INCIDENCE AND DIVERSITY AMONG COFFEE LEAF RUST PATHOGEN ISOLATES (*Hemileia vastatrix*) FROM DIFFFERENT COFFEE GROWING COUNTIES OF KENYA

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DECLARATION AND APPROVAL

Declaration

I, Ligabo George Sore, declare that the work presented in this thesis, to the best of my knowledge and belief is original, except as acknowledged in the text. The material has not been submitted, or being considered for submission, either in whole or in part, for another degree at this or any other University.

Signed…….. Date…….

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Approval

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ABSTRACT

Coffee leaf rust (CLR) is a fungal disease caused by *Hemileia vastatrix*. The pathogen is constantly evolving leading to rapid break down in resistance of once resistant coffee varieties. The disease affects Arabica coffee causing premature leaf fall, yield loss hence economic losses. In Kenya, CLR is the second most important disease after Coffee Berry Disease causing losses of up to 10-40%. The study will provide additional knowledge about the pathogen variability and differentiation that will be used to investigate the pathogen evolution and to design strategies for developing new varieties. The objectives of the study were to evaluate the disease intensity, pathogenicity and genetic diversity of CLR pathogen from coffee growing counties of Kenya. Purposeful and directional sampling methods were used to select factories and farms for disease scoring. The factories were used as the entry points from which four farms were picked through directional sampling. In each county, 120 farm units were randomly sampled. A total of seventy mature leaves were picked randomly from the selected coffee tree for disease scoring. Samples for studying pathogenicity and genetic diversity were collected from single coffee trees. The leaf samples infected with CLR pathogen were inspected for any contamination with mycophagous arthropods using a microscope. The isolates were inoculated on coffee leaf disks from different coffee genotypes. Eskes scale of 1-5 was used to score the ability to infect. DNA was extracted from the CLR pathogen spores using Diniz protocol with minor modifications using Mixed AlkylTriMethylammonium bromide (MATAB). Genetic diversity was determined using RAPD primers. Data was analyzed using EXLSTAT software 2014. Analysis of variance indicated highly significant variation ($P<0.0001$) in disease intensity among coffee growing counties of Kenya. UM2 had the highest disease intensity across all coffee growing counties. Kisii County had the highest disease intensity while Trans Nzoia County had the lowest disease intensity. Analysis of variance further revealed highly significant variation ($P<0.0001$) in pathogenicity among isolates from coffee growing counties of Kenya. Isolate 8 from Meru County was the most pathogenic on all the coffee genotypes (Mean score of 2.961). Mundo Novo was the most susceptible coffee genotype on all the CLR pathogen isolates (mean score of 3.953). No isolate sporulated on Ruiru 11, Robusta and HDT. RAPD primers revealed high genetic diversity (58%) among CLR pathogen isolates from coffee growing counties of Kenya. The number of bands produced per primer ranged from 5-14. There was dissimilarity among isolates from the same county and isolates from across the counties. Primer X-16 showed high polymorphism in CLR isolates. There is a relationship between genetic diversity and pathogenicity of CLR pathogen since both vary depending on the coffee growing County. Racetyping should be done in order to ascertain the races present in this CLR pathogen isolates.

DEDICATION

To my parents, Mr. and Mrs. Ligabo.

Table of Contents

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF APPENDICES

LIST OF ACRONYMS AND ABBREVIATIONS

a.s.l	Above Sea Level
AEZs	Agro Ecological Zones
AFLP	Amplified Fragment Length Polymorphism
AHC	Agglomerative Hierarchical Clustering
ANOVA	Analysis of Variance
BBC	Bacterial Blight of Coffee
CIFC	Centro de Investigacao das Ferrugens do Cafeeiro (Coffee Rust Research Centre)
CLR	Coffee Leaf Rust
DNA	Deoxyribonucleic Acid
dNTPs	Deoxyribonucleotidetriphosphate
FAO	Food and Agricultural Organization
GDP	Gross Domestic Product
GPS	Global Positioning System
HDT	Hibrido de Timor
HMC	Haustorical Mother Cell
ICA	International Coffee Agreement

ICO	International Coffee Organization
MATAB	Mixed Alkyltrimethylammonium Bromide
MSP-PCR	Microsatelite-Primed Polymerase Chain Reaction
RAPDs	Random Amplified Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
rpm	Revolution per minute
SCARS	Sequence Characterized Amplified Regions
SH	Resistant genes
SSA	Sub Saharan Africa
SSR	Simple Sequence Repeats
T.E	Tris Ethylenediaminetetraacetic acid
TBE	Tris Boric Ethylenediaminetetraacetic acid
UM1	Upper Midland 1
UM2	Upper Midland 2
UM3	Upper Midland 3
USA	United States of America
UV	Ultra violet rays

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CHAPTER ONE

INTRODUCTION

1.1 Background information

The *Coffea* genus contains around 130 species and belongs to the family *Rubiceae* that has over 6000 species (Davis *et al*., 2006). *Coffea* species that are under commercial cultivation are Arabica coffee (*Coffea arabica* L.) that forms 80% of world trade and Robusta (*Coffea canephora* Pierre) forms the remainder. *Coffea liberica* and *Coffea excelsa* contribute less than 1% (Pearl *et al*., 2004). C*offea arabica* plant is self-pollinating, limiting the genetic variability of the species while the Robusta coffee plant is cross-pollinating. *Coffea arabica* is the only tetraploid of the genus *coffea* with (4x=44) (Omondi 1998). It therefore does not hybridize with other species without special measures taken. *Coffea canephora* is a larger tree, with larger leaves and the laminae between veins which are more convex than in Arabica. It is self-sterile and cross-pollinates easily. Coffee is a woody perennial evergreen dicotyledonous plant. They are shrubs or small trees native to subtropical Africa, from Congo basin to the highlands of Ethiopia and Southern Asia. Most *Coffea canephora* are resistant to CLR disease caused by *Hemileia vastatrix* (Omondi, 1998).

1.2 Global Production and Economic importance of coffee

Most of the world's coffee is produced in Brazil which has led in the production since 1840. The yearly average production is estimated to be 35.7 million bags. In Africa, Ethiopia is the highest producer with an annual average of 6.4 million bags (ICO 2014).

Export of coffee from Africa to The European market in 2008/09 was US $13.5 billion and in 2009/10 it was US $15.4 billion. In 2004, coffee was the top among agricultural exports in 12 countries. In 2005, it was the world largest agricultural export (FAO 2007).

In sub-Saharan Africa (SSA), coffee is the economic backbone of more than 20 countries and central to the livelihoods of more than 20 million rural families (Oduor and Simons 2003).

Coffee is the most important cash crop for Africa contributing some 10% of the total foreign exchange earnings (FAO, 2007). It is a major source of income for millions of smallholder coffee growers who are responsible for about 80% of coffee production in Africa (Oduor and Simons 2003).

1.3 Coffee Production in Kenya

In Kenya, coffee is grown in areas with altitudes between 1200 and 2100 meters above the sea level (a.s.l). Coffee is grown both under shade or unshaded system in the 3 agro ecological zones (UM1-UM3): Coffee tea zone (UM1), main coffee zone (UM2) and marginal coffee zone (UM3). UM1 is at an altitude of 1570-1810m a.s.l with annual mean temperature of 18.4^0 C and rainfall of 1640mm; UM2 lies between 1395-1675m a.s.l and has a mean annual temperature and rainfall of 19.4^0 C and 1465mm respectively. UM3 lies between altitude of 1330-1560M a.s.l with annual temperature of 19.9^0 C and rainfall of 1270 mm (Mugo, 2012).

Coffee is grown in three regions of the country namely: East of Rift Valley (comprising areas around Mt Kenya, the Aberdare ranges and Machakos), West of Rift Valley (comprising of Kisii highlands, Mt Elgon area and the North Rift valley) and Taita Hills

at the coast. Of the estimated 160, 000 hectares of land under coffee, the East of Rift Valley region accounts for about 82%, West of the Rift Valley for 17% and the Taita Hills for only 1 % (Kathurima *et al*., 2013).

The recommended coffee varieties in Kenya are: K7 for low altitude areas (with serious CLR pathogen), SL28 and SL34 for low to medium areas with good rainfall (Mwangi, 1983) and Ruiru 11 for all coffee growing areas as it is resistant to Coffee Berry Disease (CBD) and Coffee Leaf Rust pathogen (CLR) (Opile and Agwanda 1993). Coffee is produced by two distinct sectors, the plantations (estates) and the small-holder producers. There are about 500,000 smallholder farmers organized in 500 co-operatives and about 1,200 plantation producers (Economic Survey, Central Bureau of Statistics, 2006, Nairobi, Kenya).

1.4 Economic Importance of Coffee in Kenya

Agriculture is the main contributor to the economy in Kenya to the tune of 26% of the GDP (Economic survey, 2010). From the total exports in Kenya, Agriculture alone contributes around 65%. In rural areas, those who benefit directly from agriculture are approximately 70% of the population. Coffee plays a vital role in economic development of Kenya through foreign exchange, tax income and employment opportunities. It also promotes the development of industries like the fertilizer industries and the agro chemicals and those that process coffee berries. Coffee contributes about 11% of the total foreign earnings and about 10% of Kenyans are employed in the coffee sector (Mugo, 2012) .

According to the Economic survey (2007), the coffee industry contributes so much to the economy of Kenya. This is evidenced by the increase in the production of coffee by 10%. However, the production has been fluctuating especially in 2007/2008 fiscal year due to the post election crisis and poor weather conditions (Economic survey, 2007). Other factors that contributed to the low production include increase in the prices of the farm inputs.

According to Rakuman (2005), coffee is utilized as a beverage, medicine and also as a source of food for the dairy animals. Mburu (2004) associates the final quality of coffee with the various operations involved in its processing. Consequently, due to the good cup quality of Arabica coffee, it is used to blend Robusta. According to Kathurima *et al*., (2013), Arabica coffee is highly priced due to its good cup quality.

1.5 Factors Affecting Coffee Production

Globally, approximately 350 different diseases infect coffee (Mugo, 2012). The decline in production from130, 000 metric tones in 1988/89 to 50,000 metric tones today is due to several factors: Low international market prices due to the collapse of the price support mechanism under international coffee agreement (ICA) in 1989, high cost of farm inputs, unfavourable weather conditions, pests and diseases. Pests cause losses up to 15%, pathogen up to 13% and weeds up to 13%. The pests that attack coffee include arthropods, pathogenic microorganisms and weeds. In 1998 US $ 34 billion worldwide was spent by farmers on controlling insect pests and diseases (Mugo, 2011). Coffee berry disease is an anthracnose caused by *Colleotrichum kahawae* Waller and Bridge (Gichuru *et al*., 2008). CBD infects green immature berries causing up to 80% crop loss if not controlled (Gichimu, 2010). The fungus infects all stages of the crop from flowers to ripe

fruits and occasionally leaves, but maximum crop losses occurs following infection of green berries with the formation of dark sunken lesions with sporulation, causing their premature dropping and mummification (Silva and Várzea, 2006).

However, CLR caused by *Hemileia vastatrix* Berk. et Br. is the most devastating disease on *Coffea arabica* worldwide. In Kenya, it is the second most important disease after CBD, and breeding to obtain new resistant coffee varieties has been a priority (Gichuru *et al*., 2014). The CLR fungus is found in all the coffee-growing areas of the country, causing losses between 10% and 40% (Silva *et al*. 2006). The main damages caused by the disease are premature defoliation, resulting in a reduced leaf area and withered lateral branches, leading to a gradual debilitation of the infected plant (Matiello *et al*., 2002). The result is reduced yields (Guzzo *et al.* 2009). The majority of the Arabica coffee varieties are susceptible (Van der Vossen 2001) while a few of *Coffea canephora* may be affected.

