose of sorghum under greenhouse conditions. In the present study, 101 sorghum rhizobacterial isolates, representing 34.35% of the total isolates tested demonstrated antagonism against four or more of the ten sorghum foliar fungal pathogens. Out of the antagonistic isolates, 35 inhibited at least one phytopathogenic growth *in vitro* by ≥ 70%. Production of bioactive compounds, most probably antibiotics demonstrated by the presence of clear zones of inhibition, was clearly expressed by 2 isolates grown on PDA medium using the spot inoculation method described by Loeffler *et al.* (1986). The two sorghum rhizobacterial isolates labeled KaI245 (*Aeromonas hydrophila*) and MaI254 (*Bacillus megaterium*) significantly reduced colony diameters of

more than half of the test fungal pathogens thus demonstrating a broad spectrum of activity. Further screening tests including one modelled on that of Besson *et al.* (1978) showed that *Aeromonas hydrophila* KaI245, exhibited more potency in pathogen colony size reduction compared to *Bacillus megaterium* MaI254. Microbial antagonism has similarly been reported by Walker and Abraham (1970); Broadbent *et al.* (1971); Kugler *et al.* (1990); Földes *et al.* (2000); Giuliano *et al.* (2002); Tendulkar *et al.* (2007) and Idris *et al.* (2007).

In the presence of the antagonistic sorghum rhizobacterial isolates KaI245 and MaI254, growth of *Botrytis cinerea* (the causal agent of grey mould disease), *Nigrospora oryzae* (the causal agent of ear rot) and *Colletotrichum sublineolum* (the causal agent for anthracnose) were considerably reduced. However, growth of *Fusarium moniliforme* (the causal agent of stalk rot), *Exserohilum turcicum* (the causal agent of leaf blight) and *Fusarium equiseti* (associated with *Fusarium moniliforme*; pathogenicity still questionable) were moderately sensitive. Variation in colony size reduction of the test fungal pathogens signified different levels of sensitivity to the antibiotic produced by the *Aeromonas* sp. and *Bacillus* sp. strains. Such differences have similarly been noted by other workers (Marrone *et al.,* 1998; Mette *et al.,* 1998; Giuliano *et al.,* 2002; Idris *et al.,* 2007).

The difference in sensitivity of the pathogens has been attributed to differences in cell wall composition of the plant pathogens and the type of propagules produced by the pathogen. *Fusarium, Alternaria* and *Colletotrichum* have chitin in their cell walls that has antimicrobial activity against both Gram-positive and Gram-negative bacteria likely impeding bacterial activity (Roberts, 1992; Kumirska *et al.,* 2011). *Fusarium* forms chlamydospores with thick cell walls, which are tolerant to chemicals, antibiotics and adverse environmental conditions (Campbell, 1989; Agrios, 2005). It has been reported that the mere presence of antibiotic producing bacteria within the vicinity increases the number of chlamydospores produced (Venkat, 1952). Differences in sensitivity have also been attributed to different experimental conditions as was reported by Földes *et al.* (2000) while screening *Bacillus* strains isolated from the rhizosphere, for antagonism

against phytopathogenic food-borne pathogenic and spoilage microorganisms. Results obtained in this study suggest that the two rhizobacterial isolates: *Aeromonas hydrophila* KaI245 and *Bacillus megaterium* MaI254 produced broad-spectrum antimicrobial compounds making them potential candidates for use in the biocontrol of sorghum plant fungal diseases of agricultural importance. Determination of the number of antimicrobial agents produced by this study indicated that *Aeromonas hydrophila* KaI245 produced only one antimicrobial agent.

Previous studies have demonstrated antimicrobial activity against phytopathogenic microorganisms by secondary metabolites produced by certain biocontrol PGPB of the genus *Bacillus* (Katz and Demain, 1977; Shoji, 1978; Smirnov *et al.,* 1986; Sharga, 1997; Földes *et al.,* 2000; Tendulkar *et al.,* 2007 and Idris *et al.,* 2007). In a recent study, Akgul and Mirik (2008) also reported that *Bacillus megaterium* strains could be used for biocontrol of *Phytophthora capsici*. In this light, there are many known examples of *Bacillus* antibiotic formulations with biocontrol applications (Giuliano *et al.,* 2002).

Besides *Bacillus* strains, antimicrobial activity against phytopathogenic microoganisms by other species of biocontrol PGPB have been documented. Tripathi (1999) reported the control of anthracnose of soybean caused by *Colletotrichum dematium* and sheath blight of maize caused by *Rhizoctonia solani* under *in vitro* and field conditions by fluorescent Pseudomonads. Altindag *et al.* (2006) suggested that *Burkholderia gladii* OSU 7 has the potential to be used as biopesticide for effective management of brown rot disease on apricot. In a recent study, Sang *et al.* (2008) reported that *Pseudomonas corrugata* (CCR04 and CCR80), *Chryseobacterium indologenes* (ISE14), and *Flavobacterium* sp. (GSE09) showed consistently good control efficacy against *Phytophthora capsici* the causal agent of Phytophthora blight. The combination of *Pseudomonas* strains Pf1, TDK1, and PY15 was more effective in reducing sheath rot (*Sarocladium oryzae*) disease in rice plants compared with individual strains under glasshouse and field conditions (Saravanakumar *et al.,* 2009). Hernandez-Rodriguez *et al.* (2008) indicated that *Burkholderia* sp. MBf21, MBp1, MBf15, and *P. fluorescens* MPp4 stood out for their plant growth stimulation in maize and for the biological control exerted on *Fusarium*

verticillioides M1. The strains *Burkholderia* sp. MBf21 and MBf15 showed the best results in disease suppression, which was achieved up to 80%. This study produced an *Aeromonas* sp., a Gram negative non-*Bacillus* biocontrol PGPB from the sorghum rhizobacteria which is very potent and can be formulated and used for biocontrol purposes against sorghum foliar diseases.

Members of the genus *Aeromonas* occupy a very important ecological niche in plant rhizospheres. The genus is known to; a) produce various growth promoting substances such as growth hormones, b) produce antimicrobial substances that help suppress soil-borne phytopathogenic microorganisms c) solubilize phosphorous and make it available to the plants and d) have an ability to produce enzymes that are antifungal such as chitinases (Ryley *et al.,* 1981; Inbar and Chet, 1991; del Castillo *et al.,* 2015). An *Aeromonas* sp. strain W-10 was shown to produce a mixture of up to six antifungal antibiotics from which two were isolated and identified to be cyclic octapeptides. One of the antibiotics labelled Antibiotic 20562, was active *in vitro* against *Candida albicans* at 0.075 μg/ml (Ryley *et al.,* 1981). The fungistatic activity of *Aeromonas* sp. against phytopathogenic fungi that has been reported such as the protection to cotton plants against *Rhizoctonia solani* and *Fusarium oxysporum* f.sp. *vasinfectum* was mainly attributed to chitinase production by the bacterium (Inbar and Chet, 1991). *Aeromonas* isolates have also been shown to produce growth promoting hormones such as 1-aminocyclopropane-1-caboxylate (ACC) and Indole-3-acetic acid (IAA) and at the same time solubilize phosphates (del Castillo *et al.,* 2015). To date, *Aeromonas,* among many other different bacterial genera including *Alcaligenes, Bacillus, Pseudomonas* and *Rhizobium* have shown to be capable of producing hydrogen cyanide (HCN) (Devi *et al.* 2007; Ahmad *et al.* 2008). HCN is a volatile, secondary metabolite that suppresses the development of microorganisms and that also affects negatively the growth and development of plants (Siddiqui *et al.,* 2006). HCN is a powerful inhibitor of many metal enzymes, especially copper containing cytochrome C oxidases. HCN is formed from glycine through the action of HCN synthetase enzyme, which is associated with the plasma membrane of certain rhizobacteria (Blumer and Haas, 2000). The present study

therefore present an *Aeromonas* sp. that produces very potent antifungal substances that can be used as a foliar applicant for biocontrol purposes.

5.4. Antibiotic production by fermentation in shaker-flasks

Shaker-flasks are important in fermentation development and analysis. Medium development is generally initiated at the level of the shaker-flask where compounds with exorbitant prices and which are usually not locally available are added in small quantities. Knight (1988) observed that results obtained from shake-flasks are used in construction and elaboration of sophisticated liquid fermentation facilities by industries.

Modified Farhana *et al.* (2011) liquid medium was used in this study for the production of antibiotics from the sorghum rhizobacterial isolate labeled KaI245. The medium comprised of: fructose, infusion from potatoes, $(NH_4)_2SO_4$, yeast extract, KH_2PO_4, $MgSO_4.7H_2O$, NaCl and $CaCO_3$ in 1000 ml of distilled water and pH adjusted to 7.0. Johnson *et al.* (1959) and Stowell *et al.* (1986) reported that the complex medium used for production of the antibiotic substances usually contain a source of nitrogen, several salts and certain supplementary materials such as yeast extract. The source of carbon is usually carbohydrates added in the form of glucose, mannitol, lactose, fructose, sucrose, starch etc. In this study, fructose was used as the carbon source. Fructose as a carbon source has been reported to increase the quality of antibiotics produced as opposed to glucose and glycerol by *Malikia spinosa*—a rhizosphere inhabiting bacterium—when tested against the plant pathogens; *Fusarium oxysporum* and *Colletotrichum gloeosporioides (*Farhana *et al.,* 2011).

Catabolite repression is a regulatory mechanism by which the cell coordinates the metabolism of the carbon and energy source to maximize the efficiency of nutrient utilization and control other metabolic processes. It is a well-documented fact that glucose promotes repression of the enzymes involved in the catabolism of the carbohydrates metabolites at lower rates. As a result, a hierarchy of utilization of carbon and energy sources is established. According to this, the synthesis of secondary metabolites is subjected to catabolite repression by glucose, either directly or indirectly

(Fischer and Sonenshein, 1991). Calcium carbonate ($CaCO_3$) was added to provide Ca^{2+} that increase cell wall permeability of *Aeromonas hydrophila* KaI245 to excrete the antibiotics (Petit-Glatron *et al.,* 1993).

The culture filtrate obtained by centrifuging the culture broth was variably inhibitory to *Alternaria alternata, Aspergillus candidus, Alternaria longissima, Botrytis cinerea, Colletotrichum gloeosporioides, Colletotrichum sublineolum, Exserohilum turcicum, Fusarium equiseti, Fusarium moniliforme* and *Nigrospora oryzae in vitro*. The size of inhibition zones produced was observed to depend on the pathogen tested. Variation of clear zones of inhibition among pathogens indicated sensitivity of the test pathogen to the antibiotics and the selectivity of the antibiotics produced. Several workers have reported such results from Gram negative rhizosphere dwelling bacteria. Howell and Stipanovic (1980), working with the culture filtrate of a *Pseudomonas florescens* strain Pf-5 in controlling *Pythium ultimum*, reported similar results. They reported that the bacterium produced pyoluteorin which was selective in its action. Homma *et al.* (1989) while investigating the production of antibiotics by *Burkholderia cepacia* as an agent for biological control of soilborne plant pathogens reported that the bacterium produced two antibiotics that had different effects on *Rhizoctonia solani*, the causal agent of damping-off in rice and *Pyricularia oryzae*, the causal agent of rice blast. The antibiotic pyrrolnitrin was more effective against *R. solani* whilst pseudane effectively suppressed *P. oryzae*. Phenazine antibiotics produced by *Pseudomonas fluorescens* strains 2-79 and 30-84 supressed the take-all-disease in wheat caused by *Gaeumannomyces graminis* var. *tritici* (Thomashow *et al.,* 1990). Shanahan *et al.* (1992) was able to show that the antibiotic 2,4-diacetylphloroglucinol (2,4-DAPG) produced by *Pseudomonas fluorescens* strain F113 inhibited the growth of a number of *Pythium* spp. that cause the damping off disease in a variety of crops. Actinomycetes isolates 14P, 28P, CS32 and CS35 culture filtrates were reported by Muiru (2000) to produce variable inhibition zones when tested against four filamentous fungi: *Pythium* sp*., Alternaria sesami, Fusarium oxysporum* f.sp. *phaseoli* and *Colletotrichum kahawae*.

During fermentation, the shaker flasks were incubated at 28°C on a rotary shaker at 180 rpm for 7 days. It was observed that the antibiotic-culture-filtrate of *Aeromonas hydrophila* KaI245 harvested within the 6th to the 8th day produced the largest clear zones of inhibition against *C. sublineolum* and this represented the optimal harvest time. Clear zones of inhibition were only observed from the 3rd day of incubation. Reports show that the synthesis of peptide antibiotics in liquid medium usually starts at the end of exponential growth; reaching maximum concentration after cell growth has ceased (Bodanzky and Perlman, 1969). It has been suggested that many microorganisms can synthesize antibiotics while in the growth phase (Haavik and Thomassen, 1973; Barr, 1975 and Haavik, 1976), which is in agreement with production kinetics of *Aeromonas hydrophila* KaI245. The factor triggering the onset of antibiotic synthesis is more likely the exhaustion of a limiting nutrient required for cell growth. This limitation usually stimulates differentiation, which, for the case of *Bacilli*, means endospore formation. Sporulation is associated with the synthesis of a new cell wall and the degradation of that in the mother cell. This cell wall synthesis could furnish precursors for antibiotic synthesis (Giuliano *et al.,* 2002). In other cases, antibiotic production is triggered by factors such as environmental conditions, catabolite repression, among others. For instance, the expression of phenazine biosynthetic genes responsible for the production of phenazine antibiotics produced by Pseudomonads is regulated by multiple mechanisms, which are strongly influenced by environmental conditions (Sańchez *et al.* 2010). One of the primary factors governing phenazine production is population density, and in *Pseudomonas aeruginosa*, this dependency is affected by at least three quorum-sensing systems (Van Rij *et al.,* 2004).

Catabolite repression is a type of positive control of transcription, since a regulatory protein affects an increase (up-regulation) in the rate of transcription of an operon (Todar, 2016b). It allows micro-organisms to adapt quickly to a preferred (rapidly metabolisable) carbon and energy source first. This is usually achieved through inhibition of synthesis of enzymes involved in catabolism of carbon sources other than the preferred one (Bernhard *et al.,* 2011).

In several species of *Pseudomonas*, phenazine production is affected by the carbon source. For example, the greatest production of phenazine 1-carboximide was obtained using L-pyroglutamic acid and glucose from *Pseudomonas chloraphis*. However, fructose, sucrose and ribose had negative effects on phenazine 1-carboximide production (Van Rij *et al.,* 2004). Similar results were observed in a mutant of *Pseudomonas* sp. M18G, in which the greatest production of phenazine 1-carboxylic acid was achieved with glucose and ethanol as carbon sources (Li *et al.,* 2008). Stimulation of antibiotics production by *Aeromonas hydrophila* KaI245 could have taken the path of environmental factors or/and catabolite repression.

Mechanical shaker speed, agitation and subculturing have been reported to affect antibiotic production in liquid medium. Hanson *et al.* (1965) while working with *Streptomyces rimosus*, reported that the widest inhibition zones resulted from the use of 7 to 10 day old culture. Loeffler *et al.* (1986) produced fengimycin from *Bacillus subtilis* strain F-29-3 on shake culture at 120 rpm and harvested after 7 days.

The study did not experience fungal or bacterial contamination in the shaker flasks. These results positively relate to observations made by Bruehl *et al.* (1969) that production of antibiotics by biological control agents acts as an aid to substrate possession. He further demonstrated that *Cephalosporium gramnearum* produced a wide spectrum antibiotic that enabled it to retain possession of a substrate for 2 to 3 years, whereas non-antibiotic producing strains of the same organism were overran on the same substrates by saprophytes within a few months.

5.5. Identification of rhizobacterial isolates

From the preliminary biochemical identification, this study revealed that a majority (71.4%) of the sorghum rhizobacterial isolates that were able to inhibit mycelial growth of at least one of the test sorghum fungal pathogens by $\geq$ 70% were members of the family Bacillaceae. A similar report was made by Idris *et al.* (2007) while screening sorghum rhizobacteria for biological control of Fusarium root and crown rot of sorghum in Ethiopia. He reported that 80% of the bacteria isolated and tested belonged to members

of the Genus *Bacillus* with 45% corresponding to *Bacillus cereus*. This indicates that the sorghum rhizosphere is majorly inhabited by members of this family.

Further biochemical characterization revealed that rhizobacterial isolate KaI245 was an *Aeromonas* sp. The API system confirmed the same result and identified the bacterium as *Aeromonas hydrophila*. This is unprecedented for a bacterium of the genus *Aeromonas* to be isolated from the sorghum rhizosphere. Members of the genus *Aeromonas* are predominantly aquatic in fresh water environments. Considerable populations inhabit waste waters, sludge and mud. An appreciable number of species are pathogenic to fresh water fishes, frogs as well as humans (Sakazaki and Balows, 1981; Abbott *et al.,* 2003). A recent review indicates that Aeromonads are essentially ubiquitous in the microbial biosphere. They can be isolated from virtually every environmental niche where bacterial ecosystems exist. These include aquatic habitats, fish, foods, domesticated pets, invertebrate species, birds, ticks and insects, and natural soils, although extensive investigations on the latter subject are lacking (Janda and Abbott, 2010). The lack of investigation on soil habitats notwithstanding, very recent studies indicate that members of the genus *Aeromonas* are rhizospheric and PGPR for that matter (Kaymak, 2010; del Castillo *et al.,* 2015). This is supported by earlier studies such as the one carried out by Mehnaz *et al.* (2001) who found members of the genus *Aeromonas* as part of the beneficial bacteria from the rhizosphere of rice. This study goes to emphasize the ecological niche which members of this genus occupy in the rhizosphere of cultivated plants, in this case, sorghum.

