Detection of Cassava Viruses from Elite Genotypes and Characterization of Cassava mosaic begomoviruses from farmers' fields in Kenya

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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

This work is dedicated to my parents; Mr. Ombiro and Mrs. Moige, brothers; Cliff, Bismarck and sister Cate whose immense love and support made this journey bearable. To my Redeemer, God, glory and honor for the strength, favor and perfect health during the course of this study.

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ABBREVIATIONS

ACMV	*African cassava mosaic virus*
ANOVA	Analysis of Variance
BLAST	Basic local alignment search tool
BecA	Biosciences Eastern and Central Africa
CBSD	Cassava brown streak disease
CBSV	*Cassava brown streak virus*
CMD	Cassava mosaic disease
CMBs	Cassava Mosaic Begomoviruses
CMGs	Cassava Mosaic Geminiviruses
CP	Coat protein
cDNA	Complementary deoxyribonucleic acid
CIAT	Centro International de Agriculture
CTAB	Cetyl trimethyl ammonium bromide
DNTPS	Deoxynucleotides
ELISA	Enzyme linked immune-absorbent assay
EACMV	*East African cassava mosaic virus*
EACMMV	*East African cassava mosaic Malawi virus*
EACMZV	*East African cassava mosaic Zanzibar virus*
EACMCV	*East African cassava mosaic Cameroon virus*
EACMV-UG	*East African cassava mosaic virus Uganda variant*
EDTA	Ethylene diamine tetraacetic acid
FAO	Food and Agricultural Organization
FAOSTAT	Food and Organization Statistics

GPS	Global positioning system
ICV	*Indian cassava virus*
IITA	International Institute of Tropical Agriculture
IgG	Immunoglobulin G
KARI	Kenya Agriculture Research Institute
MM	Master mix
MEGA	Molecular evolutionary genetic analysis
NCBI	National central for biotechnology information
PCR	Polymerase chain reaction
PVS	Participatory variety selection
PPB	Participatory plant breeding
PBS-T	Phosphate buffered saline Tween
RAM-AP	Rabbit anti-mouse alkaline phosphatase
RNA	Ribonucleic acid
RT-PCR	Reverse transcription polymerase chain reaction
RT-qPCR	Reverse transcription-quantitative polymerase chain reaction
SACMV	*South African cassava mosaic virus*
ssRNA	Single stranded ribonucleic acid
SLMV	*Sri-Lanka mosaic virus*
TAS	Triple antibody sandwich
UCBSV	*Ugandan Cassava brown streak virus*

ABSTRACT

Cassava is an important cash crop in the tropics that is recognized as a food security crop for over 300 million people in sub-Saharan Africa. In Kenya, it is grown for income and food covering a spread of 69,169 ha with an output of 892,122 tons. The crop faces several production challenges among which include diseases. The objectives of this study were; to determine the reaction of elite cassava genotypes to infection by Cassava mosaic disease (CMD) and Cassava brown streak disease (CBSD) in Kenya, to determine the geographical distribution and genetic diversity of cassava begomoviruses in Kenya. Five elite cassava genotypes (08/363, F10-30-R2, Nl, Tajirika, Ex-mariakani), susceptible genotype (Kibandameno) and a local check/control (Shibe) were grown under natural disease. The populations of the whitefly vector, *Bemisia tabaci* (Gennadis), severity and incidence of cassava mosaic virus were recorded during the growth period at 3, 6 and 9 months after planting (MAP). Infection was determined through disease severity, incidence, Enzyme linked immunosorbent assay (ELISA) and Polymerase Chain Reaction (PCR) assays. In another study to determine the incidence, symptom severity, prevalence and geographical distribution of cassava begomoviruses, a diagnostic survey was conducted in 2013. A total of 24 fields in Nyanza, 22 in Western, 22 in Coast and 20 in Eastern were sampled. Species specific primers were used in laboratory analysis to detect the viruses present in cassava growing regions of Kenya. The sampled regions included Coast, Nyanza, Eastern and Western areas of Kenya. Genetic diversity of cassava begomoviruses was also done through sequencing. The highest CMD incidence was recorded in Western region with 60.19% and lowest in Eastern with 34.66%. Disease prevalence was highest in Nyanza, Western (100%) and lowest in Eastern (65%) respectively. Significant differences ($p=<0.001$) in CMD severity was recorded amongst the surveyed regions. The highest CMD severity was recorded with a mean of 1.8 in Western and lowest being 1.2 in Coast region of Kenya. PCR detected five begomoviruses namely; East African mosaic virus (EACMV), East African cassava mosaic Ugandan variant (EACMV-UgV), African cassava mosaic virus (ACMV), East African cassava mosaic Kenyan variant (EACMV-KE) and DNA

satellites in the four regions under survey. The highest EACMV detection occurred in Nyanza region with 70.6% and the lowest being Western Kenya that recorded 36.6% detection. In EACMV-KE, the highest detections were recorded in Nyanza region with 26.5%, and lowest in Coast with 4.3%. However, EACMV-KE in this study was not found in Western Kenya. The highest ACMV detections were found in Nyanza with 29.4% with no detection of ACMV in the Eastern region of Kenya. EACMV-UgV was recorded in all regions surveyed in this study with Nyanza province having the highest disease detection with 41.2%. Comparison between coat protein (CP) of the Kenyan EACMV isolates showed that there was wide genetic variability in nucleotide identity forming 2 independent clusters. On the other hand, EACMV-KE Kenyan isolates did not reveal great genetic variability within themselves forming a single clade with bootstrap value of 99%. When compared with sequences in the NCBI database, the Kenyan isolates were closely related to an isolate from Tanzania (AY795988.1). The study showed high cassava mosaic begomoviruses infections in Nyanza, a cause for concern for policy makers. Urgent strategies focusing on this area can be deployed to contain the viruses. In this case strategies such as phytosanitation, breeding and promotion of disease resistant genotypes should be implemented to control crop losses. This study also tested the presence of viruses in elite genotypes through multiple detection methods namely; visual observation, serological and molecular. The Cassava mosaic and Cassava brown streak diseases incidence was highest in Kibanda meno (>70%) and lowest in Shibe and Tajirika. Mean CMD/CBSD severities in the tested genotypes was highest in Kibanda meno (>1.5). Both ELISA and PCR detected CMD and CBSD in asymptomatic and symptomatic cassava samples. Genotypes 08/363 and F10-30-R2 had greater resistance to cassava mosaic and cassava brown streak disease while Kibandameno and Ex-mariakani were highly susceptible. Further tests can also be done focusing on the virus load in order to develop a correlation between symptom severity and virus load in the cassava genotypes.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background information

Cassava (*Manihot esculenta* Crantz) is a member of Euphorbiceae family. Cassava has been known to be one of the staple foods of the African continent (Legg & Fauquet, 2004). The crop roots supply about 500 cal/day of food to over 70 million people in Africa (Chavez *et al.,* 2005). Cassava leaves provide vegetables especially in the dry spells when other vegetables are limiting (FAO, 1993). The cassava leaves can supply vitamin A, C, iron, calcium and protein (Nweke *et al*., 2002). Cassava productivity per unit area is higher compared to sweet potato (*Ipomoea batatas*) and potato (*Solanum tuberosum*) (Scott *et al.,* 2000). According to recent statistics, about 262 million tons of cassava are produced globally with Nigeria being the leading producer having 47 million tons. In East Africa, the leading cassava producer is Tanzania with 4 million tons with Kenya producing 0.9 million tons from an area of 69169 ha (FAO, 2013).