1.6 Problem Statement

The majority of coffee farmers in Kenya plant traditional coffee varieties which are susceptible to CLR pathogen. The disease causes premature leaf fall hence huge economic loses. The continued interaction of the pathogen with resistant coffee varieties has resulted into formation of new races. Breeding for resistant coffee varieties to CLR pathogen has therefore become a challenge due to the constant evolution of the pathogen and the rapid breakdown of the once resistant coffee varieties. Hibrido de Timor (HDT) is used in the breeding program as a source for resistant genes. However, the resistance of its derivatives has been broken down by CLR pathogen.

1.7 Justification of the Study

Characterization of CLR pathogen and survey of its status in coffee growing areas will provide additional knowledge of the pathogen variability and differentiation that might be used to investigate pathogen evolution and to design strategies for disease management as well as for developing new varieties. In addition, the information will be very useful to the coffee growers on the anticipated changes in distribution of coffee diseases across the different coffee growing Ago- ecological zones (AEZ). Furthermore, understanding the evolutionary forces controlling pathogen populations will help in the development and implementation of effective and durable disease control. Moreover, knowledge about the genetic diversity and structure of pathogen population will help to better understand variations observed among isolates of *Hemileia vastatrix* and the implications for disease development and control.

1.8 Objectives

1.8.1 General Objective

To improve coffee yields in Kenya.

1.8.2 Specific objectives

i. To evaluate disease intensity of CLR pathogen in coffee growing counties of Kenya.

ii. To evaluate pathogenicity of CLR pathogen from coffee growing counties of Kenya.

iii. To determine genetic diversity of CLR pathogen from coffee growing counties of Kenya using RAPD primers.

1.8.3 Hypothesis

i. There is no significant variation in disease intensity of *Hemileia vastatrix* in Kenya

ii. There is no significant variation in pathogenicity of *Hemileia vastatrix* in Kenya

iii. There is no significant genetic diversity in *Hemileia vastatrix* in Kenya

CHAPTER TWO

LITERATURE REVIEW

2.1 Coffee Leaf Rust Pathogen

Hemileia vastatrix, the causal agent of CLR, produces the uredinal, telial, and basidial stages, but only the dycariotic urediospores are responsible for the disease. *Hemileia vastatrix* infects the lower surface of the leaves where it produces large, orange colonies of uredosori (Plates 1 and 2) leading to premature leaf fall and yield losses. CLR pathogen was recorded for the first time in 1861,near Lake Victoria, but it was in Sri-Lanka that it first caused great economic impact in 1868 (Holger Hindorf and Omondi 2011).

Plate 1: SL 28 variety infected with Coffee Leaf Rust Pathogen

(Source: Author, 2014)

Plate 2: CLR pathogen symptoms on the underside of a leaf of a susceptible coffee variety (SL28)

(Source: Author, 2014)

2.1.1 Fungal Infection Process

Coffee Leaf Rust Pathogen is a biotrophic fungus hence depends on plant living cells for growth and reproduction (Shulze-Lefert and Panstruga, 2003). The initiation of the dycariotic phase of *Hemileia vastatrix* on coffee leaves, as with other rust fungi (Mendgen and Voegele, 2005) involves specific events including appressorium formation over stomata and penetration by inter- and intracellular colonization (Silva *et al*., 2002). Thus, in susceptible coffee leaves, after urediospore germination and appressorium differentiation over stomata, the fungus penetrates forming a penetration hypha that grows into the substomatal chamber. This hypha produces at the advancing tip two thick lateral branches; each hypha and its branches resemble an anchor. Each lateral branch of

the anchor bears a hypha (haustorial mother cell – HMC), the subsidiary cells being the first invaded by haustoria, whose formation starts around 36h after inoculation. The fungus pursues its growth with formation of more intercellular hyphae, including HMCs, and a large number of haustoria in the cells of the spongy and the palisade parenchyma and even of the upper epidermis. A dense mycelium is observed below the penetration area and a uredosporic sorus protrudes like a "bouquet" through the stomata about 20 days after inoculation.

After adhesion of rust urediospores to the plant surface, the development of infection structures results from a sophisticated host-surface recognition system. The tip of the dicaryotic germ tube is able to follow topographical features of the plant cuticle and thus increase the probability

of encountering a stomatal opening (Mendgen and Voegele, 2005). Host specific features, like the dimension of the outer lip of stomatal guard cells serve as inductive signals, perhaps through synergistic interaction with chemicals such as leaf alcohols (Collins *et al*., 2001). To control further fungal development within the plant, a successive sequence of signals is also required (Mendgen and Voegele, 2005).

2.1.2 Fungal Variability

The earliest characterization of *Hemileia vastatrix* was carried out in India by Mayne (1932. He categorized the local rust samples into four physiologic races. No other studies were made on the physiological specialization of *Hemileia vastatrix* until D'Oliveira initiated a world survey of coffee rust races in 1952 in Portugal (D'Oliveira, 1965). The work carried out at the Coffee Rusts Research Center (CIFC) in Portugal enabled the characterization of about 45 rust races (Várzea *et al*., 2002).

Molecular studies to detect genetic diversity in *Hemileia vastatrix* were carried out by Nandris *et al*. (1998). The Random Amplified Polymorphic DNA (RAPDS) method used revealed polymorphism among the isolates. However, a linkage between the molecular markers obtained and the pathotypes used was not established. In recent studies at CIFC, using RAPD and MSP-PCR (Microsatellite-Primed Polymerase Chain Reaction), a considerable degree of variability among the populations studied were observed, although no clear relationship was obtained between host, geographical origin and physiologic races (Gouveia *et al*., 2005).

2.1.3 Durability of Resistance

According to Varzea and Marques (2005), some improved commercial varieties from HDT and other interspecific tetraploid hybrids, like Icatú are gradually losing their resistance to CLR pathogen in some countries, due to the appearance of new virulent races. However, according to Alvarado (2005), some coffee varieties maintain their resistance while others tolerate the disease. Some Arabica varieties like Rume Sudan and Tafarikella with low yields and classified at CIFC as belonging to the susceptible group E, have a very high partial resistance in the field (Várzea *et al.*, 2002).

2.1.4 Inheritance of Resistance

Studies on inheritance of CLR pathogen resistance at CIFC demonstrated the gene-for-gene theory and are applicable to coffee-rust interactions (Noronha-Wagner and Bettencourt 1967). The resistance in the coffee plants is conditioned by at least nine major dominant genes (SH1-SH9) that act singly or are associated. By the same theory, it was possible to infer 9 genes of virulence (v1-v9) in *Hemileia vastatrix*. (Bettencourt and

Rodrigues Jr. 1988). The genes SH1, SH2, SH4 and SH5 were found in pure Arabicas originating from Ethiopia; the gene SH3 in *Coffea liberica;* and genes SH6, SH7, SH8 and SH9 found exclusively in "Hibrido de Timor" or HDT (*Coffea arabica* x *Coffea canephora*) derivatives, therefore supposedly coming from the Robusta parent(s) of the hybrid (Bettencourt and Rodrigues Jr., 1988). Besides these SH genes, it is likely that other major and minor genes might also condition the coffee-rust interactions (Bettencourt and Rodrigues Jr., 1988). The coffee genotypes are classified into physiological groups which are distinguished from each other essentially by responses of either complete resistance or susceptibility (low and high infection type) to several rust races; Group A, characterized by resistance to all the known rust races, has been found in hybrids between *Coffea arabica* x *Coffea canephora*, either spontaneously as in the HDT or man-made as in Icatú (Marques and Bettencourt 1979). Genotypes in group A have also been found in *Coffea liberica*, *Coffea dewevrei*, *Coffea eugenioides*, *Coffea congensis*, etc. (D'Oliveira and Rodrigues Jr. 1961) while the E-group, characterized by susceptibility to almost all known races, includes the traditional Typica and Bourbon cultivars (Bettencourt and Rodrigues Jr 1988). Non-specific polygenic resistance has been assessed at CIFC and more extensively in other countries (Kushalappa and Eskes 1989), mainly under laboratory conditions using different parameters, such as latency period, percentage of sporulating lesions, and spore production per lesion. Holguin (1993) suggested the existence of this type of resistance in *Coffea canephora* and interspecific hybrids and also in some *Coffea arabica* genotypes, but the mode of the inheritance of this type of resistance remains unknown.

2.1.5 Epidemiology of Coffee Leaf Rust Pathogen

In nature, uredospores are disseminated long distances, largely by wind, and over short distances, by both wind and rain-splash. Outside agents such as animals, mainly insects and humans occasionally have been shown to be involved with dissemination. The movement of CLR pathogen from one continent to another has been attributed to wind currents and the transport of contaminated seeds and/or other plant material (Kushalappa and Eskes 1989) or by man. Uredospores can withstand low temperatures, but are particularly sensitive to desiccation. Because viable uredospores have been recovered from spore traps mounted on airplanes at altitudes up to 1000m in Brazil and Kenya, it is believed that continent to continent movement may have occurred by wind. Uredospores are released diurnally and are highest at noon or midday. In spore trapping studies, more spores were trapped at 1.25 m, decreasing with increasing heights above ground up to 10m.

Rain is also an important dispersal agent. It is difficult to assess the comparative importance of rain to wind dissemination, but because of high spore numbers in rain water collected within the canopy, windblown rain or rain splash is important for within tree and within orchard disease buildup.

Although uredospores are dispersed by insects, such as thrips, larva of flies, and wasps, their importance in epidemics is considered insignificant. Of the higher animals, man is by far the most important agent for short and long distance movement of the disease with plant material, seeds, seedlings, and uredospores of the pathogen (Cooke *et al*., 2006).

2.1.6 Biology of Coffee Leaf Rust Pathogen

For infection to be successful, free water is required and is usually derived from rain. Spores germinate in 2-4 hours under optimum conditions. After uredospores germinate through germ pores in the spore, appressoria are produced which in turn produces a vesicle from which entry into the substomatal cavity is gained. Within 24-48 hours, infection is completed. If free moisture is absent, exposure to high relative humidity is not sufficient to induce spore germination (Nutman, 1963). Loss of moisture after germination inhibits the whole infection process. Recovery does not occur even when adequate moisture is reintroduced (Kushalappa and Eskes, 1989). Spore germination is better on young leaves than intermediate and old leaves. As a consequence, disease spread and development is usually limited to the rainy season, and CLR disease incidence is very low during dry periods. The incubation period or time between infection and lesion development is approximately 3-6 weeks long so that the disease is often evident in the drier seasons. Temperature is the most important factor other than moisture to influence germination and subsequent infection by the spores. This is also the most important factor influencing disease development. This relationship between temperature, moisture, and incubation period has been developed empirically and with the aid of computer modelling used to predict CLR disease severity and to schedule appropriate fungicide applications (Kushalappa and Eskes 1989).

Hemileia vastatrix life cycle is understood incompletely. The fungus survives as urediniospores, uredia and mycelia, whereas the majority of rusts usually have five spore stages and two hosts. Despite the occasional production of teliospores and basidiospores, under cool dry conditions, no alternate host has been discovered. The basidiospores

germinate in vitro but do not infect coffee leaves. Because basidiospores do not have a known function, it has been thought that physiological races arise as a result of mutation rather than genetic recombination (Rodrigues Jr. *et al*., 2001).

2.1.7 Breeding for Resistance to Coffee Leaf Rust Pathogen

Accessions and derivatives of the inter-specific hybrid Timor Hybrid (HDT) have for a long time been supplied freely by Coffee Rusts Research Centre (CIFC), in Portugal, for breeding against coffee diseases. HDTs are natural hybrids between *Coffea arabica* and *Coffea canephora* Pierre and received from the latter the genes for rust resistance, e.g. SH6, SH7, SH8 and SH9 (Várzea & Marques, 2005; Diniz *et al*., 2012).

