OCCURENCE, DIVERSITY AND BOTANICAL MANAGEMENT OF ANTHRACNOSE PATHOGEN (*Colletotrichum lindemuthianum*) IN FARM-SAVED BEAN SEED IN WESTERN KENYA.

BY

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A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF DOCTOR OF PHILOSOPHY IN SEED SCIENCE AND TECHNOLOGY, UNIVERSITY OF ELDORET, KENYA

2014

DECLARATION

Declaration by candidate

This thesis is my original work and has not been presented for a degree in any other university. No part of this thesis may be reproduced without prior permission of the author or University of Eldoret.

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This work is dedicated to my dear parents, husband and children: Eliud, Daniel and Pauline.

ABSTRACT

Common bean (*Phaseolus vulgaris* L.) crop plays an important role in human nutrition and poverty alleviation in Western Kenya. Most farmers use farm saved seed. However, seed borne anthracnose *Colletotrichum lindemuthianum* disease lowers the quality of farm-saved bean seed resulting in low bean yields. Host plant resistance and environment friendly fungicides are among the most affordable, appropriate and effective control measures of the disease. Unfortunately, *C. lindemuthianum* is highly variable resulting in breakdown of resistance in bean cultivars. The objective of this study was to investigate the occurrence, diversity and botanical management of *C. lindemuthianum* in farm-saved bean seed in western Kenya. The study was done in Bungoma, Kakamega, Trans Nzoia, Nandi, Busia, Kisii and Siaya regions of Western Kenya between December 2008 and July 2010. One hundred and ninety six farmers' farm-saved seed accessions and 122 infected bean pod samples in the field were collected from the study area using systematic random sampling. Disease incidence on the seed was determined using blotter and growing-on test. Virulence on the 12 common bean differential cultivars and BOX-AIR marker were used to assess the physiological and genetic diversity of *C. lindemuthianum* isolates from the infected pods. Eight plants extracts; *Allium cepa* L., *Allium sativum, Aloe vera, Azadirachta indica, Datura stramonium, Eucalyptus globules, Lantana camara* and *Cleome gynandra* were screened for antifungal activity against *C. lindemuthianum*. Data collected was analyzed by analysis of variance and means separated using Turkey's test at $p \leq 0.05$. Diversity data was analyzed using Numerical Taxonomy and Multivariate Analysis System for personal computer (NTSYS-pc) ®. Different seed varieties from different regions had high and significantly different ($p \leq 0.05$) anthracnose incidences. GLP 585 variety had the highest incidence (53.1%) whereas GLP 2 had the lowest (28.7%). Seventy four races of *C. lindemuthianum* were identified among 122 isolates showing high virulence diversity. Race 0 was the most frequent race and occurred frequrntly. The most virulent races were less frequent and occurred in single region only. The isolates were virulent to all bean differential cultivars with 14 races breaking the resistance of the most resistant cultivar G2333. Seventy three new races were obtained as they did not conform to the races previously obtained in Kenya. Sixty races grouped into eight clusters revealing that *C. lindemuthianum* was a highly variable pathogen. No associaton was observed between the genetic diversity and race classification of the isolates. All the *in vitro* tested water extracts were active against *C. lindemuthianum* at inhibitory concentration of 30% though *A. vera* had the highest (90.6%) whereas *L. camara* had the lowest (28.7%). *In vivo* tests showed that plants treated with *A.vera* extracts had a lower anthracnose incidence and severity (23% and 15% respectively) and compared well with disease reductions due to the application of the Mancozeb at 2 gm l^{-1} (11.1% and 10% respectively). It was concluded that in Western Kenya *C. lindemuthianum* was highly variable and 73 new races had been obtained in addition to those from previous studies in Kenya. The use of *A. vera* plant extract as foliar treatment by farmers could offer alternatives for anthracnose management for resource-poor, small holder farmers.

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ACKNOWLEDGEMENTS

I wish to recognize the several institutions and individuals who provided me with encouragement, assistance and guidance while I was carrying out this research. My deepest and sincere gratitude goes to my supervisors Prof. Julius O. Ochoudho and Dr. Linnet S. Gohole for the enthusiasm, inspiration and guidance they provided throughout the course of my study. My heartfelt gratitude goes to Kenyan Government through the Ministry of Agriculture for having nominated me for a two year study leave which gave me ample time to do this research. I am indebted to Centro International de Agricultura Tropical (CIAT), Colombia for providing the 12 defined differential common bean cultivars seed for this research. I am grateful to the farmers who generously provided samples of their farm-saved bean seed. My sincere gratitude to the University teaching Staff and post graduate students who were co-operative at all stages of this work and in particular the School of Agriculture and Biotechnology for providing the laboratory and green house facilities. Special acknowledgement to Prof. M. Kinyua, Prof. A. Onkware, Dr. E. Arunga, Dr. N. Rop, V. Anjichi, B. Makumba, Biwotti, Jane and Violet who participated in the field and laboratory activities during my research. I wish to appreciate my parents who effortlessly supported me from my basic education amidst other commitments. It is my pleasure to express my unconditional love and appreciation to my husband Mr. Elijah O. Ayiera and our children Eliud, Daniel and Pauline without whose support, encouragement and patience this work could not have been a success. Lastly but not least, I humbly thank my God for His gracious mercy, love and protection in my life.

CHAPTER ONE

INTRODUCTION

1.1 Common bean and its importance

Common bean (*Phaseolus vulgaris* L.) is an important legume food crop widely cultivated and consumed throughout the world. It originated from Central America (Purseglove, 1988) and introduced to East Africa by the Portuguese in the sixteenth century. In Kenya it is rated as one of the most important legumes grown by more than 3 million households (GOK, 2006; Kenya National Bureau of statistics (2007). Consumption of common bean is high with per capita consumption estimated at 14 kg per year in Kenya, but can be as high as 66 kg/yr in western Kenya (Spilsbury *et al*., 2004; Buruchara, 2007; FAOSTAT, 2013).

Consumed as leaves, green pods, fresh and dry grain; bean is a major source of dietary protein as it is a relatively inexpensive alternative source of protein in many households compared to animal products (FAO, 2008). It is a valuable source of protein (22%), carbohydrates, fiber, vitamins (folate) and minerals (Ca, Cu, Fe, Mg, Mn, Zn) for human diets (Pachico, 1993; Wortmann *et al*., 1998; Voysest, 2000; Broughton *et al*., 2003). It is cholesterol free and has low fat hence its regular consumption is promoted by health organizations to reduce the risk of diseases such as cancer, diabetes or coronary heart diseases (Leterme & Munoz, 2002).

Beyond its contribution to human nutrition, bean crop has considerable economic importance in providing income for smallholder farmers (ECABREN, 2000; Mwaniki, 2002; FAO, 2008). The crop therefore is particularly suitable for food security because of its nutrient composition, short growing cycle and adaptability to different cropping systems.

1.2 Bean production in Kenya

Among the major bean production areas in Kenya are the highland and midlands of Western Kenya which have adequate and well distributed rainfall and productive soils. These areas contribute about 75 percent of the annual production with Rift Valley contributing the biggest share (33 percent), followed by Nyanza and Western regions (22 percent each) (Karanja, 2006). The total area under common bean cultivation in Kenya is estimated to be an average area of 910,478 hacters with average production of about 417,000 metric tons, an equivalent of US$ 199,743,000 million (FAO, 2008; FAOSTAT, 2010;). The actual yield of 0.5 tonnes ha^{-1} (FAO, 2007) is much less compared to the yield potential of 1–2 tonnes ha^{-1} (Muasya, 2001; FAOSTAT, 2013). Further, estimate data, indicates that bean yield production rates were on the increase in 1970-1990 but the trend reversed with yield declining at an average rate of 6.8 % (MOARD, 2004; FAOSTAT, 2008) due to different constraints including diseases.

1.3 Bean production constraints

Low bean yields are attributed to a number of biotic and abiotic constraints (Otysula *et al*., 1998; Kimani *et al*., 2001; Mwaniki, 2002; Wagara, 2005). Major abiotic constraints include low soil fertility due to nitrogen and phosphorus deficiency, low pH and drought. Biotic constraints include diseases; such as common bacterial blight (*Xanthomonas campestris* pv *phaseoli*); angular leaf spot (*Phaeoisariopsis griseola*); bean rust (*Uromyces appendiculatus*); bean common mosaic virus; halo blight (*Pseudomonas syringe* pv. *phaseolicola*); anthracnose (*Colletotrichum lindemuthianum*); ascochyta blight (*Phoma exigua* var. *exigua*); root rots (*Pythium ultimatum, Rhizoctonia solani*, *Fusarium solani* f. sp. *phaseoli*) and

insect pests such as; bean stem maggot and bruchids among others (*http://www.africancrops.net/rockefeller/crops/-beans/index.htm*).

One of the most destructive diseases in wet and humid bean growing regions including Western Kenya is the seed borne bean anthracnose disease. It is caused by a cosmopolitan fungi *Colletotrichum lindemuthianum* (Sacc. & Magnus) Lams. – Scrib. (Barrus, 1911). The fungus causes dark brown sunken lesions on all above ground parts including the seeds (Jerba *et al.,* 2005). Infection of a susceptible cultivar in early growth cycle under favorable conditions leads to an epidemic which may result in 100 % yield loss (Pastor-Corrales & Tu, 1989; Fernandez *et al.,* 2000; Ansari *et al*., 2004; Sharma *et al.,* 2004). Seed infection by *C. lindemuthianum* also reduces seed quality and results in the introduction of the disease or new races into new geographic regions (Tu, 1992; Agrios, 2000). Previous results from Tanzania estimated that for each 1 percent increase in anthracnose incidence, seed yield decreased by 9 kg/ha (Allen, *et al*., 1998).

1.4 Bean seed sources in Western Kenya

Seed is the only propagation method for beans. Bean seed, among other inputs, play an important role towards achieving high yields as it determines bean productivity to a great extent. Bean seed sources in Western Kenya include certified and farm-saved seeds. Currently, more than 75% of Western Kenya farmers prefer to sow farm-saved seed because it is cheap and readily available (Opole *et al.,* 2003; Otsyula *et al*., 2004). Farm-saved seed is the harvested grain set aside by the farmer to use as seed in the following plant in season or exchanged with neighbors or purchased from the informal local grain market. Local markets consist of suppliers who buy grain from farmers in their neighborhoods at harvest time, treat and store it for resale

as seed at planting time. Farm-saved seed is of different varieties that are well adapted to farming conditions. In addition, the seed is accessible because the suppliers provide credit more easily than those stores that sell certified seed.

However, farm-saved seed have some constrains which include among others, poor seed quality due to the seed borne disease such as bean anthracnose. Anthracnose infected seed if planted, significantly lead to poor germination, blemishes on pods, distortion and discoloration of the seed (de Rio & Bradley, 2002) resulting in reduction of the crop stands, seed quality, yield and marketability (Fernandez *et al.*, 2000). It has been observed that seed infections as low as 0.01-0.1% can cause very severe yield losses exceeding 90% depending on the agro-ecological and weather conditions (Fernandez *et al*., 2000; Yesuf & Sangchote, 2007).

1.5 Control of bean anthracnose

There are several control measures of bean anthracnose including use of certified seeds, resistant varieties (Mahuku & Riascos, 2004) and chemical fungicides (Tian *et al.* 2007). The greatest setback to development and deployment of these management strategies is the high variable nature of *C. lindemuthianum* which results in continuous breakdown of resistance of both local and commercial cultivars due to easy adaptation of the pathogen to host resistance (McDermott, 1993; Mahuku & Riascos, 2004). The varieties that could be resistant in one location or year become susceptible in another (Mahuku, *et al*., 2002) and this complicates the use of host resistance genes. Further, it eventually leads to continuous and indiscriminate use of chemical fungicides in the pursuit for high bean yields.

1.6 Pathogenic and genetic variability

C. lindemuthianum is known to be a highly variable pathogen due to gene flow and mutation (Mahuku & Riascos, 2004, Sharma, 2008) brought about by the high seed exchange rate in Western Kenya. In situations where farmers lack their own farm-saved seed, they borrow from neighbors or purchase from local markets (Opole *et al*., 2003). This encourages spread and introduction of *C. lindemuthianum* races in W. Kenya hence new races keep emerging time after time (Nkalubo, 2007). In Kenya, existence of different *C. lindemuthianum* races has been reported. Ombiri *et al*., (2002) acknowledged the existence of race 485 in Rongai, Nakuru, Kenya, in addition to the previously existing six races; Beta, Alpha, Gamma, Delta, Epsilon and Lambda which had been obtained in nine parts of Kenya (Kinyua 1976; Mwangi 1986; Gathuru & Mwangi 1991). This predicts the potential of development of variable races in major bean growing areas and that race composition in any given area is continuously changing and therefore needs to be continuously monitored. There was need to carry out this study to determine the current C. *lindemuthianum* pathogenic variability in W. Kenya.

In addition to race variability, genetic variability was necessary to direct breeding efforts towards long-term resistance to anthracnose. Development of resistant cultivars depends to a large extent on levels of pathogenic and molecular variability exhibited by the pathogen. Since no work on genetic variability of the pathogen has been carried out in W. Kenya it was paramount to characterize the pathogen also using molecular markers. There is need for integration of host resistance with an effective, economical and environment friendly management strategies to control the disease. Some plant extracts have been found to be most effective in the inhibition of seed-borne pathogens and in the improvement of seed

quality and field emergence of plant seeds (Shovan *et al.,* 2008; Nwachukwu & Umechuruba, 2001; Wokocha & Okereke, 2005; Satish *et al.,* 2007; Muthusamy *et al.,* 2007; Lindomar, 2008). Plant-based antifungals are known to be one of the better alternatives to the synthetic fungicides because they have no environmental impact and danger to consumers (Varma & Dubey, 1999; Nwachukwu & Umechuruba, 2001; Kiran *et al.,* 2006). The current work was also done to determine the antifungal potential of easily available plant extracts in W. Kenya for management of anthracnose.

1.7 Statement of the problem

Bean anthracnose lowers yield, seed quality and marketability of the crop. Host resistance and chemical control methods are among the most effective integrated disease management strategies. However, the use of these methods is challenged by the high variability of the causal pathogen *Colletotrichum lindemuthianum* (Mahuku & Riasco, 2004; Sharma *et al*., 2007; Padder *et al*., 2007). The high variability results in continuous breakdown of resistance in the bean cultivars. It further complicates use of host resistance genes and encourages continuous and indiscriminate use of chemical fungicides hence accumulation of harmful substances in the seeds, soil and water and induction of resistance in pathogens. *Colletotrichum lindemuthianum* race composition and genetic variability in Western Kenya has not been studied. Control of the pathogens using plant extracts is feasible but this has not been investigated on this pathogen in W. Kenya.

1.8 Justification

The current work was done to investigate the occurrence and management of the pathogen for increased food security in Western Kenya. Results on incidence of

anthracnose in W. Kenya farm-saved seed could provide information on the susceptible varieties and regions with high disease incidence. This could enable farmers to make the right choice on the varieties to use and regions from which to borrow exchange or purchase farm-saved seed. The breeders could also be enabled to pyramid resistant genes in different bean varieties. Knowing the race composition of the pathogen will provide information on how best to manage the disease. It will give insight into gene and evolutionary potentials important information to plant breeders in their breeding work for host resistance. Organic plant extracts could be environmental friendly, easily available and affordable fungicides to control the anthracnose disease. The plant extracts are free from environment toxicity, easily biodegradable and not harmful to human consumption as compared to synthetic compound hence effective management method of bean anthracnose disease. Cumulatively knowledge on anthracnose incidence, pathogen variability and its management offer many opportunities for improvement of farm-saved seed quality. Improved farm-saved bean seed would lead to increased bean yield production.

1.9 Objectives

1.9.1 General objective: -

To improve the quality of farm-saved bean seed by investigating the occurrence and management of *Colletotrichum lindemuthianum* causal agent of bean anthracnose in Western Kenya.

1.9.2 Specific objectives:-

1. To determine the anthracnose incidence in farm-saved bean seed in Western Kenya.
1. To characterize *Colletotrichum lindemuthianum* isolates using pathogenicity, race typing and BOX-AIR marker in Western Kenya.
2. To evaluate the antifungal potential of different plant extracts against bean anthracnose.

1.10 Hypotheses

1. There is no anthracnose incidence in farm-saved seed in Western Kenya.
2. There is no pathogenic and genetic variability of *C. lindemuthianum* isolates from bean growing regions in Western Kenya.
3. Extracts from botanical plants in Western Kenya do not have antifungal properties against bean anthracnose.

CHAPTER TWO

LITERATURE REVIEW

2.1 Classification, life-cycle, morphology and etiology of *Colletotrichum lindemuthianum*

Colletotrichum lindemuthianum (Sacc. and Magn.) Scribner is the fungus responsible for anthracnose, one of the important diseases of the common bean in major bean growing regions. Recently, the pathogen has been clearly identified as a fungus that presents imperfect and perfect forms, which have been denominated *Colletotrichum lindemuthianum* and *Glomerella cyngulata* f. sp *phaseoli*, respectively (Sutton, 1992). *Colletotrichum lindemuthianum* belongs to Kingdom, Myceteae; Division, Amastigomycota; Sub Division, Deuteromycotina; Class, Deuteromycetes; Sub Class, Coelomycetidae; Order, Melanconiales; Family, Melanconiaceae (Alexopoulos *et al.*, 1962).

The imperfect form of *Colletotrichum lindemuthianum* deploys a complex life-cycle known as hemibiotroph (Fig 2.1) because it has both biotrophic and saprophytic life style. Biotrophic behavior enables it to feed on living plant cells while saprophytic behavior enables it to feed on plant detritus. In the saprophytic life style the fungus grows on any carbon source such as the plant residues in the field. The fungus produces spores inside acervulus which immerse in water soluble pre-formed mucilage. The spores absorb water and actively grow. Later, the germinating tube is formed (germinule phase), and the hyphae elongates to colonize the substrate. The aerial mycelia appear; then the fungal aerial reproductive structures are formed where the spores are stored. Finally, their saprophytic life-cycle is completed and it starts all over again.

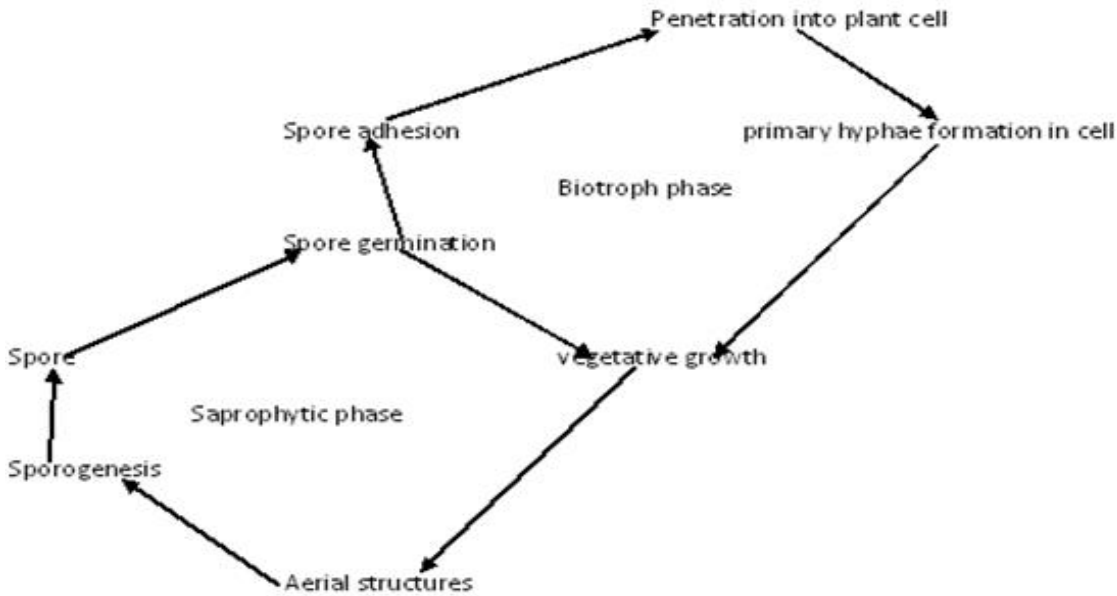

Figure 2.1: Biological life-cycle of *Colletotrichum lindemuthianum* (Martínez-Pacheco *et al*., 2009)

In the biotrophic phase the fungus has the ability to feed on nutrients in living plants. The spore germination process begins with the spore adhesion to the plant surface under adequate humidity conditions in the spore envelope (mucilage). The germinating tube is formed which penetrate into plant cell. Primary hyphae grow between the cell plasmatic membrane and the cell wall and establishes for active vegetative growth. The aerial mycelia appear; then the fungal aerial reproductive structures are formed where the spores are stored.

This biological life-cycle manifested shows the great capacity of the pathogen to survive in different environmental conditions (Dillard & Cobb, 1993). Eventually, the fungus is induced into an adaptation process to allow easy establishment in the emerging resistant bean varieties. As a result, there is a formation of stable variants of the same fungal species. These variants are of the uniform morphology but genetically

heterogeneous hence expresses differences in their virulence to different bean varieties. This brings about race pathogenic variability.

The pathogen's perfect (sexual) stage, *Glomerella cingulata* is rarely found in nature therefore the morphology of the fungus is studied in imperfect (asexual) form. Mycelia are scanty and white. The conidia are borne on orange to bright orange acervuli which are mostly in groups, coalescing and covering lesions on infected plant parts. They contain cylindric, hyaline conidia with rounded ends containing one or two guttulaes. Conidial size is about 11-20 μm X 2-6 μm (Mathur & Kongsdad, 2003).

Colletotrichum lindemuthianum is unequivocally identified by the absence of septa in germinating conidia. Any isolates that produce a conidial septum must be a different species. Acervuli also contain setae which are septate, few, longer than the conidial mass.

In an aqueous environment the conidium germinates between six and nine hours producing one to four germ tubes (Zaumeyer & Thomas, 1957). The germ tube tip swells and differentiates into a thick-walled, heavily melanized appressorium. During pathogenesis the fungi penetrates plant tissues by perforating the cuticle with the appressorium, colonizes the vascular systems and expands rapidly in the plant tissues producing lytic enzymes that cause maceration. Following infection, the symptoms begin to show after three to seven days depending on the prevailing environmental conditions (Hirst & Stedman, 1963). Western Kenya has wet and humid environmental conditions which are conducive for quick establishment and symptom manifestation.

On the bean host the pathogen affects petioles, leaf veins, stem and seeds producing characteristic anthracnose symptoms. Small, pink masses of spores are

produced in the lesions on cotyledon, stem lesions and leaves. On leaves, symptoms generally occur on the underside as linear, dark brick red to black lesions on the leaf veins. On pods the fungus manifests itself as black, sunken cankers containing millions of asexual spores, conidia (Kelly & Vallejo, 2004). Mature lesions are surrounded by a circular, reddish brown to black border with a grayish black interior (Melotto *et al.*, 2000; Jerba *et al*., 2005). Severely infected pods shrivel and the seeds they carry get infected. Infected seeds have brown to black blemishes and sunken lesions (Kelly and Vallejo, 2004).