The crop however faces many challenges of abiotic and biotic nature during growth (Ndunguru *et al.,* 2005). Some of the constraints include; pests and diseases, inadequate clean planting materials, varieties with high cyanide levels. Whiteflies and the cassava green mite (*Mononychellus tanajoa*) are the most significant pests affecting cassava production. Key diseases include Cassava Mosaic Disease (CMD) and *Cassava Brown Streak Disease* (CBSD) (Akano *et al*., 2002). Cassava mosaic disease caused by cassava begomoviruses/cassava geminiviruses has been described as the most significant cassava virus disease in sub-Saharan Africa (Legg *et al.,* 2006). The disease leads to stunted plant growth and highly reduced yield (Otim-Nape *et al.,* 2000). CMD is transmitted by whiteflies and perpetuated by stem cuttings used frequently for propagation (Maruthi *et al*., 2002). The CMD complexes in sub-Saharan Africa include

East African cassava mosaic Cameroon virus (EACMCV), *East African cassava mosaic Malawi virus* (EACMMV), *East African cassava mosaic virus (EACMV), African cassava mosaic virus* (ACMV), *East African cassava mosaic Zanzibar virus* (EACMZV) and *Southern Africa cassava mosaic virus* (SACMV) (Fauquet *et al*., 2008).

Cassava brown streak disease (CBSD) on the other hand also endangers cassava production across East Africa and is second to cassava mosaic disease in severity (Hillocks and Jennings, 2003; Rwegasira *et al.,* 2011). Cassava brown streak disease (CBSD) is caused by two distinct but related *Ipomoviruses, Cassava brown streak virus* (CBSV) and *Ugandan cassava brown streak virus* (UCBSV) found in the family *potyviridiae* (Mbanzibwa *et al.,* 2011).

Cassava brown streak disease was first reported at the foot hills of Usambara in Tanzania by Storey (1936) and thereafter in areas higher than 1000 m above sea level (Alicai *et al*., 2007; Legg *et al*., 2011). This disease has been shown to exist in all the East Africa coastal lowlands in Kenya, Uganda, Tanzania and Mozambique (Hillocks and Jennings, 2003).

Breeding for resistance is one of the strategies used in controlling these diseases (Hillocks & Jennings, 2003; Munga, 2008). Breeding has been found effective especially in areas with high disease pressure (Hillocks & Thresh, 2000).

1.1 Statement of Problem

Virus diseases infecting cassava remain a major challenge causing severe losses in yield estimated annually at US$1 billion (Legg *et al*., 2006). Before 1980s, there was little information on cassava mosaic disease (CMD) due to little data to support its presence. However with detailed surveys, its incidence, severity and prevalence has been determined in 17 out of 38 cassava growing countries in sub-Saharan Africa (Legg *et al.,* 2003). The greatest incidences have been recorded in Congo (79%), Nigeria (82%)

and Sierra Leonne (85%) (Alusaine *et al.,* 2014). The distribution and incidence of CMD in Kenya showed that the disease is highest in western at 80% and lowest in Coast province (Were *et al*., 2004). Were *et al.* (2004) reported three begomoviruses namely EACMV, ACMV and EACMZV in Kenya. Most of the EACMV and EACMV-UG were reported in the east and the west regions of Kenya with EACMZV being reported in Coastal Kenya. However, recent studies (Mwatuni *et al.,* 2015) revealed that the viruses are widely distributed in all the cassava growing regions of Kenya. From these studies, it is clear that cassava begomoviruses have spread beyond their perceived distinct territory which is a concern for their management. Recently, there has been a report of two new species of cassava mosaic begomoviruses namely *African cassava mosaic Burkina Faso virus* (ACMBFV; Tiendrebeogo *et al*., 2012) and *Cassava mosaic Madagascar virus* (CMMGV; Harimalala *et al.,* 2012). The emergence of new strains has been linked to frequent dual/multiple infections within a region leading to recombinations.

There has been a link between plant virus symptom severity and satellite molecules currently referred to as episomal Sequences Enhancing Geminivirus Symptoms (eSEGS1 and eSEGS2) in infected plants (Maredza *et al.*, 2015). Mayo *et al.* (2005) reported that these molecules are nucleic acids or viruses that do not have general nucleotide sequence homology to the helper virus but depend on the helper virus for replication. The satellite-virus (episomal Sequences) association has increased disease symptom severity leading to increased crop yield losses. In Tanzania, Nduguru *et al.* (2005) described two satellite molecules linked to CMBs namely, DNA-II and DNA-III (Nduguru *et al.,* 2008). They were reported to break down resistance to EACMV-UG2 in tolerant genotype TME 3 and also increased CMD symptom severity. Mwatuni *et al.* (2015) reported a wide presence of DNA III satellites from cassava growing regions of Kenya. For DNA II satellites however, very few samples tested positive from the cassava growing regions. This study sought to provide current information on the

cassava mosaic begomoviruses and their associated DNA satellites in Kenya to help in mapping out their spread which can help inform control strategies.

Control strategies used to manage these viruses include; phytosanitation, cultural practices like rogueing, use of clean planting materials and breeding. Cassava has greatly gained from breeding efforts (Kawano, 2003). Most cassava breeding programmes use symptom expression for selection and detection of viruses. Visual disease assessment has been found to overestimate disease severity especially when infection rates are low (Kwack *et al.*, 2005). Besides, selection using symptoms can fail to capture phenotypically mild isolates which can be severe in new cultivars or in co-infections. Another challenge with visual observation is that, some symptoms have similarities with other crop constraints like senescence and plant mineral deficiencies hence inconsistent expression. Similarly, in some varieties it takes long for the development of foliar symptoms hence the need to have multiple diagnostic methods to detect early stages of infections.

Studies by Rwegasira *et al.* (2012) determined that 22% of plants with CBSD symptoms tested negative while 7% of asymptomatic plants tested positive for the virus through reverse transcriptase polymerase chain reaction (RT-PCR). This implies that symptoms in these plants could have been caused by other causes such as biotic stress or mineral deficiency. In asymptomatic plants, the detection of the virus could be explained by presence of latent virus infection especially in tolerant cultivars that can co-exist with the virus without expressing symptoms (Fargette *et al*., 1996). Also, because of emergence of virus strains in both cassava begomoviruses and cassava brown streak disease, distinguishing the symptoms in the individual strains is difficult.