In Kenya, a breeding program undertaken at Coffee Research Foundation (CRF) at Ruiru, with a total of 35 progenitors, aimed to accumulate both the resistance existing in pure Arabica varieties and that of Robusta origin into Ruiru 11 variety (derived via the Timor Hybrid) (van der Vossen & Walyaro1981). The cultivar is a composite of 66 F1 hybrid sibs each derived from a cross between a specific female and male population (Omondi *et al*., 2001). The male parents are outstanding selections from a multiple cross programme involving Coffee Berry Disease (CBD) resistant donor parents such as Rume Sudan (R gene), HDT (T gene), K7 (k gene) and the high yielding, good quality but susceptible cultivars such as N39, SL28, SL34, Bourbon and SL4 (Omondi *et al*., 2000). The female parents are advanced generations (F3 and F4) of the cultivar Catimor from Colombia, which has HDT clone 1343/269 as one parent (Omondi *et al*., 2000). The cultivar combines resistance to major CBD and CLR with high yield, fine quality and compact growth amenable to high density planting (Omondi *et al*., 2001). The cultivar has been planted in all coffee growing areas of the country. Due to the challenges

encountered with reproducing Ruiru 11, Batian variety was released as commercial varieties. Their unique features include tall stature, true breeding and resistance CBD and CLR. The five lines were selected as individual tree from backcross progenies involving SL4, N39, Hibrido de Timor (HDT) and Rume Sudan as the donor varieties and the traditional commercial cultivars SL28, SL34 and K7 as the recurrent parents (Gichimu *et al*., 2010).

2.1.8 Management of Coffee Leaf Rust Pathogen

Most of the commercial varieties of Arabica coffee are susceptible to CLR pathogen and most management practices such as mulching, pruning and fertilizer application are associated with lower levels of CLR disease severity except intercropping which results in higher disease intensity (Bigirimana *et al*., 2012). There are two main methods of managing CLR disease. First is the use of completely or partially resistant species or varieties (Brito *et al*., 2010; Romero *et al.,* 2010). The other is the application of the environmentally hazardous fungicide, copper oxychloride (McCook 2006). Despite the use of resistant varieties and fungicides, worldwide losses and control efforts are still estimated to cost approximately US $1-2 billion annually (Hein and Gatzweiler 2006). Some researchers and producers speculate that CLR infection rates will increase due to climate-induced changes in precipitation and temperature that extend the geographic range of CLR pathogen to the higher altitudes where *Coffea arabica* is often grown (Ghini *et al.,* 2011). It has been suggested that long term pest control can best be achieved by managing the system to maximize "build-in" preventive strengths within the agro ecosystem (Lewis *et al*., 1997). This approach is similar to “conservation biological control”, where agro ecosystems are managed to provide habitat and conserve natural

enemies of pests (Letourneau *et al*., 2011). Many studies have been published on conservation biological control and the role of plant diversity, natural enemies, and reduction of pest damage (Barbosa, 1998; Fiedler *et al*., 2008; Letourneau *et al*., 2011; Tscharntke *et al.,* 2007)**.** However, varietal resistance is not stable. Varieties that were once considered "resistant" to coffee rust have since become susceptible due to the evolution of new *Hemileia vastatrix* races (Silva *et al.,* 2006).

Chemical control of Coffee Leaf Rust with protective copper and/or systemic fungicides of the triazol group has proved effective (Matiello *et al*. 2002; Zambolim *et al*. 2002). Although efficient, the effects on the environment and non-target organisms may lead to a population explosion of pests and or other coffee diseases. Moreover, the selection pressure exerted on the pathogen with this control strategy paves the way for the emergence of new fungal races resistant to the products applied (Zambolim *et al*. 2002). The most appropriate alternative to chemical control is the use of resistant cultivars, which can be obtained by conventional breeding, aided by molecular techniques (Fazuoli *et al*. 2002; Pereira *et al*. 2002; Sera *et al*. 2002; Fazuoli *et al*. 2005). Several research groups from different regions have sought to achieve durable resistance to CLR pathogen, resulting in initial success followed by disappointments because of the emergence of new virulent pathogen races able to infect the initially resistant plants (Van der Vossen 2001).

There has been some research into a more biological or organic approach to combat CLR pathogen. Certain 'hyperparasitic' fungi (that are parasitic to parasites) have been identified in nature that preys on the CLR pathogen (Muller *et al.* 2009). One such fungus is known as 'white halo'. Scientifically, there is some evidence that this and possibly

other microorganisms can reduce the viability of the rust (Jackson *et al*., 2012; Vandermeer *et al*., 2010; Haddad *et al.*, 2009).

2.2 Assessing Genetic Diversity of Coffee Leaf Rust Pathogen

Plant pathologists interested in ascertaining genetic variation in pathogen populations have adopted molecular markers techniques as population genetics tools. Motivating this shift has been the availability of a myriad of molecular techniques which makes the quantification of genetic variation a relatively straightforward endeavor (Brown 1996). Molecular markers such as allozymes (Goodwin *et al.,* 1993), Restriction Fragment Length Polymorphisms (RFLP) (Milgroom, *et al.,* 1992) and Random Amplified Polymorphic DNA (RAPD) (Peever and Milgroom 1994) have been widely used to characterize pathogen populations. More recently, Amplified Fragment Length Polymorphisms (AFLP) (Majer *et al.,* 1996) has proven to be highly polymorphic and robust markers and will likely be used extensively with plant pathogenic fungi in the future (Milgroom and Fry 1997).

Standard molecular methods such as Random Amplified Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP), Restriction Fragment Length Polymorphism (RFLP) and microsatellite analysis have been used to estimate genetic diversity and give insights into the population structure of important rusts such as *Puccinia recondita* (Kolmer *et al*., 1995), *P. striiformis* (Steele *et al*., 2001, Justesen *et al*., 2002), *Melampsora epitea* (Pei *et al.,* 1997, Samils *et al* .,2001), *Cronartium ribicola* (Hamelin *et al.,* 1998, Kinloch *et al*., 1998), *C. flaccidum* (Moricca and Ragazzi1998) and *Peridermium pini* (Hantula *et al*., 1998,Moricca and Ragazzi 1998).

The techniques differ in technical requirements, cost, sequence specificity and repeatability. RAPD, Restriction Fragment Length Polymorphism (RFLP) and Amplified Fragment Length Polymorphism (AFLP) do not require prior genetic sequence analysis for primer design and offer genome wide scanning. On the other hand, Microsatellites (Simple Sequence Repeat- SSRs) and Sequence characterized Amplified Regions (SCARs) are based on sequence specific primers with limited transferability across species but are more repeatable. RAPD are easy to use and are available (Lashermes *et al,* .1996).

2.2.1 RAPDs

The RAPD marker system has been used successfully to characterize molecular variation in other rust fungi (Steele *et al.,* 2001). It is a PCR-based marker system, jointly described by Williams *et al.,* (1990). Amplification of genomic DNA using single primers of arbitrary nucleotide sequence, in low stringency conditions, results in multiple amplification products from loci distributed throughout the genome (Williams *et al.* 1990). RAPD markers became popular because of their simplicity, applicability to any genome, no sequence information requirement, relatively small DNA quantities required, results obtained quickly and high genomic abundance. Despite this, the limitations of RAPDs are numerous and include: they are dominant markers (i.e. cannot distinguish homozygotes from heterozygotes), are sensitive to laboratory changes and have low reproducibility within and between laboratories (Rafalski 1997). The number and pattern of bands amplified can be affected with variation in template concentration and with annealing, extension and denaturing time (Bielawski and Noack 1995) and template

quality (Micheli *et al.* 1994). Lashermes *et al.* (1996) reported that genetic factors are more accurately tested by molecular markers.

RAPDs are generated by applying the polymerase chain reaction to genomic DNA samples using randomly constructed oligonucleotides as primers. Since the technique is relatively easy to apply to a wide array of plant and animal taxa, and the number of loci that can be examined is essentially unlimited, RAPDs are viewed as having several advantages over RFLPs and DNA fingerprints. When the primers are of intermediate size (on the order of 10 base pairs) multiple amplifiable fragments (from different loci) are usually present for each set of primers in each genome. The fragments can be separated by size on a standard agarose gel and visualized by ethidium bromide staining eliminating the need for radio labeled probes since the primers consist of random sequences, and do not discriminate between coding and non-coding regions. It is reasonable to expect the technique to sample the genome more randomly than conventional methods (Lashermes *et al.* 1996).

CHAPTER THREE

MATERIALS AND METHODS

3.1Survey of Disease Intensity of Coffee Leaf Rust pathogen

3.1.1 Study site

The survey was carried out in coffee growing zones in six counties in Kenya (Table 1); namely Meru, Kisii , Kericho , Trans Nzoia , Bungoma and Kiambu Counties. Meru County is located at 0^0N, $37^0$35'E, at an elevation of 1524m a.s.l. The soils are ando-humic acrisols, friable clays, strongly acidic, very low in bases and moderate in organic matter. The soils are eutric nitosols, friable clays, and weakly acidic to neutral, rich in bases, available phosphorous and moderate inorganic matter. Kisii is located at $0^0$41'S, 34^0 47'E at 1700M a.s.l. The soils are molic nitosols, friable clays with acidic pH, low to moderate bases and are high in organic matter. Kiambu County site lies within the Upper Midland 2 agro-ecological zone (UM 2) at latitude 1^o06'S and longitude 36^o 45'E and is approximately 1620m a.s.l (Kimemia *et al*., 2001). The area receives a mean annual rainfall of 1063mm and the mean annual temperature is 19^oC (minimum 12.8^oC and maximum 25.2^oC). The soils are classified as humic nitisols and plinthic ferrasols. They are well drained, deep reddish brown, slightly friable clays with murram sections occasionally interrupting. The soil pH ranges between 5 and 6 (Jaetzold and Schmidt, 2005).

Through purposeful and directional sampling, a representative factory was selected in each coffee growing zone. The selected factory was the used as entry point. At each selected factory, directional sampling was used to pick the third coffee farm.

Table 1: Study Sites

County	AEZ
Kiambu County	UM1,UM2,UM3
Trans Nzoia county	UM1,UM3
Kisii County	UM1,UM3
Meru County	UM1,UM2,UM3
Bungoma County	UM2,UM3

3.1.2 Sampling

A total of 30 coffee bushes were sampled randomly in each farm. A total of 120 coffee bushes were sampled from each coffee growing zone. Tree tagging was done in order to avoid collecting data from the same tree more than once.

3.1.3 Data collection

A total of seventy mature coffee leaves were picked from each selected coffee tree. The leaves were picked randomly from bottom, middle and top of the coffee tree, around the tree in order to have a representative sample. After collecting the leaves, both the number of diseased leaves and number of pustules per leaf were counted and recorded. A data sheet was used at each farm to record information about the farm viz age of the bushes, fertilizer/ pesticides usage and types, cultural practices, weeding and pruning. Information was also collected on whether shade trees were used and whether the coffee

was intercropped. Altitude in meters above sea level (a.s.l) was taken, using a Global Positioning System (GPS) at a central point for each farm surveyed.

3.2 Characterization of Coffee Leaf Rust Pathogen

3.2.1 Sampling of Coffee Leaf Rust Pathogen

Coffee leaves infected with *Hemileia vastatrix* were collected from various Coffee growing counties and designated as isolates (Table 2). Coffee genotypes naturally infected with CLR pathogen were used to obtain CLR isolates as shown in Table 2. The Isolates were from single tree that had high level of infection. The bulk samples from each coffee tree were kept separately, sealed and stored under ice to maintain viability. The CLR pathogen infected leaves were checked for mycophagous arthropods using a microscope in the laboratory. The contaminated leaves were discarded. The infected leaves were dried for a day on the bench at room temperature in order to remove moisture and enhance easy brushing off of CLR pathogen spores from them. After brushing, each sample was stored in autoclaved tubes at -80^0C to maintain viability.

