Characterization of coffee genotypes derived from crossing Rume Sudan and SL 28 coffee varieties against coffee berry disease (CBD) causal pathogen (*Colletotrichum kahawae*)

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other university.

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DEDICATION

To my parents, Mr. and Mrs. Kamau and to my two sisters, Anne Mwihaki Kamau and Esther Wanjiru Kamau.

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LIST OF ACRONYMS AND ABBREVIATIONS

AEZs Agro Ecological Zones.

AHC Agglomerative Hierarchical Clustering.

ANOVA Analysis of variance.

a.s.l Above sea level.

CBD Coffee Berry Disease.

CCA *Colletotrichum coffeanum Acutatum.*

CCM *Colletotrichum coffeanum* Mycelia.

CCP *Colletotrichum coffeanum* Pink.

CIFC Centro de Investigacao das Ferrugens do Cafeeiro.

CLR Coffee Leaf Rust.

CRI Coffee Research Institute.

CTAB Cetylmethylammonium bromide

dNTP Deoxyribonucleotide triphosphate.

DNA Deoxyribonucleic Acid.

EDTA Ethylenediamine tetra acetic acid.

F_1 Filial generation 1.

F_2 Filial generation 2.

HDT Hibrido de Timor.

ICO International Coffee Organization.

KALRO Kenya Agricultural and Livestock Research Organization.

MAS Marker Assisted Selection.

MATAB Mixed Alkyltrimethylammonium Bromide.

MEA Malt Extract Agar.

PCR Polymerase Chain Reaction.

RAPD Random Amplified Polymorphic DNA.

RFLP Restricted Fragment Length Polymorphism.

RS Rume Sudan.

SL 28 Scott Laboratories 28.

SSR Simple Sequence Repeats.

TAE Tris-ammonium EDTA buffer.

TBE Tris Boric Ethylenediamine tetra acetic acid.

TE Tris Ethylenediamine tetra acetic acid.

UM1 Upper Midland 1.

UM2 Upper Midland 2.

UM3 Upper Midland 3.

UV Ultra-violet rays.

ABSTRACT

Coffee is known to be one of the most important beverages in the world with a current estimated value of US$10 billion. It is ranked as the second largest traded commodity after petroleum. *Coffea arabica* is better known for its excellent cup quality but suffers from a narrow genetic base due to its history on domestication and susceptibility to coffee diseases and pests. /*Coffea Arabica*/ production in Africa is majorly constrained by Coffee Berry Disease (CBD) caused by *Colletotrichum kahawae*. Transfer of desired genes from related wild diploid *Coffea* species into the cultivated allotetraploid *C. arabica* has been known to confer better traits such as pest/disease resistance to the cultivated coffee varieties. Recently, conscious efforts have begun to integrate DNA/molecular marker based technologies, which have provided impetus, dependability and direction to the efforts on coffee genetic improvement. This study was aimed at establishing genetic diversity and microsatellite markers that co-segregate with resistance to CBD in an F_2 population derived from two coffee cultivars; Rume Sudan (resistant) and SL 28 (susceptible). Phenotypic studies using *Colletotrichum kahawae* inoculum to screen F_2 population was carried out in order to understand the segregation of Coffee Berry Disease resistance as well as their association with SSR markers. Effect of genotypes on mean infection was highly significant ($P \leq 0.0001$) and this was due to segregation in the F_2 population. Upon separation of means, the F_2 genotypes were clustered into two classes; 33 resistant and 16 susceptible genotypes. F_2 population phenotypically segregated in a 3:1 ratio for resistant and susceptible plants respectively. Among the 12 simple sequence repeats (SSR) markers tested, six markers were polymorphic but only two markers, M 24 and Sat 227 discriminated between the parents, F_1 and the F_2 population. These two SSR markers showed a "goodness-of-fit" to the expected Mendelian segregation ratio (1:2:1) for single gene effect (d.f= 1.0, $P<0.05$) in the chi-square (χ^2) analyses. The F_2 plants showed that resistance to *Colletotrichum kahawae* were putatively linked to two alleles of SSR markers, M-24 (~210 bp) and Sat-227 (~200 bp). The two SSR loci were putatively associated to CBD resistance gene in Rume Sudan. Due to SSR co-dominance nature, the F_2 genotypes were delineated into

homozygous resistant, heterozygous resistant and homozygous susceptible. This diversity among the F_2 genotypes was clearly seen in a dendogram produced by Artemis 5.0 software. Therefore, there was a correlation between phenotypic data and molecular data with regard to resistance to *Colletotrichum kahawae*. The findings of this study could be useful in CBD molecular analysis of segregating generations, breeding lines and varieties having Rume Sudan as one of the parents.

CHAPTER ONE

INTRODUCTION

1.1 Background of the study

Coffee is known to be one of the most important beverages in the world. It has a current estimated value of US$10 billion and is one of the most traded commodities second in value only to oil and a huge contributor of foreign exchange earnings for developing countries (Labouisse *et al.,* 2008). Its cultivation, processing, trading, transportation and marketing provide employment to millions of people in coffee growing countries. Arabica coffee *(Coffea arabica)* is one of the world's most valuable agricultural commodities which accounts for two-thirds of the global coffee market (Labouisse *et al.,* 2008).

In Kenya, coffee was the leading export crop since independence up to 1988. Between 1975 and 1986, it contributed over 40% of the total Kenyan export value. After the 1978/88 production peak, the international prices fell sharply in 1989 and by 1992 coffee contributed less than 9% of the total export value. Apparently, coffee plays a vital role in economic development of Kenya through foreign exchange, tax income and employment opportunities for Kenyans. It also promotes development of industries like the fertilizer industries and the agro chemicals. About 10% of Kenyans earn their livlihoods from coffee (Mugo, 2012). The crop remains a key economic venture for millions of households and is therefore fundamental to wealth creation strategies (Condliffe *et al.,* 2008).

Coffea arabica is better known for its excellent bean and cup quality but suffers from a narrow genetic base due to its domestication history and susceptibility to diseases and pests and inadaptability to various biotic/abiotic stresses.

Coffee is a major cash crop in Kenya but its production is constrained by two main diseases namely Coffee Berry Disease (*Colletotrichum kahawae* Waller & Bridge) and Coffee Leaf Rust (*Hemileia vastatrix* Berk. & Br.). Disease control measures such as

chemical methods, cultural methods and biological control have been practiced for many years. Hybridization programs have also been undertaken giving rise to resistant cultivars; composite Ruiru 11 and Batian coffee to both resistant to Coffee Berry Disease and Coffee Leaf Rust (Gichimu *et al.,* 2014).

Coffee Berry Disease (CBD) was first detected in Kenya, west of the Rift Valley around Mt. Elgon in 1922. The disease had caused losses of up to 75% almost bringing coffee cultivation in west of the Rift Valley to a stop. Due to the disease, tea plantations became predominant in the region (Omondi *et al.,* 1997).

Studies on Arabica coffee carried out in Kenya concluded that coffee resistance to CBD appears to be controlled by major genes at three different loci (Van der Vossen & Walyaro, 1980). Rume Sudan, the highly resistant variety carries the dominant *R*- and the recessive *k*-genes. Pretoria variety also has the *k*-gene. The Blue Mountain (K7) variety, a moderately resistant variety carries only the recessive *k*-gene. Hibrido de Timor carries one gene for CBD resistance at the *T*- locus with intermediate gene action (Van der Vossen & Walyaro, 1980). Unfortunately, the inherent dismally slow pace of tree breeding using conventional methods accompanied with general lack of genetic markers, universal screening and selection tools and methodologies has impeded fast development of disease-resistant coffee varieties. Therefore, incorporating modifications in the methodology of basic techniques to ensure effective disease management and utilize new breeding techniques such as molecular biology and biotechnology will enhance development of disease-resistant coffee cultivars (Aggarwal & Hendre, 2007). Concerted efforts have been undertaken in recent years to develop, use and integrate DNA marker tools/technologies in coffee genetics research. This has also provided few thoughts about future needs and perspectives to fully harness the potential of DNA marker based applications in managing and utilizing the available germplasm resources, construction of linkage maps, QTL mapping and genetic improvement of coffee (Aggarwal & Hendre, 2007). The time required for breeding by traditional methods can be shortened by use of these DNA based marker assisted selection (MAS) (Rieseberg *et al.,* 2000). These

markers assist in detecting a targeted genomic fragment, hence selecting for a desirable trait such as disease resistance (Gichuru *et al.,* 2008).

Studies in Kenya by Gichuru *et al.* (2008) was able to identify a microsatellite marker Sat 235 which was linked to CBD resistance and mapped it onto an introgressed *C. canephora* fragment which harbors the responsible Ck-1 gene synonymous to the *T* gene in Hibrido de Timor using F_2 plants from a cross between *cv.* Catimor × *cv.* SL 28 that were resistant and susceptible to CBD respectively. Gichimu *et al.* (2014) used the same SSR marker, Sat 235 in the study of occurrence of Ck-1 gene conferring resistance to Coffee berry disease in *Coffea arabica cv.* Ruiru11 and its parental genotypes. Sat 235 however, cannot be applied on Rume Sudan that has the *R* and the *k* genes, hence the need for this study. In view of the long time duration it takes to develop resistant varieties, this study was formulated with the sole objective of analyzing SSR markers that co-segregate with CBD resistance genes in coffee genotypes derived from a cross between Rume Sudan (resistant variety) and SL 28 (susceptible cultivar) for possible use in Marker Assisted Selection (MAS) that would considerably shorten the breeding process. It would also allow for large scale screening for CBD of coffee genotypes having Rume Sudan as one of the parents at any stage of development.

1.2 Statement of the problem

To widen the genetic base and introduce variability in Arabica coffee, coffee breeders have undertaken numerous hybridization programs thereby developing new coffee varieties. This has necessitated the need to determine the level and source of genetic variation within and between the new and existing coffee varieties. There is also a need to transfer disease resistance traits from related wild diploid *Coffea* species into the cultivated allotetraploid *C. arabica* that is essential in coffee breeding in order to develop disease-resistant cultivars. There is very little that has been done to identify markers that are linked to CBD resistance.

1.3 Justification of the study

There is need to determine the level and source of genetic variation within and between new and existing coffee varieties which are continuously being developed through hybridization. New insights into transfer of disease resistance genes into *C. arabica* are valuable to improve on current breeding methods. It is believed that breeding for resistance to CDB may provide a sustainable long-term management of the disease.

There is also need to increase coffee specific PCR based microsatellites/SSR markers in order to integrate them in marker based approaches for coffee genetic improvement. Therefore, this study aimed at establishing Microsatellites/SSR markers that co-segregate with CBD resistance genes from Rume Sudan coffee variety so that these markers can be used in selective breeding for CBD resistance in Arabica coffee.

1.4 Objectives

1.4.1 General objective

To characterize coffee genotypes derived from crossing Rume Sudan and SL 28 coffee varieties against CBD causal pathogen *Colletotrichum kahawae*.

1.4.2 Specific objectives

i.) To screen Rume Sudan and SL 28 parental cultivars, F_1 and F_2 progenies for resistance to CBD.

ii.) To determine genetic variation among F_1, F_2 progenies and their parents and screen for SSR markers linked to CBD resistance among F_2 progenies.

1.5 Hypothesis

1.5.1 Alternative hypotheses

i.) There is segregation for CBD resistance in F_2 population of a cross between SL 28 and Rume Sudan.

ii.) There is genetic variation among the F_2 genotypes of SL 28 × Rume Sudan and their parents.

iii.) There are DNA markers linked to CBD resistance in a cross between SL 28 and Rume Sudan.

CHAPTER TWO

LITERATURE REVIEW

2.1 Classification of coffee - taxonomy, origin and early history

Coffee belongs to the genus *Coffea*, in the family *Rubiaceae*. There are a range of published taxonomies for the genus *Coffea* with at least 100 species of coffee belonging to this genus (Fazuoli *et al*., 2000). Two species, *Coffea arabica* L. (Arabica) and *Coffea canephora* Pierre (Robusta) are the two main commercial species grown worldwide (Coste, 1992). They account for 65% and 35% of production respectively (International Coffee Organization, http://www.ico.org). *C. arabica* is the only tetraploid species (2n = 4x = 44) in the genus while all other species are diploid (2n = 2x = 22). *C. arabica* is believed to be an allotetraploid formed by hybridization between two diploid species, *C. canephora* and *C. eugenioides*, or ecotypes related to these species, based on molecular and cytological investigations, (Lashermes *et al.*, 1999).

There are two types of *C. arabica* namely, Typica and Bourbon. The Typica genetic base originated from Indonesia from a single plant which was subsequently cultivated in the Amsterdam botanical garden in the early 1715 (Anthony *et al.*, 2002). The Bourbon genetic base originated from a few coffee trees that were introduced from Mocha (Yemen) to the Bourbon Island (now La Reunion) at about the same time as Typica. *Coffee arabica* has its primary center of diversity in the highlands of southwest Ethiopia and the Boma plateau in Sudan. Some wild populations of *C. arabica* have also been reported in Mount Imatong, Sudan and Mount Marsabit, Kenya (Anthony *et al.*, 2002). The narrow geographic origin, self-fertilizing nature and the historical or selective bottlenecks in its agricultural adoption, have resulted in low genetic diversity of *C. arabica* varieties cultivated around the world (Chaparro *et al.*, 2004). This situation poses a challenge for identifying appropriate markers for cultivar identification based on DNA polymorphisms.