1.2 Justification

Forecasting of future virus spread can be enhanced by CMD surveys. There have been reports of disease spread from the Great Lakes region of Eastern Africa necessistating precise surveillance (Legg *et al.,* 2011). The regular disease diagnostics determines CMD spread in time and space (Sseruwagi *et al.,* 2004). This can help in monitoring the rate of disease spread to guard against the development of disease epidemics. Similarly, models can be developed to predict future spread and development of decision support system for disease management (Sseruwagi *et al.,* 2004). Survey data is essential in development of control mechanisms for diseases in affected regions. Because of climatic changes, the begomoviruses which were initially confined to tropical areas have spread to temperate regions. Similarly, new emerging virulent strains have been reported arising from the existence of dual/multiple cassava begomoviruses in regions (Zhou *et al.,* 1997). To trace the spread of viruses to new areas, their geographical distribution and diversity must be determined through molecular detection and characterization.

Cassava breeders have been using visual methods to screen for resistance to viruses. Visual observation of disease symptoms has not been effective in the diagnosis of viral diseases in cassava (Ogbe, 2001). This is because viruses have been detected in initially symptomless plants that have been considered CMD-free 'escapes' (Thresh & Cooter, 2005). Studies by Kaweesi *et al.* (2014) reported that multiple diagnostic techniques are essential in determining the resistance potential of genotypes, in order to eliminate disease 'escapes.' This study sought to ensure that virus diseased materials are detected through the use of multiple detection tools. For cassava geminiviruses, PCR has been used for detection of single, dual and multiple virus species reliably. On the other hand RT-PCR has been used in the detection of CBSVs (Mbanzibwa *et al*., 2011a). Therefore these molecular techniques can be used for indexing of cassava viruses allowing breeders to detect virus infection in the pre-symptomatic stage.

1.3 Objectives

1.3.1 Overall Objective

The main objective was to determine the reaction of elite cassava genotypes to virus infection and the distribution of cassava begomoviruses and associated DNA satellites in the major cassava growing areas of Kenya.

1.3.2 Specific Objectives

1. To determine the geographical distribution and genetic diversity of cassava begomoviruses and associated DNA satellites
2. To determine the reaction of elite cassava genotypes to infection by cassava mosaic begomoviruses and cassava brown streak disease

1.3.3 Hypotheses

1. There are no differences in geographical distribution and genetic diversity of cassava begomoviruses and DNA satellites in the cassava growing regions of Kenya
2. There is no presence of CBSV and CMGs in elite cassava genotypes

CHAPTER TWO

2.0 REVIEW OF LITERATURE

2.1 Origin, botany and taxonomy of cassava

Cassava crop originated from Central and South America with the centers of diversity being Central Brazil (Nassar, 2003) and Mexico (Howard *et al.,* 1994). The Portuguese sailors introduced the crop to Africa from Brazil by early 19th century and it is widely grown in East Africa (Hillocks, 2002). Cassava belongs to the family Euphorbiaceae; genus *Manihot*; sub-species *Manihot esculenta* Crantz species (spp) *esculenta* (Allem *et al.,* 2001). There are about 100 species in genus *Manihot* grouped into 19 taxanomic sections with cassava being under section Manihot (Rogers & Fleming, 1973).

Classification of cassava has been done according to morphological traits and cynogenic glycoside content. However the expression of traits varies based on environmental factors. Traits used in classification include leaf shape and size, plant height, stem and petiole colour, inflorescence and flower colour (Nassar, 2005). The cassava crop is made up of two main parts, leaves and woody shrub. Cassava grows upto a height of 3m and has adventitious roots. Out of the total tuberous root, the edible portion is about 0.8-0.9 (Nassar & Ortiz, 2006). Cassava is the only species in its genus group that serves as a food crop. Male and female flowers in cassava are borne on the same plant. The time interval from planting to flowering is dependent on the specific genotype and environmental conditions varying from 1-24 months (Byrne, 1984). Male flowers occur on tip of a single branched panicle while the female flowers occur at the base. Ceballos *et al*. (2002) has indicated that flowers begin opening around mid-day and remain open for about one day. Propagation of cassava is done via stem cuttings

which may be at least 20cm long with 4 to 5 nodes with viable buds. Propagation from seeds is advantageous as they are easy to store, transport and have long viability compared to cuttings but their heterogeneous nature and variation of seedlings limit their use (Ceballos *et al.,* 2004). Cassava root is not a tuberous root anatomically, but a true root which cannot therefore be used for vegetative propagation.

2.2 Production and utilization of cassava

Cassava has been known to be one of the staple foods of the African continent (Legg & Fauquet, 2004). In terms of productivity per unit area, cassava production is highest in comparison to sweet potato (*Ipomoea batatas*), potato (*Solanum tuberosum*) (Scott *et al*., 2000). According to recent statistics, about 262 million tons of cassava are produced globally with Nigeria being the leading producer having 47 million tons, followed by Thailand with 30 million tons and Indonesia 23 million tons from South –East Asia (FAO, 2013). In East Africa, the leading cassava producer is Tanzania followed by Uganda, Burundi, Rwanda and Kenya in that order (Table 2.1).

Table 2.1. Cassava production in selected East African countries in million tons per Year

Country	2006	2007	2008	2009	2010	2013
Tanzania	6.158	6.600	6.600	6.916	na	4.755
Uganda	4.926	4.456	5.072	5.179	5.292	2.979
Kenya	0.566	0.597	0.751	0.911	na	0.935
Burundi	0.571	0.559	0.630	0.660	na	2.234
Rwanda	0.588	0.700	0.700	0.680	na	2.948

Source: FAO (2013), na = not available.

Cassava has potential to produce in infertile soils with innate tolerance to drought conditions hence suitable for use by the poor in marginal areas (El-Sharkawy, 2007). It is an important cash crop in tropical lands recognized as a food security crop for over 300 million people in sub-Saharan Africa (Nweke, 1996). The highest consumption occurs in the Democratic Republic of Congo (FAO, 2003). Fermont (2009) reported that in Kenya, the average yield of cassava is 10.6 tons/hectare.

Cassava is primarily produced for human consumption in various forms like being pounded, dried or eaten fresh. It can be either processed into other forms like biscuits, bread, gari, and fufu from West Africa (Cleanster, 2012). Fresh cassava roots have been estimated to supply calories to more than a billion people in about 105 countries globally (FAO, 2012). The cassava root contains carbohydrates, proteins, minerals and fats (Table 2.2). Tewe (2013) also reported that cassava roots consist of glucose, fructose and maltose in minute quantities ranging between 1.5-2.8% dry weight (Aryee *et al.,* 2006). In terms of carbohydrates supply, cassava ranks third in sub-Saharan Africa (Devries *et al.,* 2011).

Table 2.2. Food energy (kcal) from cassava root

Food values of cassava	Percentage
Edible fleshy portion	80 - 90
Water	60 - 65
Carbohydrate	30 - 35
Protein	1 - 2
Fat	0 - 0.2
Fiber	1 - 2
Mineral	1 -1.5

(Adapted from Nassar & Ortiz, 2007).