Table 2: Sites for Coffee Leaf Rust Pathogen Inoculum Collection

Isolate	County	Host genotype
Isolate 1	Control	Water
Isolate 2	Kiambu	SL28
Isolate 3	Kiambu	Blue Mountain
Isolate 4	Kiambu	Clonal
Isolate 5	Kiambu	K7
Isolate 6	Trans Nzoia	SL28
Isolate 7	Kisii	SL34
Isolate 8	Meru	SL34
Isolate 9	Kericho	Batian
Isolate 10	Bungoma	K7

Table 3: Host genotypes for CLR Pathogen inoculation.

Variety	Description	Origin
HDT	Breeding Plot	Timor
Ruiru 11	Breeding Plot	Kenya
Robusta	Gene bank	Kenya
Mundo Novo	Gene bank	Latin America
Batian	Advanced Selection	Kenya
SL 28	Commercial Variety	Kenya
Pretoria	Gene bank	Guatemala
110/2	Gene bank	Portugal
Bourbon	Gene bank	Reunion

3.2.2 CLR isolate Inoculation and Evaluation

Excised pieces of leaves (1·8 cm diameter) cut with a cork borer, were taken from healthy full-grown leaves and kept in plastic boxes on sterilized foam moistened with distilled water. Nine coffee varieties (Table 3) were inoculated with the CLR pathogen isolates. Each treatment was replicated three times (Plate 3) in a completely randomized design. Each leaf disc was inoculated with one droplet of 0·025 mL *Hemileia vastatrix* spore suspensions (1 mg spores per mL). Boxes were closed with a transparent glass cover and kept at 24°C without illumination. Glass lids were removed after 24 h to allow for evaporation of the inoculation (Plate 4). Afterwards, discs were slightly wetted again with distilled water and further incubated at approximately 1000 lux intensity of artificial light, with 12 h light period, 22 ± 2°C and 100% RH. Evaluation of the reaction type was made 30 days after inoculation, scoring was done using a 6-point scale (Tamayo *et al*., 1995).

1= Absence of symptoms

2=Small chlorotic lesions

3=Median chlorotic lesions, without spores formation

4= Chlorotic lesions, with few urediniospores formation (urediniospores occupying <25% of the lesion area)

5 =Sporulation occupying between 25 and 50% of the lesion area; and

6 =Sporulation occupying >50% of the lesion area.

The genotypes were classified in two phenotyping groups: Those whose leaves scored 1-3 (absence of urediniospores) are resistant; and those with scores of 4–6 (presence of urediniospores) susceptible.

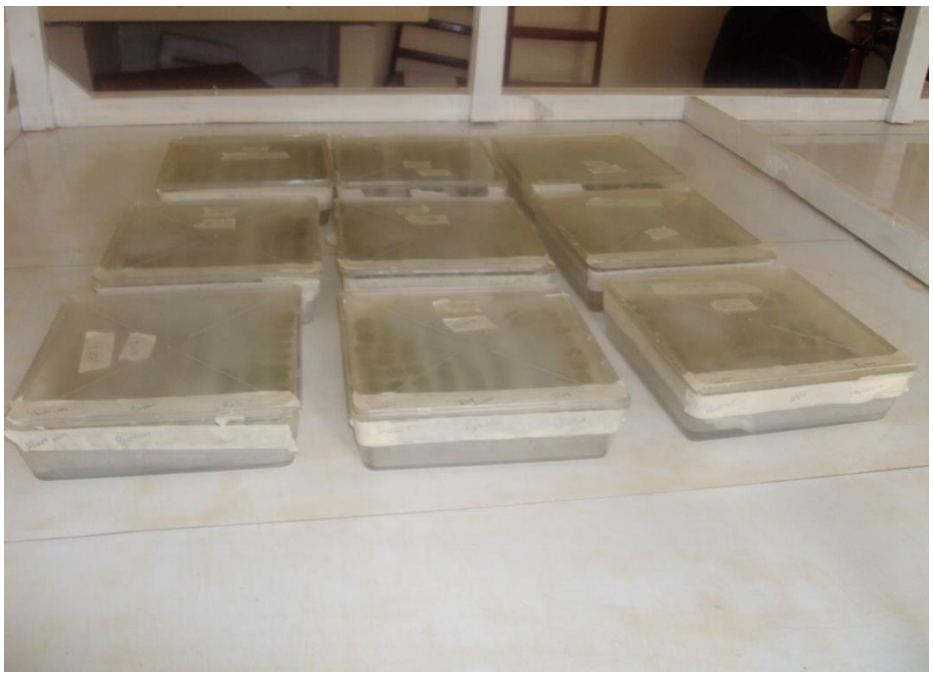

Plate: 3 Replication of CLR (*Hemileia vastatrix*) treatments

(Source: Author, 2014)

Plate 4: Regular opening of the treatments (CLR inoculations) to allow for aeration (Source: Author, 2014)

3.3 Molecular Characterization of CLR pathogen using RAPD primers.

3.3.1Sample Collection Sites

CLR pathogen samples were collected from different coffee growing counties of Kenya that include; Trans Nzoia County, Bungoma County, Kiambu County, Embu County and Kisii County. Coffee growing counties are classified into subzones (UM1, UM2 and UM 3) based on the height a.s.l (Table 4).

3.3.2 Coffee Leaf Rust Pathogen Sample Collection

Mature coffee leaves that were naturally infected with CLR Pathogen were harvested from coffee genotypes in the counties of Kenya. The Coffee leaves from each tree were bulked. An isolate constituted bulk collection of urediospores from each plant (Nunes *et*

al., 2009). CLR pathogen isolates were collected from twenty coffee genotypes as shown in Table 4.

Table 4: Source of CLR pathogen Isolates for Molecular Characterization

Sample	Genotype	Collection Site
Isolate 1	Sl28	Kitale (Plot 1269)
Isolate 2	K7	Namwela (Blk 3)
Isolate 3	K7	West Pokot (Farmer)
Isolate 4	Sl28	Kitale (Demonstration Plot)
Isolate 5	Sl6	Ruiru Plot 13
Isolate 6	Rumangabo	Ruiru (Plot13)
Isolate 7	Harar	Ruiru (Plot 5)
Isolate 8	Sl28	Azania (Farmer)
Isolate 9	Amfilo	Ruiru (Plot 5)
Isolate 10	Cumbaya	Ruiru (Plot 13)
Isolate 11	Anguistifola	Ruiru (Plot 5)
Isolate 12	SL34	Embu (Nembure Farmers)
Isolate 13	SL34	Meru (Tigania)
Isolate 14	Kp163	Ruiru (Plot 5)
Isolate 15	F53	Ruiru (Plot 5)
Isolate 16	Batian	Ruiru (Plot 16)
Isolate 17	Bourbon	Ruiru (Plot5)
Isolate 18	SL28	Kisii (Block1a)
Isolate 19	K7	Kisii (Block 7b)
Isolate 20	Clonal	Kisii (Block 5c)

3.3.3 Purification of CLR Samples

Leaves were examined under a microscope to ascertain that they were free of mycophagous arthropods specifically mites, dipteran larvae and mycoparasites . Coffee leaves with any other contaminations were discarded. Urediniospores were collected into sterile plastic tubes by gently brushing the rust pustules present on the abaxial surface of leaves with a soft camel hair brush.

3.3.4 Extraction of Genomic DNA

Five to thirty mg of *Hemileia Vastatrix* urediniospores were frozen in liquid nitrogen and ground in a microcentrifuge tube using a fitted pestle. Genomic DNA was extracted from these urediospores by the method of Diniz *et al.* (2005) with minor modifications using Mixed AlkylTriMethylammonium bromide (MATAB). Lysis and extraction buffers were added to the powder (1ml each) and grinding continued. The mixture was then transferred to a 2ml plastic bottle and incubated at 62°C in a water bath for 20-30 minutes with regular shaking. After incubation, 1 ml of chloroform/isoamyl-alcohol mixture, (24:1) was added to each bottle, vigorously mixed and then centrifuged in a desktop micro-centrifuge at 13000 rpm for 5 minutes. The supernatants were carefully pipetted out into new 2 ml plastic bottles. Twenty to thirty micro litres of RNase (10 mg/ml) was added to the supernatants and incubated at 37°C in a waterbath for 30 minutes. A volume of isopropyl alcohol equal to the volume of each supernatant was added into each bottle, and mixed gently by inverting the tubes several times to precipitate DNA. The suspended DNA was centrifuged at 13,000 rpm for 5 min and a DNA pellet was obtained and the supernatant was carefully removed. The DNA pellets were then washed with 200µl of 70% ethanol and centrifuged at 13000 rpm for 3 minutes. The ethanol was drained by

decanting or micro-pipetting, and the pellets dried in a vacuum centrifuge for 20 minutes. The pellets were dissolved overnight in 20-40 µl of TE (Tris-EDTA) (depending on pellet size) at 4°C.

3.3.5 DNA Quantification

1% agarose gel in 0.5X TBE was prepared by weighing 0.7g of Agarose in 70ml 0.5X TBE. The solution was then heated in a microwave at short intervals of 15-30 sec with occasional shaking until it was clear. The solution was then weighed again since evaporation occurs during heating after which water was added to obtain the original volume and left to cool to about 55^0C. The gel was then poured on the tray of the mini electrophoresis unit and any bubbles removed after which the combs were fixed and allowed to settle. After settling, the combs were removed and 0.5X TBE Buffer added on the mini electrophoresis unit to cover the gel.

The standard DNA was then prepared (lambda DNA/EcoR1 +Hind111 marker 500µg/ml). The lambda preparation mixture was heated at 65^0 C for 10 min and immediately chilled on ice for 5 minutes. After five minutes, 10µl of lambda and sample DNA preparations was then loaded onto the agarose gel and run at 50v for 45 min. The gel was then stained in 1mg/ml Ethidium Bromide (50µl of 10mg/ml Ethidium Bromide in 500ml dH_2O) for 20 minutes and placed into the UV transilluminator for photography. Lambda preparation table was used to estimate the quantity of DNA.

3.3.6 DNA Amplification

RAPDs were used for the study. The method of Lashermes *et al*. (1996) and modified by Agwanda *et al*. (1997) was used for RAPD analysis (Table 5). The PCR reaction mix was

in 25 µl containing, consisting of 5 µl of genomic DNA (1ng/µl), 7.5 µl of dNTPs (500 µM; 1/10 dilution of the 5 mM dNTPs), 2.5 µl of buffer (10X, Promega), 2.0 µl of MgCl2 (25 mM, Promega), 0.1µl of *Taq* DNA polymerase (Promega), 1 µl of primers (10 µM, Appligene) and 7.0 µl of PCR water. Amplification was carried out in a Flexigene thermocycler (TECHNE, USA). The amplification program started with one cycle of initial denaturation at 94°C for 5minutes followed by 45 cycles of 1 min at 94°C (denaturation), 1 min at 35°C (annealing), and 2 min at 72°C (elongation). The final extension was done at 72°C for 7 min to ensure that the primer extension reaction was completed. The RAPD products were electrophoresed in 1.8% (w/w) agarose gel and then visualized in a UV trans-illuminator after staining in ethidium bromide solution.