Given that the type species of *A. hydrophila* has predominantly been isolated from aquatic habitats, fish, foods, domesticated pets, invertebrate species and clinical specimens in the past (Sakazaki and Ballows, 1981; Abbott *et al.,* 2003; Janda and Abbott, 2010; Sarkar *et al.,* 2012), it is possible that the *A. hydrophila* strain KaI245 obtained in this study has special characteristics that makes it reside and survive in terrestial rhizosphere soil. It is these characteristics that would make it a little different from the type species. A closer examination could even likely lead to the description of a totally new species. This study therefore strongly recommends an exhaustive descriptive

examination of *A. hydrophila* strain KaI245 using such methods as typing through 16S rDNA—PCR fingerprinting among others that will bring out its distinguishing features.

5.6. Optimization of various environmental conditions for maximum antibiotic production from *Aeromonas hydrophila* KaI245

The purpose of optimizing environmental conditions for antibiotics production is to contribute towards improving the antibiotics production by *Aeromonas hydrophila* KaI245. Integrated into a broader study on the impact of environmental factors on the production of antibiotic, this work should help to build more rational control strategy, possibly involving scale-up of production of antibiotics by *Aeromonas hydrophila* KaI245.

The antibiotic produced by *Aeromonas hydrophila* KaI245 responded differently to the different environmental conditions they were subjected to. Different sizes of clear zones of inhibition produced by the antibiotic-culture-filtrate obtained when different carbon sources, fermentation incubation durations, initial liquid medium pH, incubation temperature conditions and storage conditions attest to the different responses. This showed that the antibiotic production varied in different environmental conditions, an observation that has also been reported by a number of other workers.

With regard to the effect of different carbon sources on the antibiotic production by *Aeromonas hydrophila* KaI245, fructose was found to be the best carbon source for suppressing the growth of *C. sublineolum*. This indicates that different types of carbon sources used in liquid media determine the quality of antimicrobial substances produced. Similar results were obtained by Farhana *et al.* (2011) who found fructose to be the best carbon source in basal medium for the production of antifungal substances from *Malikia spinosa*, a Gram negative bacterium, against *C. gloeosporioides*. They further went on to show that other bacterial isolates preferred other carbon sources for top quality antifungal substance production. They concluded that different types of carbon sources used in liquid media determine the quality of antimicrobial substances and that specific or special types of carbon sources are required to suppress different types of plant pathogens.

Carbon compounds constitute the major requirement for growth of bacteria as they enter in different metabolic processes that yield primary and secondary metabolites including antifungal substances (Gebreel *et al.,* 2008). Findings of this study suggest that secondary metabolite (antibiotics) production from the sorghum *Aeromonas hydrophila* KaI245 was stimulated by slowly assimilating complex carbohydrates from the production media and that it was decreased when more rapidly utilized monosaccharides such as glucose are present (Bertasso *et al.,* 2001). A possible explanation for this phenomenon is that glucose or other carbon sources cause catabolite repression in which the production of the enzyme of secondary metabolite (antibiotics) biosynthesis is inhibited (Drew and Demain, 1977; Iwai and Omura, 1982; Bernhard *et al.,* 2011).

It should be noted that many other factors besides the carbon source *per se* may represent an important role in the process of antifungal production and consequently affect the antagonistic activity of antibiotic producing bacterial species. Among them are: quantity of carbon source in liquid basal medium, quantity/density of microbial cells inoculated into the medium (inoculation volume) and amount of oxygen available to the inoculum (aeration rate/fermentation flask shaking rate). The quantity of the carbon source in the basal liquid medium affects the production of antimicrobial substances against plant pathogenic fungi. Dikin *et al.* (2005) reported that the quantity of lactose in the basal liquid medium affected the production of antimicrobial substances by *Burkholderia cepacia* RB47 and *Microbacterium testaceum* RU7 against *Schizophyllum commune*. Liquid media which were amended with 40g of lactose for the growing of *B. cepacia* RB47 and *M. testaceum* RU7 were found to produce antimicrobial substances thereby causing strong inhibition in the growth of *S. commune* mycelia as compared to the same liquid medium amended with 10g of lactose. It therefore follows that some modifications and improvements need to be done on this study to find out the most suitable quantity of carbon sources in the basal liquid medium under appropriate conditions in order to produce more effective antimicrobial substances against *C. sublineolum.*

Inoculation volume can affect the metabolites accumulation. A lower inoculum density may reduce product formation, whereas a higher inoculum may lead to the poor product

formation, especially the large accumulation of toxic substances and also cause the reduction of dissolved oxygen (Mudgetti, 1986). Wicklow *et al.* (1998) reported that optimal quality of antimicrobial substances was very much affected by the density of bacterial cells of liquid medium. Song *et al.* (2012) while optimizing fermentation conditions for antibiotic production by *Streptomyces felleus* YJ1 observed that the inoculum size and medium capacity appeared to have visible effect on inhibiting the mycelial growth of *Sclerotinia sclerotiorum*, the causal agent of root rot. When the inoculum volume and medium capacity were 5% and 75mL respectively, the inhibition rate reached the maximum (90.92%).

Delrio *et al.* (1972) noted that antibiotic production from *Pseudomonas reptilivora* in liquid medium was completely inhibited without oxygenation. Antibiotic activity against *Staphylococcus aureus*—the test microorganism—only increased as the oxygen flux increased, until a maximum was reached at an oxygen rate of 3 volumes per volume of medium per min. Song *et al.* (2012) observed that antibiotics of *Streptomyces felleus* YJ1 produced the highest growth inhibitory rate against *Sclerotinia sclerotiorum* for the culture filtrate obtained from fermentation flasks agitated at a higher speed of 180 revolutions per min. A higher agitation rate of 220 revolutions per minute was required for fermentation flasks to produce maximum antibiotics from *Xenorhabdus nematophila* using the response surface methodology (Wang *et al.* 2008). During fermentations, agitation is normally used to enhance aeration (dissolved oxygen).

There were obvious visible effects of incubation period on production of antibiotic production from *Aeromonas hydrophila* KaI245. Mycelia inhibition of *C. sublineolum* was observed between the 3rd and the 10th day. The mean maximum zone of inhibition was reached on the 6th day and then decreased slowly suggesting that the optimal incubation period for maximum production of antibiotics from *Aeromonas hydrophila* KaI245 is 6 days. This indicated that incubation period is an important factor that affects fermentation. Prolonged incubation does not necessarily yield more secondary metabolites. It is possible that increased fermentation time may produce more toxins including bacterial waste materials that can inhibit the production of antimicrobial

metabolites. Song *et al.* (2012) made similar observations when an antibiotic producing actinomycete *Streptomyces felleus* YJ1 produced active antibiotic culture filtrates between the 2nd and 4th day of incubating synthetic liquid medium and tested against *Sclerotinia sclerotiorum*. They further observed that the inhibition rate reached the maximum at day 4 and then decreased slowly.

The optimal initial pH range for antibiotic production from *Aeromonas hydrophila* KaI245 was wide and fell between pH 3 and pH 11. However, maximum antifungal activity was recorded at pH 7.0 which turned out to be the optimal pH for antibiotic production. Similar results were made by Song *et al.* (2012) who observed that the antibacterial activity of antibiotics produced by an actinomycete *Streptomyces felleus* YJ1 against *S. sclerotiorum* increased with increasing initial pH from 5.0 to 7.0, but any further increase in its values resulted in decreased antibacterial activity. He attributed this observation to the fact that too high or low initial pH decreased the production of active substance which affected the inhibitory effect. Under acidic conditions it decreased significantly. At an initial pH of 7.0, the maximum inhibitory rate of 90.69% against *S. sclerotiorum* was reached. He concluded that the optimal initial pH was around 7.0, which related to the natural growing environment of the actinomycete under review. Wang *et al.* (2008), using the response surface methodology observed that the maximum antibiotic activity for *Xenorhabdus nematophila* was achieved at the initial pH 7.64. In which case, an initial pH of 7.0 of fermentation liquid medium is good enough for a number of antibiotic producers including *Aeromonas hydrophila* KaI245.

Most bacteria are mesophilic and grow best at a temperature range of 20-45°C (Todar, 2016a). Temperature usually affects the kinetics of enzymes that are directly involved in metabolism. Antibiotic production by the *Aeromonas hydrophila* KaI245 in liquid medium was achieved at a temperature of 28°C. There was an increase albeit slight in the inhibitory effect of the mycelial growth of *C. sublineolum* and then decreased after 28°C towards 40°C. The increase and decrease were atributed to low and high temperatures than the optimal that inhibited the growth of *Aeromonas hydrophila* KaI245 and further led to the biocontrol metabolites reduction. This observation has also been made by other

workers. The optimal temperature range for antibiotic production from *Pseudomonas reptilivora* that was tested against *Staphylococcus aureus* was 28°C to 30°C (Delrio *et al.,* 1972). Song *et al.* (2012) established that maximum inhibitory rate against *S. sclerotiorum* of antibiotics produced in liquid medium from *Streptomyces felleus* YJ1 was attained when fermentation was carried out at a temperature of 28°C. They considered this as the optimal temperature for antibiotic production. Wang *et al.* (2008) using the response surface methodology, observed that the maximum antibiotic activity of *Xenorhabdus nematophila* was achieved at a temperature of 27.8^{o}C.

The two storage conditions (at refrigeration temperatures of 4°C and room temperature of 22 ± 5^oC) and duration of 8 months did not have significant effects on the activity of the antibiotic. Minor variations were recorded and these could have been brought about by fluctuations in the antibiotics activity depending on the incubation conditions. Storage can therefore be recommended at either of the two conditions for a period of 8 months.

The process of purification of the antibiotic enhanced the activity of the antibiotic culture filtrate following the prolonged persistence and greater size of inhibition zones produced by the purified antibiotic-culture-filtrate. Zones of inhibition produced were 65.47% greater compared to those of the crude antibiotic culture-filtrate.

5.7. Greenhouse evaluation of the effectiveness of *Aeromonas hydrophila* KaI245 antibiotics-culture-filtrate in controlling sorghum anthracnose

The culture filtrate obtained from *Aeromonas hydrophila* KaI245 proved effective in inhibiting growth of *C. sublineolum in vitro* and was further subjected to *in vivo* trials in the greenhouse. Despite phytotoxicity problems at high concentrations on sorghum plants, the antibiotic proved to be effective in decreasing the rate of disease progress and remarkably reduced disease severity on sorghum plants. The level of disease control compared favourably to that achieved by spraying with Folicur$^®$430SC at a rate of 1.0mL/L. Reduced disease severity was also reported by Wahome (1998) and Kamote (1999) while working with bean anthracnose and using antibiotics produced by two *Bacillus* isolates CA5 and CA10.

Effective disease control like the one achieved in this study using rhizobacterial isolate KaI245 identified as *Aeromonas hydrophila* have been reported by several researchers. Scharen and Bryan (1981) showed that metabolites of *B. licheniformis* produced in culture filtrate were antagonistic to *Pyrenophora teres*, the causal agent of net blotch of barley. When applied to the leaves of barley seedlings, *B. licheniformis* established itself and prevented infection by the fungus. Baker *et al.* (1983) found that an isolate of *B. subtilis* gave greater than 95% reduction in the subsequent number of rust pastules when it was applied in liquid culture in the greenhouse 2 to 120 hours prior to inoculation with *Uromyces phaseoli* var. *typica* uredospores. When the same isolate was applied after inoculation with uredospores, there was no effect on pastule numbers. Microscopic observation of *B. subtilis* treated bean leaves showed uredospores germination was greatly reduced and no normal germ tubes were produced. Some uredospores developed abnormal cytoplasmic protrusions. As a result of applying *B. subtilis* three times a week the level of control of bean rust was similar to that obtained with one application per week of the fungicide mancozeb. Makumba *et al.* (2012) also showed that double concentration antibiotics produced by a *B. licheniformis* MGRP1 strain were able to suppress bean anthracnose under glasshouse conditions to levels comparable to 0.1% (w/v) Benlate 50WP—a commercial fungicide.

Effective control of anthracnose by antifungal agents produced by *Aeromonas hydrophila* KaI245 renders the null hypothesis (H_0) of this study untenable. The null hypothesis (H_0) is therefore rejected in favour of the alternative hypothesis (H_1).

5.8. Phytotoxicity of *Aeromonas hydrophila* KaI245 antibiotic-culture-filtrate
There were some phytotoxic effects on sorghum plants sprayed with *Aeromonas hydrophila* KaI245 antibiotic-culture-filtrate that were observed. Phytotoxicity was manifested through change of colour (bleaching) of leaves, crinkling of leaves and stunted growth. It was observed that phytotoxicity varied with the antibiotic concentration. The culture filtrate whose concentration was doubled in particular, produced elaborate signs of toxicity on leaves of the sorghum plants. The other three

concentrations had almost no toxicity as compared to the unsprayed control plants. Pramer (1959) reported that different plants vary in their susceptibility to injury by any one antibiotic at a given concentration. Similar results had been obtained by Altman and Bachelder (1956) when testing the susceptibility of some ornamental and vegetable plants to streptomycin dust. In a quest to control Fusarium wilt in carnations caused by *Fusarium oxysporum* f.sp. *dianthi* under glasshouse conditions, Mutitu and Muthomi (1994) observed phytotoxicity that led to chlorosis and partial wilting during the first week of treatment with antibiotics extracted from a *Streptomyces* sp. on diseased carnation plants especially when applied to young and tender cuttings. The chlorosis was significantly reduced when; older plants were and well established cuttings were used and/or when the *Streptomyces* culture-filtrate was diluted to half or quarter strength.

Phytotoxic injury of sorghum leaves due to *Aeromonas hydrophila* KaI245 culture-filtrate antibiotic seemed to weaken them initially. The weakened plants also showed slow anthracnose symptoms development. Weakening the plant, the antibiotics at high concentration interfered with the host's physiological activities. Pramer (1959) noted that the effect of antibiotics in some cases was due to the action on the host, which altered the host-parasite interaction and prevented the ingress of the pathogen. Cercos (1964) studied the effect of the antibiotic Etamycin in rice seedling growth and chlorophyll formation. He observed a slowed seedling growth and reduced chlorophyll content. With the large-scale application of streptomycin formulations for the control of bacterial blight of rice, Suzuki (1966) noted its negative effect on respiration, photosynthesis, and chlorophyll content. Grain development and carbohydrate content in the grains were also affected in a different study by Suzuki *et al.* (1965). However, it was not clear whether the bleaching effect of streptomycin was due to destruction of the chloroplast or due to failure of colourless tissue to synthesize chlorophyll, or both.

A study carried out by Muthomi (1992) reported phytotoxic effects of antibiotic culture filtrates of *Actinomycete* isolates on carnation seedlings. Similar reports were made by Wahome (1998) and Kamote (1999), both of who observed phytotoxic effects on bean leaves sprayed with culture filtrates of *Bacillus subtilis* isolates CA5 and CA10. The

phytotoxic microbial antibiotic fusidic acid reduced the chlorophyll accumulation in hemp sesbania [*Sesbaniae exalta* (Raf.) Rydb.], (9 5%), wheat [*Triticum aestivum* L.] (20%), mung bean [*Vigna radiata* L.] (60%), sorghum [*Sorghurn vulgare* L.] (60%) and sicklepod [*Cassia obtusifolia* L.] (48%) in a study conducted by Hoagland (2009) who sought to establish the phytotoxicity of microbial antibiotics helvolic and fusidic acids on tissues of several plant species. Ghava *et al.* (2015) observed phytotoxic effects of three antibiotics viz: levofloxacin, tetracycline and amoxicillin on root elongation of three species of Wheat (*Triticum aestivum* L.) seeds. Concentrations of EC25s 0.1 > 10,000 mg/L were used and the strength of phytotoxicity from the most to the least phytotoxic was levofloxacin > tetracycline > amoxicillin.

The phytotoxic effect of antibiotic preparations are often reduced by additives, such as chlorophylline in the case of cycloheximide, or addition of calcium, magnesium and manganese ions in the case of streptomycin (Zahn, 1965). Several antibiotics have found real application at commercial scale in plant disease control. Some of these antibiotics are kasugamycin, polyoxins, chloramphenical, streptomycin, blasticidins and griseofulvin. The development of antibiotics seems to be confined to Europe, India, New Zealand and is more pronounced in Japan and the USA.

The results of this study have shown that it is possible to develop antibiotics that are effective against important sorghum diseases locally using local resources. This will bring about sustainable agricultural practices in the management of pests and diseases thereby increasing food production and hence food security. Developing novel antibiotics and antibiotic combinations can contain development of resistance by phytopathogens to various antibiotics.

CHAPTER SIX
CONCLUSIONS AND RECOMMENDATIONS

6.1. Conclusions

Based on the results obtained following the materials and methods employed to achieve the set objectives, this study makes its conclusions as follows:

i) The sorghum crop and its rhizosphere harbours diverse bacterial populations. A total of 294 rhizobacterial isolates were made.

ii) The sorghum crop is a host of quite a number of foliar fungal pathogens that persist/attack to/in the later stages of growth. Among the foliar fungal pathogens isolated in the sorghum growing belt in Western Kenya included *Alternaria alternata, Aspergillus candidus, Alternaria longissima, Botrytis cinerea, Colletotrichum gloeosporioides, C. sublineolum, Exserohilum turcicum, Fusarium equseti, Fusarium moniliforme* and *Nigrospora oryzae*.

iii) A considerable population of sorghum rhizobacterial are antagonistic towards sorghum foliar fungal pathogens. A few species can be harnessed for biocontrol based on the extent to which they inhibit the growth of the phytopathogens *in vitro*. Among them are members of the genera *Aeromonas* and *Bacillus*. *Aeromonas hydrophila* KaI245 and *Bacillus megaterium* MaI254 isolated and identified in this study were the most promising rhizobacterial isolates that can be used for biocontrol of sorghum foliar fungal phytopathogens.

iv) There is a promising likelihood to isolate sorghum antagonistic rhizobacteria from acrisol soils than any other type except when there are obvious differences in fertility.

v) The sorghum rhizosphere harbours a number of different bacterial populations that are antagonistic to fungal foliar sorghum phytopathogens. The majority of the antagonists belong to the family Bacillaceae.

vi) The best antagonistic sorghum rhizobacterial isolate was *Aeromonas hydrophila* KaI245.

vii) The antibiotic-culture-filtrate of *Aeromonas hydrophila* KaI245 was variably inhibitory to the growth of the test fungal pathogens including *Colletotrichum*

sublineolum in vitro. This result indicates that the active antibiotic produced by the rhizobacterial isolate is broad-spectrum. The antibiotic-culture-filtrate obtained was versatile when produced at different environmental conditions. It exhibited activity when produced; in liquid medium containing different carbon sources, over a wide range of pH, over a considerable range of temperatures and as well, showed inconsequential variability in activity when subjected to different storage conditions (refrigeration and room temperature) over a period of 8 months. This suggests that the antibiotic culture filtrate could be stored under any one of those conditions for a period not exceeding eight months.

viii)   An effective antibiotic against sorghum anthracnose can be produced from the *Aeromonas hydrophila* KaI245. Greenhouse studies showed that *Aeromonas hydrophila* KaI245 antibiotic-culture-filtrate effectively controlled sorghum anthracnose caused by *Colletotrichum sublineolum*. However, at higher concentrations, the antibiotic culture filtrate was phytotoxic to sorghum plants. Diluting the antibiotic culture filtrate with distilled water reduced phytotoxicity.