2.2 Epidemiology of *C. lindemuthianum*

Colletotrichum lindemuthianum is seedborne and seed transmitted (Yesuf & Sangchote, 2007). It survives as a dormant mycelium within the seed coat, sometimes even within cotyledons or elsewhere in the seed (Zaumeyer & Meiners, 1975). It also survives in bean crop residue (Ntahimpera *et al*., 1997). Infected seed serves an important role in the long distance distribution of the anthracnose pathogen (Tesfaye, 2003). As farmers exchange and purchase local seed within and between countries new forms of pathogens are distributed.

The disease can also spread rapidly by spores carried in splashing raindrops, through human clothing, implements or animals that come in contact with diseased plants (Ntahimpera *et al*., 1997; Tesfaye, 2003). In one growing season, one diseased plant can spread the disease to other plants within 30 m radius with an average distance of conidia spread reported to range from 3 to 4.6 m per rainstorm of 10 mm or more (Tu, 1992). From the leaves, conidia are washed down in water to the stems. Rain splash from the leaves also introduces the inocula to the soil (Tesfaye, 2003).

The diseased plant therefore acts as a source of secondary inoculum, the conidia, for spread to adjacent plants.

The amount of initial inoculum in the field is linearly related to the incidence of anthracnose on plant leaves and pods (Tesfaye, 2003). *Colletotrichum lindemuthianum* requires high relative humidity during appressorium formation and penetration as reduced humidity lower infection rates (Benard- Capelle *et al*., 2006). Therefore optimum conditions for survival, infection and development of *C. lindemuthianum* include high humidity or free moisture (> 92%), temperature of between 13^{o}C and 26^{o}C and moderate rainfall at frequent intervals (Pastor – Corrales *et al.*, 1989; Pastor – Corrales *et al*., 1995). Western Kenya has wet and humid conditions which are favorable for the pathogen to thrive making the disease both endemic and epidemic. Anthracnose results in almost 100% loss of bean production (Jerba *et al.*,2005). Quality of seed is strongly linked to the presence or absence of the pathogen in seed.

2.3 Bean anthracnose and farm-saved seed in Western Kenya

Farm-saved seed is an important source of seed for common bean farmers in Western Kenya (Mwaniki, 2002; Opole *et al*., 2003). About 75% of the farmers maintain farm-saved seed for periods ranging from less than one year to more than twenty-five years (Opole *et al.*, 2003). Farmers also consider their own seed to have good attributes such as high yield, early maturity, adapted to local conditions and good food quality. The farm-saved seed appear apparently healthy to farmers but most of them are anthracnose infected and play an important role in the transmission and spread of the disease. This is because the disease on the seeds could have originated from infected pods as observed by Tu, (1992) who found that, 10 % of cultivated

seeds without anthracnose symptoms (apparently health seeds) but originating from infected pods produced infected seedlings. Unfortunately only 18% farmers are keen on the control of anthracnose diseases during the growing period of the bean plant in W. Kenya (Opole *et al.,* 2003). This contributes towards low quality of the farm-saved bean seed that could be available for the next planting season. Bean anthracnose is one of the seed borne diseases which compromises the quality of farm-saved seeds in the region (*http://www.africancrops.net/rockefeller/crops/-beans/index.htm*). A lot of work has been done on the incidence of the disease in the bean fields but less work has bean done on it is incidence in farmers farm-saved seed in Western Kenya

There is high bean cultivar diversity of about 80 different bean seed types in different parts of Kenya (van Rheenen *et al*., 1984), but six are the most popular. These are: Red and red/purple mottled (occurring in different local names such as Roseccoco, Nyayo, Wairimu, Kitui), Purple/grey speckled (locally known as Mwezi moja) and Pinto sugars (localy known as Mwitemania). They are bush type common bean rather than climbing type and are preferred because of their low production cost requirements and convenience for market. In Western Kenya Rosecocco and Canadian wonder type were the most preferred because of their high yield and good taste. Unfortunately, the planting of these cultivars has reduced due to different factors such as increased problems of soil fertility and associated diseases; consequently they are being replaced by varieties like Mwitemania and red haricots.. However, these varieties are susceptible to seed borne anthracnose disease. Susceptible bean varieties succumb earlier to anthracnose than the resistant (Meyer *et al,* 2001; Nkalubo *et al*., 2007). Therefore a study on current status of the anthracnose disease in the farm-seed of these varieties in Western Kenya was necessary.

2.4 Pathogen variability

2.4.1 Race typing

Variation in *C. lindemuthianum* pathogenicity has been widely done. Several systems of classification were used to identify several races by earlier workers. Barrus (1911) made the first report of races β α and Y; Burkholder (1923) described γ; Andrus & Wade (1942) reported delta race; Schnock (1975) indicated κ race presence; Tu *et al*., (1984) reported ∑ race in Canada. In Kenya six races namely alpha, beta, delta, epsilon, gamma and lambda had been revealed from thirty six isolates collected from nine districts of Kenya using a system proposed by Hubbeling (1957) (Kinyua 1976; Mwangi 1986; Gathuru & Mwangi 1991). The systems of characterizing of the isolates used by earlier researchers were limited because some isolates could not be identified.

Latter researchers developed and adopted international standard procedure that could allow identical characterization of data from different research groups. The procedures involved use of international standard differential series of 12 common bean cultivars and a binary system based on the position of each cultivar within this series (CIAT, 1987; Pastor- Corrales, 1991; Buruchara, 1991). The cultivars are divided into two sets of 8 Mesoamerican and 4 Andean cultivars as follows: - (a) Andean origin (‘Michigan Dark Red kidney’; ‘Perry marrow’; ‘Widusa’; ‘Kaboon’) (b) Meso American origin (‘Michelite’; ‘Cornell 49-242’; ‘Mexico 222’;‘PI207262’; ‘To’; ‘Tu’; ‘AB136’ and ‘G2 333’) (CIAT, 1987; Pastor- Corrales, 1991; Buruchara, 1991). The sum of all binary numbers of cultivars with susceptible reactions gives a specific race number. A binary number is equal to 2n, where n is equivalent to the place of the cultivar within the differential series order. So far 300 races have been

revealed using this procedure (Kelly & Vallejo, 2004; Mahuku & Riascos, 2004; Munda *et al.,* 2009; Mwesigwa, 2009; Seher *et al.,* 2013).

Eighty five isolates of *C. lindemuthiunum* collected from different kidney bean growing areas of Himachal Pradesh state, India were characterized into 19 races (Sharma *et al*., 1999). Race 73 was identified on six *C. lindemuthiunum* isolated from six dry bean samples grown in Manitoba, Canada in 2001 season in addition to other races which had been previously obtained in the region (del Rio *et al*., 2002). Ninth races were identified among 200 *C. lindemuthianum* isolates collected from Andean and Mesoamerica bean varieties and regions revealing high level of virulence variability (Mahuku & Riascos, 2004). Ansari *et al.,* (2004), carried studies on pathogenic and genetic variability among *C. lindemuthiunum* isolates collected from a total of 10 central and South American, European and African countries. On the basis of pathogenicity tests 74 isolates were grouped into 30 different pathogenic races. In Minas Gerais, Brazil 48 isolates were inoculated on 12 differential cultivars and 10 races were identified including race 337 which had not been reported previously in the literature (Damasceno *et al*., 2007). In Turkey thirty-five races were obtained from 51 isolates collected from seven bean growing provinces from which twenty-eight races showed conformity with established *C. lindemuthianum* races throughout the world whereas 7 races did not conform (Seher *et al.,* 2013).

In East Africa 12 isolates collected from bean growing areas of Burundi, Central Africa, were characterized into 9 races. Seven of the 9 races were described for the first time in Africa (Bigirimana *et al*., 2000). Fourth one isolates collected from Kabale, Mbale, Apac, Mpigi and Wakiso districts in Uganda were characterized into 21 races of which races 1024, 1536, 1538, 1856, 1857, 1989, 3086 and 4033 were the most virulent. Highly resistant differential cultivars AB136 and G2333 showed

susceptibility (Mwesigwa, 2009). In Kenya four isolates of *C. lindemuthiunum* collected from bean seeds produced in Rongai Nakuru Disrict were found to be similar and classified as race 485 (Ombiri *et al*., 2002). This race was first reported in Kenya in addition to the previous six races (Kinyua, 1976; Mwangi, 1986; Gathuru & Mwangi, 1991).

It is apparent that *C. lindemuthiunum* is a highly variable pathogen and that race composition in any given area is continually changing, with emergence and introduction of new races. To design effective gene pyramids, breeders need information on pathogenic variability of *C. lindemuthianum* present in bean producing areas (Young *et al*., 1998). This shows the need to constantly monitor the composition of *C. lindemuthianum*. In Western Kenya several studies have been done on the pathogen but no work has been done on *C. lindemuthiunum* virulence diversity using standard differential series of 12 common bean cultivars and a binary system based on the position of each cultivar within this series.

2.4.2 Molecular variability of *C. lindemuthianum*

Virulence variability system is intended to establish uniform, consistent and comparable classification. However the system is subjective to symptoms evaluation, variations in working environment, and incubation conditions used. This may lead to misclassifications and to disagreements on classification of the same race by different research groups (Mesquita *et al.,* 1998). Characterization based on molecular differences is now encouraged alongside the traditional bean differentials. Molecular characterization is assessed using DNA-based molecular techniques (Rodriguez-Guerra *et al.,* 2003) which include Amplified Fragment Length Polymorphism

(AFLP) Restriction Fragment Length Polymorphism (RFLP), Random Amplified Polymorphism DNA (RAPD) , Simple Sequence Repeat (SSR), Inter-Simple Sequence Repeat (ISSR) and repetitive sequences such as the 35-40bp repetitive extragenic palindronic (REP) sequence, the 124-127 bp enterobacterial repetitive intergenic consensuses (ERIC) sequence and the 154bp BOX elements (Varsalovic *et al.*, 1994; Mahuku & Riascos, 2004; Bardas *et al*., 2009).

High genetic diversity was identified among 200 isolates (Mahuku & Riascos, 2004) and among 35 isolates of *C. lindemuthiunum* investigated (Bardas *et al*., 2009) both using BOX-AIR marker (Mahuku and Riascos, 2004). Also in Uganda Mwesigwa, (2009) observed high genetic variability among 74 *C. lindemuthiunum* using BOX-AIR primers. In Western Kenya despite the high seed exchange, information on molecular characterization on isolates of *C. lindemuthianum* is scanty. Studies on genetic variability in Western Kenya isolates could give clear understanding which would lead to development of durable host resistance and reduce unnecessary fungicide application. In these studies, genetic diversity of the pathogen in Western Kenya was done using BOX-AIR primers, which according to McCartney *et al*., (2003) is accurate, faster and easy to use.

2.5 Control strategies of bean anthracnose

2.5.1 Cultural practices.

The best cultural practice in bean anthracnose control is use of the disease-free seed. Unfortunately the recommended certified disease-free seeds are of high prices and not readily available to small scale farmers due to poor distribution among other factors. Seed produced under wet and humid conditions such as occur in Western

Kenya should not be planted because in most cases it is already infected (Pastor-Corrales *et al*., 1989). Other cultural practices include crop rotation and avoidance of scouting in the bean fields. A three year crop rotation will eliminate or reduce the inoculums in the fields and avoidance of scouting in the bean fields during the wet seasons will minimize spread of fungal spores from diseased to healthy plants (Tu, 1986). Removal of diseased plants is recommended so that seeds from the plants are not harvested to further disseminate the disease in future.

2.5.2 Host resistance.

Use of disease resistant hosts is one of the most effective and efficient method of anthracnose management (Esteban *et al*., 2003). Development of variable pathogen races complicates the use of resistant varieties due to breakdown of host resistance. Knowledge on diversity of *Colletotrichum lindemuthianum* races from major bean growing regions is a pre-requisite for anthracnose resistance breeding programs (Tesfaye, 2003; Buruchara, 1991) and this knowledge is needed to successfully develop and deploy resistance against the pathogen in particular regions (CIAT, 1996).

2.5.3 Chemical control.

Synthetic fungicides containing chlorothalonil, mancozeb, zineb, benomyl, captafol or folfet have been recommended at first sight of the disease and reapplied weekly during the season (Agrios, 1997) and have been reported to be effective in controlling bean anthracnoses. Tesfaye (2003) demonstrated that a benomyl seed dressing followed by a foliar difenoconazole spray, or difenoconazole application controlled the disease. The excessive use of synthetic fungicides, however, reduces

profitability as it becomes expensive; threatens the environment and is constrained by development of fungicide resistant biotypes (Milklas *et al.,* 2006). This scenario necessitates the search for and development of alternative control methods that are ecologically sustainable fungicides and effective against the target species but create minimal adversity to non-target species.

2.5.4 Alternative control methods

A lot of successful work has been done showing use of plant extracts with active antifungal property as alternative fungicides to chemical control. Azadirachtin from neem and similar alkaloids, flavonoids, terpenoids from Aloe, ginger and bitter kola respectively have been used as biopesticides and fungicides. Dubey *et al*. (2000) tested *Ocimum gratissimum*, *Zingiber cassumunar*, *Cymbopogon citratus*, and *Caesulia axilliaris* against *Aspergillus flavus* and reported that the extracts had potential antifungal activity. Lettuce (*Lactuca sativa*) incorporation into soil reduced the disease severity against root and stem rot disease of cucumber (*Cucumis sativus*) caused by *Fusarium oxysporum* f. sp. *radicis-cucumerinum* (Pavlou & Vakalounakis, 2004). Falahati *et al* 2005, reported that residues of *Melia azedarach*, *Eucalyptus citriodora* and *Alstonia scholaris* contain antifungal acids, tannins and flavonoids.

Aloe extracts has anthranol, barbaloin, chrysophanic acid, smodin, ethereal oil, ester of cinnamonic acid, isobarbaloin and resistannol which exhibit antifungal activity but are toxic at high concentrations. It is also has auxins and gibberellins and minerals such as calcium, chromium, copper, iron, manganese, potassium, sodium and zinc. Their saponins contain glycosides which have antiseptic activity. *In vitro* experiments carried out found that at 0.35% concentration *Aloe vera* gel had strong antifungal activity which completely eradicated seed borne fungi *Drechslera*

hawaiensis and *Alternaria alternate* (Uzma *et al*., 2011). Cold-water extracts of *Azadiricta indica* (neem), *Garcinia cola* (bitter kola) and *Zingiber officinale* (ginger) at various concentrations posses fungicidal activity against the mycelial growth and sclerotial germination of a soil fungus, *Sclerotium rolfsii* (Wokocha & Okereke, 2005).

Lantana camara and *Datura stromonium* plant extracts were found among other plant leaf extracts to reduce the incidence of seed-borne fungi *Aspergillus niger* and *Aspergillus awamori* and increase seed germination (Seema *et al.,* 2007). Shovan *et al.,* (2008) evaluated effect of plant extracts of garlic, ginger, onion, and neem on *Colletotrichum dematium* and found that garlic was the most effective followed by onion , ginger then neem. Leaf extracts of zimmu (combined *Allium cepa* and *Allium sativum* extracts) exhibited strong antifungal activity against *Apergillus flavus, Fusarium moniliforme, Curcularia lunata* and *Alternaria altermaria alternate* and caused *in vitro* fungal growth inhibition of 73.3%, 71.1%, 70.0% and 74.4% respectively (Muthusamy *et al.,* 2007). They also found significant incidence reduction of grain mold, increased grain weight and grain hardness on foliar application of zimmu formulatin 50 EC at $3mLL^{-1}$(v/v) concentration on sorghum 60, 75 and 90 days after sowing. *Achras zapota, Datura stramonium, Emblica officinalis, Eucalyptus globules, Lawsonia inermis, Mimusops elengi, Peltophorum pterocarpum, Polyalthia longifolia, Prosopis juliflora, Punica granatum* and *Sygigium cumini* recorded significant antifungal activity among the fifty-two plants species tested for their antifungal activity against important seed-borne pathogens of *Apergillus* species (Satish *et al.,* 2007). *Pycnoporus sanguineus* aqueous extract indicated the potential to control bean anthracnose (Lindomar, 2008). The aqueous extract (EA) had direct antimicrobial activity of up to 96% of the germination of the pathogen spores *in vitro*.

In *in vivo* assays *Pycnoporus sanguineus* aqueous extract at 20% controlled anthracnose with reduction of 70% severity on the treated and inoculated 7th leaf.

The current study was done to establish the effect of plant extract from onion (*Allium cepa* L.), garlic (*Allium sativum*), neem (*Azadirachta indica*), spider plant (*Cleome gynandra*), tick berry (*Lantana camara*), thorn apple (*Datura stramonium*), alloheh (*Aloe vera*) and red gum (*Eucalyptus globules*) based on the extracts previous literature on their ability to inhibit different fungi growth.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study area

The study was carried out in seven regions (Fig 3.1) selected purposely to represent the major common dry bean growing regions of Western Kenya. The areas have an elevation of between 1,200 and 2,100 meters above sea level. They receive bimodal rainfall with an average annual rainfall of 750 - 2,100 mm. The first rains (long rains season) start in March and end beginning of June while the second rains (short rains season) start in August and last end of November. Temperatures range from a minimum of 14 ^{o}C to 18 ^{o}C and to a maximum of 30^{o}C to 36^{o}C throughout the year. The regions are found in different agro ecological zones (Appendix 1).

3.2 Determination of anthracnose incidence on farm-saved seed.

3.2.1 Collection of farm-saved seed samples

Samples were collected from farmers who had both farm-saved seed and growing bean plants in their field. The collection was done in four seasons of both long and short rains seasons between November 2008 and July 2010. Bean farmers along accessible routes in the study regions were identified with the help of the area Agricultural extension officers. Systematic random sampling was used to select farmers at regular intervals of 4-10 km. An average of 7-15 farmers were sampled in a given region and from each 250 grams of the available farm-saved seed varieties was purchased. During the sample collections information on the region agroecological zones, climate, bean variety and season were observed and recorded (Appendix l).

Figure 3.1: Bean growing regions of Western Kenya where samples of farm-saved seed and anthracnose infected pods were collected. (Source: www.mapsofworld.com)

The same procedures were repeated for all the four season giving a total of 196 farmers sampled during the study period. In addition, certified seed of the same varieties, as those obtained from farmers were purchased from a seed company shop. The collected samples were placed in a labeled paper bag, packed in a sack and taken to University of Eldoret Seed laboratory for the study of incidence of anthracnose disease. In the laboratory all the seed samples of the same variety collected from the same season and region were mixed to form a composite seed sample of the variety. Eight common varieties were obtained from each of the seven regions in four different rains seasons (short season rains 2008, long rains season 2009, short rains season 2009 and long rains season 2010) making a total of 224 submitted samples from all bean growing regions in the study period. Certified seed were treated as a region (C0) and used as control.

3.2.2 Assessment of anthracnose incidence on farm-saved seed.

Standard blotter test (ISTA, 2008) was used to determine anthracnose incidence in the seed samples. A working sample of 400 seeds was obtained from each seed sample using random-cup method (ISTA, 2008) and divided into four replicates of 100 seeds each. The seeds were surface sterilized in a solution of 0.5% sodium hypochlorite for 10 minutes and rinsed with water. They were spread out on moistened double sheets 350 x 450 mm Whatman No. 1 filter paper and covered with another water soaked. They were then covered with a lid to maintain moisture during incubation. The seeds were incubated for seven days in darkness at 20^{o}C. The experimental design was a completely randomized design (CRD) with four replications of each treatment. There were 64 treatments in factorial combinations of

seven regions and certified seeds, four replications and eight bean varieties. The treatment combinations are shown in Appendix ll.

The factor levels were:

Factor A= Regions: C1= Bungoma; C2= Kakamega; C3= Kitale; C4= Nandi; C5= Busia; C6= Kisii; C7 Siaya; C0= certified seeds

Factor B= Variety:- V1= Rose coco (GLP 2); V2= Brown; V3=Mwezi moja (GLP1004); V4= Rose coco (GLP 288); V5= Red haricot (GLP 585); V6= Mwitemania (GLP X92); V7=White; V8=Yellow (KAT B 1).

These factors were repeated in all the four seasons: - S1 (2008) =Short rains season; S2 (2009) = Long rains season; S3 (2009) =Short rains season; S4 (2010) = Long rains season.

Seedlings were constantly moistened to enhance disease symptom development. After seven days the cotyledons and hypocotyls were examined for black depressed spots with well delimited outlines. To confirm presence of anthracnose each spot was checked for presence of acevuli with dark brown setae and conidia using 25 X magnification then 200 X magnification. The number of anthracnose infected seedlings were counted and recorded. Anthracnose incidence was determined in percentage using the following formula:-

$$I = \frac{\sum n}{\sum N} X\ 100$$

I= Incidence

$\sum N$= Total number of seeds

$\sum n$=Total number of seeds infected

3.2.2 Assessment of seed viability and vigor

To determine the effect of disease incidence on percentage emergence and emergence rate, growing-on test was done (ISTA, 2008). From each of the remaining seed sample 400 seeds were obtained using random-cup (weighted portion) method and were divided into four replicates of 100 seeds each.

All seeds were surface sterilized in a solution of 0.5% sodium hypochlorite for 10 minutes, rinsed in clean tap water. The seeds were placed about 2 cm deep in the sand and covered with a thin layer of sand. The experiment was then laid at 18-25^{o}C using a completely randomized design (CRD).

Seedling emergence was done to evaluate effect of anthracnose disease on seed viability as one of the seed quality parameter (ISTA, 2008). The emergence was considered to have occurred when the plumule had appeared on the soil surface. The emerging seedlings were constantly moistened to enhance symptom development. Emergence was determined in percentage as follows:-

$$\% \text{ Emergence} = \frac{\sum E}{\sum N} \times 100$$

Where $\sum E$ is total number of emerged seeds after nine days per treatment

$\sum N$ is the total number of the seeds planted per treatment

The rate of emergence was done to evaluate the effect of anthracnose on seed vigour. This was determined as the time the seeds took to reach 50% emergence (E_{50}) Seedling emergence counts were made at 24 hour interval and the number of newly emerged seedlings for each day recorded. The E_{50} values were computed using the equation of Orchard (1977) as shown below:

$$E_{50} = \frac{\sum Ti\ Ni}{\sum Ni}$$

Where: E_{50} = 50% emergence ; Ni = Number of seeds germinated on a day (T); Ti = serial number of the day; ∑Ni = Total number of germinated seeds.