Table 5: RAPD Primers

Primer	Base Sequence (5'-3')
N-18	GGT GAG GTC A
L-18	ACC ACC CAC C
M-4	GGC GGT TGT C
I-7	CAG CGA CAA G
J-19	GGA CAC CAC T
Y-10	CAA ACG TGG G
X-20	CCC AGC TAG A
Y-15	AGT CGC CCT T
I-20	AAA GTG CGG G
X-16	CTC TGT TCG G

3.4 Data Recording and Analysis

The data for disease intensity and pathogenicity of Coffee Leaf Rust Pathogen was subjected to analysis of variance (ANOVA) using EXLSTAT 2014 software and effects declared significant at 5% level of significance. Students-Newman Keuls (SNK5%) was used to separate the means. Graphs were generated to compare how the pathogen interacted with the coffee genotypes and to compare the different counties.

Molecular characterization data for Coffee Leaf Rust Pathogen was analyzed in order to determine the differences in fingerprinting patterns between isolates. The fingerprint patterns were assessed visually. Polymorphisms including faint bands that could be scored unequivocally were included in the analyses. Agglomerative hierarchical clustering (AHC) method of unpaired pair group method with arithmetic (UPGMA) to create the dendrograms. Euclean distance was used to check on the dissimilarity.

CHAPTER 4

RESULTS

4.1 Survey of Disease intensity of CLR pathogen in Coffee Growing Counties of Kenya

Disease intensity was significantly different (P<0.0001) among the coffee growing counties with the highest disease intensity in UM2 followed by UM3 and UM1 (Table 6).

Table 6: Separation of means of CLR pathogen disease intensity survey data from Coffee growing AEZs in coffee growing counties of Kenya

Category	Mean	Groups
UM2	72.414	a
UM3	62.849	b
UM1	47.818	c

Disease intensity was significantly different (P<0.0001) among the coffee growing Counties of Kenya with the highest disease intensity in Kisii County and the lowest disease intensity in Trans Nzoia County (Table 7).

Table 7: Separation of means of CLR Pathogen Disease Intensity Survey Data from Coffee Growing Counties of Kenya

Category	Mean	Groups
Kisii	65.315	a
Kiambu	65.159	a
Bungoma	65.048	a
Meru	57.635	b
Trans Nzoia	56.393	b

Disease intensity was significantly different (P<0.0001) among the AEZs in Kiambu County (Table 8) with the highest intensity in UM2 followed by UM3 and UM1 (Table 7).

Table 8: Separation of means of CLR Pathogen Disease Intensity Survey Data from Kiambu County

Category	Mean	Groups
UM2	76.560	a
UM3	68.976	b
UM1	49.940	c

Disease intensity was significantly different (P<0.0001) among the AEZs in Bungoma County with the highest intensity in UM2 followed by UM3 (Table 9).

Table 9: **Separation of means of CLR Pathogen Disease Intensity Survey Data from Bungoma County**

Category	Mean	Groups
UM2	69.429	a
UM3	60.667	b

Disease intensity was significantly different (P<0.0001) among the AEZs in Meru County with the highest intensity in UM2 followed by UM3 and UM1 (Table 10).

Table 10: Separation of means of CLR Pathogen Disease Intensity Survey data from Meru County

Category	Mean	Groups
UM2	69.548	a
UM3	58.905	b
UM1	44.452	c

Disease intensity was significantly different (P<0.0001) among the AEZs in Kisii County with the highest intensity in UM1 (Table 11).

Table 11: Separation of means of CLR Pathogen Disease Intensity Survey data from Kisii County

Category	Mean	Groups
UM2	78.333	a
UM1	52.298	b

Disease intensity was significantly different (P<0.0001) among the AEZs in Trans Nzoia County with the highest intensity in UM2 followed by UM1 (Table 12).

Table 12: Separation of means of CLR Pathogen Disease intensity Survey data from Trans Nzoia County

Category	Mean	Groups
UM2	68.202	a
UM1	44.583	b

4.2 Pathogenic Characterisation of CLR Pathogen Isolates

CLR pathogen isolates were significantly different in their ability to infect the coffee genotypes (P< 0.0001). The isolates were from different coffee growing regions in Kenya. Cofffee genotypes were also significantly different (P< 0.0001). The host genotypes used in the experiment were Mundo Novo, Pretoria, SL28 which is susceptible to all CLR pathogen isolates, Bourbon, 110/2 which is a CLR pathogen differential, Batian which is a commercial coffee variety and resistant to CLR pathogen, Robusta which is a Canephora and resistant to all CLR races, HDT which is used to donate

resistant genes in breeding program and Ruiru 11 which is a commercial variety and resistant to all CLR pathogen races.

The Interaction between CLR pathogen isolates and the coffee genotype was significant too (<0.0001) (Table 13)

Table 13: Separation of means of CLR pathogen isolates ability to infect coffee genotypes

Isolates	Mean	Groups
Isolate 8	2.961	a
Isolate 5	2.912	a
Isolate 10	2.825	a
Isolate 6	2.801	a
Isolate 2	2.764	a
Isolate 7	2.702	a
Isolate 9	2.381	b
Isolate 3	2.294	b
Isolate 4	2.244	b
Isolate 1	1.000	c

CLR pathogen isolates are significantly different at 0.05 level of significance. Isolate 2 (Kiambu county) , Isolate 5 (Kiambu County), isolate 6 (Trans Nzoia county) ,Isolate 7 (Kisii County) and Isolate 8 (Meru County) are significantly different from Isolate 3 (Kiambu County), Isolate 4 (Kiambu County) and Isolate 9 (Kericho County). Isolate 2 was from SL28 which is a susceptible coffee variety to all the CLR pathogen races, Isolate 5 was from K7 which is a tolerant coffee variety to CLR pathogen , Isolate 7 and 8 were from SL34 which is adapted to high altitude areas and susceptible to CLR pathogen. Isolate 9 was from Batian variety which is a true breeding variety with resistance to CLR pathogen, Isolate 3 was from Blue mountain which is susceptible to CLR pathogen and able to grow at high altitudes (Table 13).

Table 14: Separation of means of host genotype interaction of CLR Pathogen isolates

Category	Mean	Groups
Mundo Novo	3.953	a
Pretoria	3.942	a
SL28	3.776	a
Bourbon	3.353	b
110/2	2.985	c
Batian	1.387	d
Robusta	1.000	e
HDT	1.000	e
Ruiru11	1.000	e

The coffee genotypes were significantly different (P<0.0001). Mundo Novo, Pretoria and SL28 were significantly different from Bourbon , 110/2 , Batian , HDT , Robusta and Ruiru (Table 14). Robusta, HDT and Ruiru 11 are resistant to CLR pathogen. HDT is used in the breeding programme as a source for resistant genes. However,some derivatives of HDT have been reported to be susceptible to CLR pathogen. Ruiru 11 is a hybrid and used as a commercial variety. Robusta is a canephora and is resistant to CLR pathogen, Batian is a commercial coffee variety, 110/2 is a CLR pathogen differential hence used for racetyping of CLR pathogen isolates.

From Figure 1 , isolate 1 was the control (distilled water) hence did not infect coffee genotype, Isolate 2 sporulated more on SL28 with a mean score of 5, isolate 3 sporulated more on Pretoria with an average score of 3.7, isolate 4 sporulated more on Mundo Novo with an average score of 3.6, isolate 5 sporulated more on SL28 with average score of 4.5, isolate 6 sporulated more on Mundo Novo with an average score of 5, isolate 7 sporulated more on Mundo Novo with an average score of 4.6, isolate 8 sporulated more on SL28 with an average score of 4.9, isolate 9 sporulated more on Mundo Novo with an average score of 4.2, isolate 10 sporulated more on Pretoria with an average score of 4.9. All the isolates did not infect Ruiru 11, Robusta and HDT. The sporulation rate on the coffee genotypes ranged from an average of 1.0-5.0. Isolate 8 was the most pathogenic with an average score of 2.9.

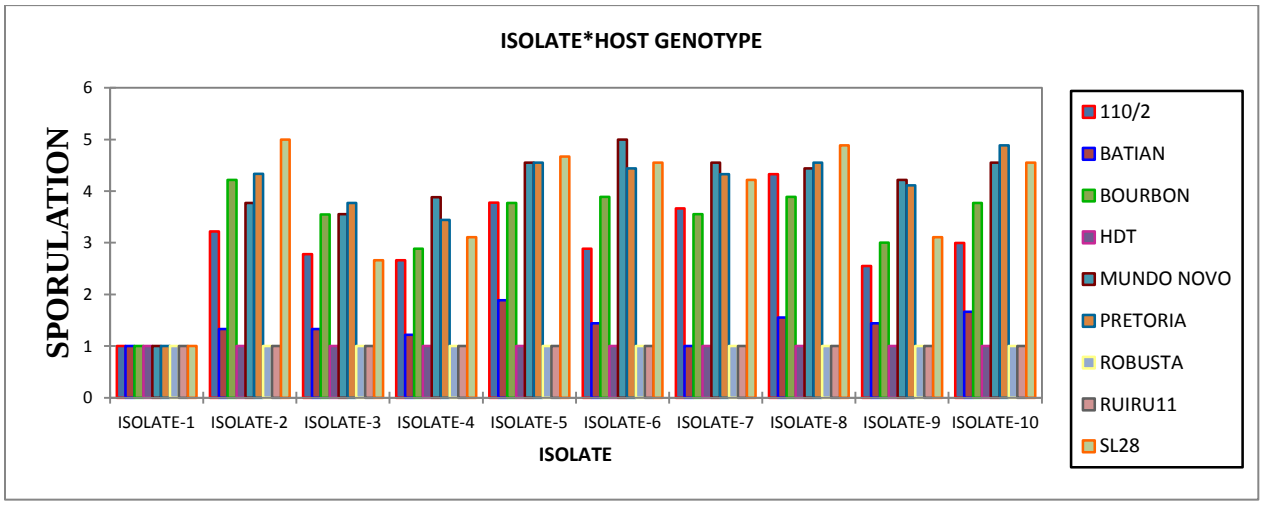

Figure 1: Interaction between CLR pathogen Isolates and the Genotypes based on the rate of sporulation of the isolates on each coffee genotype.

No CLR pathogen isolate infected Ruiru 11 and Robusta. SL28 which is universally susceptible to all CLR pathogen races was infected by all the CLR pathogen isolates. (Plate 5).

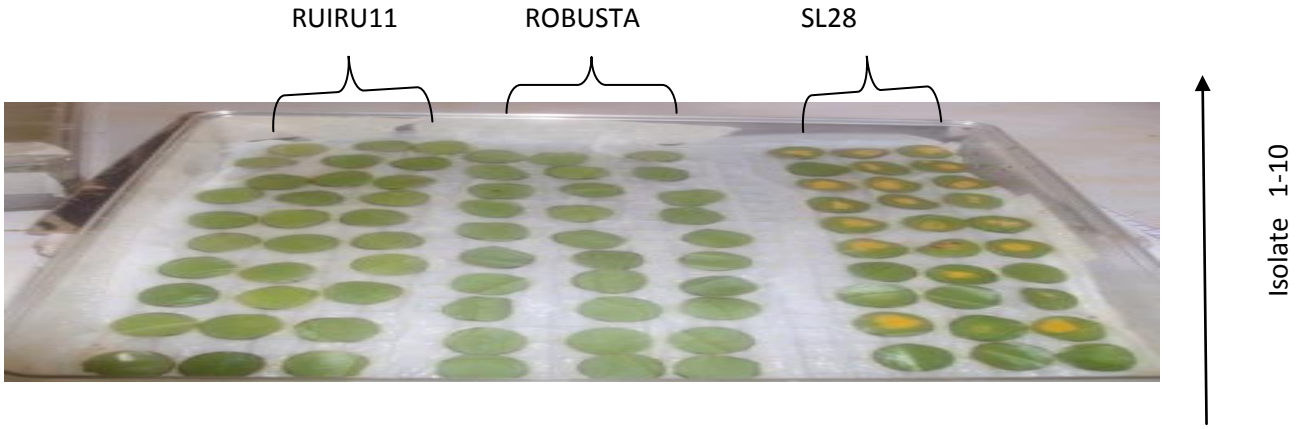

Plate 5: Interaction of CLR pathogen isolates with coffee genotypes.