Cassava leaves provide vegetables especially in the dry spells when other vegetables are limiting (FAO, 1993). The cassava leaves can supply vitamin A, C, iron, calcium and protein (Nweke et al., 2002). Henry (2000) reported that cassava starch has been used in the paper industry, food industry as thickeners, animal feed pellets and production of bioethanol. The recent interest in conversion of cassava to bioethanol is expected to increase the production of cassava in Africa (Acedo & Labana, 2008). Sporadic prices in crude oil have increased the demand for cassava as feedstock for bio-fuel production in Philippines, Thailand and China (FAO, 2007). Cassava cuttings can also be sold to earn farmers income (Alves, 2002).

In Latin America, 42% of the cassava produced is used for human food consumed as fresh, dried or roasted flour popularly referred to as *farinha de mandioca* (FAO, 1999) while in Asia, cassava for human consumption is at 40% with the remainder exported to Europe as pellets and chips for animal feed (Westby, 2002). Thailand exports a bulk of its cassava as cassava chips and processes some into pastry, cakes and noodles while in Indonesia, 57% of its production is consumed locally either as boiled or steamed or processed into gaplek which is dried chips (FAO, 1999).

2.3 Constraints to cassava production

Pests and diseases affect the production of cassava with estimated losses from cassava being in the range of 15-27 million tons/hectare (Thresh *et al*., 1997). The pests that infect cassava include mealybugs (*Phenacoccus manihot* Matile-Ferrero), green spider mites *(Mononychellus tanajoa)*, cassava green mite (*Mononychellus tanajoa* Bonder), cassava horn worm (*Erinnyis ello*), whiteflies (*Bemisia tabaci* Gennadius*)* and thrips (Montero, 2003). The diseases affecting cassava include cassava bacteria blight, cassava bud necrosis, cassava mosaic disease and *cassava brown streak virus* (Chikoti *et al.,* 2015). Globally, 17 viruses have been reported to infect cassava (Fauquet *et al.,* 2005) with 8 viruses being reported in Africa (Calvert & Thresh *et al.,* 2002). However, the most devastating amongst the cassava diseases are the viruses particularly cassava mosaic and cassava brown streak virus found in family *Geminiviridiae* and *Potyviridiae* respectively (Patil & Fauquet, 2009).

Besides the viruses, it has been determined that there are sub-viral catalyst called satellites that cause undesirable effects in cassava by increasing the severity of the symptoms and accumulation of their helper virus (Mansoor *et al.,* 2003). Cassava mosaic disease associated satellites are reported to enhance disease symptoms in CMD infected plants resulting to breakdown of CMD resistance (Ndunguru *et al.,* 2008). The fact that cassava is propagated by cuttings helps in dissemination of viruses through transportation to other places (Gibson & Otim-Nape, 1997; Chellappan *et al*., 2004). In many countries there is movement of infected materials between farmers without proper phytosanitary controls.

Another challenge facing production of this crop is the low rate of multiplication. A cassava mother plant is expected to produce a maximum of 30 cuttings at maturity compared to seed propagation in millet where a single plant can produce hundreds of seeds (Leihner, 2002). There is also rapid deterioration of roots that hampers their long

term storage in the fresh state (Okezie & Kosikowsiki, 2004). Another constraint to cassava production is the presence of toxic cyanogenic glycosides like linamarin in some varietis which can cause human deaths if not well processed (Okafor, 2004; IITA, 2007).

2.4 Viruses infecting cassava

The viruses that affect cassava include, *Cassava brown streak virus,* Cassava geminiviruses, *Cassava common mosaic virus* (CCMV), *Cassava green mottle virus*, *Cassava vein mosaic virus*, *Cassava American latent virus*, *Cassava Ivorian bacillifom virus* (Calvert & Thresh, 2002). However the viruses of economic importance in Africa are cassava geminiviruses (*Geminiviridiae:Begomoviruses)* that cause cassava mosaic disease (CMD) and *cassava brown streak virus* (CBSV) and Cassava brown streak virus Ugandan variant (UCBSV) (*Potyviridiae: Begomovirus*) that cause cassava brown streak disease (CBSD).

The first reports of CMD and CBSD in Africa are in 1894 (Warburg, 1894) and in 1936 respectively (Storey, 1936; Otim-Nape *et al.* (1994). These two diseases have been reported to attack cassava in East Africa namely. The CMGs have single stranded DNA genomes encapsulated in characteristic geminate particles. These viruses affect plants in tropical and sub-tropical areas. The cassava geminiviruses include; *East African cassava mosaic Cameroon virus* (EACMCV), *East African cassava mosaic Malawi virus* (EACMMV), *East African cassava mosaic virus* (EACMV), *African cassava mosaic virus* (ACMV), *East African cassava mosaic Zanzibar virus* (EACMZV) *South African cassava mosaic virus* (SACMV) and *East African cassava mosaic Kenya virus* (EACMKV) (Table 2.3) (Legg & Fauquet, 2004). Recently, there has been a report of two new species of cassava mosaic disease namely *African cassava mosaic Burkina Faso virus* (ACMBFV); Tiendrebeogo *et al.*, 2012) and *Cassava mosaic Madagascar*

virus (CMMGV; (Harimalala *et al.*, 2012).

Table 2.3. Cassava geminivirus species in Africa

Virus Name	Abbreviation
African Cassava mosaic virus	ACMV
East African cassava mosaic virus	EACMV
East African cassava mosaic Kenya virus	EACMKV
East African cassava mosaic Malawi virus	EACMMV
East African cassava mosaic Zanzibar virus	EACMZV
Indian cassava mosaic virus	ICV
South African cassava mosaic virus	SACMV
Sri Lankan cassava mosaic virus	SCMV
East African cassava mosaic Cameroon virus	EACMCV

(Adapted from Fauquet *et al.*, 2008)

Cassava brown streak disease (CBSD) is another disease endangering cassava production across East Africa and is second to cassava mosaic disease in severity (Hillocks & Jennings, 2003; Rwegasira *et al.*, 2011). The disease can be caused by two strains namely *cassava brown streak virus* (CBSV) and *Ugandan cassava brown streak virus* (UCBSV) which belong to family *potyviridiae* genus *ipomoviruses* (Mbanzibwa *et al*., 2011).

2.5 Cassava mosaic begomoviruses (CMBs)

2.5.1 Cassava mosaic disease (CMD) genome organization

The genome of CMD (Fig 2.1) has been classified as circular, single stranded, bipartite germinate particles ((30 x 20 nm) encapsulated in 30kDA protein coats (Stanley *et al*., 2005). They contain DNA A and DNA B genomic components which share common region of 200bp sequences and are 2700-2800bp long. In the common region are a

conserved stem-loop structure with regulatory elements nucleotide TAATATTA ↓C sequence (↓ shows nicking site to initiate virion sense DNA replication). The binding sites for the replication associated proteins are the TATA box and Iterons (Hanley *et al.,* 1999).

Genomic component DNA A (Fig 2.1) contains two open reading frames namely AV1 and AV2 with other four overlapping complementary –sense open reading frames (ORFs) namely AC1, AC2, AC3 and AC4. The function of AV1 is to encode the coat protein gene responsible for vector transmission (Harrison *et al*., 2002) and genome encapsidation. The AV2 is considered a movement protein (Rybicki, 1994). Replication of the cassava geminiviruses within the host cell is facilitated by AC1-AC4. The specific roles of the complementary sense ORFs are; AC1 encoding for replication associated protein (Rep), AC2 for transcriptional activator protein (TrAP), AC3 for replication enhancer protein (Ren) and AC4 for host activation protein (Stanley *et al.,* 2005).