6.2. Recommendations

Due to limited resources and time, certain aspects of the study were either not attempted or brought to a logical conclusion. The following is therefore recommended:

i) The assertion made in this study that there is a promising likelihood to isolate sorghum antagonistic rhizobacterial isolates from acrisol soils than any other type except when there are obvious differences in fertility needs to be verified through a more focused study.

ii) There is need to further investigate the stability of *Aeromonas hydrophila* KaI245 antibiotic under prolonged storage beyond 8 months.

iii) Further studies on the quantity of antibiotics produced by *Aeromonas hydrophila* KaI245 in different media compositions and incubation periods need to be carried out.

iv) A thorough study on the kinetics of antibiotic production and the optimization of medium design and product recovery needs to be carried out.

v) Further research is needed to identify the *Aeromonas hydrophila* KaI245 antibiotic and determine its chemical structure of the antibiotic using a combination of spectroscopy and chromatography techniques.

vi) Ways of reducing phytotoxic effects while retaining efficacy of antibiotics produced by *Aeromonas hydrophila* KaI245 need to be intensively studied.

vii) It will be of practical importance if the *Aeromonas hydrophila* KaI245 antibiotic-culture-filtrate obtained in this study was tested in the field.

viii) Formulation of the *Aeromonas hydrophila* KaI245 antibiotic for field application in disease management needs to be explored.

ix) More knowledge is required on the mechanisms of biocontrol of phytopathogens to develop rational strategies for the application of the antagonists and their metabolites within the agro-ecosystem. Once such strategies are elucidated, genetic engineering can provide an efficient way of gathering desirable characteristics from different organisms in only one organism like *Rhizobium* or other members of the rhizosphere. The cloning of such genes directly into the plant cells will be an interesting option.

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APPENDICES

Appendix i: **Soil and diseased sorghum plant samples collection sites, climatic and topographical information**

Sampling date	Site/County	Agro-ecological Zone/*Sub-Zone*	Geographical Co-ordinates	Altitude (ft a.s.l)	Average Rainfall (mm)	Average Temperature (^{o}C)	No. of soil/ Plant samples	Lab log-in prefix series
30.03.2012	Kibos/Kisumu	LM2 *l/mˆ(m/s)* or *s/m)* and *lˆ(m/s) i* [a]	00°02.342´S, 034°48.948´E	3,909	1440 [a]	21.6 [a]	4/11	KbI…..
01.06.2012	Kibos/Kisumu	LM2 *l/mˆ(m/s)* or *s/m)* and *lˆ(m/s) i* [a]	00°02.342´S, 034°48.948´E	3,909	1440 [a]	21.6 [a]	4/4	KbII…..
01.06.2012	Ureje/Siaya	LM4 *m/s + (vu)* [a]	00°01.642´S, 034°03.489´E	3,909	955 [a]	22.5 [a]	4/4	UrI…..
02.10.2012	Sega/Siaya	LM1 *lˆm i* [a]	00°14.997´N, 034°12.175´E	4,078	1700 [a]	21.35 [a]	9/3	SgI…..
19.07.2013	Bugeng'i/Busia	LM1 *lˆm i* [b]	00°27.155´N, 034°09.514´E	3,988	1900 [b]	21.6 [b]	2/3	BgI…..
19.07.2013	Burumba/Busia	LM1 *lˆm i* [b]	00°27.351´N, 034°07.135´E	3,909	1900 [b]	21.6 [b]	2/3	BrI…..
19.07.2013	Busende/Busia	LM2 *lˆ(m/s) i* [b]	00°22.521´N, 034°09.322´E	3,909	1675 [b]	21.85 [b]	2/3	BsI…..
19-07-2013	Sega/Siaya	LM1 *lˆm i* [a]	00°14.997´N, 034°12.175´E	4,078	1700 [a]	21.35 [a]	3/3	SgII…..
19.07.2013	Busire-Siriwo/ Busia	LM2 *lˆ(m/s) i* [b]	00°19.007´N, 034°11.866´E	4,173	1675 [b]	21.85 [b]	1/2	BuSiI…..
15.05.2014	Awaat/Busia	LM3 *m/lˆ (s)* [b]	00°39.298´N, 034°20.370´E	4,113	1428 [b]	21.85 [b]	2/3	AwI…..
15.05.2014	Angurai/Busia	LM3 *m/lˆ (s)* [b]	00°42.059´N, 034°21.561´E	4,627	1354 [b]	21.85 [b]	2/3	AngI…..
15-05-2014	Akiriamet/Busia	LM2 *l/mˆ (s/m) i* [b]	00°43.555´N, 034°20.312´E	4,194	1550 [b]	21.85 [b]	2/3	AkI…..
15.05.2014	Alupe B$_1$/Busia	LM1 *lˆm i* [b]	00°29.731´N, 034°08.165´E	3,869	1900 [b]	21.6 [b]	2/3	AlB$_1$I…..
15.05.2014	Alupe B$_2$/Busia	LM1 *lˆm i* [b]	00°29.840´N, 034°08.352´E	3,765	1900 [b]	21.6 [b]	2/3	AlB$_2$I…..
15.05.2014	Alupe B$_3$/Busia	LM1 *lˆm i* [b]	00°29.736´N, 034°08.237´E	3,780	1900 [b]	21.6 [b]	2/3	AlB$_3$I…..

26.08.2014	Sigor I/West Pokot	LM5 *(vs^fvs) i* [c]	01°28.907´N, 035°29.233´E	3,158	775 [c]	21.9 [c]	3/3	SrI…..
26.08.2014	Sigor II/West Pokot	LM5 *(vs^fvs) i* [c]	01°28.873´N, 035°29.193´E	3,111	775 [c]	21.9 [c]	3/3	SrII…..
26.08.2014	Sigor III/West Pokot	LM5 *(vs^fvs) i* [c]	01°29.047´N, 035°29.068´E	3,127	775 [c]	21.9 [c]	3/3	SrIII…..
26.08.2014	Sigor IV/West Pokot	LM5 *(vs^fvs) i* [c]	01°28.864´N, 035°28.969´E	3,140	775 [c]	21.9 [c]	2/3	SrIV…..
19.11.2014	Kanduyi/Bungoma	LM2 *m/l^(m/s) i* [b]	00°36.328´N, 034°32.721´E	4,849	1575 [b]	21.45 [b]	2/3	KaI…..
19.11.2014	Mayanja/Bungoma	LM3 *m/l^(s)* [b]	00°38.441´N, 034°31.209´E	4,968	1325 [b]	22 [b]	2/3	MaI…..
19.11.2014	Miti-Majambazi /Trans-Nzoia	UM4 *l/vl or two* [c]	01°03.290´N, 034°59.862´E	6,102	1100 [b]	18.65 [b]	2/3	MmI…..
19.11.2014	Misemwa /Trans-Nzoia	UM4 *l/vl or two* [c]	01°03.198´N, 034°59.575´E	6,147	1100 [b]	18.65 [b]	2/3	MiI…..

[a]Jaetzold *et al.* 2009
[b]Jaetzold *et al.* 2005
[c]Jaetzold *et al.* 2011

Appendix ii: Rhizobacterial isolates from sampled sites

Sampling date	Site/County	Agro-ecological Zone/*Sub-Zone*	Geographical Co-ordinates	Rhizobacterial isolate log No.	Comments
30.03.2012	Kibos/Kisumu	LM2 *l/m^(m/s)* or *s/m*) and *l^(m/s) i* [a]	00°02.342′S, 034°48.948′E	**KbI01**	**Antagonistic**
				KbI02	Non-antagonistic
				KbI03	Non-antagonistic
				KbI04	Non-antagonistic
				KbI05	Non-antagonistic
				KbI06	Non-antagonistic
01.06.2012	Kibos/Kisumu	LM2 *l/m^(m/s)* or *s/m*) and *l^(m/s) i* [a]	00°02.342′S, 034°48.948′E	KbII07	Non-antagonistic
				KbII08	Non-antagonistic
				KbII09	**Antagonistic**
				KbII10	Non-antagonistic
				KbII11	Non-antagonistic
				KbII12	Non-antagonistic
01.06.2012	Ureje/Siaya	LM4 *m/s + (vu)* [a]	00°01.642′S, 034°03.489′E	UrI13	Non-antagonistic
				UrI14	Non-antagonistic
				UrI15	**Antagonistic**
				UrI16	Non-antagonistic
				UrI17	**Antagonistic**
02.10.2012	Sega/Siaya	LM1 *l^m i* [a]	00°14.997′N, 034°12.175′E	**SgI18**	**Antagonistic**
				SgI19	Non-antagonistic
				SgI20	Non-antagonistic
				SgI21	Non-antagonistic
				SgI22	Non-antagonistic
				SgI23	Non-antagonistic
				SgI24	Non-antagonistic
				SgI25	Non-antagonistic
				SgI26	Non-antagonistic
				SgI27	Non-antagonistic
				SgI28	Non-antagonistic
				SgI29	**Antagonistic**
				SgI30	Non-antagonistic
				SgI31	**Antagonistic**
				SgI32	Non-antagonistic

				SgI33	Non-antagonistic
				SgI34	**Antagonistic**
				SgI35	**Antagonistic**
				SgI36	**Antagonistic**
				SgI37	Non-antagonistic
				SgI38	Non-antagonistic
				SgI39	**Antagonistic**
				SgI40	**Antagonistic**
				SgI41	**Antagonistic**
				SgI42	**Antagonistic**
				SgI43	**Antagonistic**
				SgI44	**Antagonistic**
19.07.2013	Busire-Siriwo/Busia	LM2 *l̂(m/s) i* [b]	00°19.007´N, 034°11.866´E	BuSiI45	Non-antagonistic
				BuSiI46	Non-antagonistic
				BuSiI47	Non-antagonistic
				BuSiI48	Non-antagonistic
				BuSiI49	Non-antagonistic
				BuSiI50	Non-antagonistic
				BuSiI51	Non-antagonistic
				BuSiI52	Non-antagonistic
				BuSiI53	Non-antagonistic
				BuSiI54	Non-antagonistic
				BuSiI55	**Antagonistic**
				BuSiI56	Non-antagonistic
				BuSiI57	Non-antagonistic
				BuSiI58	Non-antagonistic
				BuSi59	Non-antagonistic
				BuSiI60	Non-antagonistic
				BuSiI61	**Antagonistic**
				BuSiI62	**Antagonistic**
				BuSiI63	**Antagonistic**
				BuSiI64	Non-antagonistic
				BuSiI65	Non-antagonistic
				BuSiI66	**Antagonistic**
19-07-2013	Sega/Siaya	LM1 *l̂m i* [a]	00°14.997´N, 034°12.175´E	**SgII167**	**Antagonistic**
				SgII168	Non-antagonistic
				SgII169	Non-antagonistic

Date	Location	Code	Coordinates	Sample	Status
				SgII170	**Antagonistic**
				SgII171	Non-antagonistic
				SgII172	Non-antagonistic
				SgII173	**Antagonistic**
				SgII174	Non-antagonistic
				SgII175	**Antagonistic**
				SgII176	**Antagonistic**
				SgII177	**Antagonistic**
				SgII178	**Antagonistic**
				SgII179	Non-antagonistic
				SgII180	Non-antagonistic
				SgII181	Non-antagonistic
				SgII182	**Antagonistic**
				SgII183	Non-antagonistic
				SgII184	Non-antagonistic
				SgII185	Non-antagonistic
				SgII186	Non-antagonistic
				SgII187	Non-antagonistic
				SgII188	Non-antagonistic
				SgII189	Non-antagonistic
				SgII190	Non-antagonistic
				SgII191	Non-antagonistic
19.07.2013	Bugeng'i/Busia	LM1 $l\hat{}m\ i$ [b]	00º27.155´N, 034º09.514´E	BgI92	Non-antagonistic
				BgI93	**Antagonistic**
				BgI94	Non-antagonistic
				BgI95	Non-antagonistic
				BgI96	Non-antagonistic
				BgI97	Non-antagonistic
				BgI98	Non-antagonistic
				BgI99	Non-antagonistic
				BgI100	Non-antagonistic
				BgI101	Non-antagonistic
				BgI102	Non-antagonistic
				BgI103	**Antagonistic**
				BgI104	**Antagonistic**
19.07.2013	Busende/Busia	LM2 $l\hat{}(m/s)\ i$ [b]	00º22.521´N, 034º09.322´E	BsI105	Non-antagonistic
				BsI106	Non-antagonistic

				BsI107	Non-antagonistic
				BsI108	Non-antagonistic
				BsI109	Non-antagonistic
				BsI110	Non-antagonistic
				BsI111	Non-antagonistic
				BsI112	Non-antagonistic
				BsI113	Non-antagonistic
				BsI114	Non-antagonistic
				BsI115	Non-antagonistic
				BsI116	Non-antagonistic
				BsI117	**Antagonistic**
				BsI118	Non-antagonistic
				BsI119	Non-antagonistic
				BsI120	**Antagonistic**
				BsI121	Non-antagonistic
				BsI122	Non-antagonistic
19.07.2013	Burumba/Busia	LM1 *l̂ m i* [b]	00°27.351´N, 034°07.135´E	BrI123	Non-antagonistic
				BrI124	Non-antagonistic
				BrI125	Non-antagonistic
				BrI126	Non-antagonistic
				BrI127	Non-antagonistic
				BrI128	Non-antagonistic
				BrI129	Non-antagonistic
				BrI130	Non-antagonistic
				BrI131	**Antagonistic**
01.06.2012	Ureje/Siaya	LM4 *m/s + (vu)* [a]	00°01.642´S, 034°03.489´E	**UrI132**	**Antagonistic**
				UrI133	Non-antagonistic
				UrI134	Non-antagonistic
				UrI135	**Antagonistic**
				UrI136	Non-antagonistic
				UrI137	Non-antagonistic
				UrI138	**Antagonistic**
				UrI139	Non-antagonistic
				UrI140	Non-antagonistic
				UrI141	**Antagonistic**
				UrI142	Non-antagonistic
				UrI143	Non-antagonistic

				UrI144	Antagonistic
15.05.2014	Alupe B$_3$/Busia	LM1 *l̂m i* [b]	00°29.736′N, 034°08.237′E	AlB$_3$I45	Antagonistic
				AlB$_3$I46	Non-antagonistic
				AlB$_3$I47	Non-antagonistic
				AlB$_3$I48	Non-antagonistic
				AlB$_3$I49	Antagonistic
				AlB$_3$I50	Non-antagonistic
				AlB$_3$I51	Non-antagonistic
				AlB$_3$I52	Antagonistic
15.05.2014	Alupe B$_2$/Busia	LM1 *l̂m i* [b]	00°29.840′N, 034°08.352′E	AlB$_2$I53	Antagonistic
				AlB$_2$I54	Antagonistic
				AlB$_2$I55	Antagonistic
				AlB$_2$I56	Antagonistic
				AlB$_2$I57	Antagonistic
				AlB$_2$I58	Antagonistic
				AlB$_2$I59	Antagonistic
				AlB$_2$I60	Antagonistic
				AlB$_2$I61	Antagonistic
				AlB$_2$I62	Antagonistic
15-05-2014	Akiriamet/Busia	LM2 *l/m̂ (s/m) i* [b]	00°43.555′N, 034°20.312′E	AkI163	Antagonistic
				AkI164	Antagonistic
				AkI165	Antagonistic
				AkI166	Antagonistic
				AkI167	Antagonistic
15.05.2014	Awaat/Busia	LM3 *m/l̂ (s)* [b]	00°39.298′N, 034°20.370′E	AwI168	Antagonistic
				AwI169	Antagonistic
				AwI170	Antagonistic
				AwI171	Antagonistic
				AwI172	Antagonistic
				AwI173	Antagonistic
				AwI174	Antagonistic
				AwI175	Antagonistic
				AwI176	Antagonistic
15.05.2014	Angurai/Busia	LM3 *m/l̂ (s)* [b]	00°42.059′N, 034°21.561′E	AngI177	Antagonistic
				AngI178	Antagonistic
				AngI179	Antagonistic
				AngI180	Antagonistic

				AngI181	**Antagonistic**
				AngI181	**Antagonistic**
				AngI182	**Antagonistic**
				AngI183	Non-antagonistic
				AngI184	Non-antagonistic
				AngI185	Non-antagonistic
				AngI186	Non-antagonistic
				AngI187	Non-antagonistic
				AngI188	Non-antagonistic
				AngI189	**Antagonistic**
				AngI190	**Antagonistic**
				AngI191	Non-antagonistic
				AngI192	**Antagonistic**
				AngI193	**Antagonistic**
26.08.2014	Sigor I/West Pokot	LM5 *(vsˆfvs) i* [c]	01º28.907´N, 035º29.233´E	**SrI194**	**Antagonistic**
				SrI195	**Antagonistic**
				SrI196	Non-antagonistic
				SrI197	Non-antagonistic
				SrI198	Non-antagonistic
				SrI199	Non-antagonistic
				SrI200	Non-antagonistic
				SrI201	Non-antagonistic
				SrI202	Non-antagonistic
				SrI203	**Antagonistic**
				SrI204	**Antagonistic**
				SrI205	**Antagonistic**
				SrI206	Non-antagonistic
				SrI207	Non-antagonistic
				SrI208	**Antagonistic**
				SrI209	**Antagonistic**
15.05.2014	Alupe B$_1$/Busia	LM1 *lˆm i* [b]	00º29.731´N, 034º08.165´E	AlB$_1$I210	Non-antagonistic
				AlB$_1$I211	Non-antagonistic
				AlB$_1$I212	Non-antagonistic
				AlB$_1$I213	Non-antagonistic
				AlB$_1$I214	Non-antagonistic
				AlB$_1$I215	**Antagonistic**
				AlB$_1$I216	**Antagonistic**
				AlB$_1$I217	Non-antagonistic