(Source: Author,2014)

No isolate infected HDT. HDT is used in breeding programme to develop reistant varieties to CLR pathogen. Batian which is a commercial coffee variety was more suscptible on isolate 5. All the isolates sporulated on Pretoria (Plate 6).

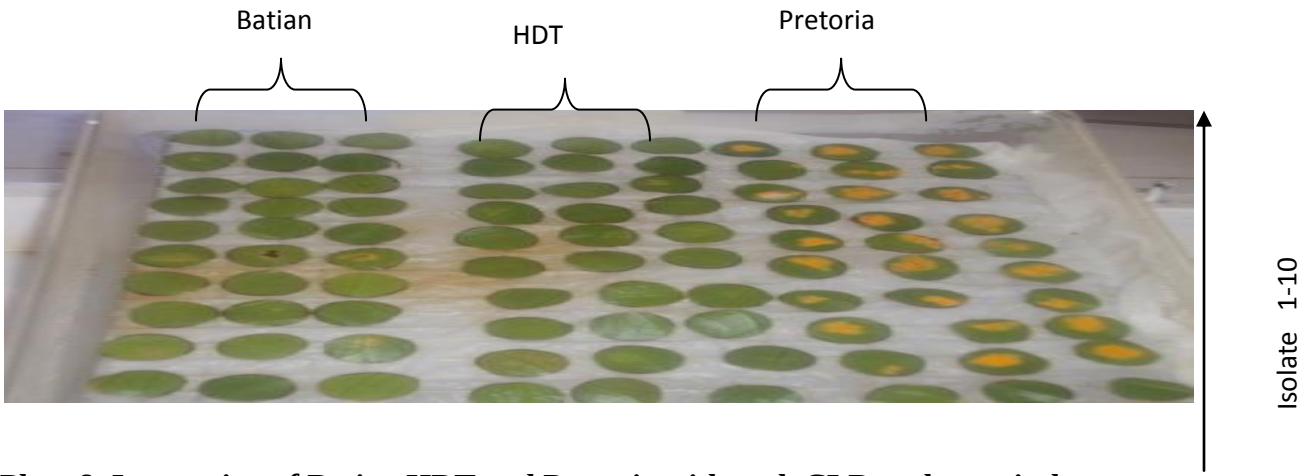

Plate 6: Interaction of Batian,HDT and Pretoria with each CLR pathogen isolate.

(Source: Author,2014)

4.3 Molecular Characterisation of CLR Pathogen

Of the 10 primers tested, 9 showed amplification with clear bands which could be scored clearly. The total bands observed was 82 (Table 15) with number of bands per primer ranging from 5-14 with 58% being polymorphic.

Table 15: RAPD primers for PCR analysis of 20 CLR pathogen isolates

Primer	Base Sequence(5′-3′)	Total Bands	Polymorphic bands	% Polymorphism
M4	GGC GGT TGT C	11	7	63
I-7-	CAG CGA CAA G	9	4	44
L-18	ACC ACC CAC C	5	2	40
Y-10	CAA ACG TGG G	8	8	100
J-19	GGA CAC CAC T	8	7	87
Y-15	GGT GAG GTC A	10	4	40
X-16	CTC TGT TCG G	14	10	71
N-18	GGT GAG GTC A	8	2	25
I-20	AAA GTG CGG G	9	4	44
Total		82	48	

The dendrogram constructed using these polymorphic bands were used to determine genetic diversity in the isolates. The Isolates separated into eight main clusters. Cluster 1 consisted of isolates 20, 7, 15, 9, 10 and 11. Isolate 20 was from clonal genotype, 7 from Harar, 15 from SL6, 9 from Amfilo, 10 from Cumbaya and 11 from Anguistifola. All the isolates were from Kiambu County.

Cluster 2 consists of isolate 4 from SL28 and Isolate 4 was from Trans Nzoia County. Cluster 3 consisted of isolates 16 and 17. Isolate 16 was from Batian cultivar, isolate 17 was from Bourbon. Both the isolates were from Kiambu County.

Cluster 4 consists of isolate 8 from SL28 genotype in Kiambu County. Cluster 5 consisted of isolate 12 from SL34 in Meru County. Cluster 6 consisted of isolates 13, 14 and 18. Isolate 13 was from SL34 in Meru County, isolate 14 was from KP163 in Kiambu County and isolate 18 was from SL28 in Kisii County.

Cluster 7 consists of isolates 1, 2, 3 and 7. Isolate 1 was from SL28 in Trans Nzoia County, isolate 2 was from K7 in Bungoma County, isolate 3 was from K7 in Pokot County and isolate 6 was from Rumangabo in Kiambu County. Cluster 8 consists of isolate 19. Isolate 19 was from K7 genotype in Kisii County (Figures 2 and 3).

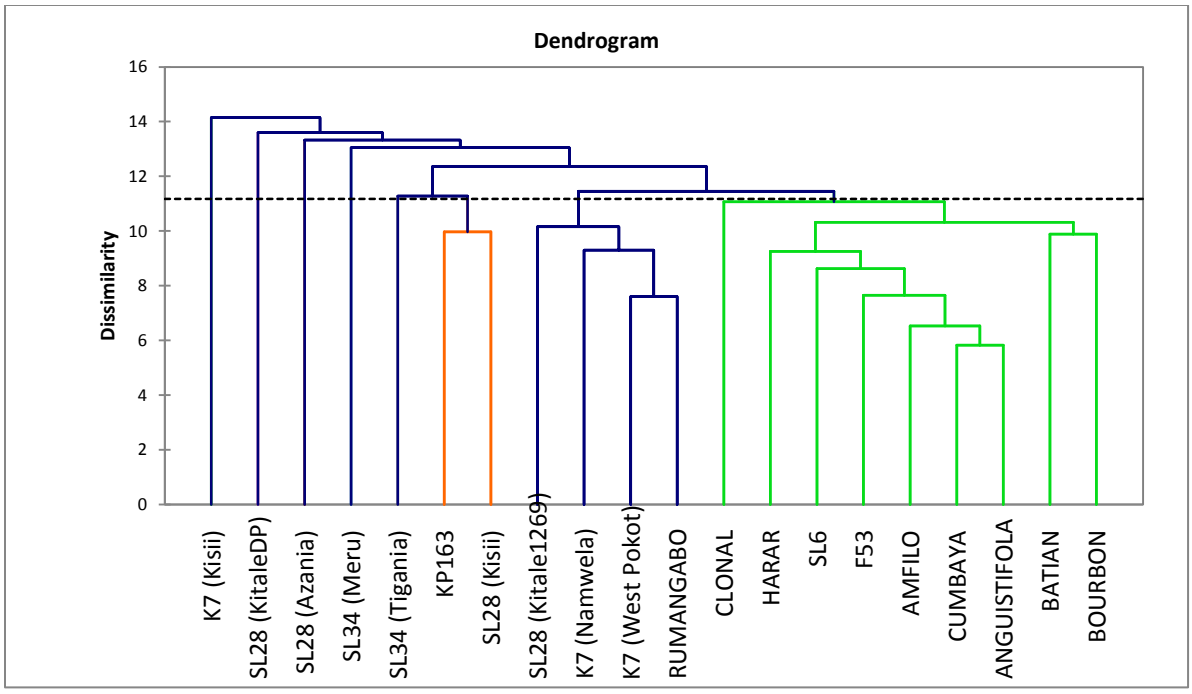

Figure 2: Clustering of CLR pathogen isolates from different coffee growing Counties of Kenya

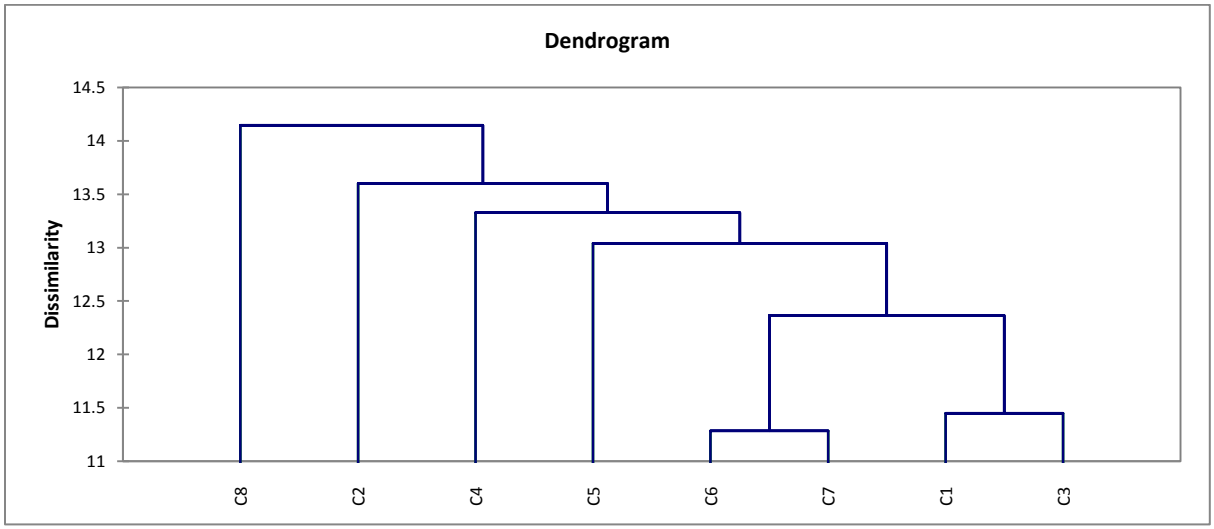

Figure 3: Clusters of CLR Pathogen isolates

Plates 7,8 and 9 show RAPD profiles generated using primer X-16 , I-7 and L-18 respectively. A ladder of 100bp was used to determine weight of the DNA bands.

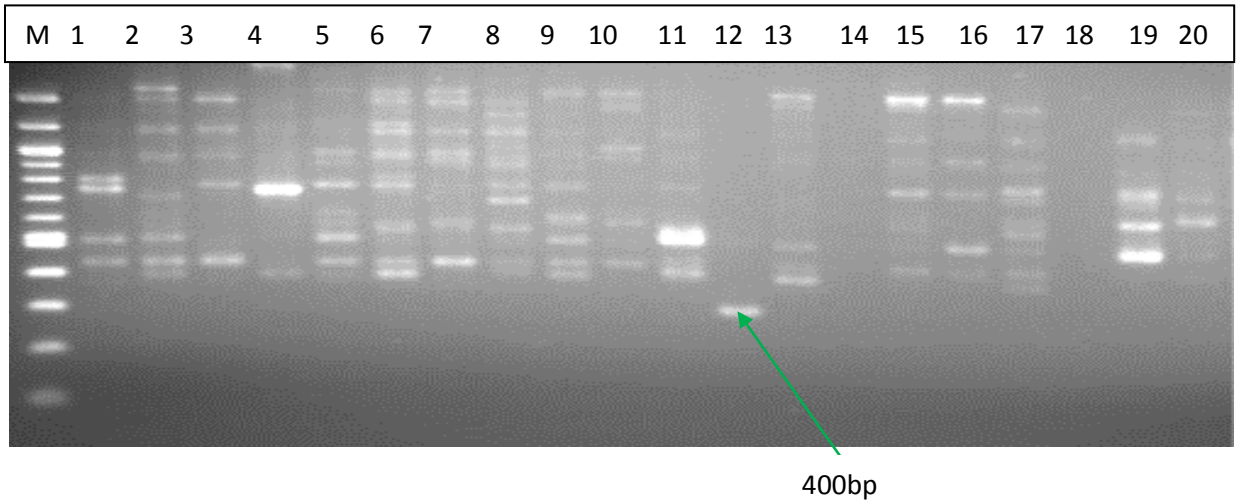

Plate 7: A panel of RAPD profiles generated by primer X-16 in CLR pathogen isolates. M is a 100 base pair marker while lanes 1–20 are CLR isolates.