2.2 Morphology and reproductive biology of *C. arabica*

Coffea arabica is a shrub or small tree that if left untended may reach a size of 4 to 5 meters. It has a dimorphic habit of branching in which vertical branches form horizontal branches, which bear flowers and fruits in clusters (Van der Vossen, 1974). Flowers of *C. arabica* have short corolla, long style and exerted stamen which is typical of the genus *Coffea*. Such floral morphology would permit natural cross-pollination but the species is predominantly autogamous. Studies on the degree of natural cross-pollination were carried out on cultivars of *C. arabica* using the recessive marker genes *Cera* (Yellow endosperm) and *Purpurascens* type (purple leaves) (Van der Vossen, 1974). A seven to fifteen percent level of natural cross-pollination was found. Most diploid species have shown to be highly self-incompatible, and allogamous (out crossing) in nature. Inflorescences develop from serial buds especially on horizontal branches. Each inflorescence normally carries 1 to 5 flowers. The flowers have a short pedicel and a rudimentary calyx and the petals are fused and form corolla with 5 lobes. The pistil consists of a long style with two stigmatic lobes and an inferior ovary. The ovary is bilocular, that is each ovary has one anatropous ovule (Van der Vossen, 1974).

Flower initiation occurs after sufficient rainfall following a dry period. The total period of flowering is normally not more than three days with the majority of flowers opening on the first and the second day (Van der Vossen, 1974). Soon after opening of the flowers early in the morning, the stigma becomes receptive and pollen shedding starts. Withering of flowers occurs in one or two days after pollination. It takes six to eight months from flowering to fruit ripening. The coffee fruit usually contains two seeds. Ripe fruits have a thick fleshy mesocarp and a hard endocarp and each seed is enveloped in a silver skin (testa), which is a remnant of the integument (perisperm) (Van der Vossen, 1974).

2.3 Ecological requirements of coffee in Kenya

Kenyan coffee is grown in areas with altitude ranging from 1200-2100 meters above the sea level (a.s.l). Coffee is mainly grown under shade or unshaded system in three agro ecological zones (AEZ) namely; Upper Midland 1 (UM1), Upper Midland 2 (UM2) and

Upper Midland 3 (UM3) (Figure 2.1). UM1 has an altitude that ranges from 1570-1810 m a.s.l with annual mean temperature of 18.4^0 C with rainfall of 1640 mm. UM2 lies between 1395-1675 m a.s.l with annual mean temperature and rainfall of 19.4ºC and 1465 mm respectively. UM3 lies between altitude of 1330-1560 m a.s.l with annual temperature of 19.9ºC and rainfall of 1270 mm (Mugo, 2012).

The optimal temperature range required by coffee is between 15 and 30ºC (Maximum day temperatures should not exceed 30ºC and night temperatures should not fall below 15°C) (Kathurima *et al.,* 2013). Otherwise, low or wide daily temperature variation may result in yellowing, distortion and cracking of leaves and tips. Soils should be free draining up to a depth of at least 1.5 m and 3 m in drier areas, fertile and slightly acidic (pH range 4.4-5.4) **(**Coffee Research Foundation, 2010a). In Kenya, coffee is mainly grown in three regions that include: 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 109, 000 hectares of land under coffee, the East of Rift Valley region accounts for about 82%, West of Rift Valley for 17% and the Taita Hills for only 1 % as shown in Table 2.1 (Kathurima *et al.,* 2013).

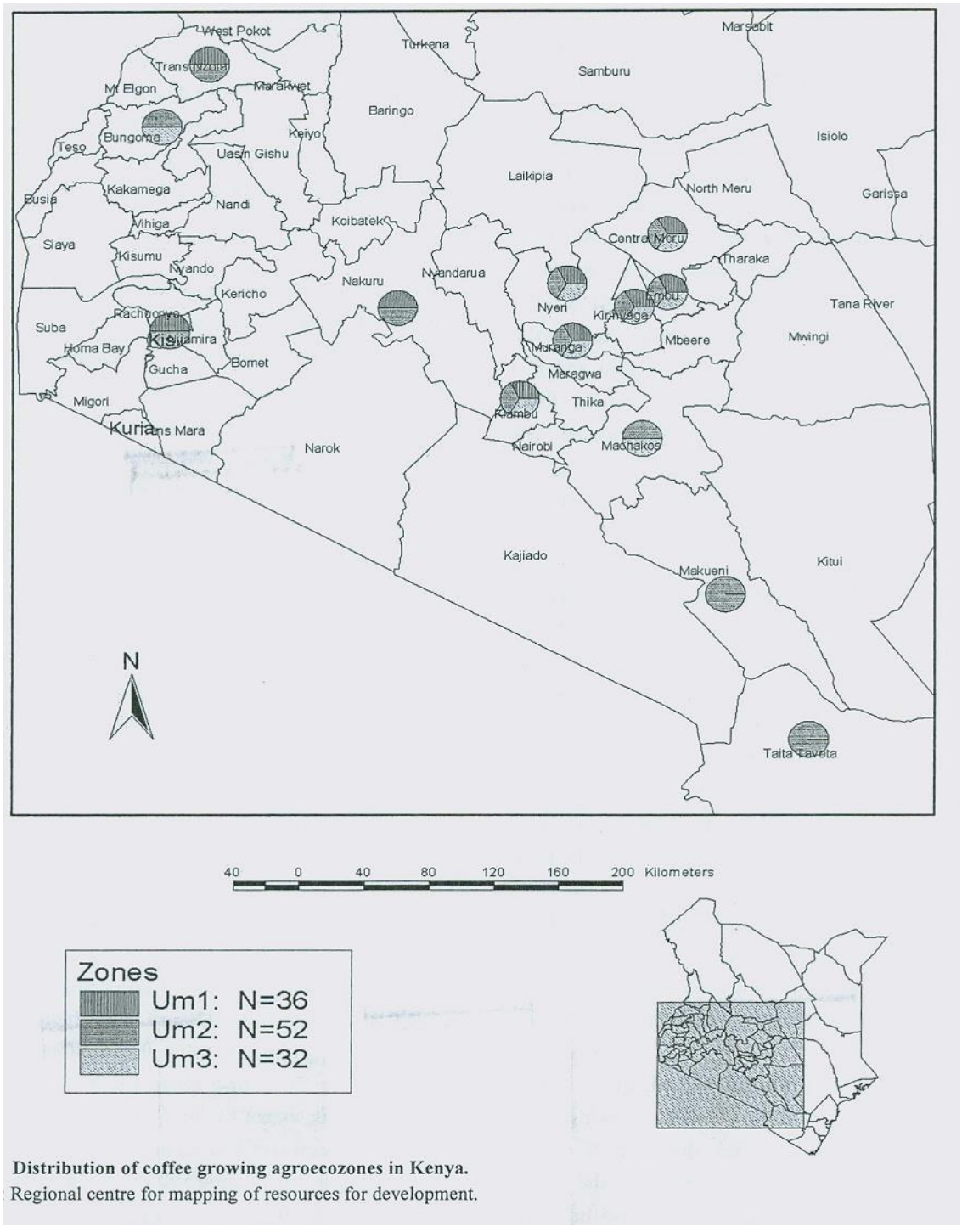


Figure 2.1: Coffee growing agro ecological zones in Kenya. (Source: Mugo, 2012)

Table 2.1: National coffee data (New estimates and current official data)

	County	Estates	Smallholders / Co-operatives	Total Area under coffee	Estates	Smallholders	Total Area under coffee
1	Baringo	-	662	662	-	662.00	662.00
2	Bungoma	-	6,010	6,010	-	6,010.00	6,010.00
3	Embu	336	6,392	6,728	336	8,499.00	8,835.00
4	Kakamega & Vihiga	-	488	488	-	2,650.00	2,650.00
5	Kericho, Nandi Hills	-	2,751	2,751	-	1,961.00	1,961.00
6	Kiambu	12,814	9,990	22,804	12,814	13,258.00	26,072.00
7	Kirinyaga	385	6,108	6,493	385	8,678.00	9,063.00
8	Kisii & Nyamira	-	5,548	5,548	-	5,550.00	5,550.00
9	Machakos	1,877	6,303	8,180	1,877	8,230.00	10,107.00
10	Makueni	-	1,711	1,711	-	1,701.00	1,701.00
11	Meru	640	7,452	8,092	640	25,317.00	25,957.00
12	Migori & Homa Bay	-	1,329	1,329	-	1,811.00	1,811.00
13	Murang'a	660	12,022	12,682	660	14,029.00	14,689.00
14	Nakuru	1,216	299	1,515	1,216.00	430.00	1,646.00
15	Nyeri	1,106	11,236	12,342	1,106	11,977.00	13,083.00
16	Taita/Taveta	-	80	80	-	80.00	80.00
17	Tharaka-Nithi	-	3,756	3,756	-	8,880.00	8,880.00
18	Trans-Nzoia, Keiyo & Marakwet	2,349	293	2,642	2,349	142.00	2,491.00
	Total	**21,383**	**82,430**	**103,813**	**21,383**	**119,865.00**	**141,248.00**

(Source; Coffee Research Foundation- Economics section, 2014)

2.4 Propagation of Coffee

C. arabica cultivars are usually propagated by seed since it is generally believed that *arabica* coffee is sufficiently true breeding. The viability of seeds is short lived and it is advisable to plant the seeds within two months of harvesting. This is because older seeds take longer to germinate and could lose viability (Clarke & Macrae, 1988). The seeds are germinated in propagators and transferred to the field after 6-12 months (Clarke & Macrae, 1988).

Vegetative propagation methods are applicable to coffee, including cuttings, grafting and tissue culture. In Kenya, vegetative propagation in coffee is done predominantly on the disease resistant cultivar, Ruiru 11. The stages in vegetative propagation include; establishment of clonal garden where selected mother plants are established in the field, construction of a propagator, rooting of cuttings, grafting and top-working which is a kind of grafting done on established traditional Arabica varieties root stock to elite varieties scions like of Ruiru 11. Propagation by cuttings is applied when few genotypes need to be propagated in large numbers. Grafting is a preferred over other methods when a small number of plants are needed from each genotype. The merit of grafting over cuttings is the vigor given to the scion by the rootstock (Clarke & Macrae, 1988), or to produce the grafts with strong rooting systems that can be drought tolerant or disease resistant and help in nutrient absorption (Bittenbender *et al.*, 2001). *In vitro* methods have also been used for propagation by two ways; micro-cutting or somatic embryogenesis. This multiplication approach is able to produce a great number of plantlets but has the limitation of requiring refined techniques and chemical media (Clarke & Macrae, 1988).

2.5 Production and economic importance of coffee

Commercial production of coffee depends on two major species, *Coffea arabica* L and *Coffea canephora* Pierre which account for 65% and 35% of production respectively. Coffee production by exporting countries has been on a gradual rise (Table 2.2). The coffee market is dominated by a few large producing countries. As of 2006, the top three producers in terms of export volume were Brazil (30%), Vietnam (15%) and Colombia (12%). Brazil is the largest Arabica producer in the world, while Vietnam, a relatively

new entrant is now the world's largest Robusta producer (International Coffee Organization, http://www.ico.org).

Table 1.2: Coffee production by exporting countries, 2008 – 2013

	2008	2009	2010	2011	2012	2013
Total production by exporting countries (000 bags).	129,260	123,023	133,065	132,207	145,323	145,194
Production in Kenya (000 bags).	541	630	641	757	875	750

(Source: International Coffee Organization, 2014)

In Kenya, the worst coffee production performance occurred in fiscal year 2007/08 due to adverse weather and the post-election crisis therefore contracting by 5.4% compared with a positive growth of 2.1% attained in 2007 as illustrated in Table 2.3 (Economic survey, 2011). Prices of fertilizers and other farm inputs also increased, further aggravating the situation in the coffee sector. Currently, Kenya coffee prices are on the rise following a rise in the global prices of Arabica and specialty markets, which Kenyan coffee happens to fall (Economic review of agriculture, 2010). Other estimates are that six million Kenyans are employed directly or indirectly in the coffee industry, (Howden & Daniel, 2012). Despite the observed decline in coffee yields, the average prices paid for 100 kg of clean coffee doubled in 2010 (Economic survey, 2011). The traditional export destinations for the Kenya coffee have been Germany (30%), Benelux (Belgium, Netherlands, and Luxembourg) (12%) USA and Canada (11%), Sweden (7%), Finland (6%) and UK (6%). However, in 2009, 3 new emerging markets were identified which included China, Japan and Russia (Economic survey, 2011).