The DNA B (Fig 2.1) component consists of BV1 and BC1 open reading frames and they encode both the movement protein and shuttle protein and they are non-overlapping. They are involved in inter (BC1) and intra (BV1) virion movement with the plant cell (Hull, 2002).

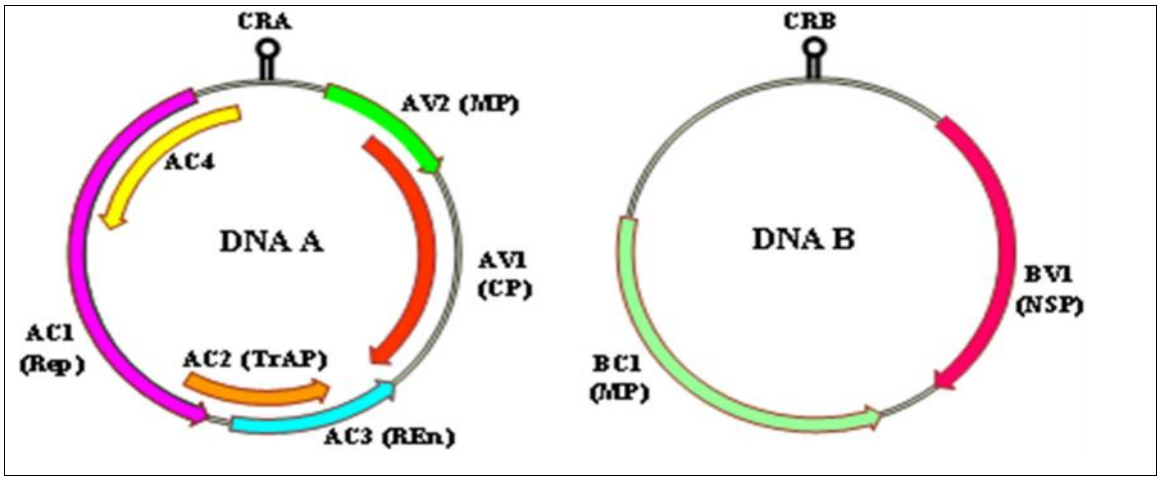

Fig 2.1. Genome organization of DNA A and DNA B components of cassava-infecting begomoviruses (Stanley *et al*., 2004)

2.5.2 Symptomatology of CMD

CMD symptoms are indicated by plants having green mosaic and yellow mosaic leaves with the green one showing contrasting regions of dark and light green tissue (Ndunguru *et al.,* 2005). Yellow mosaic is shown by conscipicous regions with normal green and yellow tissues with the chlorotic areas expanding less than the other parts of leaf lamina causing distortion of the leaflets (Plate 2.1) and rupturing of tissues (Gibson *et al.,* 1996). There is also severe chlorosis shown by premature leaf abscission with characteristic S-shaped curvature (Plate 2.1a) of the petioles and decrease in vegetative growth and yield.

Severe CMD produces a candlestick (Plate 2.1b) like structure in infected cassava plants (Legg &Thresh, 2003). According to Gibson (1994) environmental factors usually influence symptom expression with cool weather affecting symptom expression than hotter weather. Cutting back of the plant to enhance regeneration enhances symptom expression as indicated by (Verhoyen, 1979).

Plate 2.1. Infection of cassava leaves and stem with Cassava mosaic geminiviruses; The leaves show characteristic leaf distortion and yellow chlorosis typical of cassava mosaic virus (Ndunguru *et al.,* 2005).

Infection of cassava by cassava mosaic geminiviruses can either be severe or mild but there is no evidence indicating consistent differences in symptoms caused by the different cassava geminiviruses. However, dual infections with two different CMGs have been shown to cause more severe symptoms than an individual virus (Harrison *et al*., 1997; Fondong *et al.,* 2000a; Pita *et al.,* 2001). Similarity of CMD symptoms with other chlorosis factors like nutritional deficiency and pest attack make disease diagnosis difficult (Asher *et al*., 1980).

2.5.3 Distribution of Cassava mosaic disease

Early reports indicated that African cassava mosaic virus (ACMV) was confined to areas of South Africa, Central Africa and West Africa with EACMV being restricted to the Eastern part of the rift Valley that includes, Kenya, Malawi, Madagascar, Zimbabwe and Tanzania (Chikoti *et al*., 2013). India and Sri-Lanka were reported to be infected by the *Indian Cassava mosaic virus* (ICMV) and *Sri-lankan cassava mosaic virus* (SLCMV) respectively (Harrison *et al*., 1997). However, later studies indicated widespread presence of cassava *begomoviruses* (CMBs) in sub-Saharan Africa (Berry *et al*., 2001; Atiri *et al*., 2004; Bull *et al.*, 2006,) with SLCMV and ICMV only restricted to Sri-Lanka and India (Thottapily *et al.*, 2003; Patil & Fauqet, 2009).

The fact that CMBs have not been reported in the center of origin of South America, shows that the viruses are native to Africa, especially in indigenous hosts implying that cassava became their hosts accidentally (Buddenhagen, 1977). In Kenya, distribution of CMD showed that the disease is highest in western at 52% and lowest in Coast province and that there exist EACMV in combination with ACMV and this has accelerated epidemics (Were *et al*., 2004). The same findings have been recorded in Rwanda where in north eastern Umukara, infection is driven by whiteflies and a combination of EACMV and ACMV have caused severe cassava mosaic disease (Legg *et al.*, 2001).

2.5.4 Detection and characterization of Cassava begomoviruses

Viruses have been detected through molecular and biological methods that include; Enzyme linked immmunosorbent assay (ELISA), indicator plants, reverse transcriptase polymerase chain reaction (RT-PCR) immunocapture ELISA (IC), restriction fragment length polymorphism (RFLP) analysis (Tairo *et al.,* 2006). Anti-body based detection of CMBs have been reported after purification of CMBs was achieved. Double antibody sandwich enzyme linked immunosorbent assay (DAS-ELISA) (Sequira and Harrison, 1982) together with immunosorbent electron microscopy (Roberts *et al*., 1984) have been used in the detection of CMBs. ELISA can be successfully used to analyze bulky samples especially for the diagnostic surveys (Otim-Nape *et al.,* 2003) but cannot separate the different CMBs in mixed infections (Thottapily *et al.,* 2003). However, the identification of recombinant CMGs (Zhou *et al.,* 1997) necessitated the use of PCR or nucleic acid based detection and are now widely used (Legg *et al.,* 2001). In these techniques, virus specific primers obtained from full length DNA-A sequences are used (Fondong *et al*., 2000; Pita *et al*., 2001a) .