Date	Location	Code	Coordinates	Sample	Type
				AlB_1I218	Non-antagonistic
				AlB_1I219	Non-antagonistic
26.08.2014	Sigor II/West Pokot	LM5 *(vs^fvs) i* [c]	01º28.873´N, 035º29.193´E	**SrII220**	**Antagonistic**
				SrII221	Non-antagonistic
				SrII222	Non-antagonistic
				SrII223	Non-antagonistic
				SrII224	Non-antagonistic
				SrII225	**Antagonistic**
				SrII226	**Antagonistic**
				SrII227	**Antagonistic**
26.08.2014	Sigor III/West Pokot	LM5 *(vs^fvs) i* [c]	01º29.047´N, 035º29.068´E	**SrIII228**	**Antagonistic**
				SrIII229	**Antagonistic**
				SrIII230	Non-antagonistic
				SrIII231	Non-antagonistic
				SrIII232	Non-antagonistic
				SrIII233	**Antagonistic**
				SrIII234	**Antagonistic**
26.08.2014	Sigor IV/West Pokot	LM5 *(vs^fvs) i* [c]	01º28.864´N, 035º28.969´E	SrIV235	Non-antagonistic
				SrIV236	**Antagonistic**
				SrIV237	Non-antagonistic
				SrIV238	**Antagonistic**
				SrIV239	Non-antagonistic
				SrIV240	Non-antagonistic
				SrIV241	Non-antagonistic
				SrIV242	Non-antagonistic
				SrIV243	**Antagonistic**
19.11.2014	Kanduyi/Bungoma	LM2 *m/l^ (m/s) i* [b]	00º36.328´N, 034º32.721´E	**KaI244**	**Antagonistic**
				KaI245	**Antagonistic**
				KaI246	Non-antagonistic
19.11.2014	Mayanja/Bungoma	LM3 *m/l^ (s)* [b]	00º38.441´N, 034º31.209´E	MaI247	Non-antagonistic
				MaI248	Non-antagonistic
				MaI249	Non-antagonistic
				MaI250	Non-antagonistic
				MaI251	Non-antagonistic
				MaI252	Non-antagonistic
				MaI253	Non-antagonistic
				MaI254	**Antagonistic**

19.11.2014	Miti-Majambazi /Trans-Nzoia	UM4 *l/vl or two* [c]	01°03.290´N, 034°59.862´E	MmI255	Non-antagonistic
				MmI256	Non-antagonistic
				MmI257	Non-antagonistic
				MmI258	Non-antagonistic
				MmI259	Non-antagonistic
				MmI260	Non-antagonistic
				MmI261	**Antagonistic**
				MmI262	Non-antagonistic
				MmI263	Non-antagonistic
				MmI264	Non-antagonistic
				MmI265	Non-antagonistic
				MmI266	Non-antagonistic
				MmI267	Non-antagonistic
				MmI268	Non-antagonistic
				MmI269	Non-antagonistic
				MmI270	Non-antagonistic
				MmI271	Non-antagonistic
				MmI272	Non-antagonistic
				MmI273	Non-antagonistic
				MmI274	Non-antagonistic
				MmI275	Non-antagonistic
				MmI276	Non-antagonistic
19.11.2014	Misemwa /Trans-Nzoia	UM4 *l/vl or two* [c]	01°03.198´N, 034°59.575´E	MiI277	Non-antagonistic
				MiI278	Non-antagonistic
				MiI279	Non-antagonistic
				MiI280	Non-antagonistic
				MiI281	Non-antagonistic
				MiI282	Non-antagonistic
				MiI283	Non-antagonistic
				MiI284	Non-antagonistic
				MiI285	Non-antagonistic
				MiI286	Non-antagonistic
				MiI287	Non-antagonistic
				MiI288	Non-antagonistic
				MiI289	Non-antagonistic
				MiI290	Non-antagonistic
				MiI291	Non-antagonistic

	MiI292	Non-antagonistic
	MiI293	Non-antagonistic
	MiI294	Non-antagonistic

Appendix iii: **Agro-ecological zones/sub-zones descriptions of sampled sites**

Agro-ecological zones		Description	Major Crop(s)	Site(s) visited in the study
Zone	**Sub—zone**			
LM1	*LM1 lˆm i*	Lower Midland Sugar Cane Zone; with a long cropping season, followed by a medium one and intermediate rains	Sugar Cane	Alupe, Bugeng'i, Burumba and Sega
LM2	*LM2 l/mˆ (m/s or s/m) and lˆ(m/s) i*	Lower Midlands Marginal Sugar Cane Zone; with a long to medium cropping season, followed by a (weak) medium to short or short to medium one and/with a long cropping season, followed by a (weak) medium to short one and intermediate rains	Sugar Cane,	Kibos
	LM2 lˆ(m/s) i	Marginal Sugar Cane Zone; with a long cropping season, followed by a (weak) medium to short one and intermediate rains	Sugar Cane	Busende, Busire—Siriwo
	LM2 l/mˆ (s/m) i	Marginal Sugar Cane Zone; with a long to medium cropping season, followed by a (weak) short to medium one and intermediate rains	Sugar Cane	Akiriamet
	LM2 m/lˆ (m/s) i	Marginal Sugar Cane Zone with a medium to long cropping season followed by a (weak) medium to short one and intermediate rains.	Sugar Cane	Kanduyi—Makutano
LM3	*LM3 m/lˆ (s)*	Lower Midland Cotton Zone; with a medium to long cropping season, followed by a (weak) short one	Cotton	Awaat, Angurai and Mayanja
LM4	*LM4 m/s +(vu)*	Marginal Cotton Zone; with a medium to short cropping season and a very uncertain (weak) second rainy season	Cotton,	Ureje
LM5	*LM5 (vsˆ fvs) i*	Livestock-Millet Zone with a (weak) very short cropping season followed by a (weak) fully very short one and intermediate rains.	Millet	Sigor
UM4	*UM4 l/vl or two*	Maize-Sunflower Zone with a long to very long cropping season separable in two variable cropping seasons.	Sunflower	Miti-Majambazi and Misemwa

Appendix iv: Agro-ecological zones soil descriptions

Agro-ecological zones		Soil description	Site(s) associated with soil type
Zone	**Sub—zone**		
LM1	*LM1 lˆm i*	Soil type 1 (UlS1) [b]: Well drained, moderately deep to deep, dark reddish brown to strong brown, sandy clay loam to clay, over petroplinthite; in places shallow: Orthic ACRISOLS, with orthic FERRALSOLS, partly petroferric phase	Alupe and Burumba
		Soil type 2 (BXC1) [b]: Complex of: Imperfectly to poorly drained, deep to deep, very dark grey to brown, mottled, firm to very firm, sandy clay to cracking clay, in many places abruptly underlying a topsoil of friable sandy loam to sandy loam; in places saline and sodic: Dystric PLANOSOLS, dystric and vertic GLEYSOLS and pellic VERTISOLS; partly saline-sodic phases	Bugeng'i
		Soil type 3 (U1 G 3) [a]: Well drained, shallow to moderately deep, dark yellowish brown to strong brown, friable sandy clay; over petroplinthite; in places very shallow, stony or rocky: orthic and ferralo-orthic ACRISOLS, petroferric and partly stony phase, with LITHOSOLS and Rock Outcrops	Sega
LM2	*LM2 l/mˆ (m/s or s/m) and lˆ(m/s) i*	Soil type 4 (YU 1) [a]: Imperfectly to poorly drained, very deep, dark greyish brown to very dark grey, mottled, very firm, cracking clay, in many places abruptly underlying a firm topsoil of sandy clay; in places calcareous and with a sodic subsoil: veto-eutric PLANOSOLS, with chromic VERTISOLS and solodic PLANOSOLS	Kibos,
	LM2 lˆ(m/s) i	Soil type 5 (SAC1) [b]: Complex of: Imperfectly to poorly drained, deep to very deep, greyish brown to very dark grey and black, mottled, firm to very firm clay to cracking clay; in places with a saline and sodic subsoil: Eutric GLEYSOLS and pellic VERTISOLS, partly saline-sodic phase	Busende

		and: Very poorly drained, deep, dark grey to black, half ripe clay, with a humic or histic topsoil; in many places peaty: Mollic GLEYSOLS and dystric HISTOSOLS [b]	
		Soil type 6 (UlD1) [b]: Well drained, moderately deep to very deep, dark red to strong brown, friable clay; in many places shallow over petroplinthite: Chromic and orthic ACRISOLS and rhodic FERRALSOLS, partly petroferric phases, and dystric phases, with dystric NITISOLS	Busire—Siriwo
	LM2 l/mˆ (s/m) i	Soil type 7 (UmG1) [b]: Well drained, deep, reddish brown, friable, gravely sandy clay to clay, with an acid humic topsoil: Humic ACRISOLS, with humic CAMBISOLS	Akiriamet
	LM2 m/lˆ (m/s) i	Soil type 8 (UlGA1) [b]: Association of: Well drained, deep to very deep, dark yellowish brown, friable clay loam to clay; in places with an acidic humic topsoil; in places stony; on straight side slopes 50%): orthic ACRISOLS, with humic ACRISOLS, partly stony phases **and:** Well drained, shallow to moderately deep, dark yellowish brown to brown, friable sandy clay loam; over petroplinthite; in places excessively drained and sandy; on interfluves, convex slopes and near fringes to bottomlands (50%): (ferralo-)orthic ACRISOLS, petroferric phase, with ferralic ARENOSOLS	Kanduyi—Makutano,
LM3	*LM3 m/lˆ (s)*	Soil type 9 (UmG2) [b]: Well drained, deep, dark yellowish brown to dark brown, friable sandy clay to loam ; in places gravely in deeper subsoil: Ferralo-orthic ACRISOLS	Angurai and Awaat
		Soil type 10 (UmG6) [b]: Well drained, shallow to moderately deep, dark yellowish brown, friable sandy clay: orthic ACRISOLS	Mayanja
LM4	*LM4 m/s +(vu)*	Soil type 11 (Ul V 2) [a]: Well drained, moderately deep to deep, reddish brown to dark red, friable to firm clay; over petroplinthite or pisoferric material: chromic LUVISOLS and ferralo-chromic ACRISOLS, petroferric and pisoferric phase	Ureje
LM5	*LM5 (vsˆ fvs) i*	Soil type 12 (FU1) [c]: Well drained, very deep, yellowish red to dark reddish brown, loose, loamy coarse	Sigor

		sand to friable sandy clay loam: chromic LUVISOLS, rhodic FERRALSOLS and luvic to ferralic ARENOSOLS	
UM4	*UM4 l/vl or two*	Soil type 13 (Ul U 1)[c]: Well drained, very deep, red to dark red, very friable clay: rhodic FERRALSOLS	Miti-Majambazi and Misemwa

[a]Jaetzold *et al.* 2009
[b]Jaetzold *et al.* 2005
[c]Jaetzold *et al.* 2011

Appendix v: **Summarized soil sample analysis**

Date of Sampling	Soil Sampling Site	Agro-ecological Zone	Mean pH (W)	Mean % Organic C	Mean % Total N	Mean Mehlic P (ppm)	Mean C:N	Mean % Sand	Mean % Clay	Mean % Silt	Predominant Textural Class
30.03.2012	Kibos I	LM2	5.16	1.51	0.116667	8.05195	12.9428	67	22	11	Sandy clay loam
1.6.2012	Kibos II	LM2	5.74	1.55	0.315789	1.03295	4.90834	65	26	9	Sandy clay loam
1.6.2012	Ureje	LM4	5.55	1.3	0.15	1.52915	8.66667	56	34	10	Sandy clay loam
2.10.2012	Sega I	LM1	4.66	1.43	0.135965	0.69445	10.5174	56	33	11	Sandy clay loam
19.07.2013	Sega II	LM1	5.29	1.51	0.100877	8.05195	14.9687	57	33	10	Sandy clay loam
19.07.2013	Bugeng'i	LM1	5.67	1.07	0.140351	1.29105	7.62374	81	10	9	Sandy
19.07.2013	Burumba	LM1	5.83	1.89	0.180702	6.97945	10.4592	69	24	7	Sandy clay loam
19.07.2013	Busende	LM2	5.66	1.29	0.161404	0.833	7.99237	69	18	13	Sandy loam
19.07.2013	Busire-Siriwo	LM2	5.21	2.37	0.345614	0.69695	6.85736	55	36	9	Sandy clay loam
15.05.2014	Awaat	LM3	5.14	0.93	0.121053	0.8964	7.68259	88	7	5	Loamy sand
15.05.2014	Angurai	LM3	5.18	0.71	0.088596	0.65605	8.01391	88	7	5	Loamy sand
15.05.2014	Akiriamet	LM2	5.48	1.31	0.145614	0.8999	8.99639	78	13	9	Loamy sand
15.05.2014	Alupe B_1	LM1	4.74	1.15	0.10614	0.8533	10.8347	78	17	5	Sandy loam
15.05.2014	Alupe B_2	LM1	5.0	1.09	0.102632	7.37545	10.6205	80	11	9	Sandy loam
15.05.2014	Alupe B_3	LM1	6.06	2.13	0.751754	1.05295	2.83337	66	19	15	Sandy loam
26.08.2014	Sigor I	LM5	6.29	0.72	0.169298	5.08095	4.25286	71	19	10	Sandy loam
26.08.2014	Sigor II	LM5	6.5	0.78	0.047368	8.92295	16.4668	67	23	10	Sandy clay loam
26.08.2014	Sigor III	LM5	6.46	0.92	0.481579	8.92295	1.91038	67	21	12	Sandy clay loam
26.08.2014	Sigor IV	LM5	6.19	1.28	0.263158	1.30695	4.864	75	17	8	Loamy sand

19.11.2014	Kanduyi	LM2	4.76	0.83	0.065789	0.2737	12.6161	81	16	3	Loamy sand
19.11.2014	Mayanja	LM3	5.9	0.77	0.078947	0.8042	9.75338	79	15	6	Loamy sand
19.11.2014	Miti-Majambazi	UM4	5.11	1.74	0.271053	1.26775	6.41941	62	30	8	Sandy clay loam
19.11.2014	Misemwa	UM4	4.96	1.5	0.126316	0.79595	11.875	68	24	8	Sandy clay loam

Appendix vi: <u>Identification of the fungal causal agents isolated from diseased sorghum plant materials using symptomatological, cultural and morphological characteristics</u>

Sr No.	Foliar/Leaf sample description:	Fungal Cultural and Morphological characteristics on PDA/Microscopic micrographs description:	Inference:
1.	- Leaf spots, ➢ **Reference**: Navi *et al.* (1999)	- Colony olive green to dark brown in colour - Mycelium abundant and light olive green to brown in colour - Hyphae dark brown, thick, septate, and branched. - Conidiophores simple, erect, and also clustered, measuring 60 µm x 3.5 µm - Conidia produced in an acropetal succession of simple or branched chains that normally branch at the beak of a spore, or sometimes from the short lateral projection of the beak. Conidial size: 41-48 µm x 11 µm - Conidia have transverse and oblique septa, and are ovoid to obovoid, obclavate, obpyriform, ellipsoidal, uniform, with an elongated terminal cell - Conidia often have a short conical or cylindrical beak which is about one third the length of the conidium. ➢ **Reference**: Navi *et al.* (1999); Timmer *et al.* (2003); Gautam, (2013)	- Plant part suffering from leaf spot - Causal agent likely: *Alternaria alternata*
2.	- No particular symptoms but fungus found on surface of diseased plant material in association with other phytopathogens ➢ **Reference**: Navi *et al.* (1999)	- Colony white in colour - Conidiophores bearing vesicles that have sterigmata on which globose/sub-globose conidia are attached in chains ➢ **Reference**: Al-Doory and Domson (1984); Navi *et al.* (1999)	- Plant part bearing : *Aspergillus candidus*
3.	- A brownish-black fungus growing on straw lesions, ➢ **Reference**: Navi *et al.* (1999)	- Colony is brown to blackish brown, - Mycelium is partly superficial and partly immersed. - Conidiophores erect or ascending, simple or occasionally branched, straight or slightly flexuous, sometimes geniculate, somewhat swollen at the apex, septate, pale to mid-pale brown, smooth below, verruculose at and sometimes below the apex, 150 µm long, 3-5 µm thick, with one to several conidial scars.	- Plant part bearing : *Alternaria longissima*