3.2.4 Data analysis

Data on seeds anthracnose incidence, emergence and emergence rate was analyzed statistically by analysis of variance (ANOVA) procedure using GenStat® computer package (VSN International 2008) after angular transformations. Separation of means was done using Turkey's test at $p \leq 0.05$ (Mason *et al*., 1989).

3.3 Characterization of *Colletotrichum lindemuthianum*

3.3.1 Collection of infected bean pods

Anthracnose infected pods were collected from the same farmers who had been sampled for farm-saved seed in the study regions. The collection was done at the pod filling stage when the disease is conspicuous in the floral parts. Five mature bean pods with pronounced anthracnose symptoms were collected at 10-20 m intervals along the plant rows. During the sample collections information on the region agroecological zones, climate, bean variety and season were observed and recorded (Appendix l).

3.3.2 Pathogen isolation and identification.

Colletotrichum lindemuthianum was isolated from diseased part of sampled pods showing pronounced characteristic anthracnose symptoms. Isolation was done as described by del, Rio *et al.,* (2002). Small pieces of infected (0.5 cm^2) tissues were cut from diseased pods, held in running tap water for 30 seconds, surface sterilized by immersion in an aqueous solution of 0.5% sodium hypochlorite for 60 seconds and rinsed twice in sterile, distilled water. The tissues were then plated aseptically on Potato dextrose agar (PDA) in the petridishes and incubated in complete darkness at 20-21^oC for 7 days. The pathogen was identified based on its morphological characteristics using the common Laboratory Seed Health Testing Methods for detecting Fungi (Mathur & Kongsdal, 2003; ISTA, 2008). A total of 122 isolates were obtained from the submitted pod samples.

For purity of the cultures the isolates monoconidial cultures were prepared using hyphal tip transfer method as described by Ombiri *et al.,* (2002). Small pieces of hyphae from the distinct colonies that showed growth and morphology of typical *Colletotrichum lindemuthianum* fungi were transferred onto PDA media and incubated for 7 days in darkness at 20-21^oC. The plates were flooded with sterile distilled water then spores dislodged by scrapping the culture surface with a spatula. Two to three milliliter conidia suspension were aseptically spread on water agar (WA) in plate and incubated in darkness at 20-21^oC for 24 hours. The cultures were examined under a microscope at 25 X magnification for germination of conidia on WA. Three germinated conidia per culture-plate were removed using a thin isolation needle and aseptically plated separately on PDA plates. After 4 to 5 days the cultures were sub cultured on PDA plates and incubated for 14 days. A total of 122 single

spore isolates, replicated three times and stored on PDA at 4^{o}C were used in subsequent experiments.

3.3.3 Characterization *C. lindemuthianum* of isolates for virulance

Pathogenecity test was done to determine race diversity. It was conducted in a greenhouse at University of Eldoret. The recommended seeds of differential cultivar were used in the test were procured from International Centre for Tropical Agriculture (CIAT), Cali Colombia. The seeds of differential cultivars (Table 1) were multiplied under anthracnose-free green house conditions.

The 14 days old *C. lindemuthianum* single spore cultures were flooded with 5 ml distilled water and spores harvested by scrapping the culture surface with a glass rod. Spore suspension was cleared by filtration through a double layer of cheese cloth to remove the mycelia mass. Spore concentration was calibrated to 1.2 x10^{6} spores per milliliter using a haemacytometer. The anthracnose spores were suspended in Tween 80 (0.05%, v/v).

This was done according to Damasceno *et al.,* (2007). Three seedlings of each cultivar were sown 3.0 cm deep in 10 cm diameter pots filled with moist vermiculite. The experimental design was a completely randomized design (CRD) with three replications of each treatment. The pots were kept on the green house benches to germinate. The 122 isolates were used to inoculate the differential cultivars seedlings in the greenhouse at primary leaf stage about 7- 9 days after planting. Inoculation was done by spraying the inoculums suspension on both sides of primary leaves, petiole and stem until run off using a hand spray. The inoculated plants were kept in moistened chambers of 21 ± 2^{o}C temperatures and more than 95% relative humidity

for 48 hours. They were then transferred to the green house with a temperature ranging from 21 to 24^{o}C and 70% relative humidity.

After 3 to 4 days disease symptoms from the seedling were evaluated according to the 1-5 disease severity descriptive scale as described by Ombiri *et al.,* (2002).

1- Plants with no symptoms;

2- 1.5 - plants with Light infection (point lesions);

3 - plants with Moderate infection (up to 5 small to large sunken lesions-about 3mm);

5 -plants with severe infection (large deep lesions- larger than 3mm- and /or death).

There were three replicates for each isolate.

To obtain virulence data, disease severity index (DSI) of each isolate was computed using the following formula (Ombiri *et al.,* 2002):

$$DSI = (1 \times n_1 + 1.5 \times n_2 + 3 \times n_3 + 5 \times n_4) / N$$

Where 1, 1.5, 3 and 5 are the severity scores;

n_1 to n_4 refers to the number of seedlings with the respective severity scores;

N refers to the total number of seedlings examined per variety per isolate.

Plants showing the DSI between 1.0 and 1.5 were considered resistant (incompatible reaction) while those showing above 1.5 were considered susceptible (compatible reaction).

Based on the DSI of the 12 standard bean differential cultivars, different physiological races of *C. lindemuthianum* were identified on *P. vulgaris* in Western Kenya. Binary nomenclature system was used for the race designation as described by

(Mahuku & Riascos, 2004). Race values represent cumulative binary values of the cultivars showing susceptibility of a particular isolate. Each differential cultivar had an assigned number (2^n), where n corresponds to the order number of the cultivar within the 12 standard bean differential series. The designation of a race number was obtained by summing the 2^n values of all cultivars compatible reactions to the isolate used for inoculation.

Table 3.1 : Characteristics of the 12 CIAT differential common bean cultivars.

Differential cultivars (d.c.)	Binary number (2n)	d.c. gene pool	Genes present	Growth characteristics
Michelite	1	M	-	II
MDRK	2	A	*Co-1*	I
Perry Marrow	4	A	*Co-1*^{3}	II
Cornell 49-242	8	M	*Co-2*	II
Widusa	16	A	*Co-1*^{5}	I
Kaboon	32	A	*Co-1*^{2}	II
Mexico 222	64	M	*Co-3*	I
PI 207262	128	M	*Co-4*^{3},*Co-9*	III
To	256	M	*Co-4*	I
Tu	512	M	*Co-5*	III
AB 136	1024	M	*Co-6*, *Co-8*	IV
G2333	2048	M	*Co-4*^{2},*Co-5*, *Co-7*	IV

M: Mesoamerican gene pool A: Andean gene pool. Binary number (2n): where n is equivalent to the place of the cultivar within the series (0-11). I- Determinate; II- Indeterminate bush; III – Indeterminate bush with weak main stem and prostrate branches; IV = Indeterminate climber habit.

3.3.4 Molecular characterization of *Colletotrichum lindemuthianum* isolates

3.3.4.1 Mycelia mass production

For genomic characterization the 122 *C. lindemuthianum* isolates obtained from Western Kenya infected bean pods were used to prepare mycelia. The mycelia for DNA isolation were obtained as described by Mesquita *et al* (1998). Mycelia from each single-conidial isolate of *C. lindemuthianum* were grown on a petri dish containing PDA medium and incubated at 22 ^{o}C for 12 days. A culture plug from the actively growing margins of 14 days old single conidial race colonies were used to inoculate 50 ml of potato dextrose broth media (PDB) in a conical flask. The flasks were incubated under constant agitation at 108 rpm at 22 to 25 ^{o}C in the dark for 7 days. Mycelium were harvested by filtration through cheese cloth, surface dried, frozen in liquid nitrogen and ground to a fine powder using a sterilized mortar and pestle.

3.3.4.2 DNA Extraction

DNA extraction from the mycelia of *Colletotrichum lindemuthianum* isolates followed the protocol used by Balardin *et al*., (1997). Microcentrifuge tubes (1.5ml) were filled with 50-100 mg of ground mycelium that were dispersed in 400 ul of hot - 65^{o}c (2x CTAB extraction buffer (2% CTAB 100 mm Trizma base , 10 mm EDTA, 0.7M Nacl). Chloroform or isoamyl alcohol (400 ul, 24:1, v/v) were added and the mixture was agitated on a shaker for 15 minutes and centrifuged at 3,000 rpm for 5 minutes. The aqueous top layer was transferred to a new micro centrifuge tube (1.5ml) containing 400 ul of isopropanol mixed well and incubated at room

temperature for 5 minutes. The emulsion were centrifuged at 3500 rpm for 5 minutes, the supernatant discarded and tubes inverted for 5 minutes to allow complete evaporation of the isopropanol. The pellet was dissolved in 100 ul of TE (10nm Tris-HCl PH 8.0, 0.5 nm EDTA), added RNAse A at 10 ug ml^{-1} (500 ug ml^{-1}) and the emulsion incubated for 10 minutes at room temperature. Thereafter, 50 ul of 100% cold EtOH was added and DNA precipitated. Extracted DNA was dissolved in 100 ul of TE and stored at – 20^{o}C. DNA concentration was estimated in 1 % agarose gel and stained with ethidium bromide.

3.3.4.3 DNA amplification.

DNA amplification of 122 isolates was done as described by Bardas *et al*. (2009) using BOX-AIR molecular marker with BOX primer of sequence 5'-CTA CGG CAA GGC GAC GCT GAC G-3'. The amplification was conducted twice for each isolate using 150 ng DNA per reaction. Amplification reaction were performed in a final volume of 20 µl with Bioneer AccuPower ^{TM} PCR premix [1U Top DNA polymerase, 250 µM each dNTP (dATP, dCTP, dGTP, dTTP), 10 mM Tris-HCL (pH 9.0), 30 mM KCl, 1.5 mM $MgCl_2$, Stabilizer and tracking dye], 150 ng DNA, 0.2mM BOX primer and 18 µl molecular grade water. To ensure that amplification products were not primer artifacts genomic DNA was omitted from the control reaction. DNA amplification was performed in a thermal cycler programmed according to the following thermal profile: One initial denaturation cycle at 95 ^{o}C for 7 min, 30 step cycles - denaturation 94 ^{o}C for 1 min, annealing at 53 ^{o}C for 1 min and extension at 65 ^{o}C for 8 min and by a final extension step at 65 ^{o}C for 15 min.

3.3.4.4 DNA electrophoresis

The amplified PCR products were separated by electrophoresis at 70 V for 2.5 hours on 2 % agarose gel 0.5x TBE (45 mM Tris-borate, 1mM EDTA) running buffer. The products were detected by staining with 0.5 % ethidium bromide. A 100 bp DNA ladder was used as a molecular weight marker. The gels were visualized under UV light (Fotodyne Ultraviolet Trans- illuminator; Fotodyne Inc., New Berlin, WI, USA) before being photographed. The reaction was run in duplicate and only 60 of 122 isolates which had well defined and reproducible bands were analyzed.

3.3.4.5 Data analysis

BOX-AIR data analysis of the 60 isolates was done according to Bardas *et al*., (2009). To generate a BOX binary data, each polymorphic band in every sample was considered a locus with two alleles: presence or absence of amplified fragments. Each band was then scored as a binary character (1 for presence and 0 for absence). Only reproducible and most intense bands were scored. The bands detected in all samples at the same position (monomorphic) and bands that were not directly comparable between individuals because of very similar migrations were excluded from analysis. The binary data was used to calculate pair wise genetic distances with the Similarity for Qualitative Data Program (SIMQUAL) in the Numerical Taxonomy and Multivariate Analysis System for personal computer (NTSYS-pc (version 2.1 (NTSYS, New York, NY, USA). Simple Matching co-efficient (SM) were used to compute the distances and thus SM similarity coefficient data was generated. The similarity matrix was used to construct a dendrogram by the Unweighted Pair Group Method with Arithmetic Average (UPGMA) clustering method and tree program in NTSYS. The goodness of fit of the cluster analysis to the similarity data was assessed

by computing a Cophenetic (Coph) value matrix using Coph module of NTSYS and comparing it with the original SM similarity matrix using the Matrix Comparison (MXCOMP) module of NTSYS. The significance of the relationship (r) between SM similarity and Coph value matrices was determined by comparing the observed Mantel test statistic (Z) value with 1000 random permutations of Z in a one-tailed probability test (Rolf & Sokal, 1981).

3.4 Determination of efficacy of plant extracts antifungal activity against *Colletotrichum lindemuthianum*.

3.4.1 Collection of plant samples

Plant extracts from older plant parts of eight plant species were screened for antifungal activity against seed borne bean anthracnose and their effect on seed quality. The plants were onion (*Allium cepa* L.), garlic (*Allium sativum*), neem (*Azadirachta indica*), spider plant (*Cleome gynandra*), tick berry (*Lantana camara*), thorn apple (*Datura stramonium),* alloe *(Aloe vera)* and red gum (*Eucalyptus globules).* The plants were purposely selected based on the previous literature information of their antifungal property against various plant pathogens. The plant organs picked from the sampled plants were *A. cepa* (bulb), *A. sativum* (clove), *A. indica* (leaves), *C. gynandra* (leaves), *L .camara* (leaves), *D. stramonium* (leaves), *A. vera* (leaves) and *E. globules* (leaves). They were placed in a labeled paper bag and taken to University of Eldoret Seed laboratory for analysis of their antifungal potential against bean anthracnose disease.

3.4.2 Preparation of aqueous plant extract

Aqueous plant extract were prepared from the collected fresh plant samples as described by Satish *et al.*, (2007). The samples were washed first in running tap water and then in sterilized distilled water. One hundred grams of each sample were chopped and then blended in a surface sterilized blender with 100 ml sterile water (1:1 w/v) for 10 minutes. The extract was filtered through two layer of muslin cloth and the final filtrate obtained was used as stock solution. Mancozeb one of the synthetic fungicide recommended for control of bean anthracnose was used as a positive control, applied at their recommended dosage (2 gm l^{-1}). Mancozeb has chemical component of Zn-manganese ethylene bisdithiocarbamate and its common name is Dithane M-45 80WP. Water was used as negative control.

3.4.3 Assay for growth inhibition of *Colletotrichum lindemuthianum*

The *in-vitro* tests were conducted to determine effect of plant extracts on *C. lindemuthianum* mycelial growth using poison food technique (Begum and Bhiyan, 2006). Plant extract stock solution of 20 ml, 25 ml and 30 ml were mixed with 80, 75 and 70 ml of the sterilized molten (45^{o}C) PDA media, respectively to obtain 20, 25 and 30 percentage concentrations. On cooling, 15 ml of the amended medium was poured into each of the 9 ml petridish and labeled. After solidification, the plates were inoculated by placing at the centre 5 mm discs of the 14 days old PDA cultures of *C. lindemuthianum*, cut out from periphery of actively growing mycelium using a cork borer. The inoculated plates were incubated at 22 ± 2 ^{o}C for seven days. Water treatment served as negative control while Dithane M-45 80WP (2 gm l^{-1}) was used as a positive control. Each treatment was replicated 3 times. Data on inhibition of the mycelia growth was recorded on the 14 th day after incubation when the growth on the

control plates completely covered the plate. Diameter of the colonies on PDA with and without extract was measured in mm from the bottom side of the petridish. The fungi toxicity of the plant extracts in terms of percentage mycelia growth inhibition was calculated by using the following formula (Singh & Tripathi, 1999; Tegegne *et al*., 2008):

$$\% \text{ mycelia growth inhibition} = \frac{dc - dt}{dc} \times 100$$

Where dc = Average diameter increase in mycelial growth in control,

dt = Average diameter increase in mycelial growth in treatment.

The data obtained was subjected into the following 0-5 scale:- (source:- own composition)

Scale 0:- 0% - No mycelia growth inhibition

Scale 1:- 1-25%- slight mycelia growth inhibition

Scale 2:- 26-50%- lower mycelia growth inhibition

Scale3:- 51-75%- intermediate mycelia growth inhibition

Scale 4:- 76-95 %- higher mycelia growth inhibition

Scale 5:- 95-100%- complete mycelia growth inhibition

Based on the effectiveness of the plant extracts the scale was grouped as:-

Scale 0 & 1- Not effective treatment

Scale 2&3- Averagely effective treatment

Scale 4 &5- Effective treatment

3.4.4 Determination of the effect of plant extracts on incidence and severity of Anthracnose

In vivo tests were done in the green house to determine the effect of plant extracts incidence and severity of anthracnose. Bean variety GLP 585 seed variety was used in the experiment since it was widely grown in W. Kenya and one of the susceptible varieties to anthracnose. Only four plant extract treatments (*A. vera, A. cepa, A. sativum and D. stramonium*) that had a higher mycelia growth inhibition at 30 % in the in vitro tests above were used. For challenge inoculation *C. lindemuthianum* conidia isolate was randomly sampled and used during *in vivo* test because it was collected from GLP 585 variety.

The i*n vivo* assay was done as described by Lindomar, (2008). The seeds were sown 3.0 cm deep at the rate of three seeds per plastic pot (10 cm diameter) filled with moist vermiculite. The seeds were watered twice daily with tap water. Aqueous extract (EA) of *Allium cepa* L., *Allium sativum, Datura stramonium* and *Aloe vera* at 30% concentrations were sprayed on the primary leave stage seedlings (7 days old), three days before inoculation with *Colletotricum lindemuthianum.* Artificial inoculation was done by spraying 1.2 x 10^6 conidia ml $^{-1}$ inoculum suspension on both sides of primary leaves, petiole and stem until run off using a hand spray. The potted plants were replicated three times per treatment and together with control were set up in a completely randomized design in moistened chambers at 22 ± 2 ^{o} C with more than 95% relative humidity for 48 hours and then transferred to a green house with a temperature ranging from 21-24^{o} C and relative humidity of about 70%. Water and Dithane M-45 80WP fungicide (2 gm l $^{-1}$) were used as negative and positive control treatments respectively. Disease symptoms from the seedling were scored

between the sixth day and seventh day after inoculation according to the 1-5 disease severity descriptive scale (Ombiri *et al*., 2002) as follows:-

1- Plants with no symptoms;

2- 1.5 - plants with Light infection (point lesions);

3 - plants with Moderate infection (up to 5 small to large sunken lesions-about 3mm);

5 -plants with severe infection (large deep lesions- larger than 3mm- and /or death).

Further, these scales were converted to percent severity using the formula given by Wheeler, (1969).

$$S = \frac{\sum n}{N \times 5} \times 100$$

Where: S = severity of anthracnose (%); Σn = summation of individual ratings; N = total number of seedlings assessed

3.4.4 Data analysis

All the data obtained from the experiments were analyzed statistically by analysis of variance (ANOVA) procedure using GenStat computer package (VSN International 2008) after angular transformations of the data. Separation of means was done using Turkys Test at $p \leq 0.05$.

CHAPTER FOUR

RESULTS

4.1 Incidence of anthracnose on farm-saved bean seed

4.1.1 Incidence of anthracnose on bean varieties grown in Western Kenya

The most common varieties collected were Red haricot (GLP- 585), Mwitemania GLP X92, Yellow (KAT B 1), Rose coco (GLP-288), White, Rose coco (GLP-2), Brown and Mwezi moja (GLP-1004). GLP- 585 was the most predominant variety among the farmers sampled. In this study farm-saved seed collected from Western Kenya had high anthracnose incidence.

The GLP 585 variety collected from Bungoma had the highest anthracnose incidence. Results showed that Western Kenya farm-saved seed had significantly ($P \leq 0.05$) high anthracnose incidence compared to certified seed which had no pathogen in all varieties. Table 4.1 shows that disease incidence in all the seed samples were significantly ($P \leq 0.05$) different in varieties, seasons and study regions. The highest disease incidence was recorded in GLP 585 variety followed by GLP X92, white , Brown, Yellow and GLP 1004 while the lowest was recorded in variety GLP 2 followed by variety GLP 288 (Plate 4.1). However, incidence in varieties GLP 1004, Yellow, Brown, GLP X92 and white was not significantly ($P>0.05$) different.

Seed samples collected from Bungoma had highest incidence (Table 4.2) followed by Kakamega, Busia, Trans Nzoia and Nandi while those collected from Kisii had lowest incidence followed by Siaya. However, there were no significant ($P > 0.05$) differences among the mean incidence in the seed from Kakamega and Busia; Nandi and Trans Nzoia or from Kisii and Siaya.

Significant ($P \leq 0.05$) differences were observed for the anthracnose incidence in the seed on interaction of region by season by variety. GLP 585 variety, from

Bungoma region in both seasons, recorded the highest disease incidence (Plate 4.2). while long rains season, variety GLP 2 from Kisii had the lowest incidence (Appendix 10).

Table 4.1: Incidence (%) of anthracnose observed on seed varieties collected from W. Kenya.

Varieties	**Anthracnose incidence (%)**				**Variety**
	SR 2008	**LR 2009**	**SR 2009**	**LR 2010**	**Mean**
GLP2	26.8 a	25.7 a	29.8 a	30.0 a	28.7
GLP 288	29.1 ab	32.9 a	44.8 ab	30.9 a	37.0
GLP 1004	32.3 ab	35.7 ab	49.1 b	38.0 ab	40.0
Brown	35.7 ab	36.1 ab	51.1 b	42.7 ab	43.5
Yellow	44.3 bc	39.5 ab	51.4 b	44.5 ab	43.5
White	45.0 bc	41.3 ab	54.1 b	45.5 ab	44.4b
GLP X92	45.2 bc	50.0 b	54.5 b	48.2 ab	47.4
GLP 585	59.0 c	51.1 b	54.5 b	51.3 b	53.1
Season mean	39.7	39.0	48.7	41.4	
Grand mean	42.2				
S.E.D	2.9				
CV%	52.2				

Means sharing the same letters are not significantly different at $p \leq 0.05$ according to Tukey's test SR- Short Rains season; LR- Long Rains season.

Plate 4.1: Anthracnose infected bean plant of GLP – 288 variety in a field in Bungoma. Arrow showing black brown sunken lesion anthracnose symptoms (Source: Author, 2009).

Table 4.2: Incidence of anthracnose observed on seeds collected from farms in different regions of W. Kenya.

Region	Anthracnose incidence (%)				region
	SR 2008	LR 2009	SR 2009	LR 2010	Mean
KISII	28.91 a	23.28 a	30.94 a	18.91 a	25.5
SIAYA	27.50 a	25.00 a	36.88 a	27.97 ab	29.3
KITALE	39.22 ab	43.75 b	53.91 bc	42.34 bc	44.8
NANDI	42.66 ab	42.19 b	44.53 ab	44.84 cd	43.6
KAKAMEGA	47.66 b	52.66 b	54.84 bc	47.97 cd	50.7
BUSIA	45.94 b	40.00 b	54.22 bc	49.06 cd	47.3
BUNGOMA	45.78 b	46.25 b	65.31 c	58.59 d	54
Season mean	39.7	39.0	48.7	41.4	
Grand mean	42.2				
S.E.D	2.6				
CV%	49.3				

Means sharing the same letters are not different at $p \leq 0.05$ according to Tukey's test
SR- Short Rains season; LR- Long Rains season

Plate 4.2: Farm-saved seed of GLP-585 variety from Trans Nzoia: a) Healthy. b) Anthracnose infected. Arrow showing black brown sunken lesion anthracnose symptoms (Source: Author, 2009).