There has also been the use of PCR with restriction fragment length polymorphism (RFLP) in the detection of CMGs. This involves the use of cassava geminiviruses specific primers for the amplification of CMD. The amplified products are then digested using restriction enzymes (ECORV). Distinctive bands are then generated depending on the sites which the restriction enzyme cuts (Briddon *et al.,* 1993). With the use of this method, ACMV was distinguished from EACMV successfully in Uganda. Molecular diagnosis of CMBs has been successfully achieved in a number of countries including Tanzania, Kenya, Cameroon and South Africa (Fondong *et al*., 2000; Mgbechi-Ezeri *et al*., 2000; Alabi *et al*., 2008; Sserubombwe *et al.,* 2008). More than one strain or virus can now be detected simultaneously through multiplex PCR leading to amplification of products with different sizes (Alabi *et al.,* 2008).

Initially, it was considered that there was only one cassava mosaic geminivirus (Bock & Harrison, 1985). Later on, other geminiviruses species were distinguished including ACMV and EACMV. The CMD complexes in sub-Saharan Africa include *East African cassava mosaic Cameroon virus* (EACMCV), *East African cassava mosaic Malawi virus* (EACMMV), *East African cassava mosaic virus* (EACMV), *African cassava mosaic virus* (ACMV), *East African cassava mosaic Zanzibar virus* (EACMZV) and *Southern Africa cassava mosaic virus* (SACMV) (Fauquet *et al.,* 2008).

The classification of CMGs follows Fauquet et al. (2000) guidelines with species being classified on 89% pairwise similarity between DNA-A sequences, strains 85-94% pairwise identity and variants 92-100% pairwise identity between two sequences (Fauquet *et al*., 2008).

2.5.5 Epidemiology of Cassava mosaic disease

Transmission of cassava mosaic viruses can occur through infected cuttings, mechanical inoculation and vectors (Legg & Hillocks, 2003). Sap transmission and grafting have also been linked to virus transmission (Lister, 1959; Bock & Guthrie, 1976). The fact that cassava is propagated by cuttings helps in perpetuation of virus (Gibson and Otim-Nape, 1997; Chellappan *et al*., 2004). However, the spread thereafter has been linked to whiteflies (Chikoti *et al.,* 2013). Studies conducted in Uganda, Kenya and Ivory Coast, showed that whiteflies have facilitated the spread of CMD (Legg et al., 1997; Otim-Nape *et al.,* 1998a. In these studies, immigrant whiteflies have encroached into new plantings spreading the disease (Fishpool *et al.,* 1995)

Studies indicate that in Ivory Coast, up to 1.7% infectivity has been recorded when adult whiteflies obtained from severely infected fields transferred to young test seedlings of cassava (Fargette *et al*., 1990). The movement of the vector is further enhanced by the wind direction and turbulence in the crop stands. Observations show that CMD is greatest at field margins along the windward and leeward edges (Fargette

& Thresh, 1994; Colvin *et al*., 1998). Discontinuities of crop canopies also enhance alighting and establishment of viliferous vectors, incidence of disease (Fargette *et al*., 1985, 1993; Fargette and Thresh, 1994). Fauquet (1988) indicated that there are regional differences in spread with Uganda and Ivory Coast recording a more rapid spread in humid environment with greater densities of cassava cultivation (Legg *et al*., 1997).

The planting dates for cassava have influenced exposure of plants to infection. This is through the growth of plants when the whitefly vector populations are highest. Robertson (1987) reported that in Coastal Kenya, spread of CMD has been highest during the early rains of mid-May, hence planting later in the year is advantageous. A similar situation has been observed in Ivory Coast where virus spread is lower in the latter stages of the rainy season (Fargette *et al.,* 1994a). The rate of CMD spread has been slowed through the use of intercrops (Fondong *et al.,* 2002). Similarly, the combination of susceptible and resistant genotypes in Uganda has reduced the disease spread (Sserubombwe *et al.,* 2001).

2.6 Cassava brown streak disease (CBSD)

2.6.1 Genome organization of *Cassava brown streak virus* (CBSV)

The cassava brown streak disease (CBSD) is caused by two species namely, Cassava brown streak virus (CBSV) and Cassava brown streak virus Ugandan variant (UCBSV). The first species to be identified was CBSV and is considered a monopartite positive sense single stranded RNA genome that is made up of 9000 nucleotides (Winter *et al.,* 2010). Encoded in the CBSV structure is the P1 serine proteinase. Unlike other members of the family *potyviridae* such as *Sweet potato mild mottle virus* (SPMMV) and *Cucumber vein yellowing virus* (CVYV), CBSV and UCBSV do not contain coding for putative protein Helper Component Cysteine Proteinase (HC-Pro) but the 3'-proximal end contains a peculiar Maf/HAM1-like sequence (Fig 2.2) (with 226

amino acids and 678 nucleotides) found between the conserved coat protein and replicase (Mbanzibwa *et al.,* 2009b). Mbanzibwa *et al.* (2009b) observed that the P1 of CBSV is similar to the P1 of (SPMMV).

The two strains namely CBSV and UCBSV have different sizes with UCBSV lacking 11 amino acids (33 nuncleotides) at the CP-N-terminus (Fig 2.2) (Mbanzibwa *et al*., 2009a; Rwegasira *et al.*, 2011). There are differences of 94-96 nucleotides at the 3-untranslated region (Mbanzibwa *et al.*, 2011a).

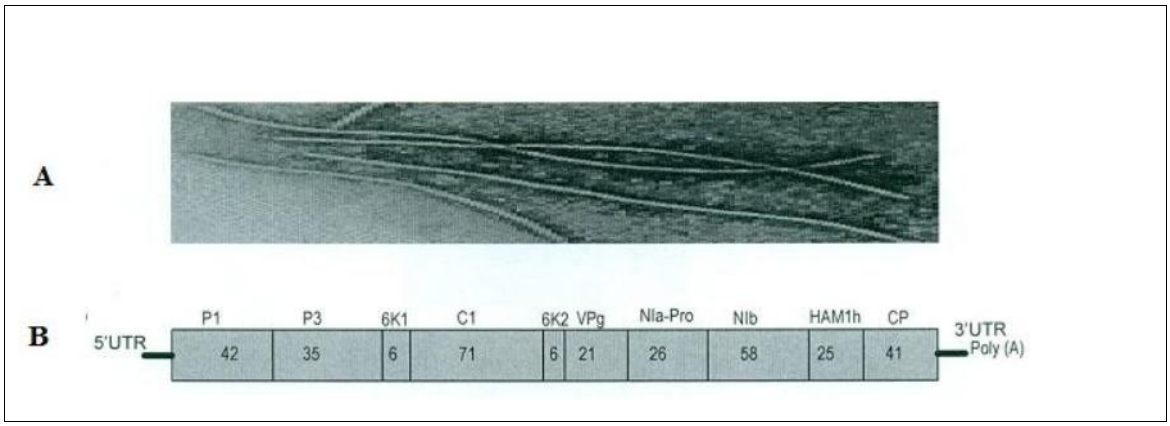

Fig 2.2: A=CBSV Particle morphology B= CBSV genome Schematic representation (Mbanzibwa *et al*., 2009a)

2.6.2 Distribution of *Cassava brown streak virus*

Early studies indicated the dominance of CBSD in the coastal lowlands of eastern and southern Africa (Calvert & Thresh, 2002). The disease was first recorded at the foot hills of Usambara in Tanzania (Storey, 1936) and has spread to areas higher than 1000 m above sea level (Alicai *et al.*, 2007). It has been shown to occur in all the East Africa Coastal lowlands (Kenya and Tanzania) and low altitude areas of Malawi (Nichols, 1950; Bock, 1994; Hillocks & Jennings, 2003;).