Table 2.2: Coffee Production in Kenya, 2005 – 2009

YEAR		2005	2006	2007	2008	2009
Production-Estates	Area (ha)	42,000	42,000	42,000	40,680	53,344
	Tons (ha)	20,745	21,257	21,257	19,740	24,650
Production- Small Holders	Area (ha)	128,000	128,000	128,000	122,040	106,656
	Tons (ha)	24,500	27,046	27,046	22,260	29,370
Yield (tons/ha)Total crop area (ha)	Tons	0.5	0.5	0.5	0.5	0.5
	Estates	0.2	0.2	0.2	0.2	0.3
Total crop area (ha)		170,000	170,000	170,000	162,720	160,000
Total Production (tons)		45,245	48,303	53,368	42,000	54,020
Price of processed coffee (per 100 kg)		11,824	10,952	10,952	-	-
Local Consumption (tons)		1,810	1,932	1,932	1,680	1,341
Exports (million Kshs.)		8,225	8,704	8,704	9,790	10,850
Total Value (billion Kshs.)		8.33	8.7	8.7	-	-

(Source: Coffee Board of Kenya, 2010)

2.6 Commercial coffee varieties in Kenya

Kenya is mostly an Arabica coffee producing country. Coffee was introduced in Kenya by missionaries at the beginning of the 20th century. The first plantations were established at Bura in the low lying coastal region of the Kenya, but because of unfavorable climatic conditions, coffee growing was relocated to higher altitudes of Kibwezi and Kikuyu. Among the first variety to be introduced and grown commercially was French Mission Coffee (Lashermes *et al.,* 1999). It is believed that almost 70% of Kenya's coffee comes from the Scott laboratory (SL) variety series root stocks, which are the main progenitors of yield and quality characters in breeding programs. Currently, varieties such as Blue Mountain, French Mission, Bourbon, Rume Sudan, Catimor, Pretoria and Hibrido de Timor (HDT) are used as sources of genes linked to resistance against Coffee Berry Disease (CBD), but Catimor and (Blue mountain) K7 are sources of resistance to leaf rust (Coffee Research Foundation, 2010a).

The main commercially grown varieties in Kenya include; SL 28, SL 34, K7, Ruiru 11 and Batian; SL 28 was selected at National Agricultural Research Laboratories (NARL) - Kabete, as a single selection from a drought resistant tree from Tanzania in 1931. The variety is best suited for medium to high altitude coffee-growing zones. It has predominantly green shoot tips, but occasionally bronze types can as well be observed. It has bold beans with fine liquor and is susceptible to coffee diseases such as, Coffee Berry Disease (CBD), Coffee Leaf Rust (CLR) and Bacterial Blight of Coffee (BBC) (Coffee Research Foundation, 2010a).

SL34 was bred from a single selection of French mission trees at Loresho estate in Kabete. It has a bronze shoot tip with few green tip strains. It produces high yields of high quality coffee in a range of climates and altitudes but susceptible to CBD, CLR and BBC (Coffee Research Foundation, 2010a).

K7 was selected from one of two French mission trees on Legetet estate-Muhoroni. It has narrow and copper leaf tips. It also has good bean liquor and quality. Ruiru 11 (F_1 hybrid) was released in 1985; the prefix "Ruiru" designates the location where it was bred. It has

broad and bronze to dark green tip leaves. It is resistant to both CBD and CLR and it also produces high yields of high quality coffee (Coffee Research Foundation, 2010a).

Batian is a tall variety and was released in 2010. It compares closely to SL28 variety. It has a bronze leaf tip and berry size is larger than that of SL28. The latter two varieties (Ruiru 11 and Batian) have quality attributes similar to the traditional varieties, like resistance to CLR and CBD, early ripening and high yields in a range of agro-ecological zones (Coffee Research Foundation, 2010a).

2.7 Constraints to coffee production in Kenya

Around the world, approximately 350 different pests infect coffee (Mugo, 2012). In Kenya, decline in coffee production from 130,000 metric tons in 1988/89 to 50,000 metric tons currently is due to; low international market coffee prices as a result of the collapse of the price support mechanism under International Coffee Agreement (ICA) in 1989, high cost of farm inputs, unfavorable weather conditions, pests and diseases. Pests cause losses of up to 15%, pathogens up to 13% and weeds up to 13% in Kenya (Mugo, 2012). The pests that attack coffee include arthropods, pathogenic micro-organisms and weeds (Mugo, 2012). Control of pests and diseases is the most important constraint to sustainable economic production of *C. arabica* in Kenya. The most important coffee diseases in Kenya are; Coffee leaf rust (CLR) (*Hemileia vastatrix*), Coffee Berry Disease (CBD) (*Colletotrichum kahawae*) and Bacterial Blight of Coffee (BBC) (*(Pseudomonas syringae Pv. Garcae*). CLR is also known as orange leaf rust. Orange patches appear on lower surface of the leaf and cause low photosynthetic capacity, defoliation, reduced vegetative and berry growth thus affecting the crops yield (Coffee Research Foundation, 2010a).

CBD is also known as green berry anthracnose. Infected berries show dark sunken pits that spread to cover the whole berry, infecting the bean thus affecting quality. BBC also referred to as Elgon/Solai dieback, attacks healthy parts of a plant leading to death of leaves, twigs, and young nodes resulting in loss of entire crop under severe conditions.

Important coffee insect pests attack berries, foliage and roots. The leaf miner, giant looper and Antestia bug attack foliage and cause yield losses in the long run. The berry

borer attacks berries leading to loss of quality of the beans. Root knot nematodes, especially *Meloidogyne* sp and the root mealy bug attack roots of the crop causing failure of beans to ripen thus loss of quality. These pests and diseases can be controlled by chemical application, introduction of resistant varieties or biological and integrated pest management (IPM) strategies (Coffee Research Foundation, 2010a).

2.8 Coffee Berry Disease (CBD)

At first, the pathogen was thought to be closely related to the fungus *Colletotrichum coffeanum* from Brazil (Noack, 1901) which causes leaf spots on Arabica coffee. However, the new disease in Kenya produced anthracnose-like symptoms on green berries. Rayner, (1950) named the pathogen *C. coffeanum var. virulans* to differentiate between leaf and berry symptoms. Several authors through morphological and pathogenicity research from 1960s to 1990s settled on the name *C. kahawae*, which represented the Kiswahili word for coffee as the species name (Waller *et al.*, 1993).

Gibbs, (1969) carried out intensive investigations on the *Colletotrichum* population in coffee and four distinct species occurring in close association with CBD on coffee were described; *Colletotrichum coffeanum* (*Var. virulans*), had slow growth, grayish-black mycelia and conidia are borne directly on hyphae; *Colletotrichum coffeanum* pink (CCP) type also has slow growth, pink mycelia and conidia are borne directly on hyphae; *Colletotrichum coffeanum* mycelia (CCM) type has fast growing profuse pace aerial mycelia and conidia are borne directly on hyphae; and *Colletotrichum coffeanum acutatum* (CCA) has a moderate growth rate, sparse, pale and aerial mycelia, conidia are produced from acervuli. In a comparative study on a range of *Colletotrichum* isolates Waller *et al.* (1993) using morphological, pathological and biochemical criteria, differentiated the CDB pathogen from other known similar *Colletotrichum* strains which occurred on coffee and suggested that it belonged to a different species which was then called *C. kahawae* species. Genus *Colletotrichum* in which the species belong is classified into *Eumycota*, which is a major subdivision of the *deutromycotina*, in the class *coelomycetes*, order *melanciniales* and family *melanconiaceae* (Owaka, 2011).

C. kahawae infect all stages of the coffee tree from flowers, ripe fruits and occasionally leaves. Maximum crop losses occur from infection of green berries with the formation of dark sunken lesions which sporulates causing their premature dropping and mummification. In the absence of berries and buds, the fungus survives in the maturing bark of secondary branches. The fungus does not attack mature coffee beans. The fungus does not spread in the berry when it attacks mature coffee beans. CBD losses mostly occur during early infestation where it destroys the beans or prevents proper wet and dry processing since the pulp cannot be completely removed causing 'stinkers' in the crop and therefore reducing the quality. CBD may cause up to 70-80% losses if no control measures are immediately adopted (Silva *et al.,* 2006).

2.9 CBD infection

The pathogenesis of *Colletotrichum* diseases is varied. This is due to nutritional and ecological diversities within the genus (Latunde-Dada, 2001). Mostly, the early stages of fungal development on the plant surface are essentially the same for all *Colletotrichum* species. Fungal conidia adhere to the cuticle and germinate producing germ tubes. Thereafter, appressoria are formed which penetrate the cuticle directly (O´Connell *et al.,* 1996, 2000). Appressorium maturation involves the formation of a penetration pore in the base of the cell, deposition of new wall layers, and secretion of extracellular matrix materials. Subsequently, melanin is deposited in a layer of the cell wall adjacent to the cell membrane. Bailey *et al.* (1992) suggested that fungal penetration into the hosts cells may be due to mechanical pressure exerted by melanized appressoria, the secretion of cutin degrading enzymes, or by a combination of both processes. The *C. kahawae* conidia germinate and differentiate into melanized appressoria both "*in vitro*" and "*in vivo*" and penetrate different coffee organs (hypocotyls, leaves and young green berries). Following penetration, *Colletotrichum* species use two main strategies to successfully colonise host tissues and avoid host defense responses: sub-cuticular intramural colonization and intracellular colonization (Figure 2.2) (Silva *et al.,* 2006).

Figure 2.2: Coffee berries infected with CBD

2.10 Variability of *C. kahawae*

Research on CBD has provided valuable information on the variability of the pathogen. In Kenya, many different coffee genotypes were tested with local *C. kahawae* isolates and differential pathogenicity was never observed as suggested by Van der Vossen *et al.* (1976). Moreover, differential interactions between host and pathogen population were never found in Ethiopia as positive effects were negligible and it was improbable they were caused by gene-for-gene specificity (Van der Graaff, 1981). Rodrigues *et al.* (1992) and Várzea *et al.* (1993) suggested the evidence for the existence of physiologic races of *C. kahawae* fungus. Further studies indicated differences only in the aggressiveness in *C. kahawae* isolates (Manga *et al.*, 1998). Omondi *et al.* 1997 and Omondi *et al.*, 2000 working with local Kenyan isolates inferred that variation in pathogenicity among isolates of *C. kahawae* was predominantly due to their aggressiveness with negligible differential pathogenicity. However, there was no positive evidence of physiologic races

for *C. kahawae*. Many authors agree that aggressiveness of the fungal population show differential variability between isolates from the same or different geographic origins (Firman & Waller, 1977; Omondi *et al.*, 1997; Manga, 1998; Varzea *et al.*, 2002).

Molecular studies carried out by Bieysse et al. (unpublished data - INCO-project ICA4-CT-2001-10008) using microsatellite markers analyzed 140 strains collected from all the geographical zones (Cameroon, Kenya, Burundi, Tanzania, Rwanda, Zimbabwe, Malawi, Angola, and Ethiopia). It was inferred that the strains studied might be classified into two groups namely; East African and Cameroon strains. The isolates showed a strong homogeneity in each geographical population suggesting a clonal multiplication of the pathogen. To detect patterns of population genetic structure and dispersal in a phylogeographical analysis Silva *et al.* (2010) used a new set of gene markers from *fl-tubulin gene 2 (fl-tub2), Internal Transcribed Spacer (ITS)* and *mating type gene (MAT1-2-1)* genes on more than fifty isolates of *C. kahawae* from nine geographical locations. To allow further inferences about the evolutionary history and origin of *C. kahawae*, additional sampling of *C. gloeosporioides* and other closely related taxa from coffee hosts was assembled. They found low genetic variability as had been previously detected in *C. kahawae.* A slight but consistent genetic structure of the sampled populations seemed to be correlated with their geographical location. The phylogenetic analysis studies revealed that populations from Angola and Cameroon are ancestral and that East African populations such as those in Kenya are derived from Angola and Cameroon (Silva *et al.*, 2010).

2.11 Inheritance of resistance to CBD

Genetic resistance appears to be partial in *C. arabica* and complete in *C. canephora*. Studies on Arabica coffee carried out in Kenya concluded that coffee resistance to CBD appears to be controlled by major genes on three different loci (Van der Vossen & Walyaro, 1980). Rume Sudan, the highly resistant variety carries the dominant *R*- and the recessive *k*-genes. Pretoria variety also has the *k*-gene. The Blue Mountain (K7) variety, a moderately resistant variety carries only the recessive *k*-gene. Hibrido de Timor carries

one gene for CBD resistance on the *T*- locus. Robinson (1974) and Van der Graaff (1981, 1985) suggested that CBD resistance is horizontal/quantitative. Ameha and Belachew (1982) suggested that 3-5 recessive genes control resistance to CBD in non-introgressed *C. arabica.* Host resistance to CBD is of a quantitative nature, but it can be complete in some Arabica coffee genotypes. There is no consensus on the genetics of CBD resistance and other authors have described oligogenes (1-3 major genes) and polygenes as determinants of CBD resistance (Van der Vossen & Walyaro, 2009).