		- Conidia solitary or catenulate, extremely variable in shape and size, pale straw coloured to brown, usually very long (up to 500 µm), *Cercospora*-like, obclavate or with a basal sub-cylindric portion of few to several cells and a very long, narrow septate beak. - Conidia have 5-40 transverse septa. Conidia are 4-17 µm thick in the broadest part and about 2.5 µm thick at the apex. Shorter conidia, variable in shape and often with a few longitudinal or oblique septa, are also formed. - Conidia are thin-walled, smooth except around the base where they are often verruculose. - Dark brown, multicellular, muriform chlamydospores 16-42 x 16-34 µm. ➢ **Reference**: Navi *et al.* (1999)	
4.	- A grey mould growing on surface of plant ➢ **Reference**: Navi *et al.* (1999)	- Colony; white/greyish brown - Conidiophores are tall, upright or nearly so, septate and branched with branches constricted at their point of origin. Measuring 750 µm to 2mm x 18 µm. - Conidia occur in clusters at the swollen rounded apices and at intervals along with conidiophores on short blunt teeth. Conidia are oval or egg-shaped, often with a slightly projecting point of attachment. Average size observed: 11 µm x 6 µm. ➢ **Reference**: Navi *et al.* (1999); *http://www.zor.zut.edu.pl/Mycota/Botrytis%20cinerea.html*	- Plant bearing the grey mould - Causal agent likely: *Botrytis cinerea*
5.	- Small elliptical to circular spots, - Up to 10mm in diameter, - Spots sharply defined , occasionally slightly depressed and reddish-brown in colour, - Black setae observed with magnifying glass ➢ **Reference**: Mathur *et al.* (1998)	- Scanty hyaline or/and brown mycelium, - Colony growth abundant, at times sparse with floccose, loose or compact, - Colonies show white to grey, to dark orange or pink-grey colour, while the reverse side of the colonies are of white, dark grey, orange or a mixture and with regular colony margins, - Sclerotia formed in black colour, - Conidia cylindrical with both apices rounded; or with one apex rounded and the other end pointed. The conidial sizes vary from 7.57-18.50 × 2.42-4.72 µm, - Conidia arising from conidiogenous cells, - Waxy acervuli bearing dark-brown septate setae, - Appressoria globose to perprolate, ovoid or obovoid or clavate, smooth or lobate or multi-lobate, apice cylindrical or obtuse, edges irregular. Average height; 12.7µm, width 7.8 µm	- Plant part suffering from anthracnose - Causal agent: Anamorph: *Colletotrichum gloeosporioides* Teleomorph: *Glomerella cingulata*

		➢ **Reference**: Munaut *et al.* (2001); Zakaria *et al.* (2009); Chowdappa *et al.* (2012); Gautam, (2014)	
6.	- Small elliptical to circular spots, - Up to 5mm in diameter, - Spots with small circular straw coloured centres, - Spots with tan to red margins, - Mid-rib infection produced elongate-elliptical red lesions - Black setae observed with magnifying glass ➢ **Reference**: Williams *et al.* (1978)	- Colonies show greyish to dark colour, - Production of yellow pigments - Sclerotia formed in black colour, - Conidia are single hyaline cells that are lunate shaped with acute apices measuring an average of 23 x 3.5 μm. - Acervuli bearing dark-brown septate setae, - Stromata/ Acervuli measuring an average of 78 μm in diameter, - Appressoria globose to perprolate, ovoid or obovoid or clavate, smooth or lobate or multi-lobate, apice cylindrical or obtuse, edges irregular. Average height; 16.5μm, width 14.8 μm ➢ **Reference**: Mims and Vaillancourt, (2002); Souza-Paccola *et al.* (2003a): Crouch *et al.* (2006)	- Plant part suffering from anthracnose - Causal agent: *Colletotrichum sublineolum*/ *Colletotrichum sublineola*
7.	- Long elliptical necrotic lesions , straw colored in the centers with dark red margins, - Lesions parallel with the veins ➢ **Reference**: Williams *et al.* (1978); Thakur *et al.* (2007)	- Black mycelium, - Conidiophore simple and erect with a swollen base bearing straight and spindle shaped conidia at the tip. - Conidia are olivaceous brown; widest at the middle; tapering at both ends; have 4—5 septa; have a truncate and protuberant hilum in their basal cell which is visible as a small thin stalk at the point of attachment to the conidiophores. - Conidia measuring an average of 50-144 x 18-33 μm, ➢ **Reference**: Ellis and Holliday, (1971a); Chidambaram *et al.* (1973); Sivanesan, (1987); Navi *et al.* (1999)	- Plant part suffering from leaf blight - Causal agent likely: *Exserohilum turcicum*
8.	- Wilting of leaves, discolouration of internal tissues of the stem close to the head, ➢ **Reference**: ICRISAT (1984)	- White colony with yellow-brown pigmentation, - No microconidia' - Macroconidia tapering towards both ends and strongly bent mainly in the central part with a more or less distinctly elongated, straight or whip-like bent apical cell and a very distinctly pedicellate basal cell, mostly 5- to 7 septa, measuring an average of 30–90 × 3.5–6.0 μm, - Sporodochia are abundant and are pale yellowish to ochraceous to salmon to honey to cinnamon-brown coloured in which macroconidia are produced,	- Plant part suffering from head blight/stalk rot - Causal agent: *Fusarium equiseti*

		- Chlamydospores abundant in hyphae, intercalary, solitary, in pairs, frequently forming chains or clusters, globose to subglobose measuring an average of 8–20 µm, smooth- or rough-walled, becoming ochraceous. ➢ **Reference**: _http://species-id.net/wiki/Fusarium_equiseti_	
9.	- Discolouration of internal tissues of the stem close to the head, ➢ **Reference**: Williams _et al._ (1978);	- White colony with pink/yellow pigmentation, - Microconidia egg/oval/club-shaped and on monophialide, some branched, bearing false heads, - Microconidia have flat bases either in beaded-chains or aggregated, catenulate and two-celled, 2-4 x 5-12 µm in size - Macroconidia with ventral and dorsal walls parallel to each other, - Macroconidia are hyaline, 3-7 septate—4 septa majorly observed, long, 1.5-4 x 20-82 µm in diameter, slender, awl-shaped, falcate to almost straight and taper towards either end. They are slightly hooked at the tip, thin-walled, with the apical cell slightly curved and tapering to a point, and are either distinctly or slightly foot-shaped at the basal cell, - Sporodochia are pale orange in colour which produce macroconidia, - Some macroconidia produced on macroconidiophores, ➢ **Reference**: Navi _et al._ (1999)	- Plant part suffering from head blight/stalk rot - Causal agent: Anamorph: _Fusarium moniliforme_ syn. _Fusarium verticillioides_ Teleomorph: _Gibberella fujikuroi_
10.	- No particular symptoms but fungus found on surface of diseased plant material in association with other phytopathogens ➢ **Reference**: Navi _et al._ (1999)	- Colony white with a striking appearance. Centre of colony appears black - Conidiophores are short, inflated and borne at right angles to hyphae, bearing conidia singly and terminally. - Conidia are smoky brown or jet black, spherical or egg-shaped with an average diameter of 15µm ➢ **Reference**: Navi _et al._ (1999); Abass and Mohammed (2014)	- Plant part bearing : _Nigrospora oryzae_

Appendix vii: **Preliminary screening of sorghum rhizobacterial isolates that produced ≥ 70% mycelia inhibition of at least one of the test sorghum foliar fungal pathogens of economic importance using the Loeffler *et al.* (1986) culture technique**

Rhizobacterial isolate	Phytopathogen	Mean % reduction in colony size, (SD)
KbI01	*Colletotrichum sublineolum*	84.40 (1.665)
	Colletotrichum gloeosporioides	69.22 (9.920)
	Fusarium moniliforme	58.58 (10.616)
	Exserohilum turcicum	72.27 (2.732)
UrI15	*Colletotrichum sublineolum*	81.60 (8.920)
	Colletotrichum gloeosporioides	65.37 (1.995)
	Fusarium moniliforme	42.31 (2.326)
	Exserohilum turcicum	72.34 (3.297)
SgI29	*Colletotrichum sublineolum*	75.42 (4.621)
	Colletotrichum gloeosporioides	74.19 (5.233)
	Fusarium moniliforme	30.38 (1.622)
	Exserohilum turcicum	63.93 (2.679)
SgI31	*Colletotrichum sublineolum*	77.07 (5.863)
	Colletotrichum gloeosporioides	79.06 (5.611)
	Fusarium moniliforme	74.77 (5.411)
	Exserohilum turcicum	77.93 (6.313)
SgI34	*Colletotrichum sublineolum*	77.70 (2.818)
	Colletotrichum gloeosporioides	73.61 (10.398)
	Fusarium moniliforme	36.36 (4.622)
	Exserohilum turcicum	66.52 (6.702)
SgI41	*Colletotrichum sublineolum*	75.42 (3.868)
	Colletotrichum gloeosporioides	67.38 (14.579)
	Fusarium moniliforme	36.83 (9.411)
	Exserohilum turcicum	57.68 (6.091)
BuSiI62	*Colletotrichum sublineolum*	74.70 (4.407)
	Colletotrichum gloeosporioides	79.34 (4.902)
	Fusarium moniliforme	50.16 (8.890)
	Exserohilum turcicum	65.48 (2.221)
SgII77	*Colletotrichum sublineolum*	65.12 (19.609)
	Colletotrichum gloeosporioides	54.76 (12.700)
	Fusarium moniliforme	70.05 (4.007)
	Exserohilum turcicum	65.76 (3.433)

SgII78	*Colletotrichum sublineolum*	62.18 (10.058)
	Colletotrichum gloeosporioides	71.16 (2.559)
	Fusarium moniliforme	55.26 (14.380)
	Exserohilum turcicum	55.66 (10.790)
BrI125	*Colletotrichum sublineolum*	51.00 (23.954)
	Colletotrichum gloeosporioides	40.18 (22.448)
	Fusarium moniliforme	63.34 (13.361)
	Exserohilum turcicum	69.75 (4.878)
BrI131	*Colletotrichum sublineolum*	78.18 (4.644)
	Colletotrichum gloeosporioides	73.58 (3.709)
	Fusarium moniliforme	48.54 (4.883)
	Exserohilum turcicum	64.88 (4.544)
UrI132	*Colletotrichum sublineolum*	67.49 (10.184)
	Colletotrichum gloeosporioides	73.81 (6.971)
	Fusarium moniliforme	53.75 (19.460)
	Exserohilum turcicum	54.94 (8.023)
UrI144	*Colletotrichum sublineolum*	75.66 (8.210
	Colletotrichum gloeosporioides	72.47 (4.663)
	Fusarium moniliforme	53.12 (0.607)
	Exserohilum turcicum	54.11 (4.990)
AlB$_2$I157	*Colletotrichum sublineolum*	54.83 (13.817)
	Colletotrichum gloeosporioides	71.36 (16.072)
	Fusarium moniliforme	56.53 (21.398)
	Exserohilum turcicum	54.97 (7.967)
AlB$_2$I159	*Colletotrichum sublineolum*	60.26 (8.130)
	Colletotrichum gloeosporioides	73.19 (4.181)
	Fusarium moniliforme	68.65 (6.040)
	Exserohilum turcicum	63.65 (5.991)
AkI163	*Colletotrichum sublineolum*	71.00 (5.217)
	Colletotrichum gloeosporioides	74.23 (2.225)
	Fusarium moniliforme	64.25 (10.135)
	Exserohilum turcicum	64.28 (5.423)
AkI164	*Colletotrichum sublineolum*	68.11 (10.153)
	Colletotrichum gloeosporioides	60.24 (14.006)
	Fusarium moniliforme	66.47 (17.076)
	Exserohilum turcicum	71.36 (7.508)
AkI165	*Colletotrichum sublineolum*	84.69 (8.548)

	Colletotrichum gloeosporioides	88.83 (7.872)
	Fusarium moniliforme	67.94 (5.156)
	Exserohilum turcicum	75.69 (6.757)
AkI166	*Colletotrichum sublineolum*	69.50 (4.671)
	Colletotrichum gloeosporioides	72.79 (1.026)
	Fusarium moniliforme	44.66 (5.071)
	Exserohilum turcicum	70.20 (2.458)
AkI167	*Colletotrichum sublineolum*	79.12 (8.790)
	Colletotrichum gloeosporioides	65.28 (22.041)
	Fusarium moniliforme	68.46 (14.360)
	Exserohilum turcicum	68.94 (9.722)
AwI171	*Colletotrichum sublineolum*	79.12 (8.790)
	Colletotrichum gloeosporioides	65.28 (22.041)
	Fusarium moniliforme	58.23 (21.846
	Exserohilum turcicum	68.94 (9.722)
AngI180	*Colletotrichum sublineolum*	58.97 (11.722)
	Colletotrichum gloeosporioides	61.55 (12.778)
	Fusarium moniliforme	54.90 (16.518)
	Exserohilum turcicum	69.84 (17.276)
AngI181	*Colletotrichum sublineolum*	66.59 (4.606)
	Colletotrichum gloeosporioides	76.85 (6.748)
	Fusarium moniliforme	67.58 (15.303)
	Exserohilum turcicum	71.38 (12.760)
AngI182	*Colletotrichum sublineolum*	68.57 (11.963)
	Colletotrichum gloeosporioides	78.11 (11.278)
	Fusarium moniliforme	48.97 (16.504)
	Exserohilum turcicum	67.17 (16.572)
SrI194	*Colletotrichum sublineolum*	70.92 (4.320)
	Colletotrichum gloeosporioides	75.65 (2.683)
	Fusarium moniliforme	45.07 (3.059)
	Exserohilum turcicum	63.48 (5.745)
SrI209	*Colletotrichum sublineolum*	75.38 (3.858)
	Colletotrichum gloeosporioides	72.03 (6.302)
	Fusarium moniliforme	44.85 (10.529)
	Exserohilum turcicum	58.61 (6.041)
AlB$_1$I215	*Colletotrichum sublineolum*	68.29 (5.801)
	Colletotrichum gloeosporioides	72.21 (4.898)

	Fusarium moniliforme	66.70 (5.523)
	Exserohilum turcicum	61.48 (6.834)
SrII225	*Colletotrichum sublineolum*	68.96 (6.005)
	Colletotrichum gloeosporioides	73.19 (4.181)
	Fusarium moniliforme	58.12 (3.487)
	Exserohilum turcicum	76.80 (8.188)
SrII226	*Colletotrichum sublineolum*	72.15 (5.495)
	Colletotrichum gloeosporioides	74.86 (5.769)
	Fusarium moniliforme	57.48 (7.619)
	Exserohilum turcicum	70.99 (7.769)
SrIV236	*Colletotrichum sublineolum*	70.02 (9.612)
	Colletotrichum gloeosporioides	78.11 (11.278)
	Fusarium moniliforme	48.97 (16.504)
	Exserohilum turcicum	66.47 (14.095)
SrIV238	*Colletotrichum sublineolum*	71.54 (5.493)
	Colletotrichum gloeosporioides	75.65 (2.683)
	Fusarium moniliforme	46.71 (5.458)
	Exserohilum turcicum	70.07 (10.54)
SrIV243	*Colletotrichum sublineolum*	75.45 (3.874)
	Colletotrichum gloeosporioides	72.03 (6.302)
	Fusarium moniliforme	44.85 (10.529)
	Exserohilum turcicum	65.28 (13.568)
KaI244	*Colletotrichum sublineolum*	68.29 (5.795)
	Colletotrichum gloeosporioides	72.21 (4.898)
	Fusarium moniliforme	66.70 (5.523)
	Exserohilum turcicum	69.74 (12.514)
KaI245	*Colletotrichum sublineolum*	68.96 (6.005)
	Colletotrichum gloeosporioides	70.62 (1.715)
	Fusarium moniliforme	57.03 (3.971)
	Exserohilum turcicum	59.37 (11.540)
MaI254	*Colletotrichum sublineolum*	72.15 (6.550)
	Colletotrichum gloeosporioides	72.13 (5.898)
	Fusarium moniliforme	63.37 (2.544)
	Exserohilum turcicum	60.33 (9.846)

Appendix viii: Analysis of Variance Tables

i) Table 3 ANOVA Table

Source Term	D.F	Sum of Squares	Mean Square	F-Ratio	Prob Level	Power ($\alpha = 0.05$)
Between phytopathogens	19	6192.739	325.9337	7.08	0.000000*	1.000000
Within phytopathogens	160	7363.318	46.02074			
Total (Adjusted)	179	13556.06				
Total	180					

* Term significant at $\alpha = 0.05$

ii) Table 4 ANOVA Table

Source Term	D.F	Sum of Squares	Mean Square	F-Ratio	Prob Level	Power ($\alpha = 0.05$)
Between phytopathogens	19	14954.07	787.0562	652.29	0.000000*	1.000000
Within phytopathogens	160	193.057	1.206606			
Total (Adjusted)	179	15147.12				
Total	180					

* Term significant at $\alpha = 0.05$

iii) Table 5 ANOVA Table

Source Term	D.F	Sum of Squares	Mean Square	F-Ratio	Prob Level	Power ($\alpha = 0.05$)
Between phytopathogens	19	7593.929	399.6805	377.56	0.000000*	1.000000
Within phytopathogens	160	169.3734	1.058584			
Total (Adjusted)	179	7763.302				
Total	180					

* Term significant at $\alpha = 0.05$

iv) Table 8 ANOVA Table

Source Term	D.F	Sum of Squares	Mean Square	F-Ratio	Prob Level	Power ($\alpha = 0.05$)
Between phytopathogens	9	11138.68	1237.631	1748.77	0.000000*	1.000000
Within phytopathogens	80	56.61711	0.7077139			
Total (Adjusted)	89	11195.29				
Total	90					

* Term significant at $\alpha = 0.05$

v) Table 10 ANOVA Table

Source Term	D.F	Sum of Squares	Mean Square	F-Ratio	Prob Level	Power (α = 0.05)
Between carbon sources	3	133.5822	44.52739	126.79	0.000000*	1.000000
Within carbon sources	32	11.23767	0.3511771			
Total (Adjusted)	35	144.8198				
Total	36					

* Term significant at α = 0.05

vi) Table 11 ANOVA Table

Source Term	D.F	Sum of Squares	Mean Square	F-Ratio	Prob Level	Power (α = 0.05)
Between incubation periods	9	12680.24	1408.916	3852.39	0.000000*	1.000000
Within incubation periods	80	29.25798	0.3657247			
Total (Adjusted)	89	12709.5				
Total	90					

* Term significant at α = 0.05

vii) Table 12 ANOVA Table

Source Term	D.F	Sum of Squares	Mean Square	F-Ratio	Prob Level	Power (α = 0.05)
Between pH values	7	6858.513	979.7876	1591.90	0.000000*	1.000000
Within pH values	64	39.39098	0.615484			
Total (Adjusted)	71	6897.904				
Total	72					