Areas previously without CBSD have reported its presence especially in high altitude areas away from the Indian coastal belt in Kenya, Mozambique and Tanzania. In

Uganda the disease has been reported in higher altitudes indicating a shift in belief that it is only a lowland disease (Alicai *et al.,* 2007). In Zanzibar, CBSD was detected in 1950 but reappeared recently (Mtunda, 2003). The disease had little economic importance in Kenya until 1994 due to local varieties exhibiting resistance to CBSD infections (Bock, 1994). Munga (2002) showed that CBSD is widely spread in coastal Kenya while Mware (2009) reported existence of the disease in other regions of Kenya. Spread of this disease to Kenya was through materials imported from Amani in Tanzania (Bock, 1994).

2.6.3 Symptomatology of CBSD

The distinguishing symptoms of CBSD include mosaic in older leaves(Plate 2.2a) together with necrosis of storage roots (Plate 2.2b). The disease distinct symptoms include necrosis and constriction in storage roots, vein clearing of diseased leaves, brown streaks on stems of cassava (Jennings, 2003). However, variants of symptoms have been found depending on crop age, variety and weather conditions (Hillocks and Jennings, 2003).

Plate 2.2. Symptoms of CBSD; A leaf chlorosis and B, necrosis of the storage roots

The economic impact of CBSD occurs in the storage roots causing corky necrosis in starch bearing tissues. There appears necrotic banding on leaves with potential to expand into large necrotic patches which become prominent on mature leaves (Hillocks

& Jennings, 2003). Symptoms associated with CBSD differ with environmental conditions, type of variety and age of the crop suggesting that there could exist CBSD variants (Patil &Fauquet, 2014).

There are challenges in detection using field symptoms as immature leaves of infected cassava often appear symptomless. The symptoms vary greatly with variety of cassava and environmental conditions (Hillocks et al., 1996). It is more difficult to detect CBSD in older plants due to senescence of lower leaves with more prominent symptoms. Occurrence of transient symptoms especially during periods of active growth resulting in symptomless tissues makes disease recognition difficult (Jennings, 1960).

2.6.4 Epidemiology of *Cassava brown streak virus*

Cassava brown streak virus (CBSV) is spread by stem cuttings and whiteflies. The whiteflies transmit the disease in a semi persistent manner (Maruthi *et al.,* 2005). High level of the disease has been linked with density of cassava cultivation making vector colonization easier, higher temperatures that have been shown to favor the whitefly populations and low altitude (Hillock et al., 1999). Fargette *et al*. (1993) has shown that low temperature lowers the whitefly population. In Malawi, among the extension workers and farmers, the cause of CBSD is unknown with some linking the disease to old age and high rainfall (Gondwe *et al.,* 2003). Another study in Malawi showed a negative relationship between altitude and CBSD.

Cultivar and environment have been found to influence severity of CSBD on infected plants according to Jennings (1960) and severity magnitude has been based on level of damage sustained by plants hence the yield loss. Environmental conditions such as direction of prevailing wind within crop stands influence distribution of immigrant whiteflies.

2.6.5 Detection of *Cassava brown streak virus*

The first and traditional method that has been used in disease detection is the use of symptoms. However, Matthews (1980) reported that expression of virus symptoms is influenced by other factors like variety, stage of crop growth and the environment. Reliability of visual symptom expression must be enhanced by supplementary confirmatory tests (Miller *et al*., 2009). There has also been use of indicator plants that respond in a characteristic and consistent manner to virus infection under greenhouse conditions (Walkey, 1991). Immunological tests that identify specific viruses through antigen-antibody reactions have been used. Winter *et al.* (2010) reported that CBSD has been detected by ELISA kits but distinction between UCBSV and CBSV has not been successful. The use of RT-PCR has become a common method in detection of RNA based viruses. Primers that bind to specific sequences are used hence multiple bands can occur if there are many sequences sharing high identities. Alabi *et al*. (2008) simultaneously detected many viruses or strains through the multiplex PCR.

Variation in CBSV relating to its genome has been observed, resulting into two species namely *Cassava brown streak virus* (CBSV) and *Ugandan cassava brown streak virus* (UCBSV). Comparisons between the CBSV and UCBSV isolates complete genomes have revealed 69%-70.3% and 73.6%-74.5% similarity at the nucleotide and polyprotein amino acid sequences respectively (Mbanzibwa *et al*., 2011b). Wider variations have been recorded between the CBSV isolates as compared to the UCBSV isolates with (70.3%-95.6%) and (86.3%-99.3%) respectively (Mbanzibwa *et al.*, 2011b).

2.7 Management of viruses infecting cassava

2.7.1 Phytosanitation

Phytosanitation is the elimination of the sources of inoculum from a cassava stand preventing further disease spread. It has been done through planting of CMD/CBSD free cuttings (Gwandu *et al*., 2015). Increased plant vigor and higher yields have been obtained when healthy stem cuttings are used as cassava propagation materials (Thresh *et al.*, 1994). The challenge facing this disease control approach is the lack of enough stocks of healthy planting materials to a majority of small holder farmers.

Virus control through rogueing is also widely practiced (Thresh, 1988). A study done by Colvin *et al*. (1999) determined that there was increased whitefly populations in CMD infected plants than healthy ones recommending that phytosanitation should be enforced. Rogueing has been used in selection and maintenance of CMD free stocks (Guthrie, 1990). In India, plants that are CMD infected have been marked with paint to prevent using them as cuttings after harvest (Malathi *et al*., 1988)

2.7.2 Breeding for resistance

According to Fehr (1987) there have been gains in productivity of crops in the twentieth century driven by crop breeding. Cassava has greatly gained from breeding efforts (Kawano, 2003). In agricultural research, plant breeding has one of the highest rates of returns. Breeding is done depending on objectives which in cassava can be geared towards industrial uses or human consumption. Among the most important achievements in cassava is improving resistance to the cassava mosaic geminiviruses. However, viruses undergo frequent genetic mutations which may break resistance bred into cassava varieties and therefore breeding efforts must always stay ahead of diseases (Nagib & Ortiz, 2010). Breeding for CMD resistance has been carried out creating

varieties such as TME 3 that are not readily infected even with exposure to large amounts of inoculum (Thresh *et al*., 1998).

To achieve breeding objectives it is necessary to use participatory research and breeding approach in cassava breeding due to farmer's preferences (DeVries & Toenniessen, 2001). To develop varieties suited to farmers, participatory crop improvement schemes like participatory variety selection (PVS) (Dorward *et al.,* 2007) and participatory plant breeding (PPB) have been recommended.