2.12 Cytological and biochemical CBD resistance mechanisms

Coffee berry disease resistance (CBD) mechanisms to *C. kahawae* in Arabica coffee are both preformed and induced, and operate at distinct stages of pathogenesis (Gichuru, 1997). The coffee berry cuticle could act as a physical barrier to the penetrating pathogen. Investigations on the occurrence and possible role of preformed antifungal compounds in the cuticle in relation to CBD resistance have been carried out, although the chemical nature of these compounds was not identified. Nutman and Roberts (1960) found that extracts from the resistant variety Blue Mountain had a stimulatory effect on the infection process in contrast to what happens with the susceptible variety Harar. Steiner (1972) concluded that surface wax extracted with chloroform from green berries of Rume Sudan and Blue Mountain contained substances that significantly decreased conidial germination and may contribute towards the high levels of field resistance to *C. kahawae* shown by these varieties. Lampard and Carter (1973) also reported the presence of antifungal compounds in the cuticular wax layers of green berries of coffee and they found a correlation between the degree of activity of cuticular wax extracts from many cultivars of Arabica coffee and their field resistance to *C. kahawae*. According to Masaba and Van der Vossen (1982), the resistance to CBD in Arabica coffee may, to a certain extent, be based on the formation of cork barriers. Phellogen was rapidly formed in some cells below the infection site and the progress of the fungal invasion was blocked by a complete barrier of suberised cells. These cork barriers corresponded macroscopically to the scab lesion, the common macroscopic expression of resistance to CBD (Gichuru, 1997). The identification of the phenolic compounds involved in the coffee - *C. kahawae*

interaction is currently under study and, preliminary results revealed an accumulation of flavonoides and hydroxycinnamic acid derivatives at infection sites. These compounds were detected earlier in resistant and partially resistant genotypes than in susceptible ones (Silva *et al.,* 2006).

2.13 Breeding for resistance to CBD

Differences in resistance of coffee trees to CBD are frequently observed both under field and laboratory conditions. In Kenya, Geisha 10, Blue Mountain (K7), Rume Sudan, and also some Hibrido de Timor (HDT) progenies have and are still being used to breed for CBD resistance (Gichimu *et al.,* 2014). High levels of resistance were found in Rume Sudan by Firman and Waller (1977) and Van der Vossen (1985). Hybridization programmes with the objective of combining both high yield and resistance to CBD and coffee leaf rust (CLR) in Kenya and Tanzania started after outbreaks of CBD in Eastern Africa. From different geographic origins, thousands of progenies of different coffee genotypes were tested against CBD isolates. However, no coffee genotypes showed 100 % resistance to all the isolates used in the hypocotyl pre-screening tests. Some lines of Rume Sudan and Hibrido de Timor (HDT) derivatives showed relatively high levels of resistance to the majority of the studied isolates. Some derivatives of interspecific tetraploid hybrids from different geographical origins were also found to have intermediate levels of resistance (Silva *et al.,* 2006). Progenitors for coffee disease resistance in other coffee growing countries were Hibrido de Timor, Rume Sudan, Kaffa and Geisha which were crossed to varieties like; SL 28, SL 34, N 39, KP 423 and H 66 which have high and good yields (Walyaro, 1983). In Zambia, a pure line, likely derived from the Colombia variety, showed a high level of resistance compared to Caturra (Teri *et al.,* 2004). Approximately 80 % of crop losses were observed when chemical control was not applied with these latter varieties while losses corresponded only to 15-20 % for the pure line derived from the Colombia variety. Eight new clones with high levels of resistance have been selected and multiplied vegetatively by somatic embryogenesis in Tanzania (Teri *et al.,* 2004) whereas in Kenya, the composite hybrid Ruiru 11 exhibits a

good level of resistance and so does the recently released Batian coffee variety (Gichimu & Omondi, 2010).

2.14 Biotechnological tools for coffee improvement

Coffee is a perennial tree crop that is difficult to improve through traditional plant breeding methods. Conventional coffee breeding methods face considerable difficulties due to limitations such as the long generation time of coffee trees, the high cost of field trials and a lack of accuracy in data collection (Etienne *et al.,* 2002). Coffee trees come into bearing four or five years after the cross is carried out. Recent advances in molecular genetics, when combined with conventional breeding, represent powerful tools for genetic improvement of coffee. The development of various biotechnological approaches such as micro-propagation techniques, embryo rescue, anther culture, molecular markers and Marker Assisted Selection (MAS), genetic map development and genetic transformation have tremendous potential for genetic improvement of coffee (Etienne *et al.,* 2002). These techniques could overcome the limitations of traditional plant breeding and greatly enhance the efforts of coffee breeders (Chaparro *et al.,* 2004). Molecular markers have advanced coffee germplasm characterization as well as our understanding on how plant genes confer specific desirable traits and more importantly transfer genes from unrelated plant species to coffee. Biotechnology will be useful in circumventing genetic barriers and accelerate release of varieties with superior traits (protected from biotic and abiotic stresses). Molecular analysis of *C. arabica* cultivars could provide knowledge on levels of genetic variation and the genetic relatedness between genotypes which can improve the efficiency of utilization of current germplasm resources. Furthermore, genetic data are important for designing effective plant breeding programs, by influencing the choice of parental genotypes to cross for the development of new populations (Coffee Research Foundation, 2010b).

2.14.1 Genetic markers for coffee improvement

These markers are either phenotypic (based on the morphology) or genotypic (based on molecular/genetic make-up) the coffee. Despite the enormous economic importance of *C.*

arabica to coffee producers, there has been little on-going research on genetic diversity in this species and its cultivated varieties. Detecting genetic variation has been hampered by *C. arabica's* limited genetic origins and self-pollination, compounded with historical and selective bottlenecks, that is susceptibility to pests and diseases. The application of molecular techniques could greatly enhance future coffee genetic improvement programs (Chaparro *et al.,* 2004) since genetic factors are more accurately tested by molecular markers (Lashermes *et al.,* 1996). Molecular techniques have been used in genetic studies of coffee including genetic diversity, identification of introgression fragments, genetic map development and cloning of economically important coffee genes (Etienne *et al.,* 2002).

Detection of genetic variation at DNA level has been made possible by the advent of molecular markers. Several DNA analysis techniques have been used in coffee studies. The techniques differ in aspects such as; technical requirements, cost, sequence specificity and repeatability. Random Amplified Polymorphic DNA (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 contrary, 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 reproducible (Kathurima *et al.,* 2011).

2.14.2 Random Amplified Polymorphic DNA (RAPD) in Coffee

Randomly Amplified Polymorphic DNA (RAPD) is a PCR-based marker system 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 makers have been used to confirm the relationships within the genus *Coffea* (Orozco Castillo *et al.,* 1996), to construct a linkage map in coffee (Lashermes *et al.,*

1996), to detect markers for resistance to coffee berry disease (Agwanda *et al.,* 1997) and coffee leaf rust (Rani *et al.,* 2000), and to study genetic diversity amongst wild accessions (Chaparro *et al.,* 2004) and cultivated varieties (Crochemore *et al.,* 2004). Apart from coffee, RAPDs have been used to; study of low correlation between genomic and morphological introgression estimates in Walnut backcross (Woeste *et al.,* 1998), linkage map of peanut based on a backcross population between two diploid species *Arachis stenosperma* and *A. cardenasii* (Garcia *et al.,* 2005) and genetic diversity in the *Gossypium.arboreum* cultivar germplasm (Azamat & Khan, 2010).

2.14.3 Microsatellites (SSR) in coffee

Microsatellites are also known as Simple Sequence Repeats (SSRs) and consist of segments of DNA containing tandem repeats typically of simple motif sequences of one to six bases. These microsatellite repeats are often flanked by unique sequences, occurring only once in the genome (Glaubitz & Moran, 2000). Microsatellites have been used in diversity studies in different crops; in the analysis of genetic diversity and population structure of rice cultivars from Korea, China and Japan (Zhao *et al.,* 2009), to characterize maize germplasm of India (Sharma *et al.,* 2010) and in the genetic diversity and population structure analysis of strawberry (*Fragaria* x *ananassa* Duch.) (Min-young yoon *et al.,* 2012). Microsatellites have also been applied in coffee to identify *C. arabica*, *C. canephora* and related species (Combes *et al.,* 2000). They have also been used to investigate polymorphisms among wild and cultivated *C. arabica* accessions (Moncada & Couch, 2004) and to analyze the introgression of DNA fragments from *C. canephora* and *C. liberica* into *C. arabica* (Lashermes *et al.,* 2010). Gichuru *et al* (2008) were able to identify a microsatellite marker, Sat 235, which was linked to CBD resistance and mapped it onto an introgressed *C. canephora* fragment which harbors the responsible Ck-1 gene using F_2 plants (*cv* Catimor × *cv* SL28) that were resistant and susceptible to CBD respectively. Gichimu *et al.* (2014) also used the same SSR marker Sat 235 in the study of, occurrence of the Ck-1 gene conferring resistance to Coffee Berry Disease (CBD) in *Coffea arabica cv*. Ruiru 11 and its parental genotypes. SSRs are powerful tools for following specific genes in assisted cross programmes (Rovelli *et al.,* 2000).

2.15 Molecular approaches to coffee breeding

The advent of DNA variations based on genetic markers and breeding approaches like Marker Assisted Selection (MAS) has provided hope and possibility for coffee genetic improvement programs. In coffee breeding these developments are impeded by; long generation cycle of 4–6 years for seed to seed generation, the narrow genetic base associated with Arabicas, the unavailability of true-to-type inbred lines or homozygous lines for the diploid genotypes because of the difficulty in selfing. Also, ploidy level variation (Arabicas are tetraploids and Robustas are diploids) and incompatibility barriers (robustas possess gametophytic self-incompatibility system), reproductive barriers for interspecific crossing due to infertility, low frequency of intergenome crossing over and zygotic counter selection, poor knowledge genetics base of coffee traits, the unavailability of multiple genetic markers, DNA marker based linkage maps with transferable landmarks across different laboratories, the unavailability of suitable screening tools/diagnostic markers for diseases and pathogens in correlation to continuous evolution of new pathotypes (Aggarwal & Hendre, 2007).

Conscious and concerted efforts have commenced globally to integrate DNA/molecular markers based technologies, which have provided impetus, dependability and direction to the efforts on coffee genetic improvement. Moreover, molecular markers linked to disease resistance provide the potential to screen for resistance in a large population of plants at any stage of plant development (Aggarwal & Hendre, 2007; Hindorf & Omondi, 2007).

The potential of DNA marker based technologies is well demonstrated (Aggarwal & Hendre, 2007). The DNA marker based technology is being utilized for coffee genetic improvement programmes such as genotyping, germplasm finger printing, construction of linkage maps, Quantitative Trait Loci (QTL), varietal identification as well as claiming intellectual property rights, and mapping in concert with Marker Assisted Selection (MAS) breeding to finally develop genetically improved coffee with desirable traits. The development and availability of coffee specific SSR markers have paved way for coffee

germplasm characterization. The merits attributable to SSRs over other marker approaches are in their ability to more efficiently detect the inherent low genetic variability of *C. arabica* (Aggarwal *et al.*, 2004; Moncada & Couch, 2004). Therefore, DNA marker technologies which provide high-genetic resolution have become important and thus the need to integrate them in research on genetics and improvement of coffee. This is in research areas such as; identification of Qualitative and Oligogenic Trait Loci and mapping in concert with Marker Assisted Selection (MAS) breeding coffee genotyping, construction of more coffee linkage maps, varietal identification and germplasm fingerprinting (Aggarwal, 2004).

Accordingly, these technologies will impact coffee research in future. However, its going to be a long way before visible gains become realized because of the slow pace of coffee research. There are now large coffee genomics and transformation programs underway in many countries. Few prominent countries being, Brazil, France, Italy, Columbia and India, where transformation studies are also being used to circumvent genetic barriers posed by *coffea* sp (Aggarwal & Hendre, 2007).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Screening for resistance to CBD

3.1.1 Study site

The study was carried out at the Kenya Agricultural and Livestock Research Organization, - Coffee Research Institute (KALRO-CRI) situated at Ruiru in Kiambu county which lies within the Upper Midland 2 agro-ecological zone (UM 2) at latitude 1º06'S and longitude 36º 45'E and is approximately 1620m above sea level (a.s.l). The area receives a mean annual rainfall of 1063 mm and the mean annual temperature is 19ºC (minimum 12.8ºC and maximum 25.2ºC). The soils are classified as humic nitisols and plinthic ferrasols. They are well drained, deep reddish brown, slightly friable clays interrupted occasionally with sections of murram. The soil pH ranges between 5 and 6 (Kathurima *et al.,* 2013).