* Term significant at α = 0.05

viii) Table 13 ANOVA Table

Source Term	D.F	Sum of Squares	Mean Square	F-Ratio	Prob Level	Power (α = 0.05)
Between temperature conditions	6	49.20833	8.201389	10.56	0.000000*	0.999996
Within temperature conditions	56	43.48107	0.7764476			
Total (Adjusted)	62	92.6894				
Total	63					

* Term significant at α = 0.05

ix) Table 14 ANOVA Table

Source Term	D.F	Sum of Squares	Mean Square	F-Ratio	Prob Level	Power (α = 0.05)
Between storage periods	13	89.66382	6.897217	8.15	0.000000*	1.000000
Within storage periods	112	94.84127	0.846797			
Total (Adjusted)	125	184.5051				
Total	126					

* Term significant at α = 0.05

Appendix ix: Screening of sorghum rhizobacterial isolates KaI245 and MaI254 against the test sorghum foliar fungal pathogens using the dual culture technique described by Paulitz *et al.* (1992) and Landa *et al.* (1997)

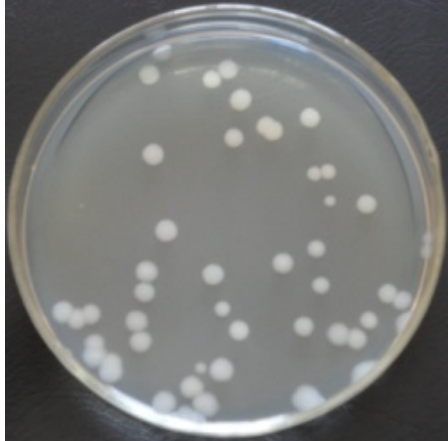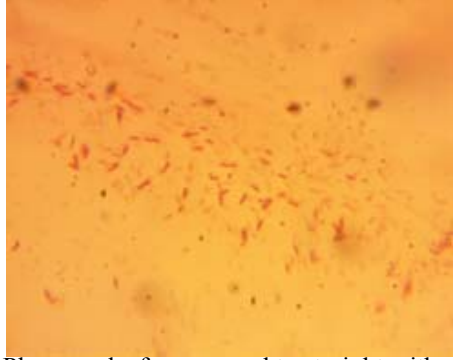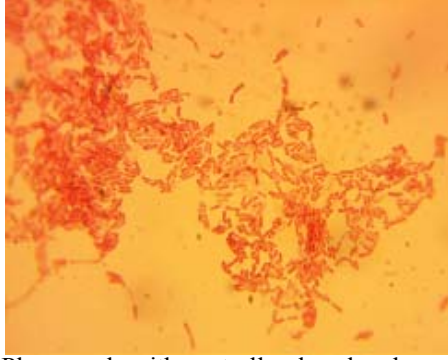| Rhizobacterial isolate	Plate rep.	% Mycelia growth inhibition									
		Alternaria alternata	*Aspergillus candidus*	*Alternaria longissima*	*Botrytis cinerea*	*Colletotrichum gloeosporioides*	*Colletotrichum sublineolum*	*Exserohilum turcicum*	*Fusarium equiseti*	*Fusarium moniliforme*	*Nigrospora oryzae*
KaI245	1	71.05	75.00	71.11	72.73	71.43	65.52	67.50	61.36	52.14	73.33
	2	68.42	71.05	68.89	71.11	71.43	63.33	62.50	62.22	57.14	71.11
	3	71.79	68.42	68.89	71.11	70.73	67.74	64.10	64.44	57.14	75.56
	4	69.89	77.78	59.52	65.85	66.67	73.33	64.71	42.86	66.67	82.22
	5	72.14	77.78	64.44	62.50	66.67	73.33	66.67	54.76	64.86	84.44
	6	71.79	80.00	60.47	61.54	67.74	76.74	71.43	52.38	64.88	82.22
	7	68.46	63.41	60.00	86.67	76.92	72.73	57.78	40.00	68.89	71.11
	8	70.19	64.29	64.44	86.49	77.50	71.11	60.00	55.00	66.67	68.89
	9	70.23	63.41	61.90	89.74	75.61	71.11	62.22	55.45	71.11	73.33
Means:		**70.44**	**71.24**	**64.41**	**74.19**	**71.63**	**70.55**	**64.10**	**54.27**	**63.28**	**75.80**
MaI254	1	72.97	76.92	66.67	69.77	69.05	68.97	46.43	72.09	59.52	75.61
	2	71.79	79.49	60.00	71.11	69.05	66.67	51.85	65.91	57.78	75.50
	3	71.05	74.36	68.75	72.73	68.29	67.74	50.00	76.74	61.90	76.48
	4	71.79	75.00	66.67	80.00	77.78	72.73	62.50	43.45	59.46	69.67
	5	69.87	72.22	66.67	75.00	75.56	68.89	67.50	41.87	62.50	70.67
	6	72.98	72.97	64.71	77.50	79.55	70.45	65.85	51.43	61.54	72.74
	7	70.19	58.14	61.54	68.89	67.50	87.18	77.78	56.45	71.79	76.92
	8	71.51	62.22	61.54	66.67	67.50	87.50	77.78	54.98	68.42	77.50
	9	71.05	65.91	58.33	68.89	66.67	90.24	77.27	49.78	71.05	71.43
Means:		**71.47**	**70.80**	**63.88**	**72.28**	**71.22**	**75.60**	**64.11**	**56.97**	**63.77**	**74.06**

Appendix x: Screening of sorghum rhizobacterial isolates KaI245 and MaI254 against test sorghum fungal plant pathogens using the Besson *et al.* (1978) culture technique

Rhizobacterial isolate	Plate rep.	Clear zones of inhibition (mm) produced against:									
		Alternaria alternata	*Aspergillus candidus*	*Alternaria longissima*	*Botrytis cinerea*	*Colletotrichum gloeosporioides*	*Colletotrichum sublineolum*	*Exserohilum turcicum*	*Fusarium equiseti*	*Fusarium moniliforme*	*Nigrospora oryzae*
KaI245	1	7.67	19.67	13.33	31.33	19.33	22.00	11.00	8.33	4.00	25.00
	2	6.67	20.67	15.00	28.33	18.00	23.33	8.33	9.33	5.00	25.33
	3	9.00	19.67	17.00	31.33	19.00	23.67	11.00	9.67	4.67	26.33
	4	8.78	19.67	14.45	30.14	19.43	20.67	7.33	8.33	4.33	26.33
	5	8.78	21.00	16.12	29.04	18.87	21.33	8.00	9.00	4.00	27.12
	6	6.67	20.14	19.10	31.34	19.27	21.67	6.00	9.33	5.00	25.89
	7	9.11	20.14	18.70	32.16	20.13	23.33	10.13	7.97	4.00	25.14
	8	8.99	20.14	16.90	31.33	18.98	24.00	10.17	8.06	4.33	28.15
	9	9.11	21.45	13.33	31.33	18.99	22.00	9.78	10.11	4.33	29.13
Means:		**8.31**	**20.28**	**15.99**	**30.70**	**19.11**	**22.44**	**9.08**	**8.90**	**4.41**	**26.49**
MaI254	1	2.67	5.67	6.33	20.00	15.67	13.00	7.67	-2.00	-2.00	19.67
	2	2.00	5.00	5.67	18.67	17.67	13.00	9.00	-1.33	-2.00	21.33
	3	2.67	5.00	6.00	22.00	17.00	14.00	8.33	-2.00	-2.00	23.33
	4	3.00	5.12	5.97	21.14	18.14	9.33	7.33	-1.33	-2.00	19.00
	5	2.87	5.00	6.00	20.58	18.15	11.33	7.00	-2.00	-2.00	21.00
	6	2.89	5.21	5.87	22.14	17.67	13.00	8.33	-2.00	-2.00	22.67
	7	3.00	4.97	7.01	23.14	17.97	14.01	9.01	-2.00	-2.00	21.00
	8	2.67	5.33	6.01	24.01	18.13	12.97	8.12	-2.00	-2.00	21.33
	9	3.11	5.13	5.03	21.02	19.12	13.01	7.00	-2.00	-2.00	23.33
Means:		**2.76**	**5.16**	**5.99**	**21.41**	**17.72**	**12.63**	**7.98**	**-1.85**	**-2.00**	**21.41**

Appendix xi: Screening of sorghum rhizobacterial isolates KaI245 and MaI254 against test sorghum fungal plant pathogens using the modified Besson *et al.* (1978) culture technique

Rhizobacterial isolate	Plate rep.	Clear zones of inhibition (mm) produced against:									
		Alternaria alternata	*Aspergillus candidus*	*Alternaria longissima*	*Botrytis cinerea*	*Colletotrichum gloeosporioides*	*Colletotrichum sublineolum*	*Exserohilum turcicum*	*Fusarium equiseti*	*Fusarium moniliforme*	*Nigrospora oryzae*
	1	10.13	7.95	2.11	11.98	10.87	8.97	3.42	7.12	1.01	15.23
	2	10.21	8.24	3.12	9.23	9.23	8.41	4.21	7.01	3.23	14.23
	3	8.97	9.76	2.89	9.97	8.12	8.23	3.12	9.23	1.24	13.99
	4	10.98	8.23	3.12	11.23	7.98	7.89	2.98	4.78	1.01	14.02
KaI245	5	11.13	7.21	3.23	11.23	9.01	8.1	2.34	5.32	2.11	13.34
	6	10.84	9.01	4.12	13.01	8.92	6.98	4.11	7.32	1.98	15.22
	7	10.73	9.32	3.11	12.98	9.02	8.67	2.11	5.34	1.11	15.22
	8	12.62	10.32	5.98	10.98	7.34	9.01	3.52	9.23	2.1	14.42
	9	12.43	10.12	7.43	14.23	8.97	11.23	4.22	9.14	2.02	13.99
Means:		**10.89**	**8.91**	**3.90**	**11.65**	**8.83**	**8.61**	**3.34**	**7.17**	**1.76**	**14.41**
	1	0	0	0	5.11	10.09	6.23	-8	-9	-8	4.34
	2	0	1.22	0	4.23	8.67	5.97	-8	-9	-8	7.65
	3	-2.1	1.34	0	4.34	8.78	8.33	-8	-9	-8	8.67
	4	0	0	0	2.11	9.33	6.33	-8	-9	-8	4.56
MaI254	5	0	1	0	3.23	8.33	6.33	-8	-9	-8	5.46
	6	-1.22	1.33	0	3.56	8.67	7.12	-8	-9	-8	6.67
	7	-2.11	1.33	1.67	3.67	8.23	6.99	-8	-9	-8	8.33
	8	-1	1.33	0	4.23	10.13	8.33	-8	-9	-8	7.32
	9	0	1.67	0	4.33	9.89	8.56	-8	-9	-8	6.33
Means:		**-0.71**	**1.02**	**0.19**	**3.87**	**9.12**	**7.13**	**-8**	**-9**	**-8**	**6.59**

Appendix xii: Pictorial morphological and biochemical characteristics of rhizobacterial isolates KaI245 and MaI254

Sr. No.	Test	Isolate KaI245 test image/comments	Isolate MaI254 test image/comments
1.	Colonial characters on NA/PDA	Small, copiously mucoid, convex with entire margins	Large, non-mucoid, flat with erose margins
2.	Pigmentation	Nil	Nil
3.	Microscopic characters	Plump rods, from curved to straight with rounded edges	Plump rods with centrally placed endospores

4.	Acid production from glucose	+ve	+ve
		KaI245, Control	MaI254, Control
5.	Gas production from glucose	+ve	–ve
		KaI245 gas bubble fomation	MaI254 No gas bubble fomation
6.	Casein hydrolysis	–ve	+ve
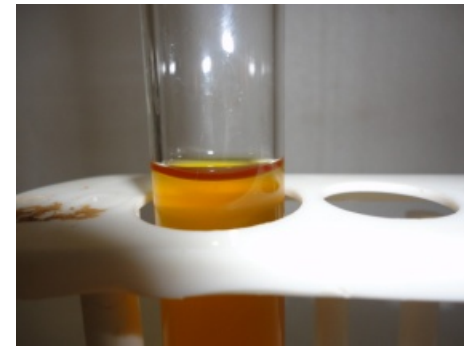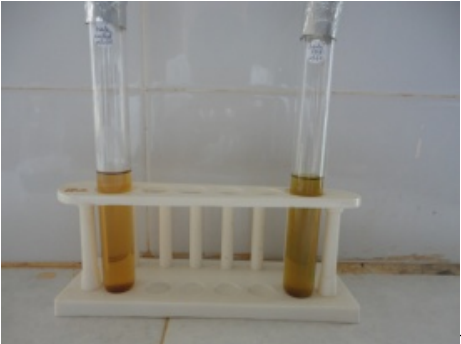
7.	Citrate utilization		

+ve

Control, MaI254, KaI245

+ve

Control, MaI254, KaI245

8.	Gelatin liquefaction

+ve

KaI245   Control

+ve

MaI254   Control

9.	Growth at 50°C	Nd	–ve
10.	Growth at 65°C	Nd	–ve
11.	Growth in 7% NaCl	Nd	+ve
12.	Growth in anaerobic agar	Nd	–ve
13.	Indole production		

–ve

Control,   KaI245

–ve

Control,   MaI254

14.   Glucose fermentation

+ve
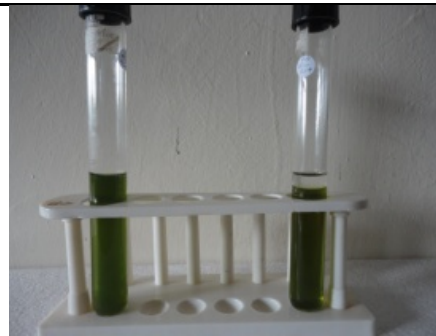
–ve

15.   Fructose fermentation

–ve

–ve

16.   Lactose fermentation

+ve
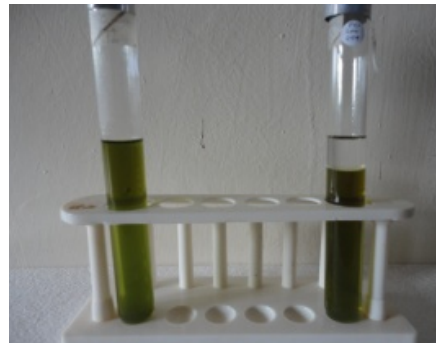
–ve

17.	Sucrose fermentation		
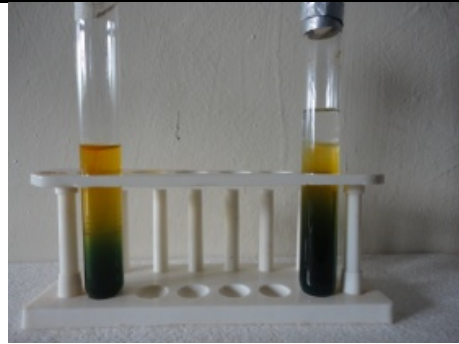
+ve
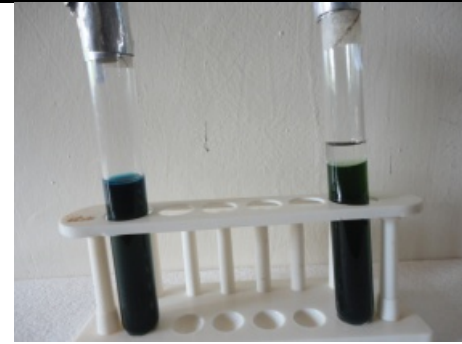
−ve

18.	Nitrate reduction		

+ve
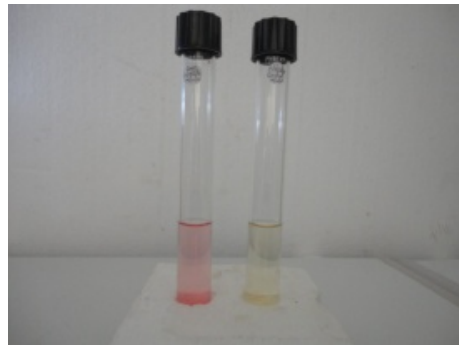
KaI245,   Control
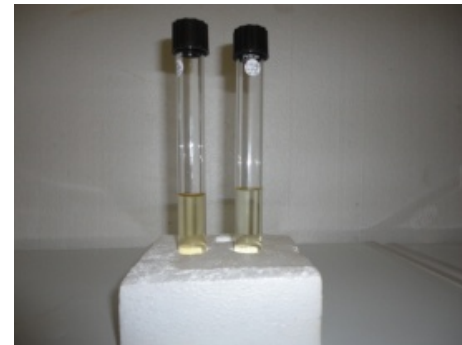
−ve