4.1.2 The quality of farm-saved seed sampled during survey.

The farm-saved seed from Western Kenya exhibited low percentage emergence than the certified seed which showed between 99-100% germination. There was significant ($P \leq 0.05$) difference in percent emergence for varieties and regions. GPL-2 variety seeds had the highest emergence of (Table 4.3), followed by GLP 288 variety seeds. GLP 585 variety seeds had the lowest emergence followed by GLP X92, Brown, Yellow, GLP 1004 and White (Plate 4.3).

Seed from Siaya had the highest emergence (Table 4.4), followed by Kisii and Nandi, Bungoma and Kakamega. Seed from Busia had the lowest emergence followed with those from T. Nzoia.

Days to 50% emergence (E_{50}) was used as an indicator of seed lot vigour. Seeds of all the varieties including those of the certified seed which were used as control experiment, obtained a high emergence rate of E_{50}, by day 5. There was no significant ($P>0.05$) difference in germination rate for varieties (Table 4.3).

Table 4.3: Seed viability seedlings and vigor of bean seed collected from W. Kenya measured as % emergence and rate of emergence.

	Seed viability and vigor	
Varieties	**% emergence**	**Rate of emergence**
GLP 2	69.4 d	5.2a
GLP288	66.9 cd	5.1 a
Brown	62.2 bc	5.0 a
Yellow	58.1 b	5.1 a
GLP 1004	59.1 b	5.1 a
white	58.1 b	5.1 a
GLP X92	50.9 a	5.1 a
GLP 585	50.0 a	5.1 a
SED	2.2	0.1
CV%	27.8	16.7

Plate 4.3: Growing-on test on clean sand (a) Healthy seedlings (b) Anthracnose infected seedlings with arrows showing the anthracnose dark brown lesions on cotyledons (Source: Author, 2009).

Table 4. 4: Seed viability and vigor of bean seed collected from different regions of W. Kenya.

	Seed viability and vigor	
Region	**% emergence**	**Rate of emergence**
Kisii	66.0de	5.0 a
Siaya	67.3e	5.0 a
Nandi	60.3cd	5.1 a
T. Nzoia	53.0ab	5.2 a
Busia	50.1a	5.1 a
Kakamega	58.1bc	5.1 a
Bungoma	59.1 bc	5.1 a
SED	2.1	0.1
CV%	28.2	16.6

Means sharing the same letters are not different at p≤ 0.05 according to Turkeys' test

4.2 Characterization of *Colletotrichum lindemuthianum*

4.2.1 Cultural characterization

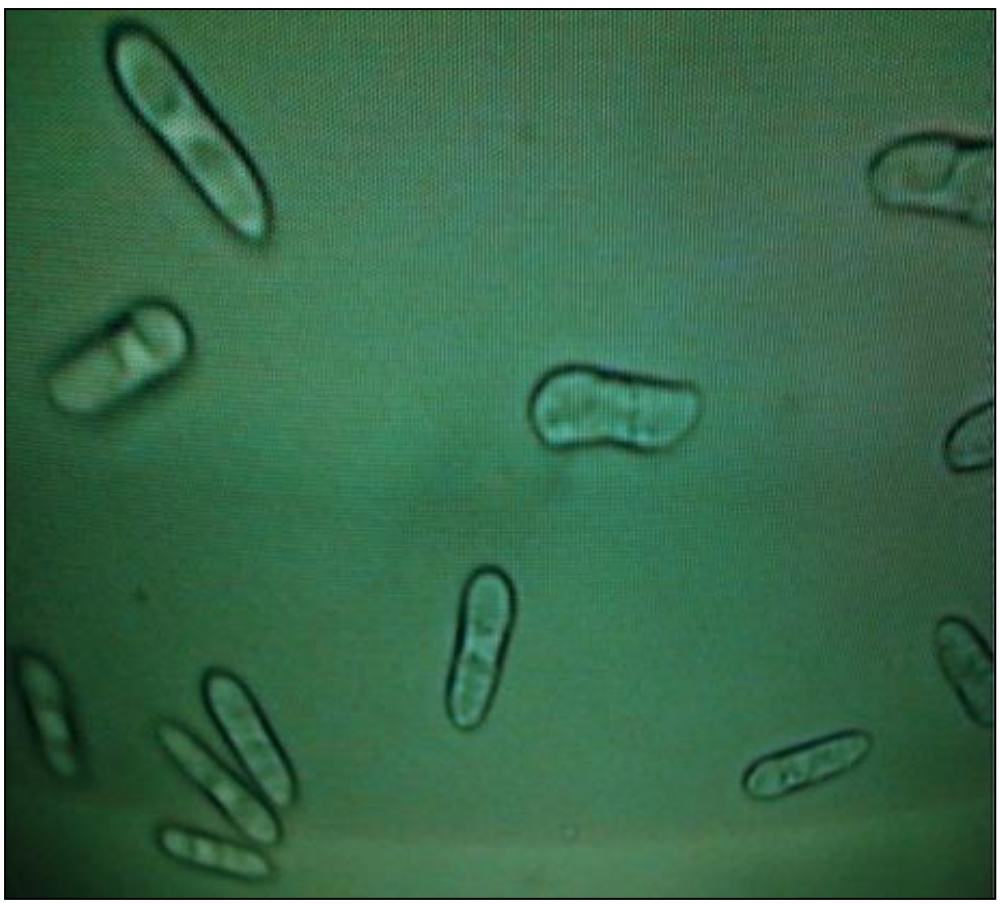

Plate 4.4: *C. lindemuthianum* conidia 200 X magnification from an infected pod of GLP – 585 bean variety (Source: Author, 2009).

After 14 days of culture incubation, it was observed that the 122 single spores isolates (Plate 4.4) exhibited diversity in cultural characteristics in terms of the mycelium colour. The colonies exhibited eight different mycelia colours (Plate 4.5) which included: - dark brown colony with light brown concentric rings was of the highest number of isolates and from all regions of study followed by black with brown

concentric rings, brown concentric rings colony with brown batches and black colony with white outer concentric rings (Table 4.5). Black colony with yellow batches had the lowest number of isolates followed by Black concentric rings colony, yellow colony with outer black concentric ring and several black spherical portions with black concentric rings.

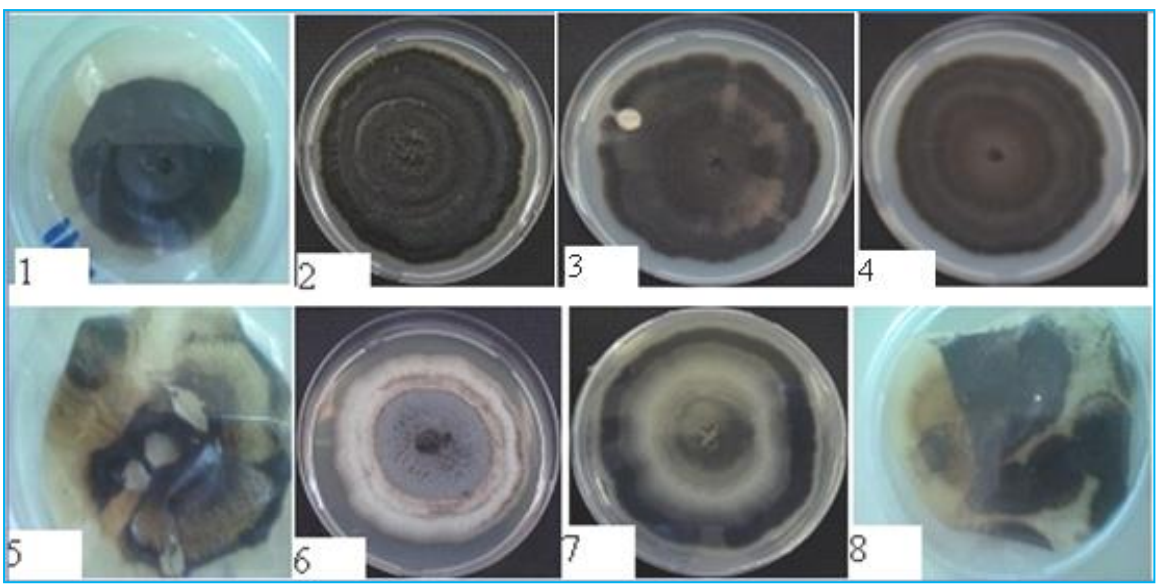

Plate 4.5: Different mycelia colour of *C. lindemuthianum* single spore colonies cultured on PDA and observed on the 14th day after incubation in darkness at 20 – 21 °C. 1. Black concentric rings colony 2.Black with brown concentric rings 3. Brown concentric rings colony with brown batches 4. Dark brown colony with light brown concentric rings 5. Black colony with yellow batches 6. black colony with white outer concentric rings 7. Yellow colony with outer black concentric ring 8. Several black spherical portions with black concentric rings (Source: Author, 2009).

Table 4.5: Mycelia Colour variation exhibited by the 122 isolates of *C. lindemuthianum*.

	Mycelia colours								Total
	A	B	C	D	E	F	G	H	isolates
Bungoma	12	3	2	4	0	0	2	0	23
Kakamega	10	4	3	4	0	0	0	0	21
T. Nzoia	13	1	2	1	0	0	0	0	17
Nandi	12	3	2	0	0	0	0	0	17
Busia	8	5	2	4	0	0	0	0	19
Siaya	6	2	2	3	1	0	0	0	14
Kisii	2	0	0	0	1	3	3	2	11
Total	66	22	13	9	3	3	4	2	122

Mycelia colour :- A. Dark brown colony with light brown concentric rings B. Black with brown concentric rings. C. Brown concentric rings colony with brown batches D. Black colony with white outer concentric rings E. Black concentric rings F. Yellow colony with outer black concentric ring G. Several black spherical portions with black concentric rings. H. Black colony with yellow batches.

4.2.2 Characterization of the pathogen using virulence and race typing on standard differential cultivars

All the 12 differential bean cultivars were susceptible to the W. Kenya isolates as indicated by the disease severity scale (Plate 4.6) indicating presence of 12 virulence factors in Western Kenya. There was variation in susceptibility of the 12 differential cultivars to the 122 isolates. Mexico 222 was the most susceptible to the highest number of isolates followed by Perry Marrow then MDRK, Cornell 49242, Kaboon, PI 207262, TO, Widusa, TU, AB 136, G2333, and finally Michelite was the least susceptible with the lowest number of isolates (Fig. 4.1).

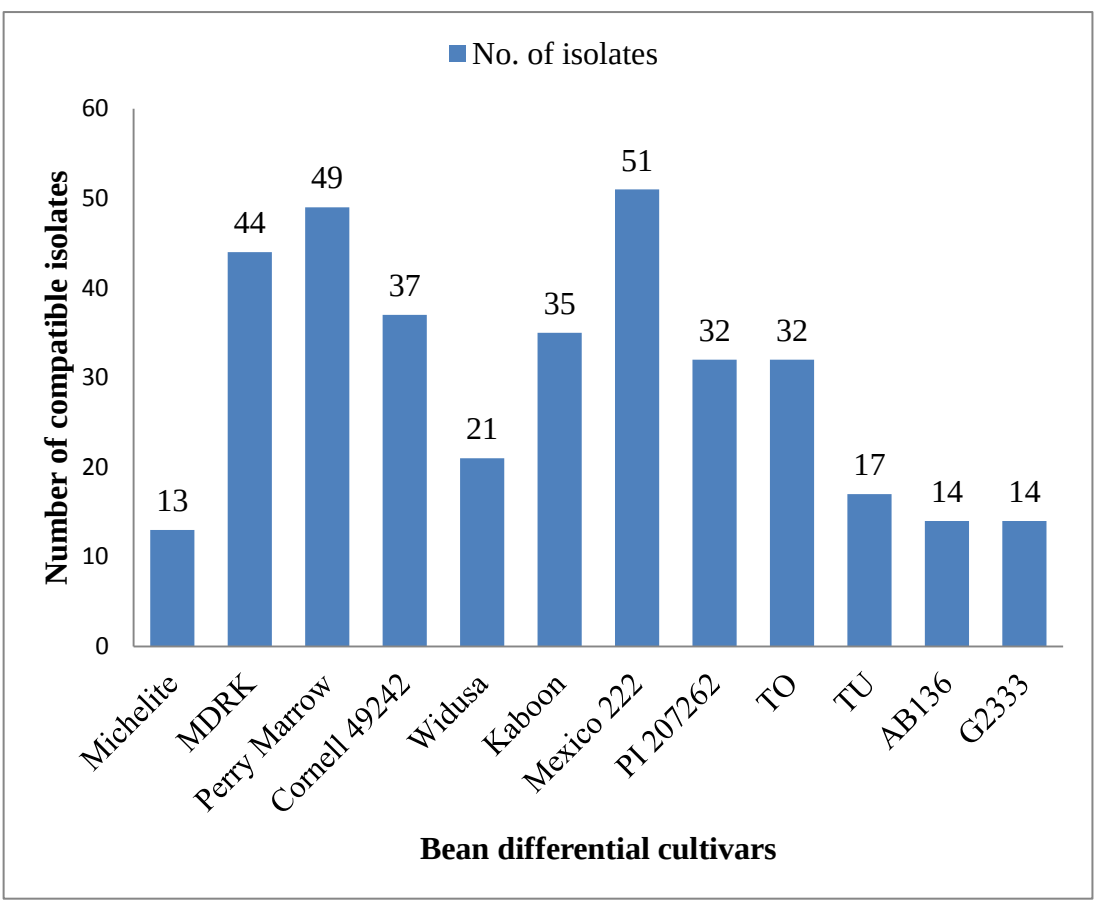

Figure 4.1: Susceptibility of common bean differential cultivars to the 122 isolates collected from W. Kenya.

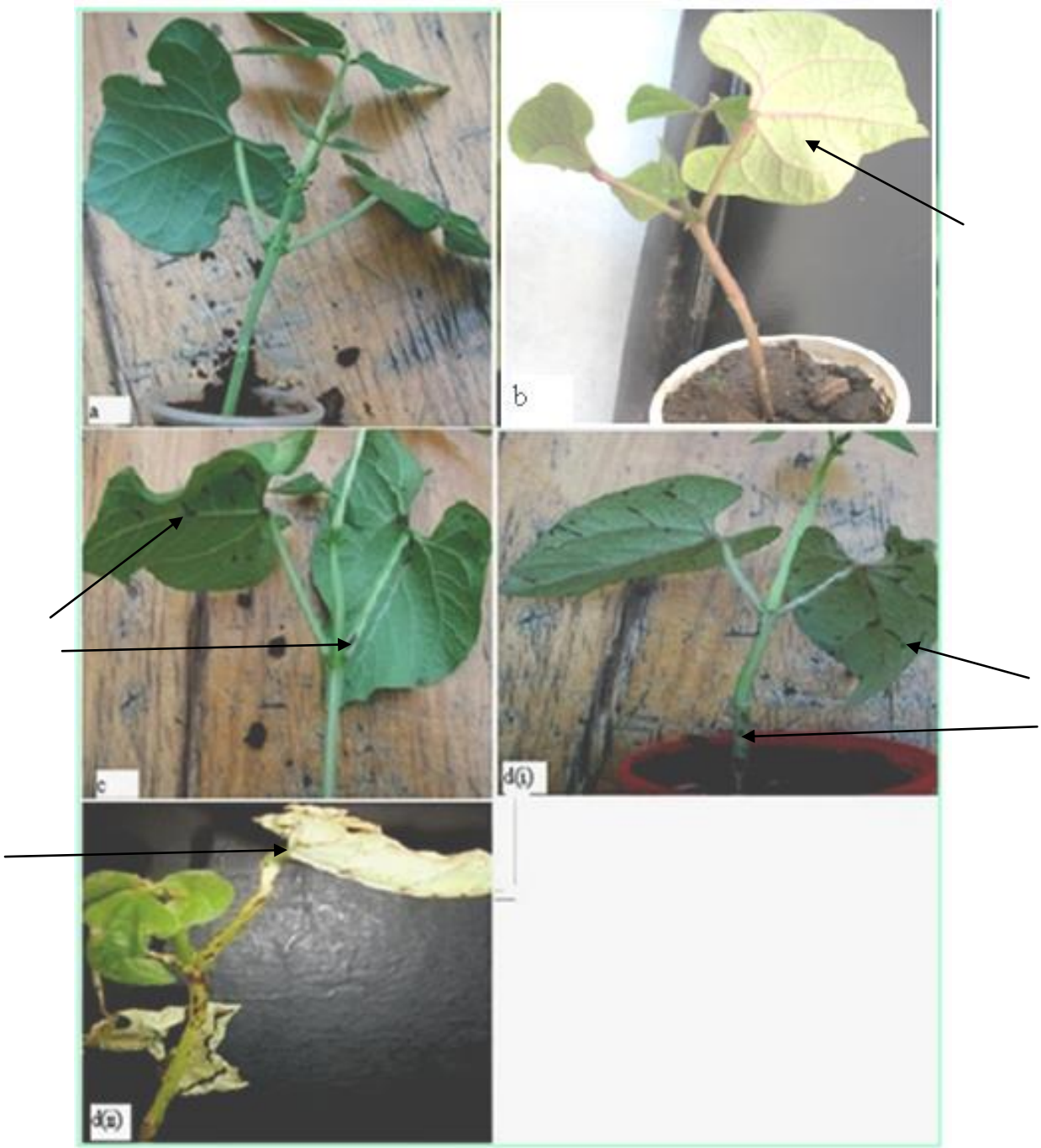

Plate 4.6: Disease symptoms on bean differentials in green house conditions showing severity scale 0 - 5:- (a) 1- Plants with no symptoms**, (b) 1.5 -** plants with Light infection (point lesions)**, (c) 3 -** plants with Moderate infection (up to 5 small to large sunken lesions-about 3mm)**, (d) 5 -** plants with severe infection (large deep lesions- larger than 3mm**,** (e) 5 - plants with severe infection (death). Arrows showing black brown sunken lesion anthracnose symptoms **(Source: Author, 2009).**

Table 4.6:- The reaction of the differential bean cultivars to the *C. lindemuthianum* isolates collected from W. Kenya.

Race[(z)]	Differential cultivars												Region collected from (no. of isolates)
	a	b	c	d	e	f	g	h	i	j	k	l	
0	r	r	r	r	r	r	r	r	r	r	r	r	Bungoma(3); Trans Nzoia(3); Busia(1); Siaya(1)
3	s	s	r	r	r	r	r	r	r	r	r	r	Nandi(1)
4	r	r	s	r	r	r	r	r	r	r	r	r	Bungoma(2); Nandi(1); Siaya(1)
6	r	s	s	r	r	r	r	r	r	r	r	r	Kisii(1)
8	r	r	r	s	r	r	r	r	r	r	r	r	Siaya(1); ransNzoia(2); Bungoma(1);
14	r	s	s	s	r	r	r	r	r	r	r	r	Nandi(2)
16	r	r	r	r	s	r	r	r	r	r	r	r	Nandi(2); Bungoma(1);
18	r	s	r	r	s	r	r	r	r	r	r	r	Trans Nzoia(1)
21	s	r	s	r	s	r	r	r	r	r	r	r	Trans Nzoia(1)
22	r	r	s	r	s	r	r	r	r	r	r	r	Busia(1)
23	s	s	s	r	s	r	r	r	r	r	r	r	previous
36	r	r	r	r	r	s	r	r	r	r	r	r	Kisii(1); Busia(1)
38	r	s	s	r	r	s	r	r	r	r	r	r	Siaya(3); Busia(2); Kisii(1)
46	r	s	s	s	r	s	r	r	r	r	r	r	Siaya(1); Busia(1)
48	r	r	r	r	s	s	r	r	r	r	r	r	Kakamega(1)
55	s	s	s	r	s	s	r	r	r	r	r	r	Previous data
64	r	r	r	r	r	r	s	r	r	r	r	r	Kakamega(1); Bungoma(3)
68	r	r	s	r	r	r	s	r	r	r	r	r	Trans Nzoia(1)
70	r	s	s	r	r	r	s	r	r	r	r	r	Siaya(1); Busia(1); Bungoma(2); Kakamega(1)
72	r	r	r	s	r	r	s	r	r	r	r	r	Kisii(1)
80	r	r	r	r	s	r	s	r	r	r	r	r	Nandi(1)
86	r	s	s	r	s	r	s	r	r	r	r	r	Kakamega(1)
92	r	r	s	s	s	r	s	r	r	r	r	r	Busia(1)
94	r	s	s	s	s	r	s	r	r	r	r	r	Bungoma(2)
96	r	r	r	r	r	s	s	r	r	r	r	r	Nandi(1); Kakamega(1)
98	r	s	r	r	r	s	s	r	r	r	r	r	Kakamega(1)
102	r	s	s	r	r	s	s	r	r	r	r	r	Kisii(1)
104	r	r	r	s	r	s	r	s	r	r	r	r	Nandi(1)
110	r	s	s	s	r	s	s	r	r	r	r	r	Bungoma(1)
130	r	s	r	r	r	r	r	s	r	r	r	r	Bungoma(1)
134	r	s	s	r	r	r	r	s	r	r	r	r	Siaya(1); Kisii(1)
162	r	s	r	r	r	s	r	s	r	r	r	r	Bungoma(1)
192	r	r	r	r	r	r	s	s	r	r	r	r	Bungoma(1)
200	r	r	r	s	r	r	s	s	r	r	r	r	Siaya(2); Busia(1)
256	r	r	r	r	r	r	r	r	s	r	r	r	Siaya(2); Kisii(1)
258	r	s	r	r	r	r	r	r	s	r	r	r	Kisii(1)
267	s	s	r	s	r	r	r	r	s	r	r	r	Nandi(1)
284	r	r	s	s	s	r	r	r	s	r	r	r	Nandi(1)

Cont.