(Source: Author, 2014)

Primer X-16 amplified a total of 10 polymorphic bands from the 20 CLR isolates representing 40%. Isolate 1 from Trans Nzoia County amplified into 4 polymorphic bands (900bp, 850bp, 500bp, 350bp). Isolate 2 from Bungoma County amplified into 8 polymorphic bands (1550bp, 1500bp, 1200bp, 1100bp, 800bp, 600bp, 450bp, 400bp), isolate 3 from Trans Nzoia County amplified into 5 polymorphic bands (1500bp, 1200bp, 1000bp, 550bp), isolate 4 from Trans Nzoia County amplified into two polymorphic bands (1800bp, 800bp), isolate five from Kiambu County amplified into two polymorphic bands (1000bp, 800bp), isolate 6 from Kiambu County amplified into 9 polymorphic bands (1500bp, 1400bp, 1200bp, 1000bp, 800bp, 650bp, 600bp, 400bp, 350bp), isolate 7 from Kiambu County amplified into five polymorphic bands (1500bp, 1400bp, 1200bp, 1000bp, 650bp), isolate 8 from Kiambu County amplified into seven

polymorphic bands (1500bp, 1400bp, 1200bp, 1000bp, 800bp, 750bp, 650bp), isolate 9 from Kiambu County amplified into four polymorphic bands (1500bp, 800bp, 650bp, 600bp), isolate ten from Kiambu County amplified into three polymorphic bands (1500bp, 1400bp, 650bp), isolate eleven from Kiambu County amplified into one polymorphic band (600bp), isolate 12 from Embu County amplified into one polymorphic band (350bp), isolate thirteeen from Meru County amplified into three polymorphic bands (1500bp, 1400bp, 600bp), isolate fourteen from Kiambu County amplified did not amplify, isolate fifteen from Kiambu County amplified into two polymorphic bands (1500bp,800bp), isolate sixteen amplified into two polymorphic bands (1500bp , 800bp), isolate seventeen from Kiambu County amplified into two polymorphic bands (1400bp, 800bp), isolate eighteen from Kiambu County did not amplify, isolate nineteen from Kisii County amplified into two polymorphic bands (1400bp, 650bp) and isolate twenty from Kisii County amplified into one polymorphic band (650bp) (Plate 7).

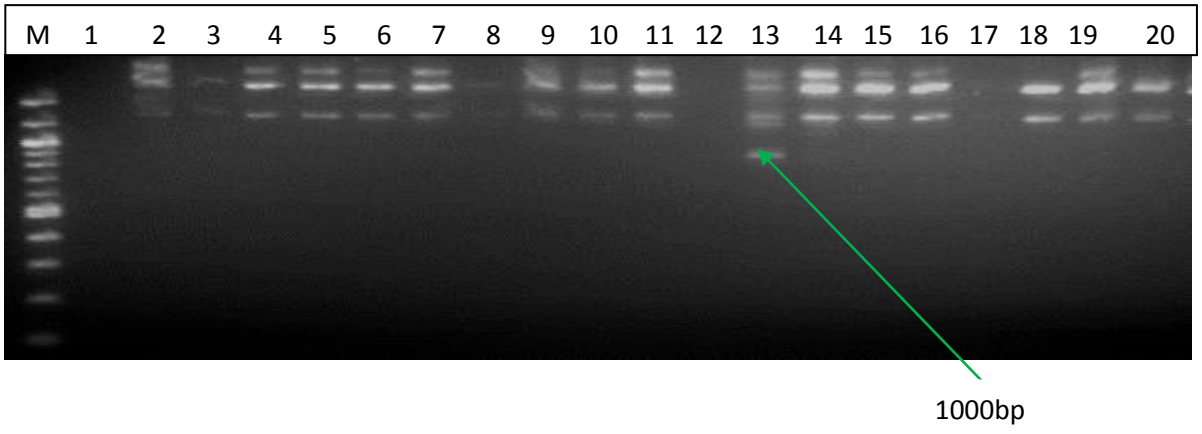

Plate 8: A panel of RAPD profiles generated by primer L-18 in CLR pathogen isolates. M is a 100 base pair marker while lanes 1–20 are CLR isolates

(Source: Author,2014)

Primer L-18 amplified a total of 5 bands with two polymorphic bands representing (40%). The primer amplified a unique band of 1000bp in isoloate 13 from Meru County.The primer did not amplify isolate 1,3,8,12 and 17. Isolate 4 from Trans Nzoia County, Isolates 5,6,11, 7,16 from Kiambu County, Isolate 19 from Kisii County and Isolate 14 from Meru County had common bands (1700bp,1600bp and 1250bp). Isolate 18 and 20 from Kisii County had two bands (1500bp and 1200bp). The primer also revealed similarity on isolates from within the same County: isolate 5,6,7,11,14 and 15 from Kiambu County had bands in common (1700bp,1600bp and 1250bp) (Plate 8).

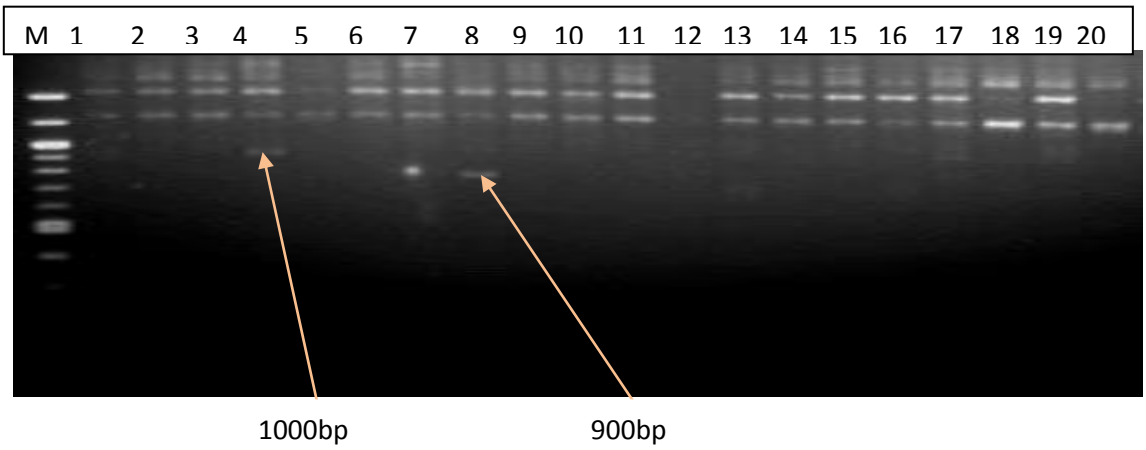

Plate 9: A panel of RAPD profiles generated by primer I-7 in CLR pathogen isolates. M is a 100 base pair marker while lanes 1–20 are CLR pathogen isolates. (Source: Author, 2014)

Primer I-7 amplified a total of 9 bands and 4 polymorphic bands which represent 44%. The primer amplified a unique band of 1000bp in isolate 4 and 900bp in isolate 8. Isolate 12 did not amplify into any band. Isolate 5, 12, 18 and 20 each amplified into 2 polymorphic bands (1500bp and 1200bp) (Plate 9).

CHAPTER 5

DISCUSSION

5.1 Survey of Disease Intensity of CLR Pathogen

Disease intensity of CLR pathogen is highly significant (P<0.0001) in the coffee growing counties of Kenya. This finding agrees with the findings of Bigirimana *et al*., 2012 who reported high incidences of CLR pathogen in different provinces with some provinces reporting as high as 100% severity. Kisii County had the highest disease intensity with an average of 65.315. According to Prakash *et al*., (2005), the differences in disease intensity of CLR pathogen in the coffee growing counties is attributed to the alternating wet and dry conditions which favor high build up of CLR pathogen. Furthermore, the farmers in most of the counties plant traditional coffee varieties. According to Bigirimana *et al*., 2012, high disease incidences in coffee growing counties are as a result of the susceptible commercial varieties.

Upper midland 2 had the highest disease intensity in all the coffee growing counties of Kenya (72.414). UM2 is a medium altitude (1580-1700M a.s.l) and it's the main coffee growing zone. High disease intensity in UM2 is as a result of favorable weather condition that promotes sporulation of CLR pathogen. This finding agrees with the findings of Mugo (2012) who reported high distribution of coffee diseases in UM2 and that distribution of key diseases depends on agro ecological zones. Rayner (1961) found out that warmer temperatures in lower altitudes permit greater infection during wet periods and a shorter latent period.

UM1 (47.818) had the lowest disease intensity. The low disease intensity in upper midland 1 is due to the changes in the climatic conditions. This agrees with the findings of Bigirimana *et al*., 2012 who reported that coffee leaf rust pathogen severity is so high at higher altitudes.

Upper midland 3 had low incidences of disease intensity with an average score of 62.849. UM3 is a marginal zone with low temperatures (over 1700M a.s.l). Presence of low disease intensity in UM3 is proved by Riveira (1984) and Bigirimana *et al*., 2012 who observed a low level of disease intensity at high altitudes. Kulashappa and Eskes also found out that higher altitudes are associated with lower disease severity. Since altitudinal range of coffee has been increasing, it is believed that this will increase disease pressure in coffee growing areas (Mugo, 2012). The study was conducted in the month of April and May when the rains were high and the temperatures warm. CLR pathogen sporulates in the presence of water and warm temperatures. Kulashappa *et al*., (1983) reported that very low temperatures or very high temperatures limit lesion development of CLR pathogen. According to Bayet (2001), high rainfall, high humidity or wetness and relatively low temperature that persist for long periods favor fungal development.

5.2 Pathogenic Characterization of Coffee Leaf Rust pathogen (CLR)

The ability of CLR pathogen from different coffee growing counties to infect coffee genotypes was significantly different ($P<0.0001$). This agrees with the findings of Herrera *et al*., (2009) who reprted that the ability of coffee leaf rust pathogen to infect coffee genotypes depends on the climatic conditions, susceptibility of the coffee genotype and virulence of the pathogen. Isolate 8 from Meru County was significantly different

from all the other isolates with a mean score of 2.961. The isolate sporulated more on SL28 with a mean score of 4.9. According to Gichuru *et al*., 2012, virulence genes in CLR pathogen isolates are highly evolving leading to formation of new races. This change in the virulence genes is associated with continued interaction with resistant coffee genotypes leading to formation of new races. . He further reported that there is emergence of new CLR pathogen races which are attributed to resistant varieties which exert pressure on the pathogen.

Isolate 2 from Bungoma County was so virulent on SL28 with an average score of 5. Robusta, Ruiru 11 and HDT were resistant to all the CLR isolates. According to Omondi et al.,(1998), Ruiru 11 is resistant to CLR pathogen since it contains resistant genes from both Arabica and Robusta (Gichimu, 2012) whereas HDT is the main source for resistant gene to race 11 (Caxieta *et al*., 2003 and Britol *et al.*, 2010). In addition, Pereira (1995) in his study reported that the resistant spectra in HDT can only be annulled by a combination of virulence genes (V5-V9) present in different races of the fungi. Furthermore, according to Bettencourt and Rodriguez (1988), resistance of coffee genotypes to CLR pathogen varies and is determined by resistance genes in the coffee genotype (SH1-SH9) hence this allows coffee genotypes to be classified in resistant groups according to the physiological races of the rust pathogen (Herrera *et al*., 2009).