MaI254,   Control

19.	Voges-Proskaeur test		
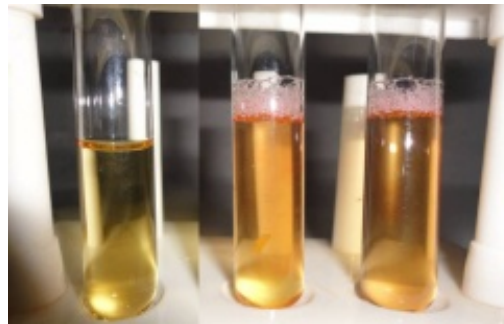
+ve

Control,   KaI245,   KaI245
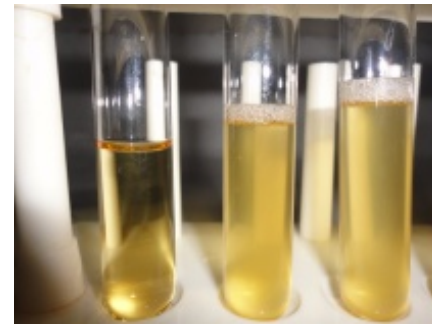
−ve

Control,   MaI254,   MaI254

20.	H₂S production		
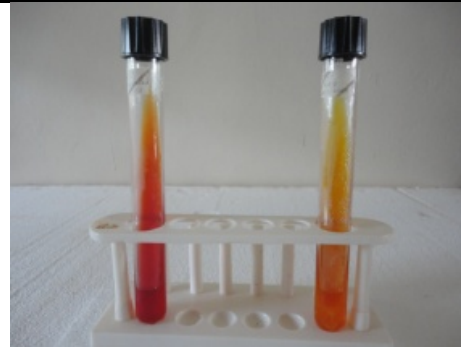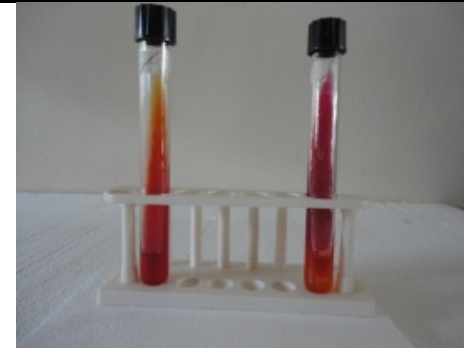
–ve

Control,　　　KaI245

–ve

Control,　　　MaI254

21.	Urease production		

–ve

Control, MaI254, KaI245

–ve
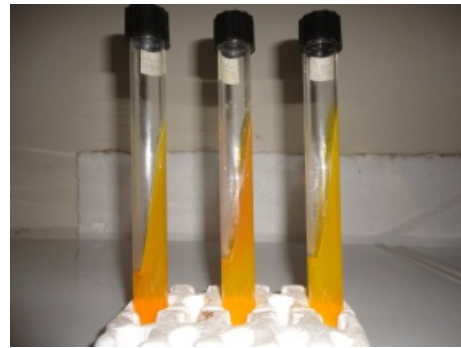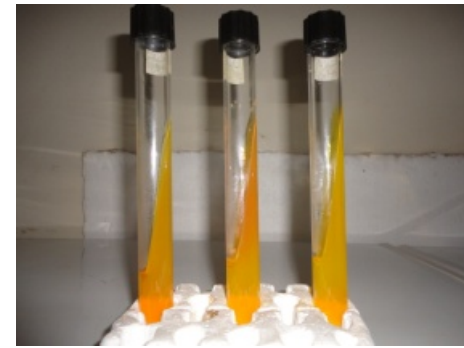
Control, MaI254, KaI245

22.	Starch hydrolysis		

+ve

+ve

Nd—Not determined

Appendix xiii: Clear zones of growth inhibition produced by the antibiotic-culture-filtrate (glucose as the source of carbon) of *Aeromonas hydrophila* **KaI245** against the test sorghum fungal pathogens 3 days after inoculation

Rhizobacterial isolate	Disc rep.	Clear zones of inhibition (mm) produced against:									
		Alternaria alternata	*Aspergillus candidus*	*Alternaria longissima*	*Botrytis cinerea*	*Colletotrichum gloeosporioides*	*Colletotrichum sublineolum*	*Exserohilum turcicum*	*Fusarium equiseti*	*Fusarium moniliforme*	*Nigrospora oryzae*
	1	2.52	7.59	15.52	34.21	21.02	29.01	0.00	18.02	16.01	31.58
	2	3.01	7.00	16.10	33.54	21.53	29.01	0.54	18.01	16.53	32.52
	3	2.54	7.54	16.51	34.21	22.14	28.53	0.56	18.53	17.00	31.56
	4	3.21	8.53	14.03	34.14	24.05	29.34	1.03	18.09	16.24	32.54
KaI245	5	2.52	8.02	15.52	33.12	23.51	29.13	0.55	18.52	16.52	32.09
	6	2.54	8.13	16.21	32.15	24.53	29.17	0.00	19.52	16.51	32.16
	7	3.13	8.50	14.52	31.54	20.56	29.53	0.00	17.05	17.54	34.22
	8	3.42	8.50	14.03	34.23	20.58	29.01	0.00	17.52	17.21	33.57
	9	2.54	8.52	14.56	31.57	21.46	28.53	0.00	17.56	17.52	34.09
Means:		**2.83**	**8.04**	**15.22**	**33.19**	**22.15**	**29.03**	**0.30**	**18.09**	**16.79**	**32.70**

Appendix xiv: Clear zones of mycelia growth inhibition (mm) produced by antibiotic-culture-filtrate[†] of *Aeromonas hydrophila* KaI245 subjected to refrigeration conditions (+4ᵒC) and room temperature conditions (22 ± 5ᵒC) for 8 months and tested against *Colletotrichum sublineolum*

Storage conditions	Paper disc replicate	Clear zones of mycelia growth inhibition (mm) produced by KaI245 culture filtrate assayed at day:						
		0	**40**	**80**	**120**	**160**	**200**	**240**
Refrigeration (+4ᵒC)	**1**	30.74	31.29	30.84	30.93	32.67	31.04	32.45
	2	30.91	31.12	30.11	30.41	32.51	32.56	32.75
	3	30.96	31.18	30.78	30.98	32.89	30.76	32.12
	4	31.88	33.13	30.85	30.89	33.58	32.87	32.92
	5	31.97	31.43	31.65	30.02	32.94	30.04	32.65
	6	31.94	30.51	30.94	30.98	33.64	31.98	33.43
	7	33.88	31.71	32.98	30.89	32.49	30.78	33.42
	8	33.66	31.57	32.99	30.96	31.96	32.12	32.67
	9	33.79	30.99	32.65	30.9	32.79	33.11	33.22
	Means:	**32.19**	**31.44**	**31.53**	**30.77**	**32.83**	**31.70**	**32.85**
	1	30.74	30.56	30.71	31.74	33.13	30.14	32.23
Room temperature (22 ± 5ᵒC)	**2**	30.91	30.88	30.76	30.08	30.98	30.91	33.41
	3	30.96	30.88	30.74	30.44	33.55	31.11	32.76
	4	31.88	30.58	30.29	30.86	32.76	31.8	32.28
	5	31.97	30.78	30.43	30.77	33.78	31.86	33.17
	6	31.94	30.86	31.45	30.54	32.74	32.24	31.67
	7	33.88	31.8	33.43	30.76	33.08	33.86	33.99
	8	33.66	31.97	33.46	32.99	32.69	32.16	32.87
	9	33.79	32.11	32.83	30.41	32.61	33.79	33.56
	Means:	**32.19**	**31.16**	**31.57**	**30.95**	**32.81**	**31.99**	**32.88**

Appendix xv: **Effect of storage and prevailing storage temperature on the antibiotic activity of** *Aeromonas hydrophila* **KaI245 culture-filtrate tested against** *Colletotrichum sublineolum*

218
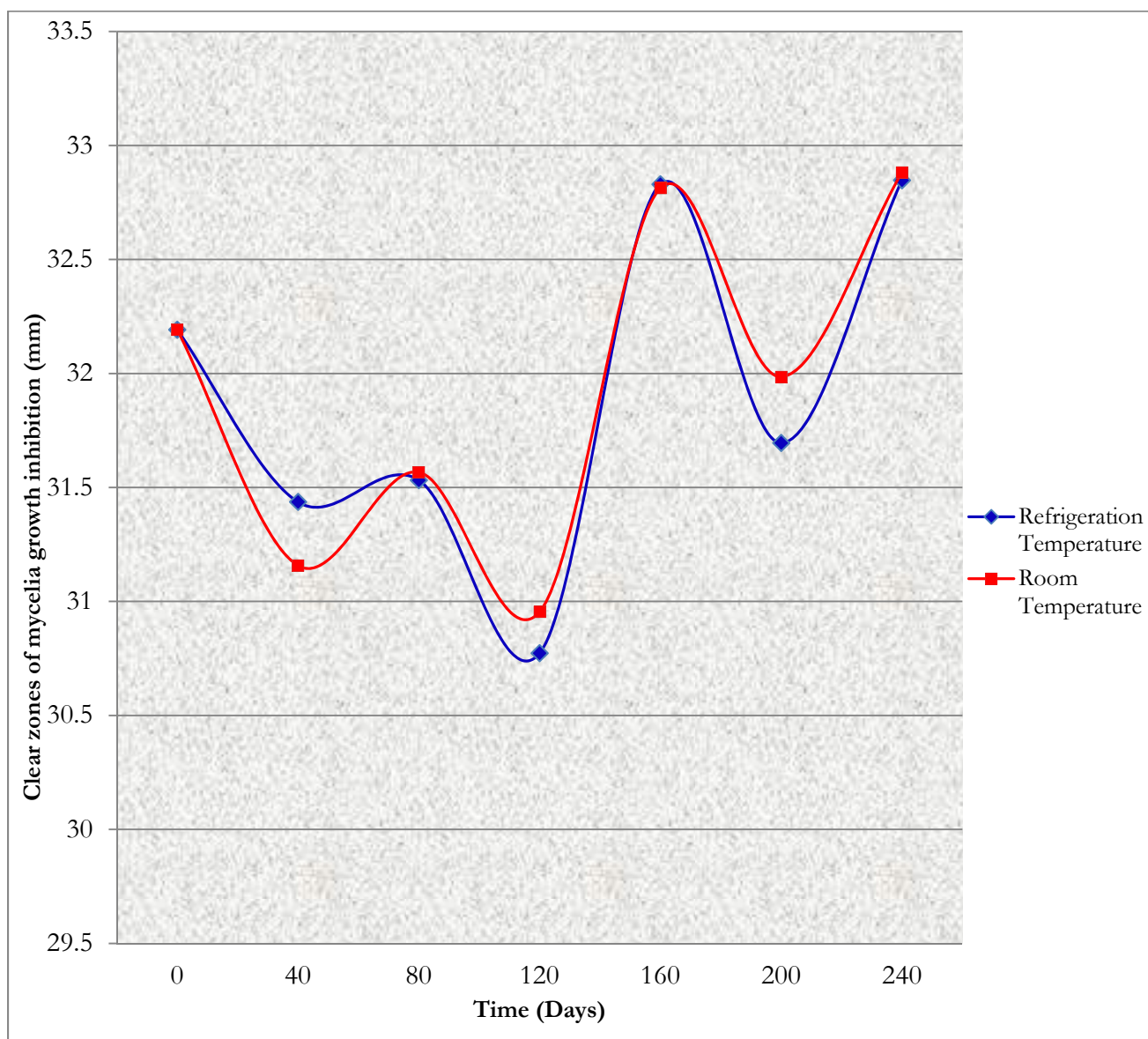
Appendix xvi: Effectiveness of *Aeromonas hydrophila* KaI245 active-culture-filtrate at different concentrations in controlling sorghum anthracnose *in vivo* compared to Folicur® (0.1%) and Ballad®Plus (0.2%).

Treatment	Leaf replicate	Sorghum anthracnose disease severity at the end of:				
		Week 1	Week2	Week 3	Week 4	Week 5
Aeromonas hydrophila KaI245 original antibiotic-culture-filtrate	1	2	2	2	3	3
	2	1	2	3	3	3
	3	2	2	3	3	3
	4	1	1	1	3	3
	5	2	3	4	4	4
	6	1	2	3	2	3
Means		1.5[b]	2.0[d]	2.7[i]	3.0[n]	3.2[t]
Aeromonas hydrophila KaI245 ½ the original concentration antibiotic-culture-filtrate	1	2	2	3	4	4
	2	2	3	3	3	3
	3	2	3	4	4	4
	4	1	2	2	3	3
	5	2	3	3	4	4
	6	1	2	3	3	3
Means		1.7[b]	2.5[f]	3.0[i]	3.5[o]	3.5[t]
Aeromonas hydrophila KaI245 ¼ the original concentration antibiotic-culture-filtrate	1	3	4	4	4	5
	2	2	3	3	4	4
	3	3	4	4	4	4
	4	2	3	3	3	4
	5	2	2	3	4	4
	6	3	4	4	5	4
Means		2.5[c]	3.3[g]	3.5[j]	4.0[o]	4.2[u]

Aeromonas hydrophila **KaI245 double the original concentration antibiotic-culture-filtrate**	1	2	2	2	2	3
	2	2	2	2	3	3
	3	2	2	3	3	3
	4	1	2	2	3	3
	5	2	3	3	3	3
	6	1	2	2	2	2
Means		1.7[b]	2.2[e]	2.3[i]	2.7[m]	2.8[s]
Biofungicide Ballad®Plus (0.2%)	1	3	3	4	5	5
	2	2	3	3	3	4
	3	2	2	3	3	3
	4	2	2	2	3	4
	5	3	4	4	5	5
	6	2	3	3	4	5
Means		2.3[c]	2.8[f]	3.2[i]	3.8[o]	4.3[u]
Conventional fungicide Folicur® (0.1%)	1	1	2	2	3	3
	2	1	1	2	2	2
	3	1	2	2	2	2
	4	1	1	2	2	2
	5	2	2	3	3	3
	6	1	2	2	2	2
Means		1.2[a]	1.7[d]	2.2[h]	2.3[l]	2.3[r]
+ve control (*Colletotrichum sublineolum* suspension: 4.0 x 10⁴ conidia ml⁻¹)	1	1	3	4	5	6
	2	1	3	5	6	7
	3	3	4	6	7	8
	4	3	4	5	6	7
	5	3	4	5	6	7

		6	3	4	4	7	8
	Means		**2.3ᶜ**	**3.7ᵍ**	**4.8ᵏ**	**6.2�q**	**7.2ʷ**
-ve control (Sterile distilled water)		**1**	2	2	3	4	5
		2	2	3	3	5	7
		3	2	3	3	4	5
		4	2	2	3	4	6
		5	3	4	4	5	7
		6	2	3	4	6	7
	Means		**2.2ᵇ**	**2.8ᶠ**	**3.3ⁱ**	**4.7ᵖ**	**6.2ᵛ**

Vertical means per week for the various treatments against sorghum anthracnose symptoms followed by different letters are honestly significantly different (HSD) at $p \leq 0.05$. Significant differences obtained by Tukey-Kramer Multiple-Comparison Test.

Analysis of Variance Table—Week 1

Source Term	D.F	Sum of Squares	Mean Square	F-Ratio	Prob Level	Power ($\alpha = 0.05$)
Between treatments	7	9.666667	1.380952	3.95	0.002316*	0.962582
Within treatments	40	14	0.35			
Total (Adjusted)	47	23.66667				
Total	48					

* Term significant at $\alpha = 0.05$

Analysis of Variance Table—Week 2

| Source Term	D.F	Sum of Squares	Mean Square	F-Ratio	Prob Level	Power (α = 0.05)
Between storage periods	7	19.25	2.75	6.88	0.000022*	0.999348
Within storage periods	40	16	0.4			
Total (Adjusted)	47	35.25				
Total	48					

* Term significant at α = 0.05

Analysis of Variance Table—Week 3

Source Term	D.F	Sum of Squares	Mean Square	F-Ratio	Prob Level	Power (α = 0.05)
Between storage periods	7	29.25	4.178571	9.29	0.000001*	0.999986
Within storage periods	40	18	0.45			
Total (Adjusted)	47	47.25				
Total	48					

* Term significant at α = 0.05

Analysis of Variance Table—Week 4

Source Term	D.F	Sum of Squares	Mean Square	F-Ratio	Prob Level	Power (α = 0.05)
Between storage periods	7	63.3125	9.044642	18.88	0.000000*	1.000000
Within storage periods	40	19.16667	0.4791667			
Total (Adjusted)	47	82.47916				
Total	48					

* Term significant at α = 0.05

Analysis of Variance Table—Week 5

| Source Term	D.F	Sum of Squares	Mean Square	F-Ratio	Prob Level	Power (α = 0.05)
Between storage periods	7	117.5833	16.79762	41.14	0.000000*	1.000000
Within storage periods	40	16.33333	0.4083333			
Total (Adjusted)	47	133.9167				
Total	48					