Race [z]	Differential cultivars												Region collected from (no. of isolates)
	a	b	c	d	e	f	g	h	i	j	k	l	
320	r	r	r	r	r	r	s	r	s	r	r	r	Bungoma(1)
324	r	r	s	r	r	r	s	r	s	r	r	r	Trans Nzoia(1)
358	r	s	s	r	r	s	s	r	s	r	r	r	Nandi(1)
379	s	s	r	s	s	s	s	r	s	r	r	r	Trans Nzoia(1)
448	r	r	r	r	r	r	s	s	s	r	r	r	Busia(3); Siaya(1)
449	s	r	r	r	r	r	s	s	s	r	r	r	Bungoma(1)
456	r	r	r	s	r	r	s	s	s	r	r	r	Siaya(2); Busia(3)
485	s	r	s	r	r	s	s	s	s	r	r	r	Previous
531	s	s	r	r	s	r	r	r	r	s	r	r	Kakamega(1)
544	r	r	r	r	r	s	r	r	r	s	r	r	Bungoma(2)
548	r	r	s	r	r	s	r	r	r	s	r	r	Bungoma(2)
550	r	s	s	r	r	s	r	r	r	s	r	r	Kisii(1); Busia(2);
552	r	r	r	s	r	s	r	r	r	s	r	r	Nandi(1)
558	r	s	s	s	r	s	r	r	r	s	r	r	Kakamega(1)
608	r	r	r	r	r	s	s	r	r	s	r	r	Trans Nzoia(1)
622	r	s	s	s	r	s	s	r	r	s	r	r	Trans Nzoia(1)
648	r	r	r	s	r	r	r	s	r	s	r	r	Kakamega(1)
776	r	r	r	s	r	r	r	r	s	s	r	r	Trans Nzoia(1)
968	s	r	r	s	r	r	s	s	s	s	r	r	Kakamega(1)
1028	r	r	s	r	r	r	r	r	r	r	s	r	Kakamega(1)
1110	r	s	s	r	s	r	s	r	r	r	s	r	Kakamega(1)
1189	s	r	s	r	r	s	r	s	r	r	s	r	Kakamega(1)
1280	r	r	r	r	r	r	r	r	s	r	s	r	Trans Nzoia(1)
1463	s	s	s	r	s	s	r	s	s	r	s	r	Bungoma(1)
2054	r	s	s	r	r	r	r	r	r	r	r	s	Kakamega(1)
2124	r	s	r	s	r	r	s	r	r	r	r	s	Siaya(1)
2504	r	r	r	s	r	r	s	s	s	r	r	s	Kakamega(1)
2540	r	r	s	s	r	s	s	s	s	r	r	s	Kakamega(1)
2816	r	r	r	r	r	r	r	r	s	s	r	s	Trans Nzoia(1)
3073	s	r	r	r	r	r	r	r	r	r	s	s	Busia(1)
3080	r	r	r	s	r	r	r	r	r	r	s	s	Siaya(1)
3136	r	r	r	r	r	r	s	r	r	r	s	s	Siaya(1)
3216	r	r	r	r	s	r	r	s	r	r	s	s	Trans Nzoia(1)
3447	s	s	s	r	s	s	s	r	s	r	s	s	Nandi(1)
3529	s	r	r	s	r	r	s	s	s	r	s	s	Busia(1)
3546	r	s	r	s	s	r	s	s	s	r	s	s	Bungoma(1)
4045	r	r	s	s	r	r	s	s	s	s	s	s	Kisii(1)
4049	s	r	r	r	s	r	s	s	s	s	s	s	Kisii(1)

a-Michelite (1); b-MDRK(2); c-Perry Marrow(4); d-Cornell 49-242(8); e-Widusa(16); f-Kaboon(32); g-Mexico 222(64); h-PI 207262(128); i-TO(256); j-TU(512); k-AB136(1024); l-G2333(2047). s- Compatible reaction; r- incompatible reaction. z- Race designation based on sum of binary values assigned to susceptible differential cultivars (CIAT, 1988)

The binary system of race classification based on pathogenicity testing assigned the 122 isolates to 74 physiological races (Table 4.6). The 74 races identified showed high race diversity in Western Kenya. Bungoma region had the highest number of isolates (Table 4.7), followed by Kakamega, Busia and Siaya. Kisii had the lowest followed by Trans Nzoia and Nandi.

Based on the race distribution in the study regions there are two categories of races. Category one contained 57 races which were found to occur in only one region for example race 4045 was only found in Kisii whereas race 3080 was found in Siaya only. Category two consist of 17 races each found in two or more regions of the study area for example race 0 which was assigned to nine isolates distributed in five different regions; Bungoma, Kakamega, Trans Nzoia, Siaya and Kisii. Races 4, 8, 16, 36, 38, 46, 64, 70, 96, 134, 200, 256, 448, 456, 548 and 550 were spread in between two and four.

Pathogenicity of the pathogen on the bean differentials categorized the races into four. Category one contained 9 races including race 6. Their isolates were of Andean origin because they predominantly attacked bean differentials of Andean gene pool. Category two had 16 races including race 64. Their isolates were of Meso American origin because they predominantly attacked bean differentials of Meso American gene pool. Category three consisted 97 races including race 21. Their isolates were of a wider range of virulence because they predominantly infected bean differentials of both Andean and Meso American origin. Category four contained 9 races all identified as race 0. Their isolates had no compatible interaction with any of the bean differential cultivars.

Table 4.7: Distribution of *C. lindemuthianum* races detected among the isolates collected from Western Kenya.

Region	Total Isolates	Races detected (Race classification)
Bungoma	24	18 races (0; 0; 0; 4; 4; 8; 16; 64; 64; 70; 70; 94; 110; 130; 162; 192; 320; 449; 544; 544; 548; 1189; 1463; 3546)
Kakamega	20	17 races (0; 0; 0; 48; 64; 70; 96; 98; ; 200; 200; 304; 558; 648; 968; 1028; 1110; 1189; 2054; 2504; 2540)
Trans Nzoia	14	13 races (0; 8; 8; 18; 21; 68; 324; 379; 608; 622; 776; 1280; 2816; 3216)
Nandi	15	13 races (3; 4; 14; 14; 16; 16; 80; 96; 104; 267; 284; 344; 358; 552; 3447)
Busia	19	13 races (0; 22; 36; 38; 38; 46; 70; 92; 200; 448; 448; 448; 456; 456; 456; 550; 550; 3073; 3529)
Kisii	11	11 races (6; 36; 38; 72; 102; 134; ; 256; 258; 550; 4045; 4049).
Siaya	19	15 races (0; 4; 8; 38; 38; 38; 46; 70; 86; 134; 256; 256; 448; 456; 456; 531; 2124; 3080; 3136).

Cluster analysis (Fig. 4.2) assigned the 74 races of 122 isolates *Colletotrichum lindemuthianum* into two clusters : a minor (1) and a major (2). The major cluster was further subdivided into major divsions 1 and 2. Cluster 2 was further subdivided into three sub clusters 2a, 2b and 2c. The clusters had races from all the regions of study.

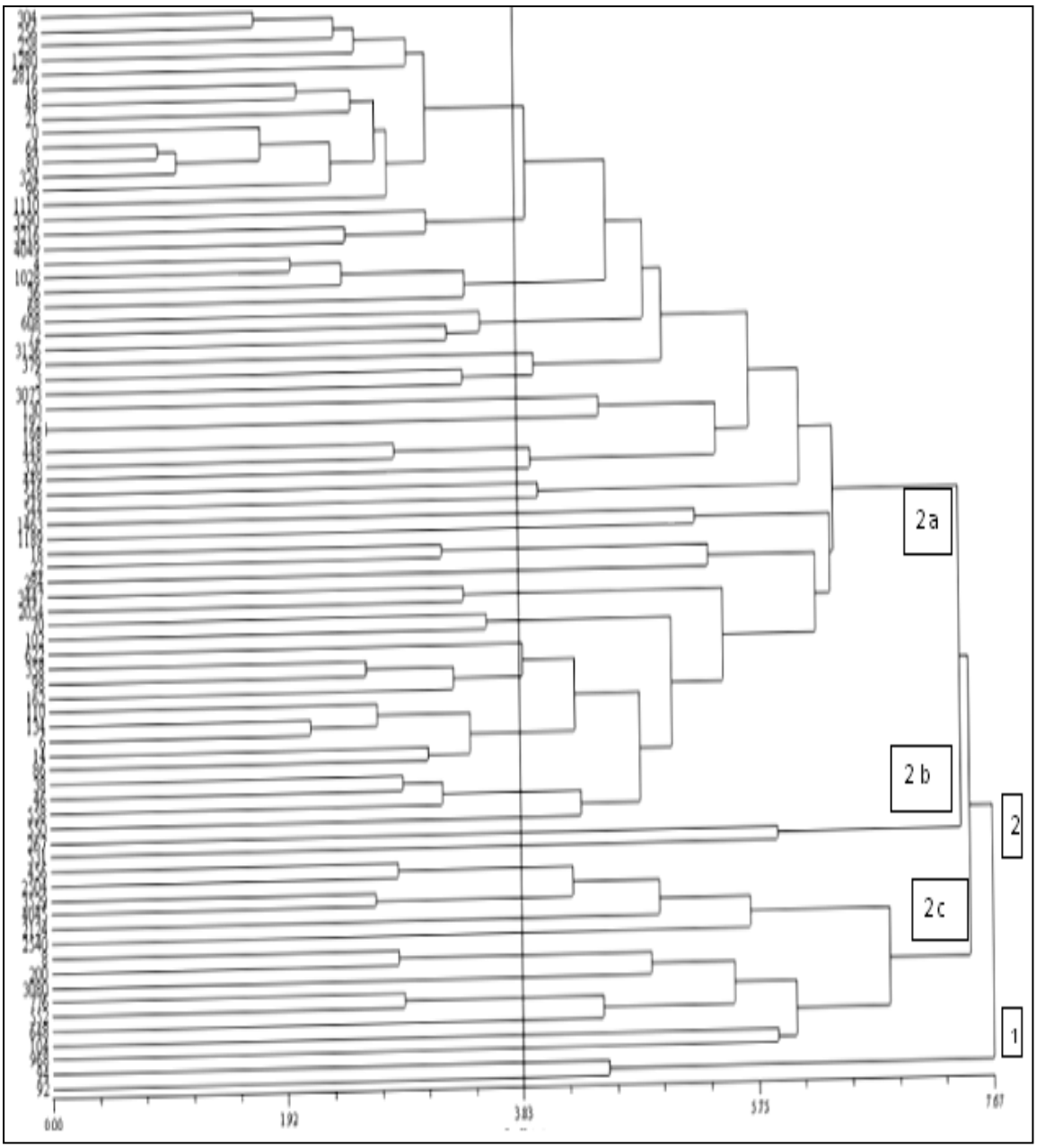

Figure 4.2: Cluster analysis of Western Kenya *C. lindemuthianum* races.

4.2.3 Molecular characterization of *C. lindemuthianum* races from W. Kenya.

From the 122 isolates that were characterized by virulence tests, only 60 isolates were successfully analyzed using BOX-AIR molecular markers. BOX primer was used at least twice for each isolate and the replicates yielded the same banding profiles (Plate 4.7).

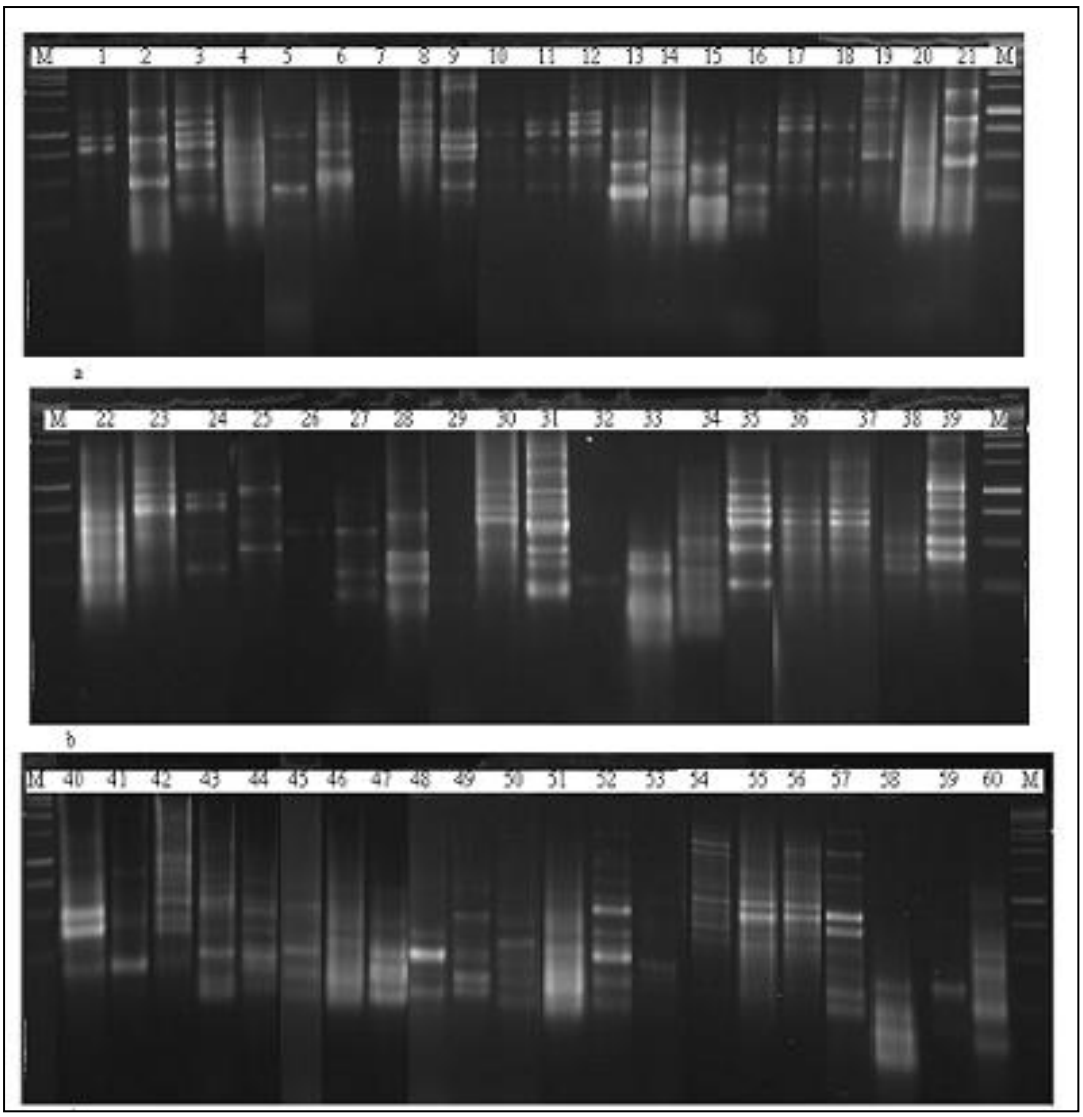

Plate 4.7 : PCR amplification products of genomic DNA of 60 selected races characterized from W. Kenya isolates of *C. lindemuthianum*. M- Moleclr weight Markers of 100kb DNA ladder. 1. 1. **Race 16,** 2. **Race 324,** 3. **Race 38,** 4. **Race 622,** 5. **Race 608,** 6. **Race 18**, 7. **Race 1280,** 8. **Race 3229**, 9. **Race 21**, 10. **Race 448,** 11. **Race 96,** 12. **Race 64**, 13. **Race 2816**, 14. **Race 38** 15. **Race 0,** 16. **Race 38,** 17. **Race 1028,** 18 . **Race 16,** 19. **Race 134**, 20. **Race 86,** 21. **Race 0,** 22. **Race 46**, 23. **Race 448,** 24. **Race 256**, 25. **Race 64**, 26. **Race 162** 27. **Race 448** ,28. **Race 320,** 29. **Race 256,** 30. **Race 550,** 31. **Race 6**, 32. **Race 102**, 33. **Race 0** 34. **Race 3** 35. **Race** 64 36. **Race 64**, 37. **Race 130** 38. **Race 548**, 39. **Race 544**, 40. **Race 22**, 41. **Race 456**, 42. **Race 1189**, 43. **Race 379** ,44. **Race 110**, 45. **Race 192,** 46. **Race 2054**, 47. **Race 284**, 48. **Race 3**, 49. **Race 550,** 50. **Race 104**, 51. **Race 284**, 52. **Race 3080**, 53**. Race 4049**, 54. **Race 14,** 55. **Race 80,** 56. **Race 80**, 57. **Race 552**, 58. **Race 531**, 59. **Race 96**, 60. **Race 14 (Source: Author, 2010).**

A total of 318 bands were obtained within the range of 800 to 2072 base pairs. From the total bands only 292 bands were polymorphic. The polymorphic bands were scored and a dendrogram was derived using the UPGMA analysis (Fig. 4.3). The genetic varations (s) among the isolates ranged from 0.65 to 1.0. The BOX data distinguished 8 separate clusters. The number of isolates within a cluster ranged from 41 in cluster 2 to 1 for cluster 3, 6, 7 and 8. The cut-off line showed average variation value at s=0.61 for all isolates. The cluster 1 consisted of 10 different isolates assigned races; 16, 448, 448, 550, 18, 64, 130, 8, 0 and 14. Cluster 4 had 2 races 4049 and 3080 while cluster 5 had 3 races 21, 96 and 64. Clusters 3, 6, 7 and 8 had races; 531, 3229, 544 and 6 respectively. All the remaining races (41) were grouped into cluster 2 which was further divided into 2 sub clusters 2a (32) and 2b (9).

The clusters contained isolates from different regions of W. Kenya and of different races. The dendrogram showed that there was no congruence between the BOX and virulance characterization. Isolates that were identical for virulence that is, the same race were most often dissimilar for BOX marker. The three isolates (ClY4, ClS6, ClS11) that were classified as race 38 were distributed across three clusters (1, 2a and 5 respectively) within the molecular dendrogram. This also applies to race 448 distributed in clusters 1, 2a and 2b and race 64 distributed in cluster 5 and 2b. All isolate races exhibited polymorphism except isolates ClN4 and ClN5 classified as race 80 which were monomorphic. The 2 races identified as race 80 were of isolates from the same infected tissue.

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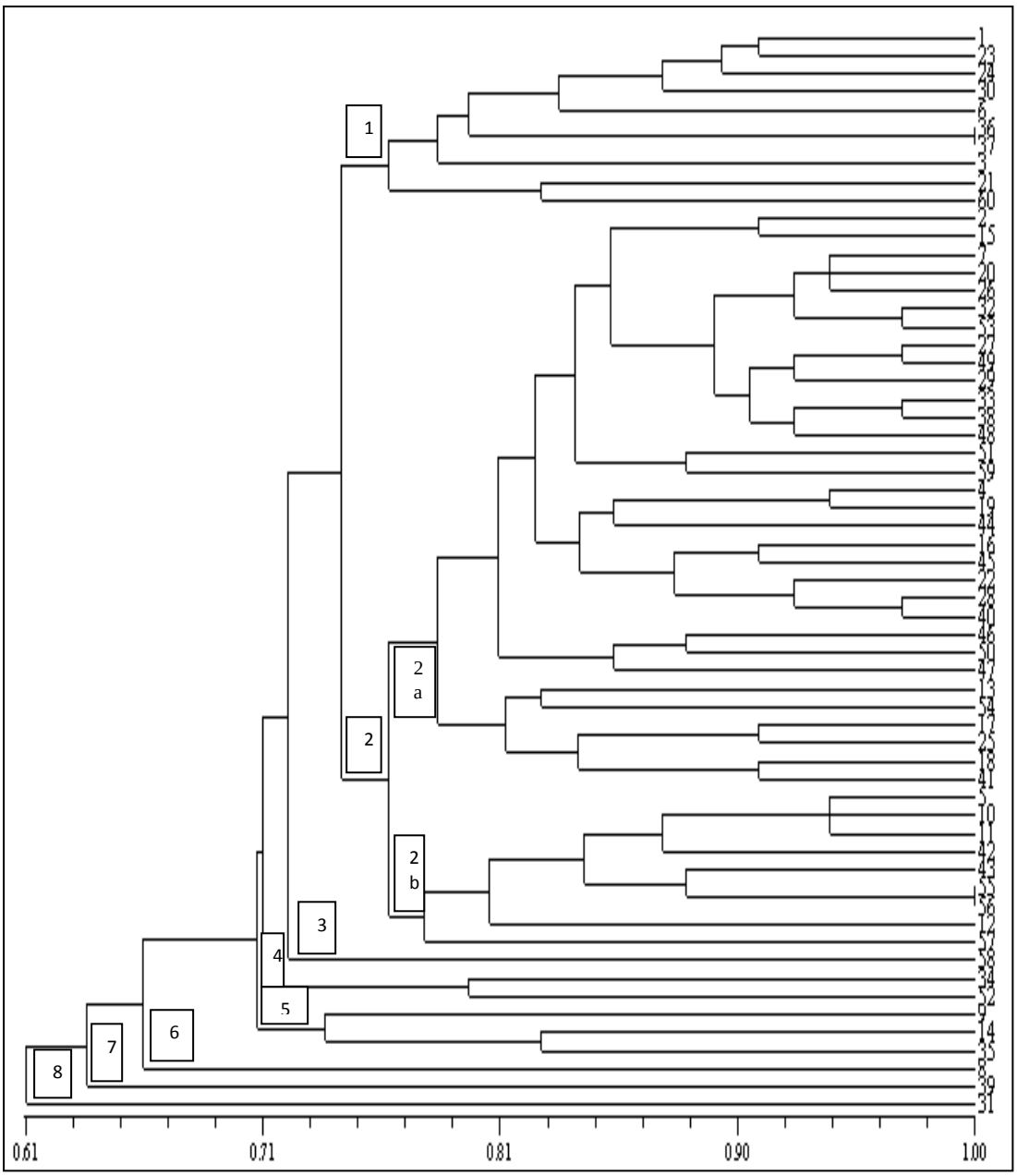

Euclidean distance

Figure 4.3 : Dendrogram showing similarities of 60 *C. lindemuthianum* races based on molecular polymorphism generated by BOX-AIR DNA analysis. 1. **Race 16,** 23. **Race 448,** 24. **Race 256**, 30. **Race 550,** 6. **Race 18**, 36. **Race 64**, 37. **Race 130,** 3. **Race 38,** 21. **Race 0,** 60. **Race 14,** 2. **Race 324,** 15. **Race 0,** 7. **Race 1280,** 20. **Race 86,** 26. **Race 162,** 32. **Race 102** 53**. Race 4049**, 27. **Race 448** 49. **Race 550,** 29. **Race 256,** 33. **Race 0,** 38. **Race 548**, 48. **Race 3**, 51. **Race 284**, 59. **Race 96**, 4. **Race 622,** 19. **Race 134**, 44. **Race 110**, 16. **Race 38,** 45. **Race 192,** 22. **Race 46**, 28. **Race 320,** 40. **Race 22**, 46. **Race 2054**, 50. **Race 104**, 47. **Race 284**, 13. **Race 2816,** 54. **Race 14,** 17. **Race 1028,** 25. **Race 64**, 18. **Race 16,** 41. **Race 456**, 5. **Race 608,** 10. **Race 448,** 11. **Race 96,** 42. **Race 1189**, 43. **Race 379**, 55. **Race 80,** 56. **Race 80**, 12. **Race 64**, 57. **Race 552**, 58. **Race 531**, 34. **Race 3** 52. **Race 3080**, 9. **Race 21**, 14. **Race 38** , 35. **Race** 6**4,** 8. **Race 3229**, 39. **Race 544**, 31. **Race 6**.