3.1.2 Plant materials

C. arabica parental genotypes;

i.) Rume Sudan - Introduced in Kenya from Boma plateau in Sudan and resistant to CBD at the dominant *R*- and the recessive *k*- loci (Figure 3.1)

ii.) SL 28 - Selected at National Agricultural Laboratories (former Scott laboratories, Kenya). It is highly susceptible to CBD and Coffee Leaf Rust (CLR) and high yielding. It has good cup quality and adaptable to local conditions (Figure 3.2).

iii.) One F_1 progeny from a cross between Rume Sudan and SL 28 coffee cultivars (RS × SL 28).

iv.) Forty nine F_2 progenies selfed from F_1 progenies (RS × SL 28) × (RS × SL 28).

The above genotypes used are in *Ex-situ* gene banks apart from SL 28 which is grown on commercial plots at KALRO-CRI in Ruiru, Kiambu County.

Figure 3.1: Rume Sudan coffee tree

Figure 3.2: SL28 coffee tree

3.1.3 Isolation of *Colletotrichum kahawae* in the laboratory

In order to obtain *C. kahawae* inoculum, detached green coffee berries infected with CBD at the black lesion stage were collected from infected SL 28 mature trees in upper Kiambu County. The berries were washed in a conical flask using 0.01% of liquid soap and rinsed twice with double-distilled water and incubated at 24°C for 48 hrs in Petri dishes containing moist cellulose wadding to promote sporulation. A culture from each berry was obtained by inoculating on malt extract agar (MEA: BD Diagnostics, Franklin Lakes, NJ, USA) and subsequently sub-cultured on the same media. Confirmatory tests of *C. kahawae* isolates on MEA were based on mycological colour chart developed by Rayner (1970), where *C. kahawae* colonies usually have a grayish colony texture. After a period of 24 hrs, advancing hyphae and germinating conidia of each isolate were re-isolated and sub-cultured on 3.4 MEA (oxoid) containing 0.04% streptomycin to eliminate any bacterial growth. Each isolate was inoculated in 5 replicates and incubated at room temperature for 10 days. *C. kahawae* colonies from two uncontaminated petri dishes from the five replicates were mixed together in a conical flask using double distilled water. They were thoroughly shaken to mix the colonies and break the solid MEA from which the colonies were embedded. Using a muslin cloth, the suspension was sieved to isolate the *C. kahawae* spores and later standardized to a concentration of $2x10^6$ conidia/ ml using a haemocytometer through counting and serial dilutions.

3.1.4 Collection of green coffee berries from trees

A total of 52 mature coffee trees were randomly selected in their respective plots for green berries collection and evaluation of CBD resistance (Table 3.1). The mature coffee trees were also selected on the basis of green berry maturity (3 months). Parental coffee genotypes, Rume Sudan and SL 28, one F_1 progeny and 49 F_2 progenies were tagged in order to avoid collecting data from the same tree more than once and for collection of green berries.

Table 3.1: List of coffee genotypes evaluated for CBD resistance

Lab serial No;	Genotypes		Source/plots
1	Rume Sudan	KALRO-CRI	Plot 4
2	SL 28	"	Plot 3
3	F1 49	"	Plot 14
4	F2 8	"	Plot 16
5	F2 9	"	Plot 16
6	F2 13	"	Plot 16
7	F2 14	"	Plot 16
8	F2 15	"	Plot 16
9	F2 16	"	Plot 16
10	F2 18	"	Plot 16
11	F2 20	"	Plot 16
12	F2 22	"	Plot 16
13	F2 25	"	Plot 16
14	F2 26	"	Plot 16
15	F2 27	"	Plot 16
16	F2 28	"	Plot 16
17	F2 29	"	Plot 16
18	F2 30	"	Plot 16
19	F2 33	"	Plot 16
20	F2 44	"	Plot 16
21	F2 46	"	Plot 16
22	F2 49	"	Plot 16
23	F2 72	"	Plot 16
24	F2 75	"	Plot 16
25	F2 76	"	Plot 16
26	F2 77	"	Plot 16
27	F2 78	"	Plot 16
28	F2 80	"	Plot 16
29	F2 82	"	Plot 16
30	F2 83	"	Plot 16
31	F2 86	"	Plot 16
32	F2 87	"	Plot 16
33	F2 92	"	Plot 16
34	F2 93	"	Plot 16
35	F2 97	"	Plot 16
36	F2 98	"	Plot 16
37	F2 99	"	Plot 16
38	F2 104	"	Plot 16
39	F2 105	"	Plot 16
40	F2 108	"	Plot 16
41	F2 110	"	Plot 16
42	F2 111	"	Plot 16
43	F2 115	"	Plot 16
44	F2 116	"	Plot 16
45	F2 117	"	Plot 16
46	F2 121	"	Plot 16
47	F2 122	"	Plot 16
48	F2 123	"	Plot 16
49	F2 124	"	Plot 16
50	F2 125	"	Plot 16
51	F2 454	"	Plot 16
52	F2 468	"	Plot 16

3.1.5 Inoculation of berries with *C. kahawae*

Thirty expanding berries that were 15 weeks old (Pinard *et al.*, 2012) from the 49 F_2 trees, one F_1 tree and parental trees; Rume Sudan and SL 28 were collected during the mid-week of March 2014. The berries were picked randomly from bottom, middle and top of the coffee tree in order to have a representative sample. Clean plastic boxes were partially filled with distilled water and a grid supporting absorbent paper was placed inside to bear the berries and create a humid atmosphere. The berries were cleaned with liquid soap (0.01%), rinsed twice with double-distilled water and dried with a sterile cotton cloth. The wounded stalk end of the berries was removed with a sterile scalpel to avoid contamination and to limit the development of saprophytic fungi. A total of 10 berries per genotype were placed in 3 rows in each box (Figure 3.3) with three replications per genotype and arranged in a completely randomized block design. Each replication had 19 plastic boxes and the berries were inoculated with a freshly prepared CBD inoculum from a standard CBD pathogen isolate on MEA in the laboratory.

A *C. kahawae* inoculum concentration of $2x10^6$ conidia/ml was prepared and 20 µl of suspension were deposited on the berries using a pipette while shaking time to time when drawing the inoculum. The boxes were sealed to provide the saturated humid conditions necessary for disease development. Control treatments of Rume Sudan and SL 28 were inoculated with sterile distilled water. The sterile inoculation room was maintained at 21°C. After 24 hrs post inoculation, the drops of *C. kahawae* suspension were blotted with a sterile absorbent paper to prevent spilling and infecting adjacent berries. Regular opening after every three days was done for 10 min to allow for aeration of the berries.

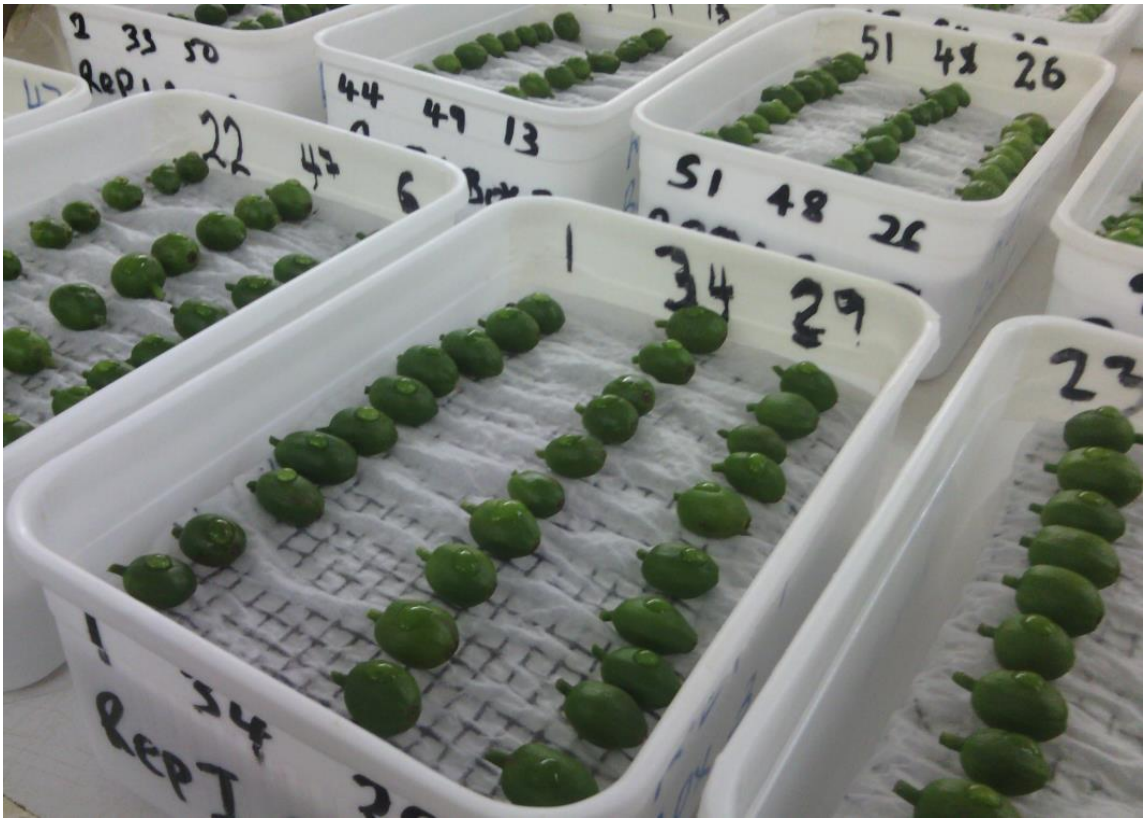


Figure 3.3: Berry inoculation with *C. kahawae*

3.1.6 Data Collection and Evaluation

The first infection data collection was performed seven days post inoculation since it is the period when CBD symptoms are visible (Pinard *et al.*, 2012). Other data collections were performed after every three days for two weeks for CBD assessment using a berry inoculation sheet to determine CBD progression. CBD development was assessed and scored using a visual scale from 0% to 100% of the total berry surface affected on a scale of 1-5, where;

0-5% = 1, 6-10% = 2, 11-25% = 3, 26-50% = 4 and 51-100% = 5.

A score range of ≤2 was considered resistant while that of ≥3 was regarded to be susceptible in accordance with their standard deviations. After scoring each coffee berry individually, average infection (AI) on each genotype across the replicates was calculated as follows:

$$AI = \Sigma\,[Ir1 + Ir2 + Ir3 + \cdots Irn]/N$$

Where, **I** is the sum of disease score; *n* is the number of replication; **I***rn* is the sum of disease score in replication *n; N* is the total number of berries scored in the replications.

The last data at the end of 3 weeks period was used for downstream analysis. Scored data was subjected to analysis of variance (ANOVA) using XLstat software 2014 version and effects declared significant at 5% level using Fisher (Least Significance Difference) method. For data analysis, the F_1 and F_2 coffee genotypes showing a score that was not significantly different from Rume Sudan were considered to be resistant (R) while the rest were considered to be susceptible (S). Segregation data was analyzed by the chi-square ($\chi 2$) test. All chi-square analysis was calculated using the formula, $\chi 2 = (O - E)^2 / E$, where O is the observed value and E is the expected value. Each chi-square value was considered to be significant ($P \leq 0.05$) (3.84).

3.2 Identification of DNA Markers Linked to CBD resistance

3.2.1 Leaf Sampling

Disease-free leaves from the tagged total 52 mature trees; Parental coffee genotypes, Rume Sudan and SL 28, one F_1 progeny and 49 F_2 progenies were randomly picked from first and second nodes from the growing tips of coffee branches. The leaves were packed individually as per the genotypes and taken to the molecular laboratory for genomic DNA extraction.

3.2.2 Extraction of Genomic DNA

Genomic DNA was extracted from the fresh leaf material by the method of Diniz *et al.* (2005) with minor modifications, where mixed alkyltrimethylammonium bromide (MATAB) was used instead of cetyltrimethylammonium bromide (CTAB). Five grams of the leaves were macerated in liquid nitrogen. One ml each of lysis and extraction buffers (Appendix 2) was added to the powder in the mortar. The macerated tissue was distributed into two 1.5 ml tubes and incubated at 65°C in a water bath for 30 min with regular shaking. After incubation, 1ml of chloroform/ isoamylalcohol mixture in the ratio of 24:1 was added to each tube, then mixed gently by shaking and

centrifuged at 13000 rpm for 10 min in a micro-centrifuge. The supernatant was pipetted out into clean 1.5 ml tubes. Twenty µl of RNase were added to the supernatants and incubated at 37°C in a water-bath for 30 min to remove RNA. An equal volume of isopropyl alcohol was added into each tube and mixed gently by inverting the tubes several times to precipitate DNA. The mixture was centrifuged at 13000 rpm for 5 min to obtain DNA pellets after the supernatant was carefully discarded. The DNA pellets were then washed with 200 µl of 70% ethanol and centrifuged at 13000 rpm for 3 min. The ethanol was drained by decanting and the pellets dried in a vacuum centrifuge for 20 min. The pellets were dissolved in 40 µl of Tris-EDTA (TE) buffer and stored at 4°C.