Batian which is grown as a commercial variety was most susceptible to isolate 5 from Kiambu County with a mean score of 1.9. The same line of Batian cultivar was used in the experiment. Susceptibility of Batian to CLR pathogen could be due to lack of purity during individual tree selection process from backcross progenies. Back cross involves

crossing of F1 hybrid with any of its parents. Lack of purity during selection might have led to segregation. Segregation follows the law of independent assortment where characters separate independently from each other during gamete formation (Davis 2005).

Furthermore the CLR pathogen isolates formed different clusters. Clustering together of CLR pathogen isolates from different coffee growing regions is as a result of spores that are dispersed from one coffee growing county to the other. This agrees with the findings of Kulashappa and Eskes (1989) that movement of CLR pathogen spores leads to introduction of new virulent races in different coffee regions. He further reported that, the movement of rust from one continent to another is attributed to wind currents and the transport of contaminated seeds and/or other plant material by man.

5.3 Genetic diversity of CLR Pathogen

The CLR pathogen isolates from coffee growing counties in Kenya were highly polymorphic. RAPD primer yielded upto 58% polymorphism. All the primers showed polymorphism in all the CLR pathogen isolates. High polymorphism is CLR pathogen could be due to high rate of evolution and mutation which changes the genetic makeup of the pathogen. This agrees with the findings of Varzea and Marques (2005) who observed high genetic diversity in CLR and that mutation is one of the reasons leading to genetic changes in CLR pathogen. Polymorphism was so high which is uncommon in rust fungi as reported by Lie *et al*., (2001) who found out less than 50% polymorphism in determining variability in gall rust.

The RAPD primers amplified unique bands in the isolates from different coffee growing counties. The unique bands include: 400bp in isolate 12 from Embu County by primer X-16,1000bp in isolate 13 from Meru County by Primer L-18 and 1000bp in isolate 4 from Trans Nzoia County by primer I-7. This presence of high genetic diversity among CLR pathogen isolates in Kenya concurs with the findings of Manuela and Gouveia (2005) in their study on genetic diversity on *Hemileia vastatrix* based on RAPD markers where they reported that genetic diversity of CLR pathogen from different geographical regions was high.

Moreover, the dendrogram separated the CLR pathogen isolates into eight main clusters. The isolates from different coffee growing counties clustered together. Grouping together of the isolates from different coffee growing regions could be due to dispersal of the pathogen by wind or man from one county to the other. The transfer of isolates from one geographical region to the other is evidenced by Hovmoller and Brown (2002) in their study in aerial dispersal of pathogens on the global and continental space and its impacts on plant disease. They reported that long distance dispersal of fungal spores by wind can spread plant diseases across and between continents.

However, some isolates from within the same County clustered together e.g. Cluster 1 consisted of isolates 20,7,15,9,10 and 11 from Kiambu County. Genetic similarity is attributed to clonal reproduction in *Hemileia vastatrix* population. Rodriguez Jr.*et al.*, (2001) reported absence of biological evidence for sexual behavior under natural and green house conditions for rust fungi.

In addition, within a county, isolates were unique hence formed their own clusters e.g. Cluster 5 which consist of isolate 12 from Meru County and cluster 8 which consist of isolates from Kisii County. This agrees with Brown and Hovmoller (2002) who found out that in agricultural ecosystems, pathogen populations evolve adapting to constant changes in environment conditions such as resistant varieties and use of resistant varieties.

CHAPTER 6

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusions

The severity of CLR pathogen depends on the virulence of the pathogen, susceptibility of the varieties and the climatic conditions. Isolate 2 from Bungoma County in Upper midland 2 was more aggressive on SL28 which is a susceptible coffee variety.

The characterization of CLR pathogen using molecular technique demonstrated the isolate variability. This confirms what was found through the conventional method of characterizing CLR pathogen. Thus use of conventional method showed that, the CLR pathogen from different coffee growing counties varied in their ability to infect the coffee genotypes whereas the molecular technique grouped the isolates into different clusters.

Disease intensity of CLR pathogen depends on the coffee growing county. UM2 had the highest disease intensity whereas UM1 had the lowest disease intensity in all the coffee growing counties of Kenya.

6.2 Recommendations

1. Isolate 2 from Bungoma County is more virulent on SL28. Consequently, farmers from this County should not be advised to plant SL28 variety.

2. The research established high disease intensity in Upper midland 2 in all the coffee growing Counties in Kenya. However, further research should be done using more farms in order to make more findings on the disease intensity in these counties.

3. Racetyping should be done to ascertain the races present in CLR pathogen isolates especially Isolate 12 from Embu County with unique bands of 400bp, isolate 13 from Meru County with unique band of 1000bp, isolate 4 from Trans Nzoia County with unique band of 1000bp and isolate 8 from Kiambu County with unique band of 900bp.

4. RAPD primer X-16 should be used in the study of genetic diversity of CLR (*Hemileia vastatrix*). In addition, molecular primers should be used to study virulence of CLR pathogen.

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APPENDICES

Appendix I: Data Collection Sheet

Study of Disease Intensity of CLR in different Coffee growing Counties of Kenya

1. Name of the farm……

2. Name of the factory……

3. Date of data collection……

4. The altitude…….

5. Latitude……..

6. Agro ecological zone…….

7. Slope orientation……

8. Map of the farm…….

9. Coffee varieties found on the farm……

10. Variety sampled……..

11. Age of the bushes……

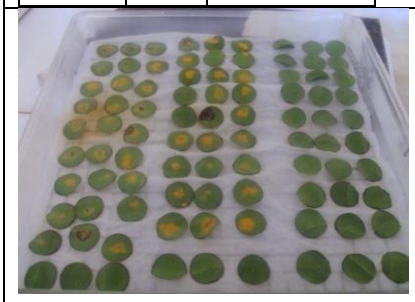
12. Whether there is shade and the type of shade……...

13. Level of management…….

14. Status of the bushes……

Appendix II: Sporulation of CLR Pathogen Isolates on Coffee Genotypes

REP 1 BOX			REP 1 BOX 2			REP 1 BOX 3		
BATIAN	HDT	PRETORIA	SL28	RUIRU 11	110/2	M.NOVO	ROBUSTA	BOURBON
								
REP 2 BOX 1			REP 2 BOX 2			REP 2 BOX 3		
110/2	HDT	M.NOVO	RUIRU 11	ROBUSTA	SL28	BATIAN	BOURBON	PREORIA
								
REP3 BOX 1			REP 3 BOX 2			REP3 BOX 3		

110/2	SL 28	ROBUSTA		M.NOVO	BOURBON	RUIRU11		BATIAN	HDT	PRETORIA
										

Appendix III: ANOVA Tables for Host Genotype Interaction

ANOVA of CLR disease intensity survey data from coffee growing regions in Kenya

Source	DF	Sum of squares	Mean squares	F	Pr > F
Model	4	22819.837	5704.959	8.827	< 0.0001
Error	1435	927503.518	646.344		
Corrected Total	1439	950323.355			

ANOVA of CLR disease intensity survey data from Meru County

Source	DF	Sum of squares	Mean squares	F	Pr > F
Model	2	38076.508	19038.254	37.812	< 0.0001
Error	357	179750.612	503.503		
Corrected Total	359	217827.120			

Computed against model Y=Mean(Y)

ANOVA of CLR disease intensity survey data from Trans Nzoia County

Source	DF	Sum of squares	Mean squares	F	Pr > F
Model	1	33471.565	33471.565	87.894	< 0.0001
Error	238	90634.252	380.816		
Corrected Total	239	124105.816			

Computed against model Y=Mean(Y)

Type I Sum of Squares analysis:

Source	DF	Sum of squares	Mean squares	F	Pr > F
ISOLATE	9	82.624	9.180	28.264	< 0.0001
HOST GENOTYPE	8	443.016	55.377	170.492	< 0.0001
ISOLATE*HOST GENOTYPE	72	76.059	1.056	3.252	< 0.0001

Type II Sum of Squares analysis:

Source	DF	Sum of squares	Mean squares	F	Pr > F
ISOLATE	9	82.624	9.180	28.264	< 0.0001
HOST GENOTYPE	8	443.016	55.377	170.492	< 0.0001
ISOLATE*HOST GENOTYPE	72	76.059	1.056	3.252	< 0.0001

Type III Sum of Squares analysis:

Source	DF	Sum of squares	Mean squares	F	Pr > F
ISOLATE	9	82.624	9.180	28.264	< 0.0001
HOST GENOTYPE	8	443.016	55.377	170.492	< 0.0001
ISOLATE*HOST GENOTYPE	72	76.059	1.056	3.252	< 0.0001

ANOVA of CLR disease intensity survey data from AEZs in coffee growing counties in Kenya

Source	DF	Sum of squares	Mean squares	F	Pr > F
Model	2	161825.443	80912.721	147.460	< 0.0001
Error	1437	788497.912	548.711		
Corrected Total	1439	950323.355			

ANOVA of CLR disease intensity survey data from Bungoma County

Source	DF	Sum of squares	Mean squares	F	Pr > F
Model	1	4606.259	4606.259	7.313	0.007
Error	238	149919.728	629.915		
Corrected Total	239	154525.986			

Computed against model Y=Mean(Y)

ANOVA of CLR disease intensity survey data from Kiambu County

Source	DF	Sum of squares	Mean squares	F	Pr > F
Model	2	45137.562	22568.781	36.123	< 0.0001
Error	357	223045.204	624.776		
Corrected Total	359	268182.766			

Computed against model Y=Mean(Y

ANOVA of CLR disease intensity survey data from Kisii County

Source	DF	Sum of squares	Mean squares	F	Pr > F
Model	1	40671.505	40671.505	79.219	< 0.0001
Error	238	122190.323	513.405		
Corrected Total	239	162861.828			

Computed against model Y=Mean(Y)

Appendix IV: DNA Extraction Buffers

(i) Extraction buffer

- NaCl 8.77g
- MATAB 2% (2g, added just before extraction) (Mixed Alkyltri-methylammonium Bromide)
- Sarcosil 3% (9.5ml of 5% solution) (N-Lauroyl-Sarcosine)
- Sodium bisulphite 1% (1g, added just before extraction)
- Tris HCl 0.20M (20ml of 1 M, pH=8.0)
- EDTA 40mM (1.49g)

(ii) Lysis buffer

- Sorbitol 0.35M (6.38g)
- Tris-HCl 0.20M (20ml of 1 M, pH=8.0)
- EDTA 40mM (1.49g)
- PVP 2% (2g) (polyvinyl pyrrolidone, added just before extraction)

❖ Volume up to 100ml with distilled water

(iii) EDTA 0.5M pH 8 at 25^{o}C (1L)

- EDTA 186g
- NaOH 20g

❖ Add distilled water, dissolve, adjust pH and adjust final volume to 2L

(iv) Formamide Blue (for loading in denaturing acrylamide gels)

- Formamide 98% 49ml
- EDTA 10mM 186mg

- Bromophenol Blue 125mg
- Xylene cyanol a pinch

(v)TAE 50X (1L)

- Tris 242g
- Glacial acetic acid 57.1ml
- EDTA 0.5M pH 8 100ml

❖ Make volume to 1 L

(VI) TBE 10X (2L) (Tris Boric acid EDTA)

- Tris 216g
- Boric acid 110g
- EDTA 0.5M pH 8 80ml
- Distilled water top to 2L
- TE (Tris –EDTA buffer)
- 1ml of Tris HCl 1M pH=8
- 200µl of EDTA 0.5 M pH=8, volume make to 100ml

(VII) dNTPs 5mM

- dATP 100mM 50µl
- dGTP 100mM 50µl
- dTTP 100mM 50µl
- dCTP 100mM 50µl

❖ Added double distilled water to make 1000µl