There was no grouping or clustering of the isolates according to the geographical origin. The two large clusters, cluster 1 and 2 were made up of isolates that were from different geographic regions. Even the smaller clusters (4 and 5) had variable isolates based on geographical regions. Cluster 4 contained isolates ClN7 and ClY4 from Nandi and Siaya that were designated as races 3 and 3080 respectively. Cluster 5 had isolates ClT9, ClS5 and ClB11 from Trans Nzoia, Busia and Bungoma that were designated as races 21, 38 and 64 respectively. The eight genomic clusters contained different isolates.

4.3 Antifungal activity of the plant extracts.

4.3.1 Laboratory tests

The results showed that plant extracts had ability to inhibit the mycelial gowth of *C. lindemuthianum*. There was significant ($P \leq 0.05$) difference in mycelia growth inhibition for plant extracts, positive control (Dithane M-45 80WP) and negative control (water). The positive control exhibited maximum growth inhibition of 92.6% while mycelia growth was not inhibited in negative control of water (0.0%) (Plate 4.8).

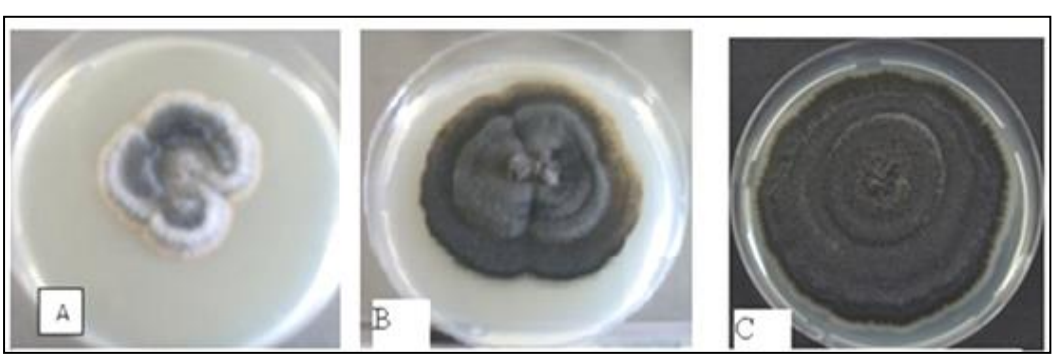

Plate 4.8: Inhibition of mycelium growth by plant extracts 14 days after incubation. A. effective; *A. vera* 90% inhibition, B. Averagely effective; *L. camara* 50% C. Not effective; Control 0% inhibition **(Source: Author, 2011).**

However there was significant (P≤ 0.05) difference in the mycelia growth inhibition by the plant extracts at different concentrations. At 30 % concentration, *Aloe vera* had significantly higher mycelia growth inhibition (Figure 4.4) followed by *Datura stramonium*, *Allium sativum*, *Allium cepa*, *Azadirachta indica* and *Eucalyptus globules*. Lowest inhibition of mycelia growth at 30 % concentration was recorded in *Lantana camara* followed by *C. gynandra*. The least mycelia growth inhibition was exhibited with *C. gynandra* at 20% concentration.

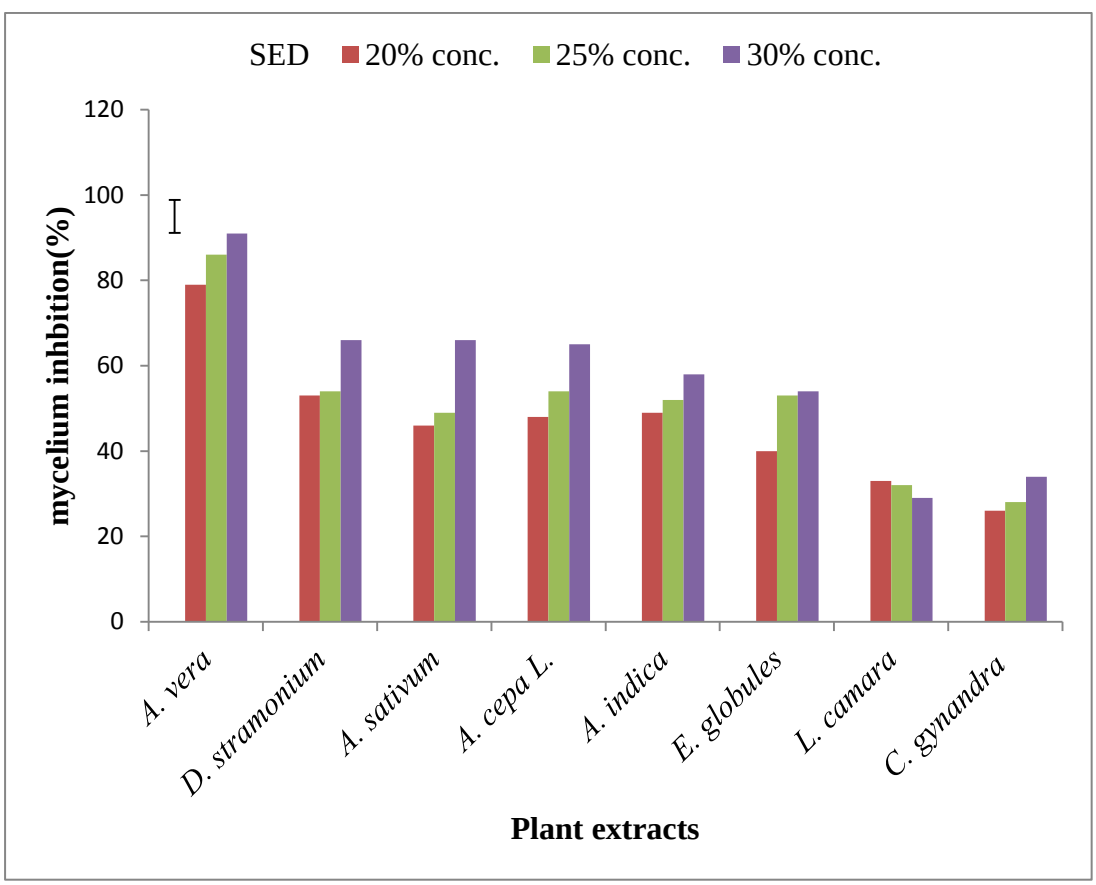

Figure 4.4: Effect of different plant extracts at varying concentrations on *C. lindemuthianum* mycelia growth.

There was a significant (P ≤ 0.05) difference in the efficacy of the plant extracts at different concentrations. All the extracts had their highest mycelia growth

inhibition at 30% concentration (Figure 6). *A. vera* was the most effective extract in all concentrations, however at 30% concentration the extract exhibited the highest mycelia growth inhibition followed by 25% while 20% was the least effective.

4.3.2 *In- vivo* tests.

Green house tests were done to investigate the effect of four plant extracts; *Aloe vera, Datura stramonuim, Allium sativa* and *Allium cepa;* on incidence and severity of anthracnose disease. These plant extract had higher percent mycelia growth inhibition in laboratory tests at 30% concentration (Figure 4.5).

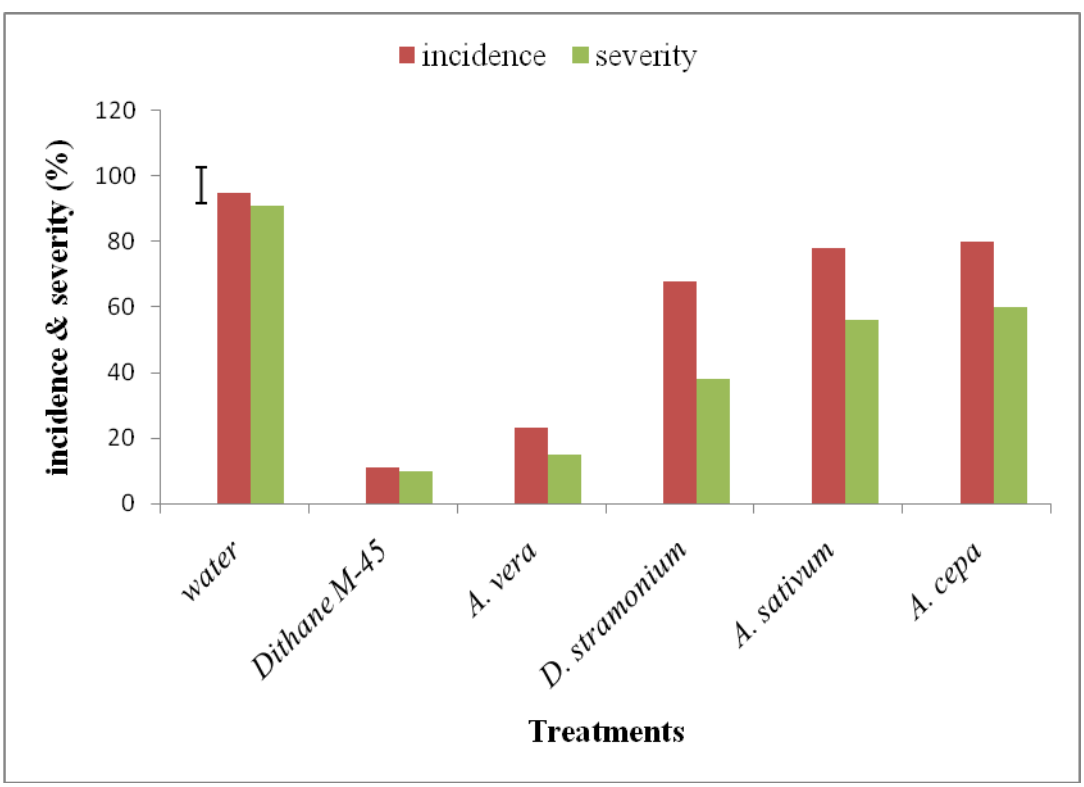

Figure 4.5: The effects of some plant extracts on the incidence and severity of anthracnose disease on beans.

Significant (P≤0.05) difference in incidence and severity of the disease in the different extracts was observed. Plants treated with *A.vera* extracts had a lower

incidence and severity (23% and 15%, respectively) followed by *D. stramonium* (68% and 38%); then *Allium sativum* (77.8% and 56%) while *Allium cepa* had the incidence and highest severity (80% and 60%, respectively) over negative control, water, (95% and 91%). Positive control, Dithane M-45 80WP had the lowest incidence and severity (11.1% and 10%, respectively).

CHAPTER FIVE

DISCUSSION

5.1 Incidence of anthracnose on farm-saved bean seed.

Results from this study showed that anthracnose incidence was generally higher in farm-saved seed than in the certified seed which was used as control. This implies that the apparently clean healthy farm-saved seed was of low quality in terms of its health status due to anthracnose infection. These variations in the disease incidence in different regions of study could be attributed to the fact that the regions had diverse seed varieties, agro-climatic conditions, agronomic practices and agro-ecological conditions which bring about different influences on disease development. GLP 285 variety had the highest disease incidence while GLP 2 had the lowest. This study shows differences in susceptibility of different varieties to the disease as also observed by Ombiri *et al*., 2003 & Nkalubo, 2007. They concluded that variety susceptibility depends on type and number of resistance genes present in each cultivar. This encourages cultivation of many bean varieties on the same farm since different varieties have different resistance genes to the disease (Mwaniki, (2002) leading to production farm-seed of low disease incidence. GLP 2 variety is known to have genes resistant to anthracnose although still there was incidence of infection by the disease. Farmers in Kisii predominantly planted GLP 2 and GLP 288 varieties which had low disease incidence. This led to low disease incidence in the region.

In this study it has been observed that variation in climatic conditions from one season to another influences the occurrence and severity of the disease. During the collection of the farm-saved seed the temperature ranged between 15 and 18^{o}C in the short rains season and 24 and 30^{o}c in the long rain season. Rainfall ranged

between 1600 and 2100 mm in short rain season and 1200 and 1800 mm in the long rain season. Seed collected during long rains season had generally lower anthracnose incidence level while short rains season had higher incidence in all the study regions. It confirms that rainfall and temperature contribute to the level of infection and development of anthracnose. Anthracnose is reported to develop well in high altitudes, moderate temperature ranges of 18 - 24 ^{o}C, more rainy days and high relative humidity of above 95% (Yesuf & Sangchote, 2007).

Variation of disease incidence during the collection period could also be attributed to observed different agronomic practices in different seasons (Appendix l). Most Western Kenya farmers predominantly practiced pure stand cropping during the short rain season while in long season they practiced intercropping with other crops especially maize. In mono cropping practice bean crops are very closely spaced than in the intercrop. Closely spaced crops retain high humidity and temperature in between their population, a condition conducive for the diseases to develop and spread from one plant to another. Close spacing of bean plants increase chance of contact within plant populations or use of farm implements by farmers during different agronomic practices in the field such as weeding leading to spread of the disease from infected plants to health plants. Rain splashes between closely spaced plants are other factors that could increase dissemination of the pathogen in the field. This contributes to high incidence of anthracnose in seeds harvested on short rain season than long rain season seeds. However farmers in Kisii regions predominantly practiced intercropping in both seasons. This could also explain why Kisii consistently had lower incidence in both seasons.

Anthracnose infections on bean seeds play an important role in transmission of the causal pathogen to the seedlings and thus enhance disease development (Yesuf &

Sangchote, 2007). In this study growing-on test confirmed that the farm-saved seed had the pathogen and could transmit the pathogen to the seedlings if sown. The farm-saved seed used in this study produced significant number of diseased seedlings. This implies that infected seeds consistently produced diseased seedlings as indicated by previous studies that prevalence of anthracnose was high in infected populations (Benard-Capelle *et al*., 2006). They also had a lower emergence over certified seeds. This confirmed that farm-saved seed was of low viability, one of the parameters indicating low quality seed. Higher emergence observed in GLP2 showed that the seeds were of higher quality than the other farm-saved varieties collected. There was no significant difference in the emergence rate at E_{50} of the different seed varieties used in this study indicating that the anthracnose diseased seeds with a viable embryo germinate with same vigour as the certified seeds. This confirms why the farmers use the farm-saved seeds unsuspecting that their seeds are of low quality in terms of health status. In this study both blotter and growing test confirm that Western Kenya farm-saved seed had high anthracnose incidence which contributes to their low quality. Also variation of disease incidence in the farm-seed confirmed the necessity to characterize the causal pathogen, *C. lindemuthianum*.

5.2 Characterization of *Colletotrichum lindemuthianum*

5.2.1 Cultural variation of *Colletotrichum lindemuthianum*

During the preparation of the pathogen for characterization the mycelia cultures of the isolates exhibited variation in colour despite the fact that all the 122 isolates were cultured in the same media PDA at the same period and incubation conditions. This shows that the isolates were highly diverse in nature. Most of the colour clusters contained isolates from different regions and of different races an

indication of high pathogen diversity. This means that clustering of the isolates was not according to region of origin or the races.

5.2.2 Virulence characterization of *Colletotrichum lindemuthianum*

Virulence characterization was done based on the susceptibility of the 12 differential cultivars to the 122 isolates. The differential cultivars used comprised four Andean and eight Meso American cultivars. All the 12 bean differential cultivars showed susceptibility responses to the 122 isolates hence there were 12 virulence factors within the 122 isolates. This demonistrates that virulence of *C. lindemuthianum* in W. Kenya was very diverse and that the genes present in the differential cultivars were susceptible to the isolates.

Previous studies in Kenya had only 10 of the differential cultivars susceptible to the disease as shown by Ombiri *et al*., (2002) who discovered a new race 485. Race 485 was as a result of additional break of resistance in cultivars Mexico 222, PI 207262 and TO to the previous five Michelite, MDRK, Perry Marrow, Cornell 49-242, Widusa and Kaboon which had lost their resistance to the disease (Kinyua 1976; Mwangi 1986; Gathuru & Mwangi 1991). The races such as race 4049 and 3080 obtained in this study had more virulent genes against the differential cultivars because even the most resistant bean cultivars of Meso American gene pool were attacked. Susceptibility of cultivars TU, AB 136 and G2333 to *C. lindemuthianum* had rarely been reported around the world but in this study each of these cultivars were found to be susceptible to atleast 14 isolates. This shows the high virulent nature of the isolates. Mahuku and Riascos, 2004 and Seher et al., 2013 have also observed races that break the resistance of AB136 while G2333 cultivars. The cultivar AB 136 with resistance genes *Co-6 and Co-8* (Mahuku *et al*., 2002) was found to be resistant

to many isolates from different parts of the World because gene *Co-8* is a conditional recessive gene, and virulence factors towards this gene are rare (Alzate-Marin *et al*., 1997; Gonzalez *et al*., 1998). This study showed that stable resistance to *Colletotrichum lindemuthianum* in Western Kenya might not be found in the primary gene pool in the bean varietieties grown in the region. Mutation, within the pathogen population can lead to a loss of avirulence factors producing a compatible reaction with previously resistant genes such as those in cultivars AB136 and G2333. The pathotype diversity displayed is a reflection of the dynamic nature of preexisting races and continued evolution of pathoypes in response to the introduction of new resistantance genes as also found by Fabre *et al*., 1995; McDonald & Linde, 2002. Therefore more alternative sources of resistance must be sought. In addition there could be adequate planning and observation in the regions with meso American races and host cultivars, to delay the dissemination or arrival of new races, and consequently, prolong the useful life of the cultivars indicated for cropping.

Further, nine isolates had no compatible interaction with any of the differential cultivars and were assigned race zero (0). This means that the isolates did not fit into any of the two host gene groups. This reveals that there are more susceptible genes in the bean cultivars of Western Kenya that have not been incorporated in the differential cultivars. The breeders need to consider incorporating more genes to the differential cultivars used in the race characterization.

Using binary system of race classification the 122 isolates studied were designated into 74 races based on different patterns of virulence on the 12 differential cultivars. This was a high number of races which indicates high pathogen diversity in the region. Race analysis of the pathogenicity data found that there was high race diversity in the entire study region indicating the high variable nature of the pathogen

in Western Kenya. Kisii had 11 isolates all assigned 11 different races while Trans Nzoia had 14 isolates assigned 13 races. Busia had 19 isolates which were assigned 13 races. These results confirm that *Colletotrichum lindemuthianum* is a pathogenically highly variable in the study area. This is in agreement with Damasceno *et al*., (2007); Mahuku & Riascos, 2004) and Ansari *et al*., (2004) who identified high levels of pathogenicity diversity of 10 races among 48 isolates, 90 races among 200 isolates and 30 races among 74 isolates respectively in different countries. In Kenya Ombiri *et al*., (2002) found one race in 4 isolates collected from Rongai Kenya.

Further more the number of races detected in this study was high than in the previous studies conducted in Kenya. Previously using a system proposed by Hubbeling, (1957) seven races in addition to uncharacterised ones had been reported in Kenya namely alpha, beta, deita, epsilon, gamma and lambda (Kinyua 1976; Mwangi 1986; Gathuru and Mwangi 1991; Ombiri *et al.* 2002). The races were designated as race 17, 2, 38, 23, 1, 55 and 485 respectively and were collected from nine regions of Kenya. Only race 38 identified in the present study resembled the races of *C. lindemuthianum* characterized from the previous studies in Kenya. All the other races obtained in this study did not conform to those obtained in the previous studies in Kenya. Latter race 485 was found in Rongai , Nakuru using binary system (Ombiri *et al.*, 2002). This implies that the pathogen in Western Kenya has increasingly become diverse in virulence. Mahuku *et al.* (2004); Kelly *et al*. (2004) and Vidigal *et al*. (2007) have also indicated that *C. lindemuthianum* has high rates of evolution and new races keep emerging from time to time. It also extends that very virulent races such as 4045 and 4049 obtained from Kisii had not been previously reported in any parts of the world. This bring about a great challenge to management

of anthracnose of common bean because there is a potential appearance of new virulence alleles capable of breaking the resistance of those alleles widely used in bean breeding programmes. For this reason, it is necessary to keep monitoring the pathogen population to impact on any races that may emerge and thus to minimize the effect on the local bean in W. Kenya. Therefore more resistance genes need to be generated to overcome the new virulence genes emerging in W. Kenya.

The 74 races of *C. lindemuthianum* identified were unevenly distributed in W. Kenya. W. Kenya has variable agro-ecological zones (LH3, LH2; LH1, UM4, UM3, UM2, UM1, LM3, LM2 and LM1) with different agro- climatic conditions (Appendix 1). These geographical differences could influence the observed high pathogen variability.

Races of lower numbers were the most frequent and widely distributed. Races 0 was the most widely distributed geographical (5 regions) and most frequent (9 isolates) followed by race 70 (4 regions) races and 4, 8 and 38 (three regions each). Races 16, 36, 46, 64, 96, 134, 200, 256, 448, 456, 548 and 550 distributed in two regions each. This agrees with the previous studies by Damasceno *et al.*, (2007) who reported that widely spread races 5, 12, 73 and 81 of *Colletotrichum lindemuthianum* infected a smaller number of differential cultivars. Other studies by Pastor-Corrales, (1996) and Ansari *et al*., (2004) reported the same. These results emphasize on the need of breeders to develop cultivars with resistance genes to these races by pyramiding resistance genes. The wide distribution of these races is also generated by the free grain trade and exchange (Opole *et al.,* 2003) within Western Kenya and other neighboring regions as also found by Damasceno *et al*., (2007).

The 74 races obtained from the different regions of W. Kenya were dispersed in the cluster analysis. Results from virulence study reveal that *Colletotrichum*

lindemuthianum is a highly diverse pathogen that mantains high levels of virulence diversity.This is crucial information for designing anthracnose management strategies, deploying resistance genes and developing resistant on-farm bean cultivars.

5.2.3 Molecular characterization of *Colletotrichum lindemuthianum*

Molecular analysis using BOX-AIR markers technique showed high genetic variabilty in W. Kenya. The BOX-AIR primer generated products of 800-2072 bp and DNA polymorphism among 60 isolates. This BOX DNA amplicons pattern shows high polymorphism indicating the highly variable nature of the pathogen. The high molecular diversity was also observed by Mahuku & Riascos, (2004); Bardas *et al*. (2009).

The dendrogram derived from the UPGMA analysis was divided into seven groups at 61 % genetic dissimilarity. The races that clustered together were not the same nor from same region of collection. This indicates that the genetic structure of *Colletotrichum lindemuthianum* reveals no geographical differentiation in Western Kenya. These results are supported by the conclusion of Balardins *et al*. (1997), Fabre *et al*. (1995) Mahuku & Riascos, 2004, Damasceno *et al*., (2007) and Bardas *et al*. (2009) all who reported that genetic structure of *Colletotrichum lindemuthianum* reveal no geographical differentiation. This has important implications in deployment of resistace genes and directing development of anthracnose resistant cultivars. The clustering of isolates by region of collection could not occur because of high free interchange of farm-saved seed infected with *Colletotrichum lindemuthianum* spores among regions of Western Kenya. Introduction of new genes into bean varieties through breeding programmes brings about pathogen mutation as they strive to adopt to the new environment leading to high variabilty.