3.2.3 Quantification of DNA

One percent (1%) agarose gel in 0.5X Tris Boric Ethylenediaminetetraacetic acid (TBE) was prepared by adding 0.7 g of Agarose to 70 ml 0.5X TBE and weighed. The solution was then heated in a microwave at short intervals of 15-30 sec with occasional shaking until it boiled and became clear. Due to evaporation that occurs during heating, the solution was weighed again, after which water was added to obtain the original weighed volume and left to cool to about 55^0C.

The gel was then poured on the tray of the mini electrophoresis unit (MUPID) to solidify and bubbles were removed after which the combs were fixed and the gel allowed to set. After solidifying, 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 500 µg/ml). The lambda preparation mixture was heated at 65^0C for 10 min and immediately chilled on ice for 5 minutes before use. After five minutes, 10 µl of lambda and 12 µl of sample DNA preparations were loaded onto the 1 % agarose gel and run at 50 V for 45 min. The gel was stained in 1mg/ml Ethidium Bromide (50 µl of 10 mg/ml Ethidium Bromide in 500 ml dH_2O) for 20 minutes and placed into the UV transilluminator to be

viewed and photographed. Lambda preparation table was used to estimate the quantity of DNA.

3.2.4 PCR Amplification with SSR primers

PCR reactions were performed in a final volume of 25 µl containing 5.4µl of double distilled water; 10 ng (10 ng/µl) of template genomic DNA, 2.5 µl of 10X PCR buffer (16 mM $MgCl_2$, Dongsheng), 1.0 µl of $MgCl_2$ (25 mM, Dongsheng), 3.75 µl of dNTPs (500 µM, Eurogentec), 1.0 µl each of forward and reverse Primer (10 µM, Eurogentec), 0.3 µl of Taq DNA polymerase (5 U/µl, Dongsheng) (Appendix 3). Amplification was carried out in a Eurogene thermocycler (TECHNE, UK). The SSR amplification program started with one cycle of initial denaturation at 94°C for 5 minutes followed by 35 cycles of 45 seconds at 94ºC (denaturation), 30 seconds at 55ºC for primer annealing, and 90 seconds at 72ºC for elongation. The final extension was done at 72ºC for 10 minutes and final hold at 4ºC. Twelve SSR primer pairs were used for DNA PCR amplification (Table 3.2). Selection of these primers was guided by previous work done by Omondi and Pinard (2006) for M 24 primer and Lashermes *et al.* (2010) for other primers. For electrophoretic analysis, the PCR products were mixed with 3.0 µL 6X loading dye (0.2 % bromophenol blue, 0.2 % xylene cyanol dye and 30 % glycerol in a Tris-EDTA buffer). A ladder was loaded in the first well to confirm the allele sizes. Ten µl of amplification products were loaded in the sample wells and electrophoresis was run using a 2.3 % agarose gel in 1X TBE buffer (0.05 M Tris, 0.05 M boric acid, 1 mM EDTA, pH 8.0) for 3 hours. Bands were visualized and photographed on a UV- Transilluminator after ethidium bromide staining for 30 minutes.

Table 3.2: SSR forward and reverse primer sequence used in the PCR analysis

Locus	Forward primer (5'-3').	Reverse primer (5'-3').
Sat11	ACCCGAAAGAAAGAACCAA	CCACACAACTCTCCTCATTC.
Sat32	AACTCTCCATTCCCGCATTC	CTGGGTTTTCTGTGTTCTCG.
Sat172	ACGCAGGTGGTAGAAGAATG	TCAAAGCAGTAGTAGCGGATG.
Sat207	GAAGCCGTTTCAAGCC	CAATCTCTTTCCGATGCTCT.
Sat227	TGCTTGGTATCCTCACATTCA	ATCCAATGGAGTGTGTTGCT.
Sat229	TTCTAAGTTGTTAAACGAGACGCTTA	TTCCTCCATGCCCATATTG.
Sat240	TGCACCCTTCAAGATACATTCA	GGTAAATCACCGAGCATCCA.
Sat254	ATGTTCTTCGCTTCGCTAAC	AAGTGTGGGAGTGTCTGCAT.
Sat255	AAAACCACACAACTCTCCTCA	GGGAAAGGGAGAAAAGCTC.
Sat262	CTGCGAGGAGGAGTTAAAGATACCAC	GCCGGGAGTCTAGGGTTCTGTG.
Sat283	GCACACACCCATACTCTCTCTT	GTGTGTGATTGTGTGTGAGAG.
M 24	GGCTCGAGATATCTGTTTAG	TTTAATGGGCATAGGGTCC.

3.2.5 Data Recording and Analysis

Alleles were scored based on the parental and F_1 bands. The plants that showed a pattern similar to the resistant parent alleles were scored as (1) and those with a banding pattern similar to the susceptible parent alleles were scored as (0), and the heterozygous plants were scored as (2).

Segregation data were analyzed by the chi-square ($\chi 2$) test. The chi-square analysis for the genotypic and phenotypic ratio was calculated using the formula, $\chi 2 = (O - E)^2 / E$, where O is the observed value and E is the expected value. Each chi-square value was considered to be significant ($P \leq 0.05$) (3.84).

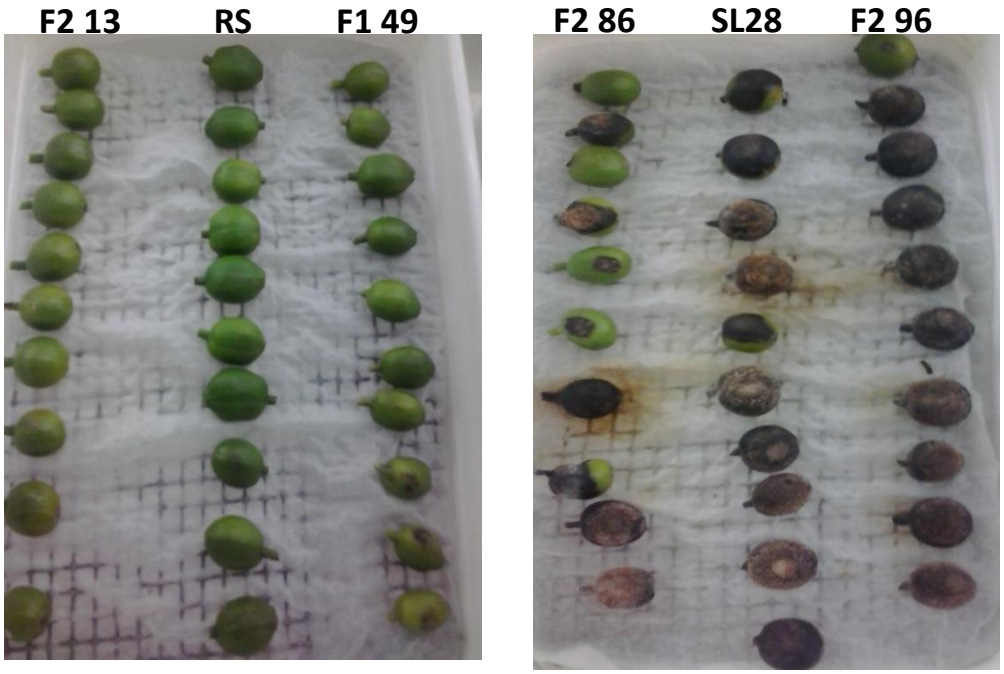
Using the SSR binary data, Agglomerative Hierarchical Clustering (AHC) method of Neighbor-Joining (NJ) analysis was used to construct a dendogram. Jaccard coefficient was used to check on the dissimilarity using Artemis 5.0 Software.

CHAPTER FOUR

RESULTS

4.1 Resistance to CBD in the F_2 population

CBD infection was expressed as black lesions on different genotypes, some were not infected by the pathogen. The susceptible genotypes had their berry surfaces entirely covered by black sunken lesions. Rume Sudan, F_1 and some F_2 genotypes formed restricted black scab lesions that hindered further penetration of the pathogen into the intercellular parts of the berry hence, resistance to CBD (Figure 4.1).



A **B**

Figure 4.1: Coffee berries of Rume Sudan (RS) (resistant) (A), SL 28 (susceptible) (B) and an F_1 and F_2 progenies inoculated with conidia of *Colletotrichum kahawae* after 21 days at 21°C.

Coffee berry disease progression on the genotypes over three weeks was of roughly linear. Disease severity increased with increasing time from the day seven to day 21, when the last scoring was done as shown in Figure 4.2.

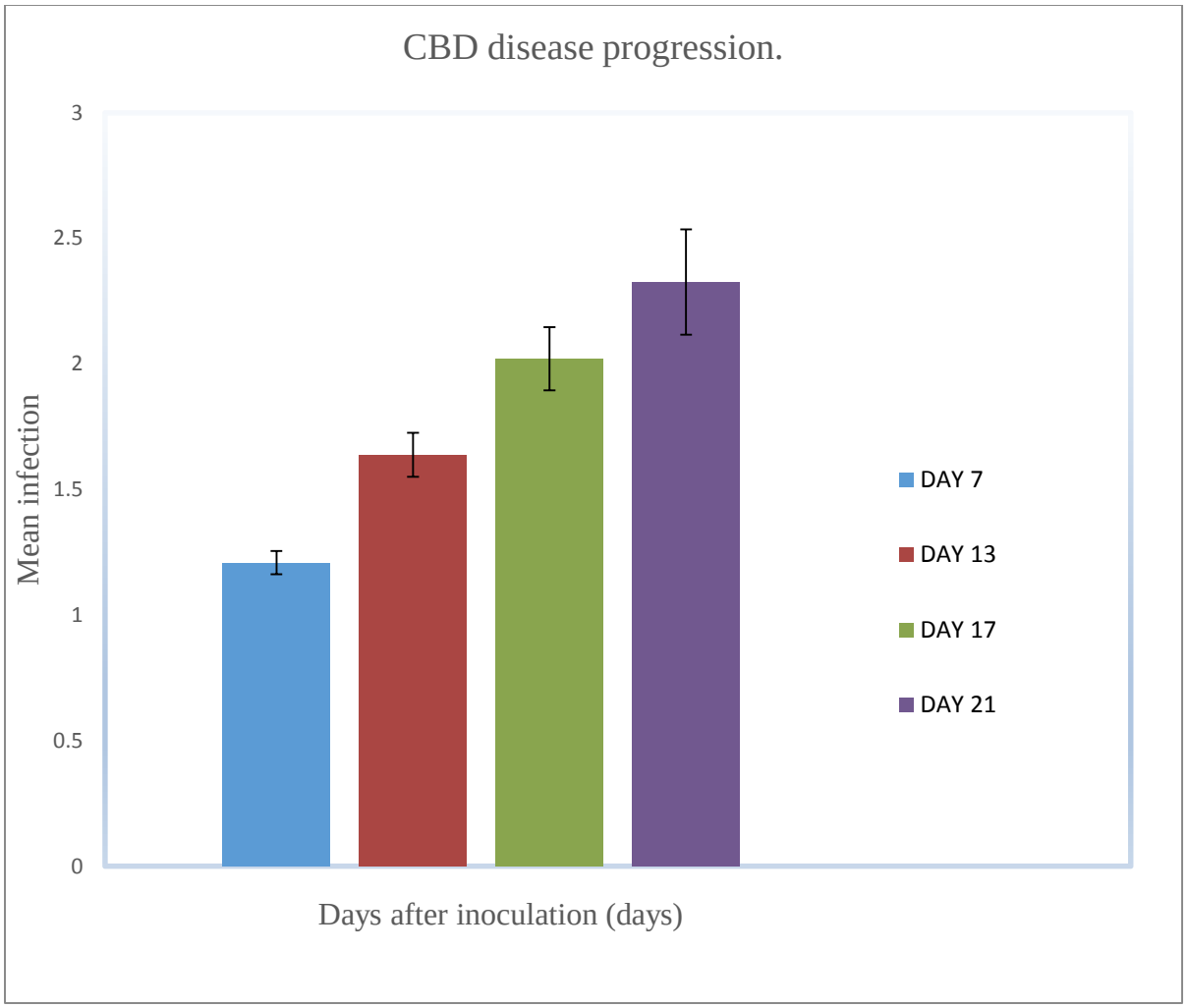


Figure 4.2: Coffee berry disease progression

Analysis of variance indicated that the effect of genotypes was highly significant ($p \leq 0.0001$). The genotype means were separated using Fisher (Least significance difference $LSD_{5\%}$) method as illustrated in Table 4.1. There were 33 F_2 genotypes in the resistant class and 16 F_2 genotypes in the susceptible class. Rume Sudan recorded an average infection score of 1.87, F_1 tree had 1.80 and SL 28 had 4.57.