* Term significant at α = 0.05

Appendix xvii: **Anthracnose disease progress on sorghum leaves treated with undiluted (original concentration) antibiotic-culture-filtrate from *Aeromonas hydrophila* KaI245 compared with Folicur® as a standard, sprayed 48 h after inoculating with *C. sublineolum* spore suspension**
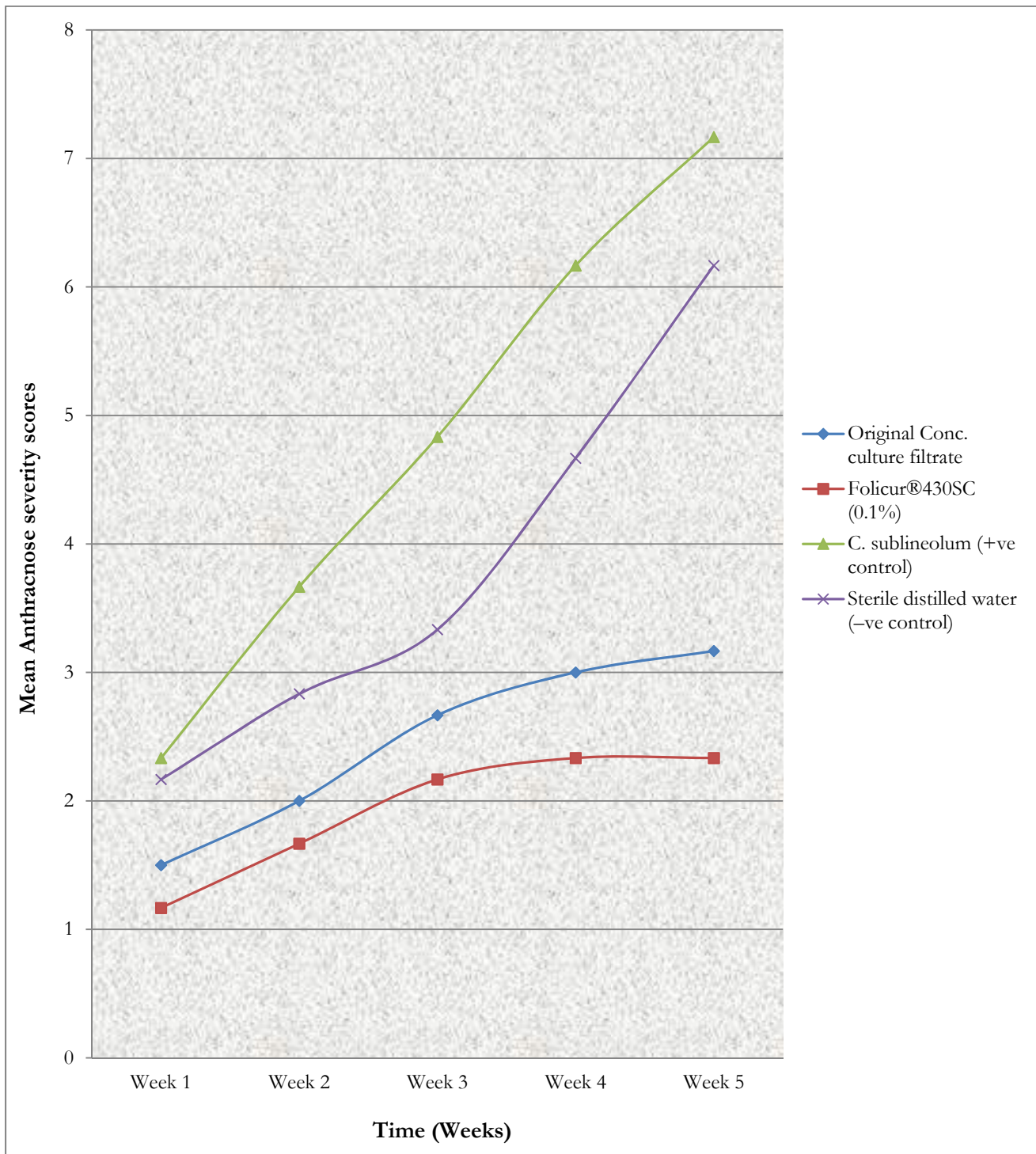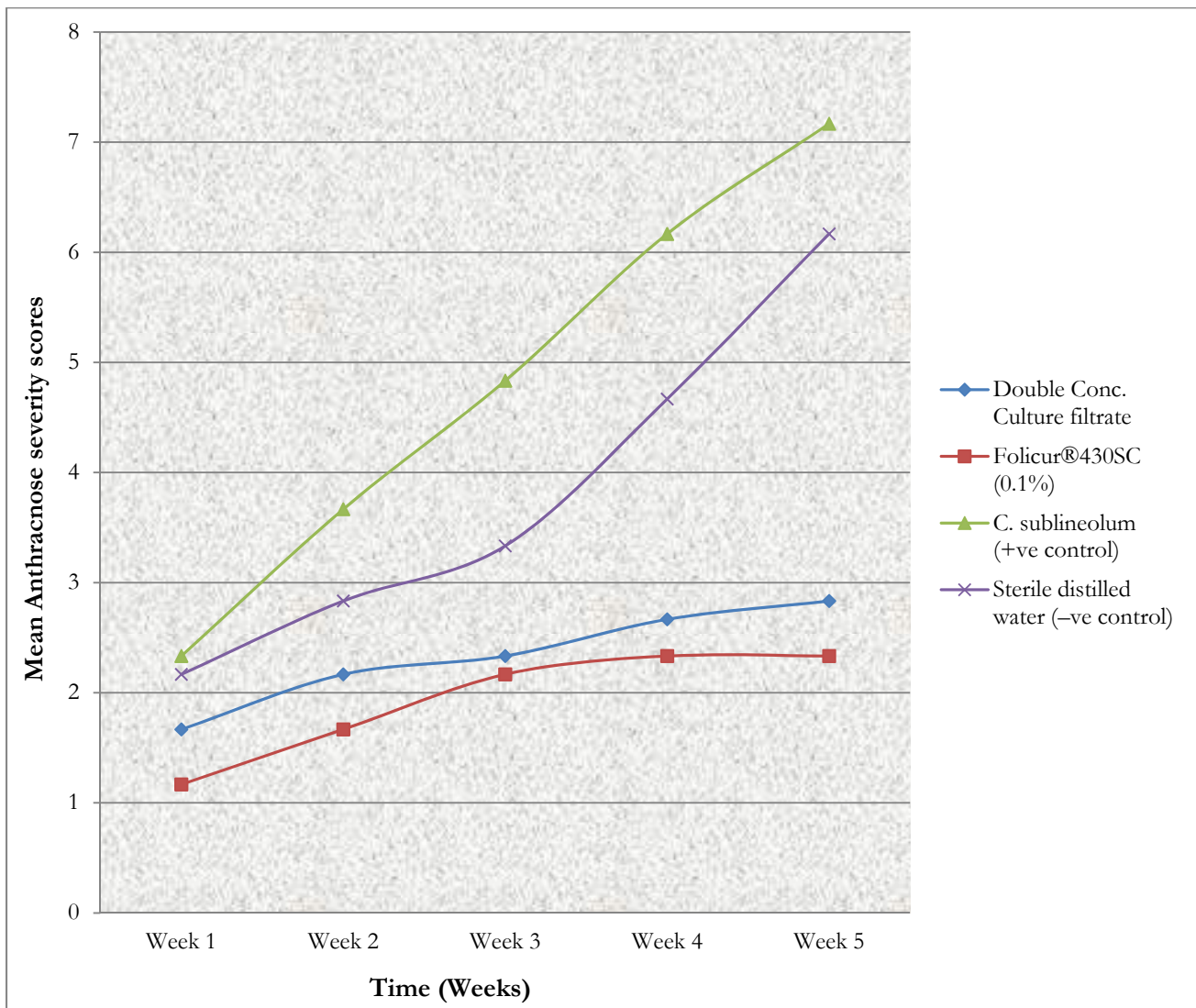
Appendix xviii: Anthracnose disease progress on sorghum leaves treated with diluted (half the original concentration) antibiotic-culture-filtrate from *Aeromonas hydrophila* KaI245 compared with Folicur® as a standard, sprayed 48 h after inoculating with *C. sublineolum* spore suspension
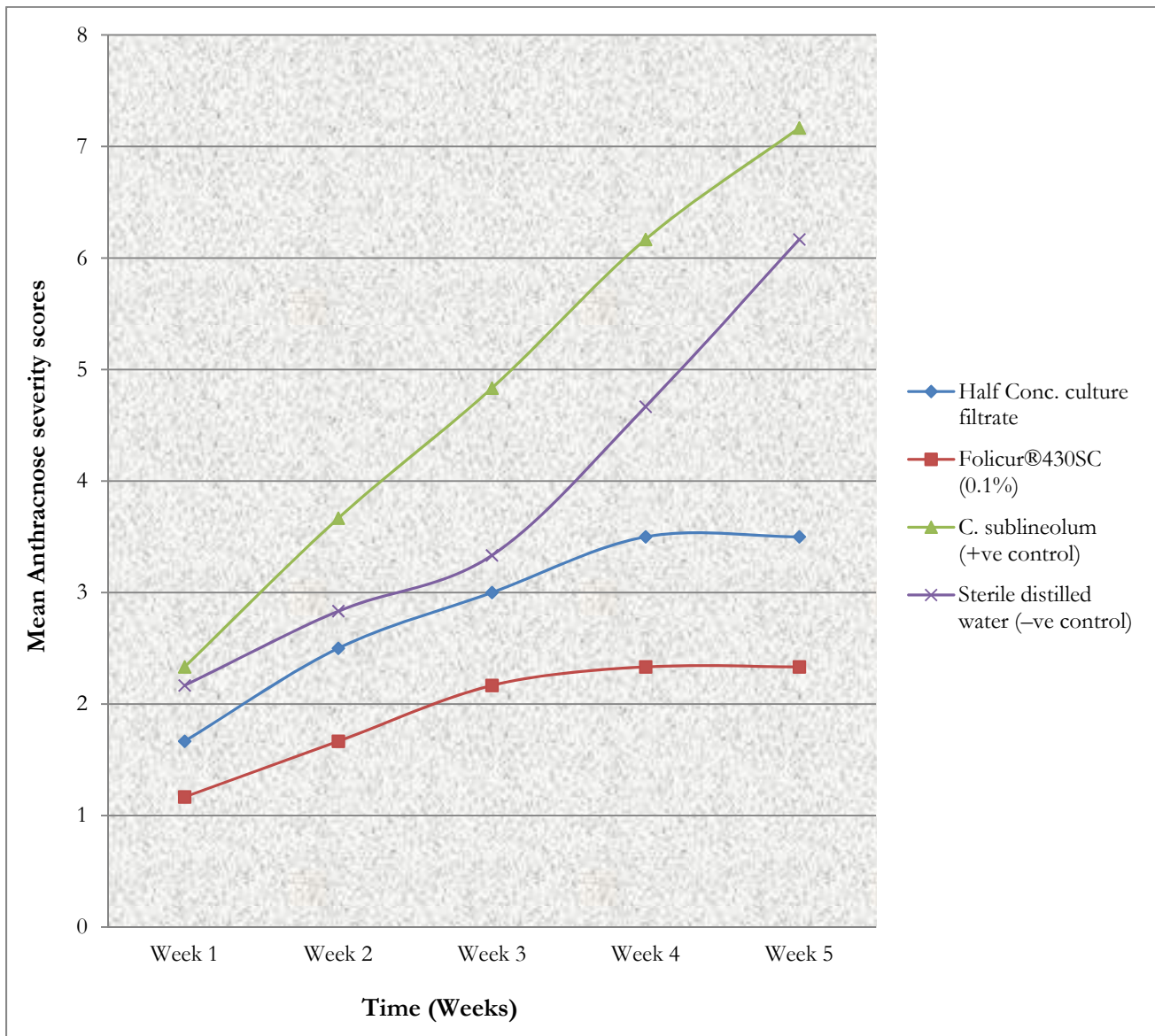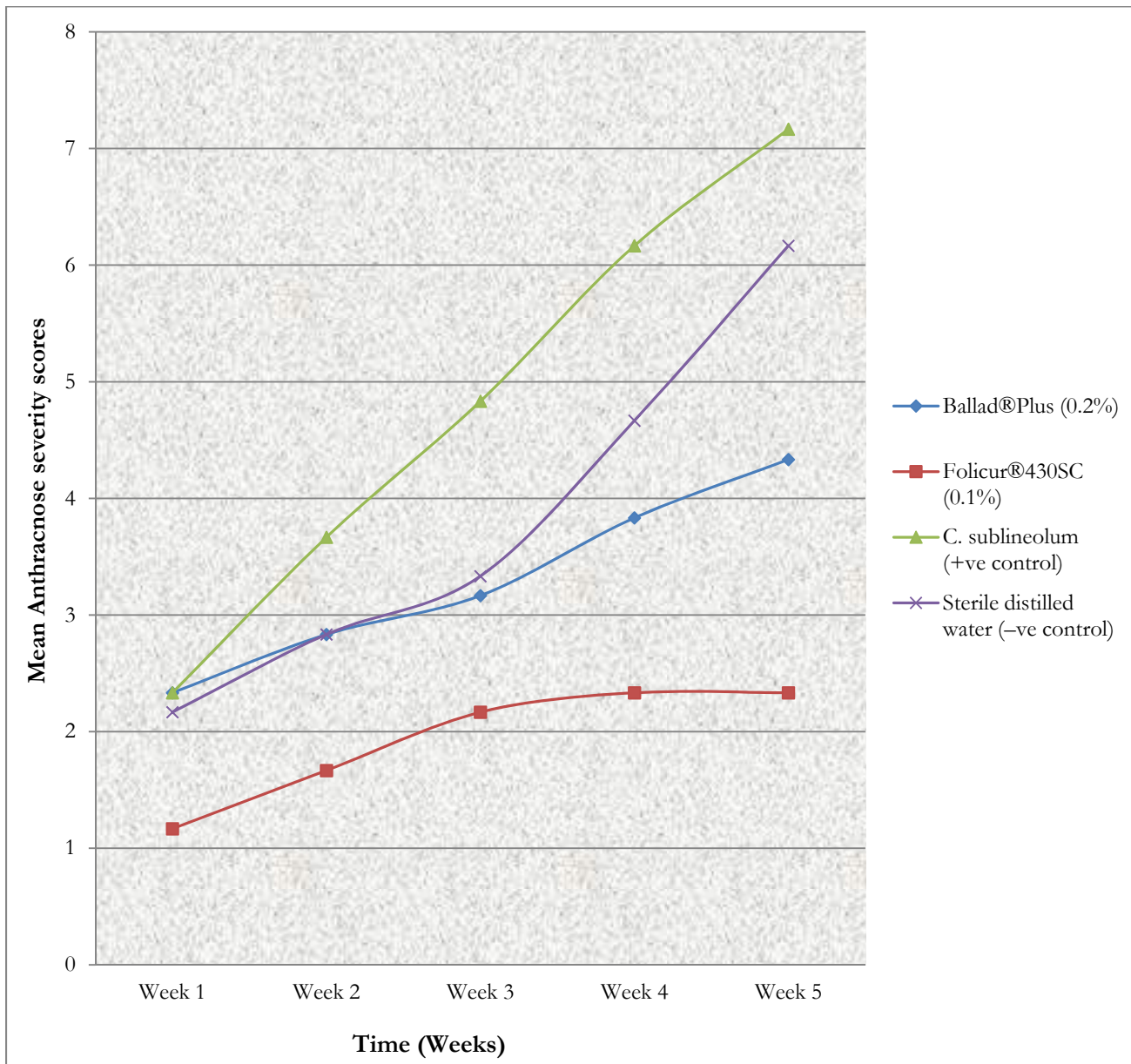
Appendix xix: Anthracnose disease progress on sorghum leaves treated with diluted (quater the original concentration) antibiotic-culture-filtrate from *Aeromonas hydrophila* KaI245 compared with Folicur® as a standard, sprayed 48 h after inoculating with *C. sublineolum* spore suspension
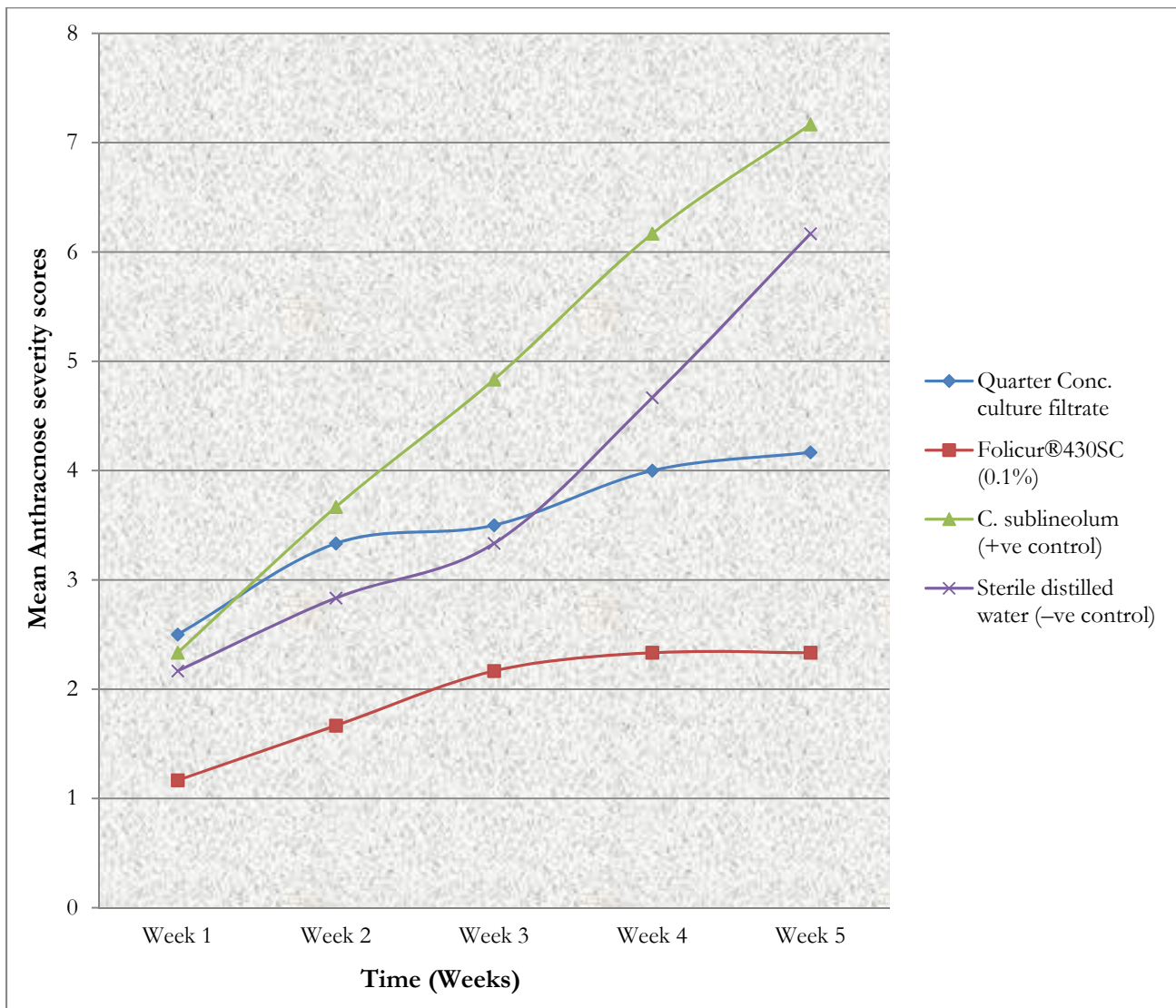
Appendix xx: Anthracnose disease progress on sorghum leaves treated with concentrated (double the original concentration) antibiotic-culture-filtrate from *Aeromonas hydrophila* KaI245 compared with Folicur® as a standard, sprayed 48 h after inoculating with *C. sublineolum* spore suspension

Appendix xxi: **Anthracnose disease progress on sorghum leaves treated with a commercial biofungicide (Ballad®Plus) compared with Folicur® as a standard, sprayed 48 h after inoculating with _C. sublineolum_ spore suspension**

Appendix xxii: A comparison of anthracnose disease progress on sorghum leaves treated with different concentrations of *Aeromonas hydrophila* KaI245 antibiotic-culture-filtrate compared with a commercial biofungicide (Ballad®Plus), sprayed 48 h after inoculating with *C. sublineolum* spore suspension.