The sexual form of *Colletotrichum lindemuthianum* in natural populations has been identified although it is rare (Damasceno *et al*., 2007). This means that *C. lindemuthianum* is not exclusively asexual and have significant advantages over strictly asexual or sexual pathogens. During sexual cycle, many new combinations of genes are produced and mutations recombined leading to diverse genetic characteristics (McDonald & Linde, 2002). Both sexual and asexual reproduction in *Colletotrichum lindemuthianum* play an important role in enhancing genetic diversity.

Also *Colletotrichum lindemuthianum* is a seedborne disease (Yesuf and Sangchote, 2007) and seed is the only primary means of dissemination. Due to high rate of seed exchange and movement in Western Kenya, new races through contaminated seed bring about gene flow. The movement of seeds also facilitate introduction of new seed varieties some of which may be having resistance to the pathogen. The new resistance could be challenged by pathogen mutation to adapt to new environment hence causing genetic diversity. This agrees with previous studies that gene flow, sexual, asexual and parasexual reproduction coupled with selection over time and introduction of new varieties with new sources of resistance (Damasceno *et al*., 2007) play major roles in generating and mantaining the high genetic variation observed in this pathogen.

The molecular study revealed that *C. lindemuthianum* is a highly variable pathogen that mantains high levels of genetic diversity. This is crucial information for designing anthracnose management strategies, deploying resistance genes and developing resistance in bean cultivars.

Molecular markers have combined with virulence data have been used to generate pathogen genetic diversity and evolutionary relationships of the plant pathogens (Mesquita *et al*., 1998; Mahuku & Riascos, 2004; Bardas *et al*., 2009). This

informaton provide a better view of the specific pathogen's variability, leading to more efficient control strategy (Mahuku & Riascos, 2004). The current study used BOX-AIR and virulence markers to show that there exists high virulence and molecular diversity of *Colletotrichum lindemuthianum* Western Kenya. Overall, the results of this research showed that their was no association between genetic diversity and race classification of the isolates. Cultural features also show variation of the pathogen.

5.3 Efficacy of plant extracts antifungal activity against bean anthracnose.

Current investigation clearly indicates that the antifungal activity against *C. lindemuthianum* vary with the species of the plants tested. There was no tested plant extract that had no inhibitory activities because their inhibitory mycelia radial growth compared with the control as also observed by Wokocha & Okereke, (2005).

Current investigation clearly indicates that the antifungal activity against *Colletotricum lindemuthianum* vary with the species of the plants tested. The *in-vitro* tests showed that *Aloe vera* leaf extract was effective in inhibiting mycelia growth. This indicates that *Aloe* has inherent ability to induce toxic effects on mycelia growth and proliferation of *Colletotricum lindemuthianum*. The toxic effect is attributed to presence of main active constituent, the aloine, an anthraquinone heteroside (Bruneton, 1993). This study is in agreement with several other workers who tested the activities of *A. vera* with the aim of assessing their activity against *Colletotrichum gloeosporioides* and *Colletotrichum capsici* using food poison technique. The extracts showed higher activity against the *Colletotrichum* species. They identified that aloine and aloe-emodin chemical components in *Aloe vera* plant were the active reagents with activity against *Colletotrichum gloeosporioides* and *Colletotrichum capsici*. Also

Jaya & Dhananjay (2012) worked on six plants, *Azadirachta indica, Aloe vera, Ocimum sanctum, Ocimum basilicum, Lantana camara and Asparagus* antifungal activity against the *Aspergillus niger, Aspergillus flavus, Rhizoctonia solani, Rhizoctonia bataticola* and found that *Aloe vera* was the most effective.

Datura stramonium, Allium sativum and *Allium cepa* showed intermediate mycelia inhibitory after *Aloe vera. Datura* plants extracts contain tropane alkaloids such as scopolamine, hyoscyamine, and atropine (Preissel & Preissel, 2002). Because of presence of these substances *Datura* has been used as an antifungal against many plant pathogens. Rajesh & Sharma, (2002) the antimycotic properties of *Datura spp* on different species of *Aspergillus.* Sharma *et al.,* (2009) studied antibacterial and antifungal activities of some common plants and weeds; they found weed plant *Datura stramonium* showed pronounced antibacterial and antifungal activity against *Escherichia coli, Staphylococcus aureus, Bacillus subtilis, Klebsiella pneumonia, Aspergillus niger and Candida albicans.* The effectiveness of *Allium cepa* and *Allium sativum* is well supported by Muthusamy *et al.,* (2007). Alliums were known to possess powerful organosulfur-containing compounds, allicin, and other numerous phenolic compounds (Rivlin, 2001; Griffiths *et al.,* 2002). However, garlic contains much sulfur-containing compounds than onions (11–35 mg/100 g fresh weight) (Lawson, 1996). Other investigators also found that garlic extract was effective in controlling the anthracnose pathogen in different crop species (Singh *et al*., 1997).

Garlic and neem products have also shown some antimicrobial properties and have been used in the control of fungal pathogens (Obagwu *et al.* 1997). In this study *A. indica* had an averagely effective inhibitory activity. The fungicidal spectrum of *Azadirachta indica* has been attributed to Azadiractrachin which belong to terpeniodes (Raheja *et al.,* (2002).

Eucalyptus globules gave moderate control of the disease. Other studies show that *E. globules* does not have strong antifungal properties. *C. gynandra* had the least inhibitory activity followed by with *L. camara.* *C. gynandra* leaves, leaf juice, and seeds have antifungal activity due to the presence of glucosinolates. *L. camara* has antifungal property due to presence of Lantadene (Sarita *et al*. 1999). Lantadene is a pentacyclic triterpenoid present in the leaves of *Lantana* plant. Previous studies show *L. camara* significantly suppressed the growth of *Colletotrichum falcatum, Fusarium* spp. and *Aspergillus* sp. (Lingayya *et al.*, 2011). However despite the presence of antifungal properties in *L. camara* and *C. gynandra*, the current studies are known to have low inhibitory activities on *C. lindemuthianum*.

All the tested plant extracts had high inhibitory activity at 30% concentration than at 25% and 20%. This indicates that there was an increase in inhibition of fungal mycelia growth as the extract concentrations increased. This could be due to increased availability of the antifungal chemicals in the media. Other studies have also shown that there was increase in inhibition of fungal colony when extracts concentrations increased (Cao & van Bruggen, 2001; Shovan *et.al*,. 2008; Masangwa *et al*., 2012). Masoko *et al*., 2005 speculated it to be due to solubility of compounds in water and stated that water fails to extract non-polar active compounds in plant materials and hence need for higher plant extracts concentration to achieve high fungal toxic level.

The *in-vivo* results confirm that aqueous extract of *A. vera* is the most effective antifungal extract. The artificial inoculation of the plants had low disease incidence and severity. This shows that the extract also reduces the incidence and severity of anthracnose in the green house as also observed by Emua *et al*., (2009). Plant extracts as an alternative control method if integrated in anthracnose management could reduce over reliance on the synthetic fungicides by the farmers, as

well as cut down cost of bean production. The plants used in the study are readily available and is of easy in extraction hence they can be exploited in the control of bean anthracnose.

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusion

Farm-saved seed in Western Kenya has high incidence of bean anthracnose. The causal organism, *Colletotrichum lindemuthianum* in this region is highly variable physiologically and genetically. All the 12 bean differential cultivars are susceptible to the isolates bringing about 12 virulence factors are available. Nine of the isolates were not virulent to any of the 12 differential cultivars. Seventy three new virulent which had not been found in Kenya were obtained. In addition more virulent races such as race 4045 and race 4049 that did not conform to any of the previously obtained races in Kenya or in any part of the world were obtained. There is no associaton between genetic diversity and race classification of the isolates. *Aloe vera* extract was the most effective at 30% concentration that could be used as an alternative antifungal fungicide.

6.2 Recommendations

1. More work should be done by pathologist to evaluate current amount of the *C. lindemuthianum* in the bean fields' plant residues so that to determine its contribution to the incidence and severity of anthracnose disease in W. Kenya.
2. There is need to expand the scope of this study to cover the whole country so as to know the diversity of the *C. lindemuthianum* in Kenya because of high seed exchange rate within bean farmers in or outside the country.
3. Further work should be done on the *Aloe vera* plant extract to investigate its durability as systemic fungicide in the plants for increased efficiency against *C. lindemuthianum*.

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APPENDINCES

Appendix l: Table showing altitude range, average mean temperature range, average means rainfall and agroecological zones of the 7 counties surveyed between 2008 and 2010.

S. N.	region	SEASON and YEAR	GPS site location	ALT.	AEZ	Ave. (mm) range mean rainfall	Mean temp.	SEED CODE	ISOLATE CODE	ISOLATE race no.
1	Bungoma	SR 2008	0° 34' 0" N, 34° 34' 0" E	1200-2000	UM1	1200-1800	30-15	B1	Clb1	0
2	Bungoma	SR 2008	0° 34' 0" N, 34° 34' 0" E	1200-2000	LM3	1200-1800	30-15	B2	Clb2	0
3	Bungoma	SR 2008	0° 34' 0" N, 34° 34' 0" E	1200-2000	LM2	1200-1800	30-15	B3		
4	Bungoma	SR 2008	0° 34' 0" N, 34° 34' 0" E	1200-2000	UM2	1200-1800	30-15	B4		
5	Bungoma	SR 2008	0° 34' 0" N, 34° 34' 0" E	1200-2000	LM3	1200-1800	30-15	B5	Clb3	4
6	Bungoma	SR 2008	0° 34' 0" N, 34° 34' 0" E	1200-2000	UM2	1200-1800	30-15	B6	Clb4	8
7	Bungoma	SR 2008	0° 34' 0" N, 34° 34' 0" E	1200-2000	UM1	1200-1800	30-15	B7		
1	Bungoma	LR 2009	0° 34' 0" N, 34° 34' 0" E	1200-2000	LM3	1200-1800	30-15	B8		
2	Bungoma	LR 2009	0° 34' 0" N, 34° 34' 0" E	1200-2000	LM2	1200-1800	30-15	B9		
3	Bungoma	LR 2009	0° 34' 0" N, 34° 34' 0" E	1200-2000	UM2	1200-1800	30-15	B10		
4	Bungoma	LR 2009	0° 34' 0" N, 34° 34' 0" E	1200-2000	LM3	1200-1800	30-15	B11	Clb5	544
5	Bungoma	LR 2009	0° 34' 0" N, 34° 34' 0" E	1200-2000	UM2	1200-1800	30-15	B12	Clb6	548
6	Bungoma	LR 2009	0° 34' 0" N, 34° 34' 0" E	1200-2000	UM1	1200-1800	30-15	B13		
7	Bungoma	LR 2009	0° 34' 0" N, 34° 34' 0" E	1200-2000	LM3	1200-1800	30-15	B14	Clb7	3550
1	Bungoma	SR 2009	0° 34' 0" N, 34° 34' 0" E	1200-2000	UM1	1200-1800	30-15	B15		
2	Bungoma	SR 2009	0° 34' 0" N, 34° 34' 0" E	1200-2000	LM3	1200-1800	30-15	B16		
3	Bungoma	SR 2009	0° 34' 0" N, 34° 34' 0" E	1200-2000	LM2	1200-1800	30-15	B17	Clb8	0
4	Bungoma	SR 2009	0° 34' 0" N, 34° 34' 0" E	1200-2000	UM2	1200-1800	30-15	B18	Clb9	4
5	Bungoma	SR 2009	0° 34' 0" N, 34° 34' 0" E	1200-2000	LM3	1200-1800	30-15	B19		
6	Bungoma	SR 2009	0° 34' 0" N, 34° 34' 0" E	1200-2000	UM2	1200-1800	30-15	B20		
7	Bungoma	SR 2009	0° 34' 0" N, 34° 34' 0" E	1200-2000	UM1	1200-1800	30-15	B21	Clb10	16
1	Bungoma	LR 2010	0° 34' 0" N, 34° 34' 0" E	1200-2000	LM3	1200-1800	30-15	B22	Clb11	64
2	Bungoma	LR 2010	0° 34' 0" N, 34° 34' 0" E	1200-2000	LM2	1200-1800	30-15	B23	clb12	80
3	Bungoma	LR 2010	0° 34' 0" N, 34° 34' 0" E	1200-2000	UM2	1200-1800	30-15	B24	Clb13	130
4	Bungoma	LR 2010	0° 34' 0" N, 34° 34' 0" E	1200-2000	LM3	1200-1800	30-15	B25		
5	Bungoma	LR 2010	0° 34' 0" N, 34° 34' 0" E	1200-2000	UM2	1200-1800	30-15	B26		
6	Bungoma	LR 2010	0° 34' 0" N, 34° 34' 0" E	1200-2000	UM1	1200-1800	30-15	B27	Clb14	1463
7	Bungoma	LR 2010	0° 34' 0" N, 34° 34' 0" E	1200-2000	LM3	1200-1800	30-15	B28		
1	Kakamega	SR 2008	0° 17' 1" N, 34° 44' 58" E	1250-1750	UM4	750-1800	28-15	K1	Clk1	0
2	Kakamega	SR 2008	0° 17' 1" N, 34° 44' 58" E	1250-1750	LM2	750-1800	28-15	K2		
3	Kakamega	SR 2008	0° 17' 1" N, 34° 44' 58" E	1250-1750	LM1	750-1800	28-15	K3		

4	Kakamega	SR 2008	0° 17' 1" N, 34° 44' 58" E	1250-1750	LM1	750-1800	28-15	K4	Clk2	48
5	Kakamega	SR 2008	0° 17' 1" N, 34° 44' 58" E	1250-1750	UM1	750-1800	28-15	K5		
6	Kakamega	SR 2008	0° 17' 1" N, 34° 44' 58" E	1250-1750	UM1	750-1800	28-15	K6		
7	Kakamega	SR 2008	0° 17' 1" N, 34° 44' 58" E	1250-1750	UM1	750-1800	28-15	K7		
1	Kakamega	LR 2009	0° 17' 1" N, 34° 44' 58" E	1250-1750	UM4	750-1800	28-15	K8		
2	Kakamega	LR 2009	0° 17' 1" N, 34° 44' 58" E	1250-1750	LM2	750-1800	28-15	K9	Clk3	0
3	Kakamega	LR 2009	0° 17' 1" N, 34° 44' 58" E	1250-1750	LM1	750-1800	28-15	K10	Clk4	0
4	Kakamega	LR 2009	0° 17' 1" N, 34° 44' 58" E	1250-1750	LM1	750-1800	28-15	K11		
5	Kakamega	LR 2009	0° 17' 1" N, 34° 44' 58" E	1250-1750	UM1	750-1800	28-15	K12		
6	Kakamega	LR 2009	0° 17' 1" N, 34° 44' 58" E	1250-1750	UM1	750-1800	28-15	K13		
7	Kakamega	LR 2009	0° 17' 1" N, 34° 44' 58" E	1250-1750	UM1	750-1800	28-15	K14		
1	Kakamega	SR 2009	0° 17' 1" N, 34° 44' 58" E	1250-1750	UM4	750-1800	28-15	K15		
2	Kakamega	SR 2009	0° 17' 1" N, 34° 44' 58" E	1250-1750	LM2	750-1800	28-15	K16		
3	Kakamega	SR 2009	0° 17' 1" N, 34° 44' 58" E	1250-1750	LM1	750-1800	28-15	K17		
4	Kakamega	SR 2009	0° 17' 1" N, 34° 44' 58" E	1250-1750	LM1	750-1800	28-15	K18		
5	Kakamega	SR 2009	0° 17' 1" N, 34° 44' 58" E	1250-1750	UM1	750-1800	28-15	K19	Clk5	64
6	Kakamega	SR 2009	0° 17' 1" N, 34° 44' 58" E	1250-1750	UM1	750-1800	28-15	K20		
7	Kakamega	SR 2009	0° 17' 1" N, 34° 44' 58" E	1250-1750	UM1	750-1800	28-15	K21		
1	Kakamega	LR 2010	0° 17' 1" N, 34° 44' 58" E	1250-1750	UM4	750-1800	28-15	K22		
2	Kakamega	LR 2010	0° 17' 1" N, 34° 44' 58" E	1250-1750	LM2	750-1800	28-15	K23		
3	Kakamega	LR 2010	0° 17' 1" N, 34° 44' 58" E	1250-1750	LM1	750-1800	28-15	K24		
4	Kakamega	LR 2010	0° 17' 1" N, 34° 44' 58" E	1250-1750	LM1	750-1800	28-15	K25		
5	Kakamega	LR 2010	0° 17' 1" N, 34° 44' 58" E	1250-1750	UM1	750-1800	28-15	K26		
6	Kakamega	LR 2010	0° 17' 1" N, 34° 44' 58" E	1250-1750	UM1	750-1800	28-15	K27	Clk6	96
7	Kakamega	LR 2010	0° 17' 1" N, 34° 44' 58" E	1250-1750	UM1	750-1800	28-15	K28	Clk7	1028
1	Kitale	SR 2008	1° 1' 0" N, 35° 0' 0" E	1296-2100	UM4	1300-1850	28-11	T1	Clt1	0
2	Kitale	SR 2008	1° 1' 0" N, 35° 0' 0" E	1296-2100	UM4	1300-1850	28-11	T2	clt2	8
3	Kitale	SR 2008	1° 1' 0" N, 35° 0' 0" E	1296-2100	UM4	1300-1850	28-11	T3	Clt3	8
4	Kitale	SR 2008	1° 1' 0" N, 35° 0' 0" E	1296-2100	UM3	1300-1850	28-11	T4		
5	Kitale	SR 2008	1° 1' 0" N, 35° 0' 0" E	1296-2100	UM3	1300-1850	28-11	T5		
6	Kitale	SR 2008	1° 1' 0" N, 35° 0' 0" E	1296-2100	LH3	1300-1850	28-11	T6	Clt4	68
7	Kitale	SR 2008	1° 1' 0" N, 35° 0' 0" E	1296-2100	LH3	1300-1850	28-11	T7	Clt5	324
1	Kitale	LR 2009	1° 1' 0" N, 35° 0' 0" E	1296-2100	UM4	1300-1850	28-11	T8		
2	Kitale	LR 2009	1° 1' 0" N, 35° 0' 0" E	1296-2100	UM4	1300-1850	28-11	T9		
3	Kitale	LR 2009	1° 1' 0" N, 35° 0' 0" E	1296-2100	UM4	1300-1850	28-11	T10		
4	Kitale	LR 2009	1° 1' 0" N, 35° 0' 0" E	1296-2100	UM3	1300-1850	28-11	T11	Clt6	776
5	Kitale	LR 2009	1° 1' 0" N, 35° 0' 0" E	1296-2100	UM3	1300-1850	28-11	T12	Clt7	
6	Kitale	LR 2009	1° 1' 0" N, 35° 0' 0" E	1296-2100	LH3	1300-1850	28-11	T13	Clt8	
7	Kitale	LR 2009	1° 1' 0" N, 35° 0' 0" E	1296-2100	LH3	1300-1850	28-11	T14	Clt9	2816

1	Kitale	SR 2009	1° 1' 0" N, 35° 0' 0" E	1296-2100	UM1	1300-1850	28-11	T15	clt10	3216
2	Kitale	SR 2009	1° 1' 0" N, 35° 0' 0" E	1296-2100	UM4	1300-1850	28-11	T16		
3	Kitale	SR 2009	1° 1' 0" N, 35° 0' 0" E	1296-2100	UM4	1300-1850	28-11	T17		
4	Kitale	SR 2009	1° 1' 0" N, 35° 0' 0" E	1296-2100	UM4	1300-1850	28-11	T18		
5	Kitale	SR 2009	1° 1' 0" N, 35° 0' 0" E	1296-2100	UM3	1300-1850	28-11	T19	Clt11	18
6	Kitale	SR 2009	1° 1' 0" N, 35° 0' 0" E	1296-2100	UM3	1300-1850	28-11	T20	Clt12	21
7	Kitale	SR 2009	1° 1' 0" N, 35° 0' 0" E	1296-2100	LH3	1300-1850	28-11	T21		
1	Kitale	LR 2010	1° 1' 0" N, 35° 0' 0" E	1296-2100	LH3	1300-1850	28-11	T22		
2	Kitale	LR 2010	1° 1' 0" N, 35° 0' 0" E	1296-2100	UM4	1300-1850	28-11	T23	Clt13	379
3	Kitale	LR 2010	1° 1' 0" N, 35° 0' 0" E	1296-2100	UM4	1300-1850	28-11	T24	clt14	608
4	Kitale	LR 2010	1° 1' 0" N, 35° 0' 0" E	1296-2100	UM4	1300-1850	28-11	T25	clt15	622
5	Kitale	LR 2010	1° 1' 0" N, 35° 0' 0" E	1296-2100	UM3	1300-1850	28-11	T26		
6	Kitale	LR 2010	1° 1' 0" N, 35° 0' 0" E	1296-2100	UM3	1300-1850	28-11	T27	Clt16	1110
7	Kitale	LR 2010	1° 1' 0" N, 35° 0' 0" E	1296-2100	LH3	1300-1850	28-11	T28	Clt17	1280
1	Busia	SR 2008	0° 28' 0" N, 34° 6' 0" E	1200-1700	LM2	750-1800	22-18	S1	cls1	0
2	Busia	SR 2008	0° 28' 0" N, 34° 6' 0" E	1200-1700	LM2	750-1800	22-18	S2	Cls2	22
3	Busia	SR 2008	0° 28' 0" N, 34° 6' 0" E	1200-1700	LM3	750-1800	22-18	S3	Cls3	36
4	Busia	SR 2008	0° 28' 0" N, 34° 6' 0" E	1200-1700	LM3	750-1800	22-18	S4	Cls4	38
5	Busia	SR 2008	0° 28' 0" N, 34° 6' 0" E	1200-1700	LM3	750-1800	22-18	S5	Cls5	38
6	Busia	SR 2008	0° 28' 0" N, 34° 6' 0" E	1200-1700	LM2	750-1800	22-18	S6	Cls6	46
7	Busia	SR 2008	0° 28' 0" N, 34° 6' 0" E	1200-1700	LM2	750-1800	22-18	S7	Cls7	64
8	Busia	SR 2008	0° 28' 0" N, 34° 6' 0" E	1200-1700	LM1	750-1800	22-18	S8	Cls8	70
9	Busia	SR 2008	0° 28' 0" N, 34° 6' 0" E	1200-1700	LM1	750-1800	22-18	S9	Cls9	70
1	Busia	LR 2009	0° 28' 0" N, 34° 6' 0" E	1200-1700	LM2	750-1800	22-18	S10	Cls10	70
2	Busia	LR 2009	0° 28' 0" N, 34° 6' 0" E	1200-1700	LM2	750-1800	22-18	S11	cls11	70
3	Busia	LR 2009	0° 28' 0" N, 34° 6' 0" E	1200-1700	LM3	750-1800	22-18	S12	Cls12	92
4	Busia	LR 2009	0° 28' 0" N, 34° 6' 0" E	1200-1700	LM3	750-1800	22-18	S13	cls13	94
5	Busia	LR 2009	0° 28' 0" N, 34° 6' 0" E	1200-1700	LM3	750-1800	22-18	S14	Cls14	98
6	Busia	LR 2009	0° 28' 0" N, 34° 6' 0" E	1200-1700	LM2	750-1800	22-18	S15	cls15	162
7	Busia	LR 2009	0° 28' 0" N, 34° 6' 0" E	1200-1700	LM2	750-1800	22-18	S16	Cls16	192
8	Busia	LR 2009	0° 28' 0" N, 34° 6' 0" E	1200-1700	LM1	750-1800	22-18	S17	Cls17	198
9	Busia	LR 2009	0° 28' 0" N, 34° 6' 0" E	1200-1700	LM1	750-1800	22-18	S18	Cls18	200
10	Busia	LR2009	0° 28' 0" N, 34° 6' 0" E	1200-1700	LM1	750-1800	22-18	S19	Cls19	110
1	Busia	SR 2009	0° 28' 0" N, 34° 6' 0" E	1200-1700	LM2	750-1800	22-18	S20	Cls20	320
2	Busia	SR 2009	0° 28' 0" N, 34° 6' 0" E	1200-1700	LM2	750-1800	22-18	S21	Cls21	448
3	Busia	SR 2009	0° 28' 0" N, 34° 6' 0" E	1200-1700	LM3	750-1800	22-18	S22	Cls22	448
4	Busia	SR 2009	0° 28' 0" N, 34° 6' 0" E	1200-1700	LM3	750-1800	22-18	S23	Cls23	448
5	Busia	SR 2009	0° 28' 0" N, 34° 6' 0" E	1200-1700	LM3	750-1800	22-18	S24	cls24	449
6	Busia	SR 2009	0° 28' 0" N, 34° 6' 0" E	1200-1700	LM2	750-1800	22-18	S25	cls25	456