Table 4.1: Variation in CBD infection among F_2 genotypes

Genotypes	Mean score CBD infection	Genotypes	Mean score CBD infection
Rume Sudan	1.867^{k-o}	F_2 78	3.100^{d-g}
SL 28	4.567^{a}	F_2 80	2.300^{h-m}
F_1 49	1.800^{k-p}	F_2 82	1.700^{k-p}
F_2 8	2.733^{f-j}	F_2 83	3.733^{b-d}
F_2 9	1.567^{m-p}	F_2 86	2.200^{h-n}
F_2 13	1.133^{o-p}	F_2 87	1.900^{k-n}
F_2 14	1.067^{p}	F_2 92	2.933^{e-h}
F_2 15	1.700^{k-p}	F_2 93	2.000^{j-n}
F_2 16	2.333^{h-l}	F_2 97	1.733^{k-p}
F_2 18	1.800^{k-p}	F_2 98	4.367^{a-b}
F_2 20	1.800^{k-p}	F_2 99	1.867^{k-o}
F_2 22	2.333^{h-l}	F_2 104	1.967^{k-n}
F_2 25	1.633^{l-p}	F_2 105	1.733^{k-p}
F_2 26	3.533^{c-e}	F_2 108	1.600^{l-p}
F_2 27	1.833^{k-o}	F_2 110	2.767^{f-i}
F_2 28	1.933^{k-n}	F_2 111	2.000^{j-n}
F_2 29	3.300^{c-f}	F_2 115	3.333^{c-f}
F_2 30	3.333^{c-f}	F_2 116	3.533^{c-e}
F_2 33	1.533^{n-p}	F_2 117	3.467^{c-f}
F_2 44	1.567^{m-p}	F_2 121	1.467^{n-p}
F_2 46	2.400^{g-k}	F_2 122	1.833^{k-o}
F_2 49	1.833^{k-o}	F_2 123	2.033^{i-n}
F_2 72	4.300^{a-b}	F_2 124	1.733^{k-p}
F_2 75	1.833^{k-o}	F_2 125	2.733^{f-j}
F_2 76	1.633^{l-p}	F_2 454	4.033^{a-c}
F_2 77	1.500^{n-p}	F_2 468	2.033^{i-n}

Means followed by the same letter(s) within the column are not significantly different at $P \leq 0.05$ according to Fisher (LSD) method. (=MEAN 2.3265, =STDEV 0.8906). **Key**: The hyphen (-) represents the alphabetical range between the letters.

The effect of replication was non-significant ($p \leq 0.05$) and interaction between replications and genotypes was also non-significant ($p \leq 0.05$).

There were also visual observations on the resistant F_2 genotypes having restricted scab lesions. The restricted scab lesions never progressed on the berry surfaces of these resistant F_2 genotypes during CBD development up to the last day of data collection (21 days) as shown in Figure 4.3.

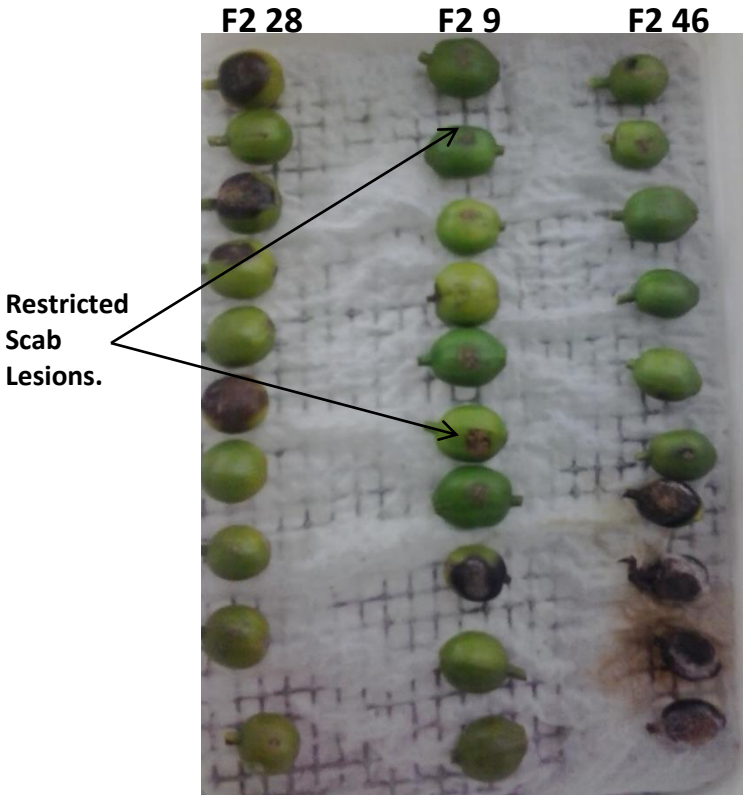


Figure 4.3: Presence of restricted scab lesions on a resistant F_2 genotype inoculated with conidia of *Colletotrichum kahawae* after 21 days at 21°C.

The F_2 coffee genotypes showed that resistance in this population segregated in a 3:1 ratio for major gene effect for plants with resistance and susceptibility as illustrated in Table 4.2.

Table 4.2: Observed and expected segregation ratios of resistant and susceptible berries in the F_2 generation from a cross between the Rume Sudan × SL 28 inoculated with *C. kahawae.*

Generation	Pathogenicity assay			$\chi 2$ (3:1)	P
	Category	Observed number.	Expected number		
F_2	Resistant	33	36.75		
	Susceptible	16	12.25		
	Total	49	49	1.5307*	0.25

d.f. = 1.0; $\chi 2$ (0.05, 1) = 3.84. Significantly different (*).

4.2 DNA Markers linked to CBD resistance

4.2.1 Marker segregation analysis

Among the 12 SSR markers used, six markers showed polymorphism among two parents, F_1 and F_2 populations but could not discriminate among parental genotypes F_1 and F_2 progenies as illustrated by (Figure 4.4).

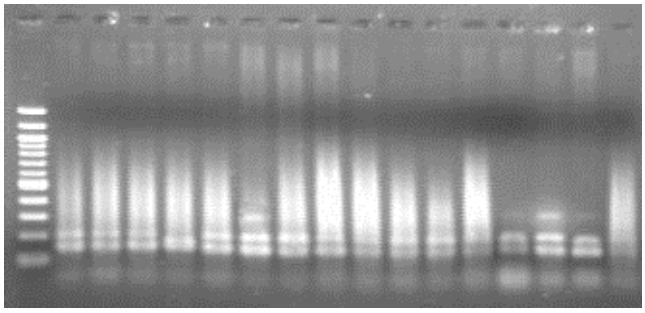


Figure 4.4: SSR marker polymorphism on parental genotypes, F_1 and F_2 progenies

Two SSR markers, M 24 and Sat 227 showed polymorphism between susceptible and resistant parents and corresponding F_1 and F_2 genotypes upon permutative testing indicating their possible association with CBD resistance in the segregating population. Therefore, the F_2 population was genotyped with these two markers to study their possible association with CBD resistance.

Segregation study with marker M 24 recorded an allele of resistant parent (~210 bp) amplified in 16 F_2 plants, whereas an allele of susceptible parent (~180 bp) was amplified in 12 F_2 plants. (Table 4.3 and Figure 4.5). Twenty-one F_2 plants exhibited both the alleles (heterozygous) like the F_1 plant. Genetic analysis with chi-square test indicated "goodness-of-fit" to the expected ratio of 1:2:1.

Table 4.3: Evaluation of the F_2 population with SSR marker M 24

Category	Observed genotype	Expected genotype	χ2(1:2:1)	P
Resistant	16	12.25		
Heterozygote	21	24.50		
Susceptible	12	12.25		
Total	49	49	1.6684*	0.10

Significantly different (*).

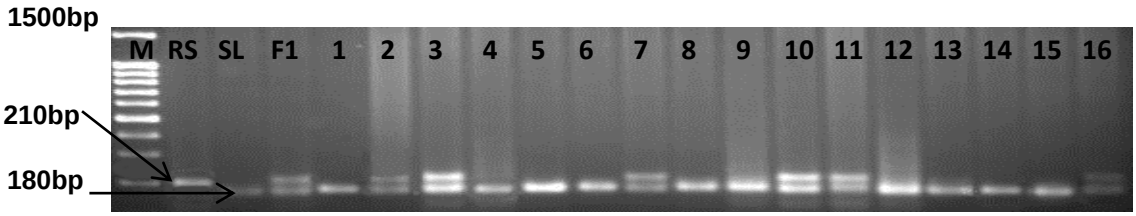


Figure 4.5: DNA banding patterns in an F_2 population of coffee derived from a cross between Rume Sudan (RS) × SL 28 (SL) for SSR marker M 24. M = 100-bp ladder; RS= Rume Sudan; SL= SL 28; F_1 and lanes 1-16 = F_2 progenies.

Segregation study with marker Sat 227 recorded an allele of resistant parent (~200 bp) amplified in 11 F_2 plants, whereas an allele of susceptible parent (~220 bp) was amplified in 17 F_2 plants. (Table 4.4 and Figure 4.6). Twenty-one F_2 plants exhibited both the alleles (heterozygous) like the F_1 plant. Genetic analysis with chi-square test indicated "goodness-of-fit" to the expected ratio of 1:2:1 for single gene model indicating the

association of Sat 227 with CBD resistance gene. The ratio 1:2:1 observed by the co-dominant SSR markers corresponds to the 3:1 ratio observed in the phenotypic data.

Table 4.4: Evaluation of the F_2 population with SSR marker Sat 227

Category	Observed genotype	Expected genotype (1:2:1)	$\chi 2$	P
Resistant	11	12.25		
Heterozygote	21	24.50		
Susceptible	17	12.25		
Total	49	49	2.4694*	0.10

Significantly different (*).

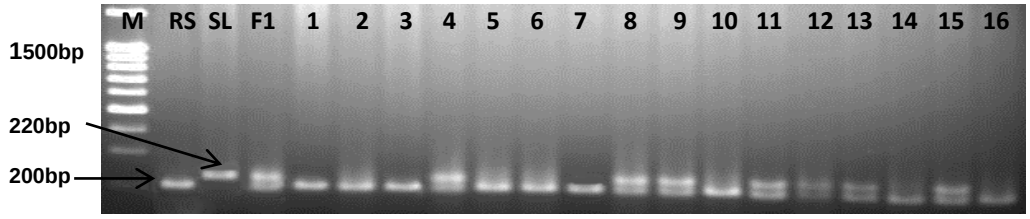


Figure 4.6: DNA banding patterns in an F_2 population of coffee derived from a cross between Rume Sudan (RS) × SL 28 (SL) for SSR marker Sat 227. M = 100-bp ladder; RS= Rume Sudan; SL= SL 28; F_1 and lanes 1-16 = F_2 progenies.

The dendrogram constructed using SSR markers, M 24 and Sat 227 binary data was used to determine genetic diversity of the F_2 genotypes. The F_2 genotypes separated into three main clusters. The F_2 genotypes clustered depending on the parental genotypes; Rume Sudan and SL 28 and the F_1 genotype banding patterns in regard to *C. kahawae* resistance. Cluster one consisted of F_2 genotypes that resembled F_1 banding pattern (colour green). Cluster two consisted of two sub-clusters that were closely related

comprising of those F_2 genotypes that resemble Rume Sudan banding pattern (colour red). Cluster three also consisted of two sub-clusters that were closely linked and comprised of those genotypes that resembled SL 28 banding pattern (colour blue) as illustrated in Figure 4.7.

Figure 4.7: Dendogram showing clustering of parental genotypes,F1 and F2 progenies

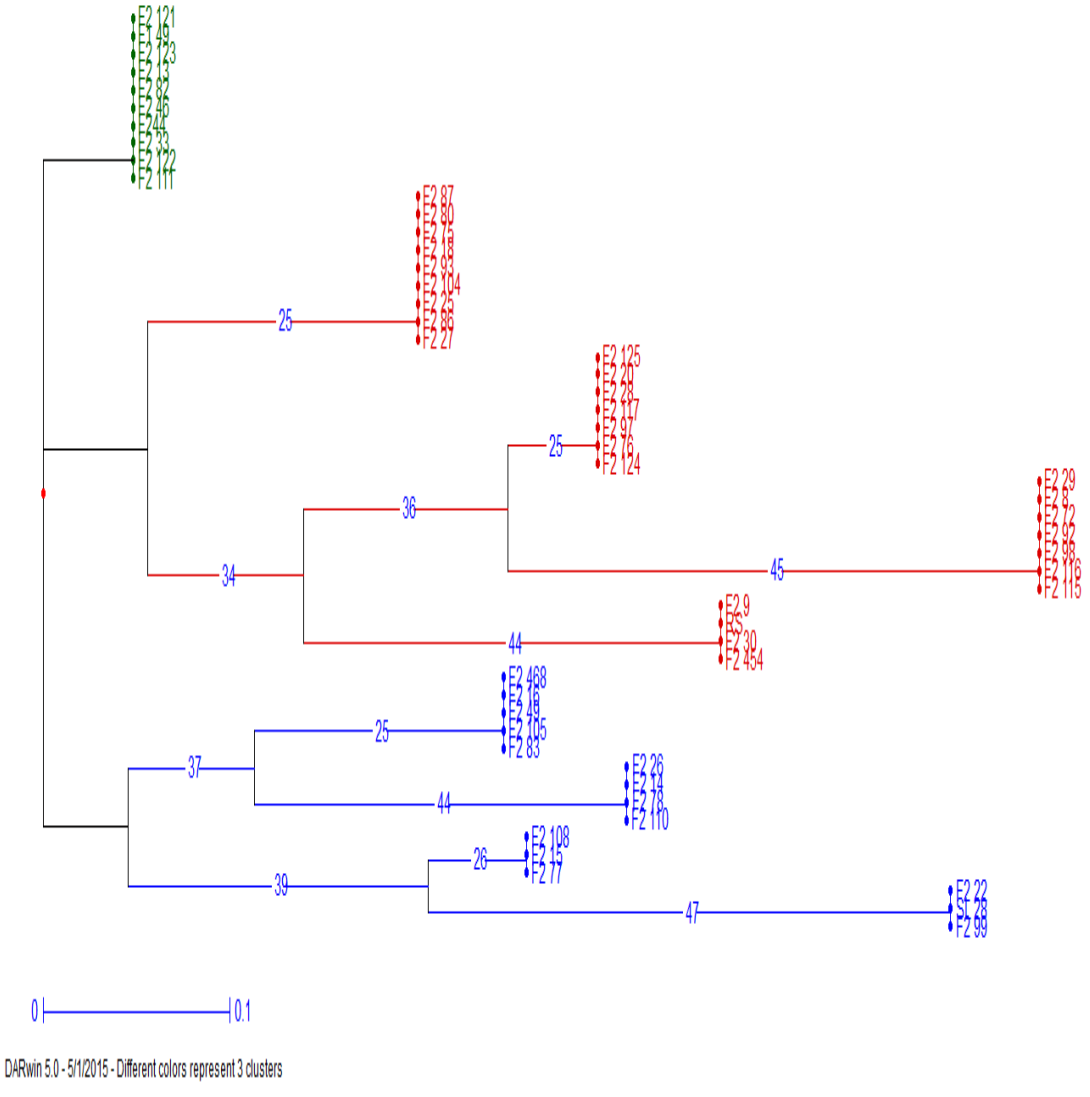


Figure 4.7: Clustering of parents, F_1 and F_2 progenies using markers M 24 and Sat 227

CHAPTER FIVE

DISCUSSION

5.1 Phenotypic analysis of the F_2 population

Detached green berry inoculation test was used to screen for resistance against CBD. The inoculation of detached berries under artificial conditions offer the merit of direct assessment of *C. arabica* and *C. kahawae* interaction at fruit level, making the characterization of resistant genotype under field conditions (Bock 1956, Pinard *et al.*, 2012). Screening for CBD resistance was successful as there was phenotypic diversity in that, there were genotypes that were resistant (statistically similar to Rume Sudan) and other genotypes susceptible (statistically similar to SL 28).

Coffee berry disease progression was of a roughly linear nature (allowing for some minor deviations which were considered random error). This can be attributed to the fact that *C. kahawae* under the prevailing favourable conditions of temperature and humidity was able to infect on host genotype tissues. It can also be concluded to be because of *C. kahawae* aggressiveness. This was because the infected green berries used for inoculum preparation was collected at a time when CBD disease pressure was high, hence presence more of *C. kahawae* isolates. According to Omondi *et al.* 1997 and Omondi *et al.*, 2000) working with local Kenyan *C. kahawae* isolates concluded that variation in pathogenicity was predominantly due to the pathogen population aggressiveness.

Variation for resistance to *C. kahawae* among F_2 population of a cross between coffee cultivars, Rume Sudan (resistant) and SL 28 (susceptible) was mainly due to segregation. This conclusion was supported by the fact that the main effect of genotypes was significant ($P \leq 0.0001$). The F_2 progenies segregated into two groups; 33 of them had disease scores that were statistically similar to the resistant parent, Rume Sudan and F_1 progeny. Sixteen F_2 genotypes exhibited scores that were similar statistically to the susceptible parent, SL 28. It can be concluded that the resistance genes in Rume Sudan were passed onto the resistant F_2 progenies. The presence of these resistance genes in Rume Sudan at the dominant R- and recessive k- loci were previously reported by Van

der Vossen and Walyaro (1980). Hence, Rume Sudan has been used for multiple cross programs with other varieties since it confers resistance to CBD.

Visual observation showed that Rume Sudan CBD lesions compared to the susceptible SL 28 had not progressed on the entire berry surface, meaning infection took place but its progression was curtailed by resistance in Rume Sudan. This was due to the antifungal compounds in the cuticular wax layer of the Rume Sudan berries. This is in agreement with studies reported by Steiner (1972) that surface wax extracted with chloroform from green berries of Rume Sudan and Blue Mountain contained substances that significantly decreased conidial germination and may contribute towards the high levels of field resistance to *C. kahawae* shown by these varieties. Lampard and Carter (1973) reported the presence of antifungal compounds in the cuticular wax layers of green berries of coffee and they found a correlation between the degree of activity of cuticular wax extracts from many cultivars of Arabica coffee and their field resistance to *C. kahawae*. Resistance reaction in the F_2 genotypes to *C. kahawae* was presented as restricted scab lesions. Restricted scab lesions hinder intercellular penetration and further colonization of the CBD pathogen inside the coffee beans. These results agree with the findings of Gichuru (1997) that scab lesions are the common macroscopic expression of resistance to CBD. This resistance to CBD is preformed and induced, and it operates at distinct stages of pathogenesis (Gichuru, 1997). Pinard *et al.* (2012) also came to a deduction that berry resistance could be separated into two types; one against the pathogen penetration and the other against its growth in berries through scab lesion formation. These were the possible explanation why there was phenotypic variation among the F_2 genotypes with regard to CBD resistance. Coffee variety SL 28 is categorized among the CBD susceptible commercial varieties though it has good cup quality and is high yielding (Gichimu *et al.,* 2014).

There was controlled uniformity in the inoculation conditions and therefore the effect of replication and interaction between replications and genotypes were non-significant ($p \leq 0.05$). The uniform conditions were attained by having water inside the closed containers to increase humidity and maintaining temperatures at 21 ^{o}C in the cold room.

Pinard *et al.* (2012) reported that presence of water (rain, mist or dew) on berry surfaces and favorable temperatures between 21 and 23ºC are necessary conditions for infection and development of epidemics. Uniformity was also achieved by using berries that were collected at the same time of maturity, four months post flowering and at their soft stage which is most susceptible to *C. kahawae*. Mulinge (1970) reported that during the first four weeks, the berry does not increase in size instead it remains at the "pinhead". This stage is resistant to CBD. During the next 4-16 weeks after flowering, the berry is expanding and at this stage, it is the most susceptible this is unlike fully expanded green berries, which are resistant. Further uniformity was also attained by standardizing the incubation period to seven days and inoculum concentration to $2x10^6$ spores ml^{-1}. During the course of the experiment, the controls remained symptomless, an indication of the absence of latent infections which are frequent with anthracnose diseases of other fruit tree species.

5.2 Microsatellite markers linked to CBD resistance

Genetic diversity with regard to CBD among F_1, F_2 progenies and the parental genotypes was evidenced by different banding patterns using SSR markers M 24 and Sat 227. The two SSR markers; M 24 and Sat 227 were found to be putatively linked to CBD resistance genes at loci/alleles of ~210bp and ~200bp respectively.

Chi-square analysis of the markers M 24 and Sat 227 in the F_2 population segregated in a simple Mendelian fashion 3:1 (1:2:1) ratio. This was an indication of a single dominant gene effect which was reported by Van der Vossen and Walyaro (1980). Segregation study with marker M 24 and Sat 227 recorded 21 F_2 genotypes each exhibiting both the alleles (heterozygous) similar to the F_1 banding pattern. This was due to the co-dominant nature of SSR markers as suggested by Rovelli *et al.* (2000). Microsatellites offer the merits of identification of many alleles at a single locus, they are also distributed all over the genome and they are co-dominant in nature, which enables distinction of heterozygous samples from homozygotes. SSRs are therefore powerful tools for following specific genes in marker assisted cross programmes. An analysis of the SSR

data using marker M 24 and Sat 227 clearly delineated the F_2 plants into three categories; homozygous resistant, heterozygous resistant and homozygous susceptible.

Chi-square analysis revealed a strong correlation between the phenotypic data and the SSR data. A segregation ratio of 3:1 for resistant: susceptible plants was observed with phenotypic data while SSR data revealed a segregation ratio of 1:2:1 representing homozygous resistant, heterozygous resistant and homozygous susceptible respectively. Due to the co-dominance nature of SSR markers, the resistant genotypes scored by the phenotypic assay were further disaggregated into homozygous resistant and heterozygous resistant with SSR markers, M 24 and Sat 227. The M 24 SSR locus that was putatively associated to CBD resistance in the study was also reported by Omondi *et al.* (2000) who concluded that one of the resistance genes from Rume Sudan might have been carried by the marker which could be used for marker assisted selection for resistance to CBD.

The genetic variation in the F_2 population was observed in the dendogram. The first cluster composed of heterozygotes in F_2 genotypes similar to F_1. The second cluster had two sub-clusters which were closely linked since the confidence level was less than 50% and composed of homozygous resistance F_2 genotypes similar to Rume Sudan. One sub-cluster had Rume Sudan and three F_2 genotypes. It can be concluded that the three F_2 genotypes may be having the R- dominant and k- recessive genes while the rest of the F_2 genotypes in the other sub-cluster only the dominant R- gene alone. The third cluster also had two sub-clusters which were also closely linked since the confidence level was below 50%. Both sub-clusters were composed of homozygous susceptible F_2 genotypes similar to SL 28.

5.3 Conclusion and recommendations

5.3.1 Conclusion

From phenotypic data it showed that resistance to CBD was controlled by one dominant gene that gave a phenotypic ratio of 3:1. From genotypic data the two SSR markers co-segregated with the CBD resistance genes from Rume Sudan in a ratio of 1:2:1. The phenotypic and the SSR data were closely correlated with regard to CBD resistance due

to their respective segregation ratios of 3:1 and 1:2:1 respectively. This indicates that the SSR markers co-segregated with the CBD resistance genes from Rume Sudan, suggesting that the markers and the resistance genes could be putatively linked. The findings of this study could be useful in molecular analysis of large-scale segregating populations, breeding lines and varieties at any stage of development which have Rume Sudan as one of the parents.

5.3.2 Recommendations

1. A linkage analysis to determine the actual distances between the two SSR markers and the resistance genes requires to be done to establish the most suitable marker that can aid in selection for resistance. Since, the more closely linked the marker to the gene, the better it is for Marker Assisted Selection (MAS).
2. SSR analysis appears to be useful in genotyping for resistance. Other robust DNA analysis methods such as Inter-Sequence Simple Repeats (ISSR), Single Nucleotide Polymorphism (SNP), Sequence Related Amplified Polymorphism (SRAP) and Genotyping by Sequencing (GBS) could be also tested in Kenyan Coffee.
3. There is also need to use a larger number of markers to enhance chances of getting more markers linked to CBD resistance and subsequent mapping.

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APPENDICES

Appendix 1: DNA Extraction buffers

(Before use, the buffers were kept for 20-30min at 62 °C).

Extraction buffer*

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

***- The solution was viscous. It was dissolved at 40°C and stored at 4°C**

Lysis buffer

- Sorbitol 0.35 M (6.38 g).
- Tris-HCl 0.20 M (20 ml of 1 M, pH=8.0).
- EDTA 40 mM (1.49 g).
- PVP 2% (2 g) (polyvinyl pyrrolidone, added just before extraction).
  Volume up to 100 ml with distilled water.

Appendix 2: ANOVA Tables

Table 1: ANOVA to determine genotype interaction with *C. kahawae*.

Source	DF	Sum of squares	Mean squares	F	Pr > F
Genotypes	51	117.882	2.311	10.317	< 0.0001
Error	104	23.300	0.224		
Corrected Total	155	141.182			

Computed against model Y=Mean(Y).

Table 2: ANOVA to determine effect of replications on CBD infections.

Source	DF	Sum of squares	Mean squares	F	Pr > F
Reps	2	0.834	0.417	0.455	0.635
Error	153	140.348	0.917		
Corrected Total	155	141.182			

Computed against model Y=Mean(Y)

Table 3: ANOVA of interaction between replications and genotypes.

Source	DF	Sum of squares	Mean squares	F	Pr > F
Genotypes*Reps	154	141.160	0.917	40.739	0.124
Error	1	0.022	0.022		
Corrected Total	155	141.182			

Computed against model Y=Mean(Y)

Appendix 3: Preparation of amplification master mix for SSRs

Master Mix	1Rxn (µl)
Dd H_2O	13.05
Buffer (10 x)	2.50
DNTPs (500 µM)	3.75
Mg^{2+} (25 mM)	2.50
Primer	1.00
Taq (5 U/µl)s	0.20
DNA (1 ng /µl)	2.00
Total	**25.00**

Appendix 4: Manuscript