7	Busia	SR 2009	0° 28' 0" N, 34° 6' 0" E	1200-1700	LM2	750-1800	22-18	S26	cls26	456
8	Busia	SR 2009	0° 28' 0" N, 34° 6' 0" E	1200-1700	LM1	750-1800	22-18	S27	Cls27	456
9	Busia	SR 2009	0° 28' 0" N, 34° 6' 0" E	1200-1700	LM1	750-1800	22-18	S28	Cls28	544
1	Busia	LR 2010	0° 28' 0" N, 34° 6' 0" E	1200-1700	LM2	750-1800	22-18	S29	cls29	550
2	Busia	LR 2010	0° 28' 0" N, 34° 6' 0" E	1200-1700	LM2	750-1800	22-18	S30	Cls30	550
3	Busia	LR 2010	0° 28' 0" N, 34° 6' 0" E	1200-1700	LM3	750-1800	22-18	S31	Cls31	1189
4	Busia	LR 2010	0° 28' 0" N, 34° 6' 0" E	1200-1700	LM3	750-1800	22-18	S32	Cls32	2054
5	Busia	LR 2010	0° 28' 0" N, 34° 6' 0" E	1200-1700	LM3	750-1800	22-18	S33	Cls33	2504
6	Busia	LR 2010	0° 28' 0" N, 34° 6' 0" E	1200-1700	LM2	750-1800	22-18	S34	ClS34	3073
7	Busia	LR 2010	0° 28' 0" N, 34° 6' 0" E	1200-1700	LM2	750-1800	22-18	S35	Cls35	3529
1	Siaya	SR 2008	0° 33' 36" N, 34° 17' 10" E	1200-1700	LM1	750-1800	22-20	Y1	Cly1	0
2	Siaya	SR 2008	0° 33' 36" N, 34° 17' 10" E	1200-1700	LM2	750-1800	22-20	Y2	Cly2	4
3	Siaya	SR 2008	0° 33' 36" N, 34° 17' 10" E	1200-1700	LM1	750-1800	22-20	Y3		
4	Siaya	SR 2008	0° 33' 36" N, 34° 17' 10" E	1200-1700	LM2	750-1800	22-20	Y4	Cly3	8
5	Siaya	SR 2008	0° 33' 36" N, 34° 17' 10" E	1200-1700	LM1	750-1800	22-20	Y5	Cly4	38
6	Siaya	SR 2008	0° 33' 36" N, 34° 17' 10" E	1200-1700	LM1	750-1800	22-20	Y6	Cly5	38
7	Siaya	SR 2008	0° 33' 36" N, 34° 17' 10" E	1200-1700	LM1	750-1800	22-20	Y7	Cly6	38
1	Siaya	LR 2009	0° 33' 36" N, 34° 17' 10" E	1200-1700	LM1	750-1800	22-20	Y8	Cly7	46
2	Siaya	LR 2009	0° 33' 36" N, 34° 17' 10" E	1200-1700	LM2	750-1800	22-20	Y9	Cly8	70
3	Siaya	LR 2009	0° 33' 36" N, 34° 17' 10" E	1200-1700	LM1	750-1800	22-20	Y10	Cly9	86
4	Siaya	LR 2009	0° 33' 36" N, 34° 17' 10" E	1200-1700	LM2	750-1800	22-20	Y11	Cly10	134
5	Siaya	LR 2009	0° 33' 36" N, 34° 17' 10" E	1200-1700	LM1	750-1800	22-20	Y12	cly11	134
6	Siaya	LR 2009	0° 33' 36" N, 34° 17' 10" E	1200-1700	LM1	750-1800	22-20	Y13	Cly12	200
7	Siaya	LR 2009	0° 33' 36" N, 34° 17' 10" E	1200-1700	LM1	750-1800	22-20	Y14	cly13	200
1	Siaya	SR 2009	0° 33' 36" N, 34° 17' 10" E	1200-1700	LM1	750-1800	22-20	Y15	Cly14	256
2	Siaya	SR 2009	0° 33' 36" N, 34° 17' 10" E	1200-1700	LM2	750-1800	22-20	Y16		
3	Siaya	SR 2009	0° 33' 36" N, 34° 17' 10" E	1200-1700	LM1	750-1800	22-20	Y17	cly15	256
4	Siaya	SR 2009	0° 33' 36" N, 34° 17' 10" E	1200-1700	LM2	750-1800	22-20	Y18	Cly16	448
5	Siaya	SR 2009	0° 33' 36" N, 34° 17' 10" E	1200-1700	LM1	750-1800	22-20	Y19	Cly18	456
6	Siaya	SR 2009	0° 33' 36" N, 34° 17' 10" E	1200-1700	LM1	750-1800	22-20	Y20	Cly19	456
7	Siaya	SR 2009	0° 33' 36" N, 34° 17' 10" E	1200-1700	LM1	750-1800	22-20	Y21	cly20	531
1	Siaya	LR 2010	0° 33' 36" N, 34° 17' 10" E	1200-1700	LM1	750-1800	22-20	Y22	Cly21	558
2	Siaya	LR 2010	0° 33' 36" N, 34° 17' 10" E	1200-1700	LM2	750-1800	22-20	Y23	Cly22	648
3	Siaya	LR 2010	0° 33' 36" N, 34° 17' 10" E	1200-1700	LM1	750-1800	22-20	Y24	cl723	968
4	Siaya	LR 2010	0° 33' 36" N, 34° 17' 10" E	1200-1700	LM2	750-1800	22-20	Y25	Cly24	2124
5	Siaya	LR 2010	0° 33' 36" N, 34° 17' 10" E	1200-1700	LM1	750-1800	22-20	Y26	Cly25	2540
6	Siaya	LR 2010	0° 33' 36" N, 34° 17' 10" E	1200-1700	LM1	750-1800	22-20	Y27	Cly26	3080
7	Siaya	LR 2010	0° 33' 36" N, 34° 17' 10" E	1200-1700	LM1	750-1800	22-20	Y28	Cly27	3136
1	Nandi	SR 2008	0° 70' 0" N, 35° 11' 0" E	1883-2093	UM4	1300-1850	18-10	N1	Cln1	3

2	Nandi	SR 2008	0° 70' 0" N, 35° 11' 0" E	1883-2093	UM4	1300-1850	18-10	N2	Cln2	4
3	Nandi	SR 2008	0° 70' 0" N, 35° 11' 0" E	1883-2093	LH3	1300-1850	18-10	N3		
4	Nandi	SR 2008	0° 70' 0" N, 35° 11' 0" E	1883-2093	LH3	1300-1850	18-10	N4		
5	Nandi	SR 2008	0° 70' 0" N, 35° 11' 0" E	1883-2093	LH3	1300-1850	18-10	N5	Cln3	16
6	Nandi	SR 2008	0° 70' 0" N, 35° 11' 0" E	1883-2093	LH3	1300-1850	18-10	N6		
7	Nandi	SR 2008	0° 70' 0" N, 35° 11' 0" E	1883-2093	LH3	1300-1850	18-10	N7		
1	Nandi	LR 2009	0° 70' 0" N, 35° 11' 0" E	1883-2093	UM4	1300-1850	18-10	N8	cln4	96
2	Nandi	LR 2009	0° 70' 0" N, 35° 11' 0" E	1883-2093	UM4	1300-1850	18-10	N9	Cln5	104
3	Nandi	LR 2009	0° 70' 0" N, 35° 11' 0" E	1883-2093	LH3	1300-1850	18-10	N10	Cln6	267
4	Nandi	LR 2009	0° 70' 0" N, 35° 11' 0" E	1883-2093	LH3	1300-1850	18-10	N11	Cln7	284
5	Nandi	LR 2009	0° 70' 0" N, 35° 11' 0" E	1883-2093	LH3	1300-1850	18-10	N12		
6	Nandi	LR 2009	0° 70' 0" N, 35° 11' 0" E	1883-2093	LH3	1300-1850	18-10	N13		
7	Nandi	LR 2009	0° 70' 0" N, 35° 11' 0" E	1883-2093	LH3	1300-1850	18-10	N14		
1	Nandi	SR 2009	0° 70' 0" N, 35° 11' 0" E	1883-2093	UM4	1300-1850	18-10	N15		
2	Nandi	SR 2009	0° 70' 0" N, 35° 11' 0" E	1883-2093	UM4	1300-1850	18-10	N16		
3	Nandi	SR 2009	0° 70' 0" N, 35° 11' 0" E	1883-2093	LH3	1300-1850	18-10	N17	Cln8	14
4	Nandi	SR 2009	0° 70' 0" N, 35° 11' 0" E	1883-2093	LH3	1300-1850	18-10	N18	Cln9	14
5	Nandi	SR 2009	0° 70' 0" N, 35° 11' 0" E	1883-2093	LH3	1300-1850	18-10	N19		
6	Nandi	SR 2009	0° 70' 0" N, 35° 11' 0" E	1883-2093	LH3	1300-1850	18-10	N20	cln10	16
7	Nandi	SR 2009	0° 70' 0" N, 35° 11' 0" E	1883-2093	LH3	1300-1850	18-10	N21	Cln11	80
1	Nandi	LR 2010	0° 70' 0" N, 35° 11' 0" E	1883-2093	UM4	1300-1850	18-10	N22		
2	Nandi	LR 2010	0° 70' 0" N, 35° 11' 0" E	1883-2093	UM4	1300-1850	18-10	N23		
3	Nandi	LR 2010	0° 70' 0" N, 35° 11' 0" E	1883-2093	LH3	1300-1850	18-10	N24		
4	Nandi	LR 2010	0° 70' 0" N, 35° 11' 0" E	1883-2093	LH3	1300-1850	18-10	N25		
5	Nandi	LR 2010	0° 70' 0" N, 35° 11' 0" E	1883-2093	LH3	1300-1850	18-10	N26	cln12	358
6	Nandi	LR 2010	0° 70' 0" N, 35° 11' 0" E	1883-2093	LH3	1300-1850	18-10	N27	Cln13	548
7	Nandi	LR 2010	0° 70' 0" N, 35° 11' 0" E	1883-2093	LH3	1300-1850	18-10	N28	cln14	3447
1	Kisii	SR 2008	0° 41' 0" S, 34° 46' 0" E	1582-1899	UM1	1400-2100	18-15	L1		
2	Kisii	SR 2008	0° 41' 0" S, 34° 46' 0" E	1582-1899	UM1	1400-2100	18-15	L2		
3	Kisii	SR 2008	0° 41' 0" S, 34° 46' 0" E	1582-1899	UM1	1400-2100	18-15	L3	Cli1	38
4	Kisii	SR 2008	0° 41' 0" S, 34° 46' 0" E	1582-1899	UM1	1400-2100	18-15	L4		
5	Kisii	SR 2008	0° 41' 0" S, 34° 46' 0" E	1582-1899	LH2	1400-2100	18-15	L5		
6	Kisii	SR 2008	0° 41' 0" S, 34° 46' 0" E	1582-1899	LH2	1400-2100	18-15	L6	Cli2	134
7	Kisii	SR 2008	0° 41' 0" S, 34° 46' 0" E	1582-1899	LH3	1400-2100	18-15	L7		
1	Kisii	LR 2009	0° 41' 0" S, 34° 46' 0" E	1582-1899	UM1	1400-2100	18-15	L8	Cli3	550
2	Kisii	LR 2009	0° 41' 0" S, 34° 46' 0" E	1582-1899	UM1	1400-2100	18-15	L9		
3	Kisii	LR 2009	0° 41' 0" S, 34° 46' 0" E	1582-1899	UM1	1400-2100	18-15	L10		
4	Kisii	LR 2009	0° 41' 0" S, 34° 46' 0" E	1582-1899	UM1	1400-2100	18-15	L11		
5	Kisii	LR 2009	0° 41' 0" S, 34° 46' 0" E	1582-1899	LH2	1400-2100	18-15	L12		

6	Kisii	LR 2009	0° 41' 0" S, 34° 46' 0" E	1582-1899	LH2	1400-2100	18-15	L13	Cli4	72
7	Kisii	LR 2009	0° 41' 0" S, 34° 46' 0" E	1582-1899	LH3	1400-2100	18-15	L14	Cli5	102
1	Kisii	SR 2009	0° 41' 0" S, 34° 46' 0" E	1582-1899	UM1	1400-2100	18-15	L15		
2	Kisii	SR 2009	0° 41' 0" S, 34° 46' 0" E	1582-1899	UM1	1400-2100	18-15	L16	cli6	256
3	Kisii	SR 2009	0° 41' 0" S, 34° 46' 0" E	1582-1899	UM1	1400-2100	18-15	L17	Cli7	258
4	Kisii	SR 2009	0° 41' 0" S, 34° 46' 0" E	1582-1899	UM1	1400-2100	18-15	L18		
5	Kisii	SR 2009	0° 41' 0" S, 34° 46' 0" E	1582-1899	LH2	1400-2100	18-15	L19	Cli8	4045
6	Kisii	SR 2009	0° 41' 0" S, 34° 46' 0" E	1582-1899	LH2	1400-2100	18-15	L20	cli9	6
7	Kisii	SR 2009	0° 41' 0" S, 34° 46' 0" E	1582-1899	LH3	1400-2100	18-15	L21	Cli10	36
1	Kisii	LR 2010	0° 41' 0" S, 34° 46' 0" E	1582-1899	UM1	1400-2100	18-15	L22		
2	Kisii	LR 2010	0° 41' 0" S, 34° 46' 0" E	1582-1899	UM1	1400-2100	18-15	L23		
3	Kisii	LR 2010	0° 41' 0" S, 34° 46' 0" E	1582-1899	UM1	1400-2100	18-15	L24		
4	Kisii	LR 2010	0° 41' 0" S, 34° 46' 0" E	1582-1899	UM1	1400-2100	18-15	L25		
5	Kisii	LR 2010	0° 41' 0" S, 34° 46' 0" E	1582-1899	LH2	1400-2100	18-15	L26		
6	Kisii	LR 2010	0° 41' 0" S, 34° 46' 0" E	1582-1899	LH2	1400-2100	18-15	L27	cli11	4049
7	Kisii	LR 2010	0° 41' 0" S, 34° 46' 0" E	1582-1899	LH3	1400-2100	18-15	L28		

Appendix ll. Treatment Randomization and layout (Complete Randomized Design) for the laboratory blotter test on bean seed anthracnose incidence study.

C1S1V1	C1S1V2	C7S2V1	C2S2V5	C5S1V5	C4S1V4
C2S2V2	C0S1V1	C4S2V4	C3S1V1	C5S2V1	C1S2V4
C5S2V3	C3S1V2	C5S1V3	C6S1V1	C0S1V4	C6S2V1
C3S2V3	C7S2V2	C2S2V4	C4S2V5	C3S1V3	C5S1V4
C4S1V3	C5S2V2	C6S2V3	C1S1V6	C4S2V3	C1S2V2
C1S2V3	C2S2V3	C4S1V2	C2S2V6	C6S2V4	C2S2V1
C1S1V7	C2S2V4	C3S2V5	C4S2V2	C5S2V4	C7S2V3
C6S2V5	C6S1V2	C7S2V4	C5S1V7	C1S2V3	C1S1V5
C5S1V1	C4S2V6	C4S1V7	C0S2V5	C0S1V3	C2S1V4
C1S2V1	C5S1V6	C3S1V6	C6SIV7	C3S2V6	C6S1V3
C2S1V7	C7S1V6	C7S1V7	C0S1V7	C4S1V5	C1S2V7
C7S1V2	C0S2V6	C3S2V6	C2S2V5	C5S1V2	C1S1V3
C3S1V7	C1S1V4	C2S2V7	C0S2V4	C3S1V4	C3S2V7
C0S1V2	C6S1V4	C6S2V6	C3S2V2	C4S2V7	C5S2V7
C1S1V8	C2S1V2	C4S1V8	C7S2V7	C1S2V4	C0S2V8
C3S1V8	C4S1V6	C5S1V6	C0S1V5	C4S2V1	C0S2V7
C5S2V6	C7S2V5	C1S2V1	C6S1V5	C7S1V4	C7S1V8
C2S1V6	C4S1V1	C6S2V7	C5S1V8	C2S1V1	C1S2V8
C2S2V8	C0S2V3	C2S1V3	C0S1V6	C6S1V8	C1S2V6
C2S2V1	C1S2V5	C3S1V5	C5S2V2	C1S1V2	C4S1V6
C2S1V8	C7S1V5	C3S2V8	C4S2V8	C0S2V2	C5S2V8
C7S2V8	C1S1V6				

Appendix lll: Means of anthracnose incidence (%) in seed varieties collected from W. Kenya.

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
variety	7	41376.1	5910.9	12.17	<.001
Error	888	431133.3	485.5		
Total	895	472509.3			

Appendix IV: Means of anthracnose incidence (%) in W. Kenya regions.

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
COUNTY	6	88486.1	14747.7	34.14	<.001
Error	889	384023.2	432		
Total	895	472509.3			

Appendix V: Incidence of anthracnose (%) on the eight bean cultivars from various Western Kenya regions during the long and short seasons of Dec 2008 and Jul 2010.

Source of variation	D.F	S.S	M.S	F value	F pr.
VARIETY	7	22626.6	3232.4	16.64	<.001
COUNTY	6	50197.3	8366.2	43.06	<.001
VARIETY.COUNTY	42	85197.7	2028.5	10.44	<.001
Error	1960	380771.2	194.3		
Total	2015	538792.8			

Appendix VI: Means of seed germination (%) in seed varieties collected from W. Kenya.

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
VARIETY	7	37242.4	5320.3	19.76	<.001
Error	888	239107.1	269.3		
Total	895	276349.6			

Appendix VII: Means of seed germination (%) in seeds collected from W. Kenya.

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
COUNTY	6	88486.1	14747.7	34.14	<.001
Error	889	384023.2	432		
Total	895	472509.3			

Appendix VIII: Means of germination rate in seeds collected from W. Kenya.

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
COUNTY	6	2.7090	0.4515	0.63	0.705
Error	889	635.8014	0.7152		
Total	895	638.5104			

Appendix IX: Effect of different plant extracts and their concentrations on mycelia growth inhibition against *C. lindemuthianum*.

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
plant_extract	10	61556.36	6155.64	69.55	<.001
plant_extract	10	61556.36	6155.64	69.55	<.001
plant_extract.treatment	20	2281.79	114.09	1.29	0.218
Error	66	5841.24	88.5		
Total	98	70609.95			

Appendix X : Incidence of anthracnose (%) disease on the eight bean cultivars from Western Kenya during the long and short seasons between Dec 2008 and Jul 2010.

cultivar	Season 1 (long rains)								Season 2 (short rains)								G.m
	Region								Region								
	Bungoma	Kakamega	Trans Nzoia	Nandi	Busia	Kisii	Siaya	Mean	Bungoma	Kakamega	Trans Nzoia	Nandi	Busia	Bisii	Siaya	Mean	
GLP 2	30 a-l	43 a-p	27 a-k	38a-p	26a-j	5a	26a-j	28	30 a-l	53c-r	18a-d	38a-p	26a-j	8 ab	26 a-j	29	29
GLP 288	42a-p	40 a-p	33 a-m	36a-p	26a-j	18 a-d	26 a-j	33	43b-p	37a-p	63 i-r	54 c-r	33a-n	27a-k	26 a-j	41	34
BROWN	31a-m	59 g-r	48c-r	47d-r	39a-p	32a-m	21 a-f	40	25 a-i	60g-r	28a-k	47c-r	39a-p	47d-r	36a-p	40	44
WHITE	69 m-r	30 a-l	36a-p	49c-r	48c-r	24a-h	20a-d	39	56d-r	43b-p	60 g-r	33a-m	64j-r	37a-p	54 c-r	49	37
GLP 585	83 qr	58 e-r	43b-p	53c-r	64j-r	23a-g	35a-o	51	83 r	61h-r	44b-p	53c-r	73p-r	23a-g	48 c-r	55	53
YELLOW	32a-m	41 b-p	46c-r	45b-q	44b-p	26a-j	33a-n	49	63 j-r	49c-r	46c-r	45b-q	54c-r	49c-r	33a-m	48	44
X 92	64 k-r	83 qr	54 c-r	34a-o	60g-r	19a-d	21a-e	48	71n-r	71o-r	53 c-r	34 a-o	60f-r	23a-g	19a-d	47	47
GLP 1004	68 l-r	36a-p	58e-r	45b-q	49c-r	23a-g	29a-k	44	68l -r	33a-m	61h-r	45b-q	53c-r	28a-k	51c-r	43	45
MEAN	53	50	43	44	45	21	27		56	51	47	44	50	30	32		
G.mean	42.2																
SED	8.5																
CV%	40.0																