It has been realized that participatory breeding incorporates farmer's perspectives, influencing acceptance of released improved lines. The goal for the development of CBSD resistant varieties has been to increase yields and also meet farmers' needs urgently. Interspecific hybridization followed by several backcrosses began at Amani, Tanzania resulting to development of CBSD resistant cultivars like Namikonga, Kiroba and Nanchinyaya (Jennings & Iglesias, 2002; Hillocks & Jennings, 2003). In Kenya advanced lines (Guze, Kibiriti mweusi and Gushe) have been tested in various sites learning from the Tanzania experience.

Currently gaining momentum worldwide is the concept of transgenic resistance. This concept is based on inducing resistance to viruses through genetic engineering also called pathogen derived resistance (Taylor *et al*., 2012). This concept can enhance resistance in susceptible cassava plants popular with farmers. There have been successes in using this approach to control CMGs in both cassava and some herbaceous plants (Legg & Fauquet, 2004). In this approach, transformation techniques use *Agrobacterium tumefaciens* in integrating transgenes into target tissues generating new plants (Taylor *et al*., 2012).

Other than disease resistance, genetic engineering successfully produced plants with elevated protein and low cyanide levels (Liu *et al.,* 2011). Success in production of genetically engineered cassava crops have been successful in confined trials and

greenhouse conditions (Yadav *et al.,* 2011; Taylor *et al.,* 2012). However, field trials have not been done mainly because of challenges involved in obtaining government approvals and acceptability by the public. The utilization of resistant varieties in the control of viruses is encouraged though, caution should be undertaken to avoid erosion of the genetic diversity of African cassava cultivation (Thresh *et al*., 1998).

CHAPTER THREE

3.0 Distribution and characterization of Cassava begomoviruses and associated DNA satellites in main cassava growing regions in Kenya

Abstract

A diagnostic survey was done in 2013 to determine the incidence, symptom severity, prevalence, geographical distribution and genetic diversity of cassava begomoviruses. A total of 24 fields in Nyanza, 22 in Western, 22 in Coast and 20 in Eastern were sampled. Species specific primers were used in laboratory analysis to detect the viruses present in cassava growing regions of Kenya. The sampled regions included Coast, Nyanza, Eastern and Western areas of Kenya. Genetic diversity of cassava begomoviruses was also done through sequencing. The highest CMD incidence was recorded in Western region with 60.19% and lowest in Eastern with 34.66%. Disease prevalence was highest in Nyanza, Western (100%) and lowest in Eastern (65%) respectively. Significant differences ($p=<0.001$) in CMD severity was recorded amongst the surveyed regions. The highest CMD severity was recorded with a mean of 1.8 in Western and lowest being 1.2 in Coast region of Kenya. PCR detected five begomoviruses namely; East African mosaic virus (EACMV), East African cassava mosaic Ugandan variant (EACMV-UgV), African cassava mosaic virus (ACMV), East African cassava mosaic Kenyan variant (EACMV-KE) and DNA satellites in the four regions under survey. The highest EACMV detection occurred in Nyanza region with 70.6% and the lowest being Western Kenya that recorded 36.6% detection. In EACMV-KE, the highest detections were recorded in Nyanza region with 26.5%, and lowest in Coast with 4.3%. However, EACMV-KE in this study was not found in Western Kenya. The highest ACMV detections were found in Nyanza with 29.4% with no detection of ACMV in the Eastern region of Kenya. EACMV-UgV was recorded in all regions surveyed in this study with Nyanza province having the highest disease detection with 41.2%. Comparison between

coat protein (CP) of the Kenyan EACMV isolates showed that there was wide genetic variability in nucleotide identity forming 2 independent clusters. On the other hand, EACMV-KE Kenyan isolates did not reveal great genetic variability within themselves forming a single clade with bootstrap value of 99%. When compared with sequences in the NCBI database, the Kenyan isolates were closely related to an isolate from Tanzania (AY795988.1). The study showed high cassava mosaic begomoviruses infections in Nyanza, a cause for concern for policy makers. Urgent strategies focusing on this area can be deployed to contain the viruses.

3.1 Introduction

Cassava is an important crop in sub-Saharan Africa which is recognized as a food security crop for over 300 million people in tropical lands (Nweke, 1996). It is reported that the highest consumption of cassava for starch occurs in the Democratic Republic of Congo (FAO, 2003). Pests and diseases especially viruses affect the production of cassava with estimated losses from cassava being in the range of 15-27 million tones/hectare (Thresh *et al*., 1997). Cassava is affected by viruses in the family *Geminiviridae* and genus *Begomoviruses*. In tracing the changes in disease levels, annual surveys have been utilized (Sseruwagi *et al.*, 2004).

In Kenya, Were *et al*. (2004a) reported three begomoviruses infecting cassava, namely EACMV, ACMV and EACMZV. Bull *et al.* (2006) reported that these viruses have got distinct geographical patterns. However, recent studies in 2009 (Mwatuni *et al.,* 2015) revealed that the viruses are widely distributed in all the cassava growing regions of Kenya. There has also been a link between plant virus symptom severity and satellite molecules currently referred to as episomal Sequences Enhancing Geminivirus Symptoms (eSEGS1 and eSEGS2) in infected plants (Maredza *et al.,* 2015). The

satellite-virus association has increased disease symptom severity leading to increased crop yield losses. New surveys can help map the spread of these viruses to new agro-ecological zones in order to help institute management measures.

Genetic variations of viruses have been determined to be caused by recombination and mutations (Roossinck, 1997). Recombinations have been determined in many viruses (Chare & Holmes, 2006; Valli *et al.*, 2007; Tugume *et al.*, 2010). In recombination, a portion of the coat protein (CP) of one virus is replaced with a homologous CP of another virus, like it happened when recombination between EACMV and ACMV led to the emergence of a severe strain in Uganda called EACMV-UG (Zhou *et al.*, 1997). Recombination occurs frequently due to co-existence of multiple CMB species in one geographical area. The generation and emergence of highly fit and new recombinant variants can affect cassava productivity.

Understanding how viruses evolve requires the knowledge of genetic variability dynamics of the viruses through characterization. Through characterization, virus genetic imprints, migration and dissemination routes across the African continent can be traced through sequence analysis. This study sought to provide current information on the distribution of cassava mosaic begomoviruses and their associated DNA satellites in Kenya in order to help in designing control strategies. The study also aimed at determining whether the overlapping of CMBs across geographical ranges and recombination events have yielded any epidemiologically significant lineage.

3.2 Materials and methods

3.2.1 Study sites

Cassava mosaic begomoviruses and associated DNA satellites were assessed in a country-wide survey in four regions of Kenya in 2013. The sites surveyed have high cassava production as a food crop. Fields were randomly selected along motorable

roads at regular intervals of 5-10 km. In the selected fields, disease severity, incidence and prevalence were determined. Infected cassava leaf samples were also collected in silica gel-filled containers lined with cotton wool for laboratory analysis. The field co-ordinates and altitudes were determined by the global positioning system (GPS). The sampling sites are shown in the map below (Fig 3.1).

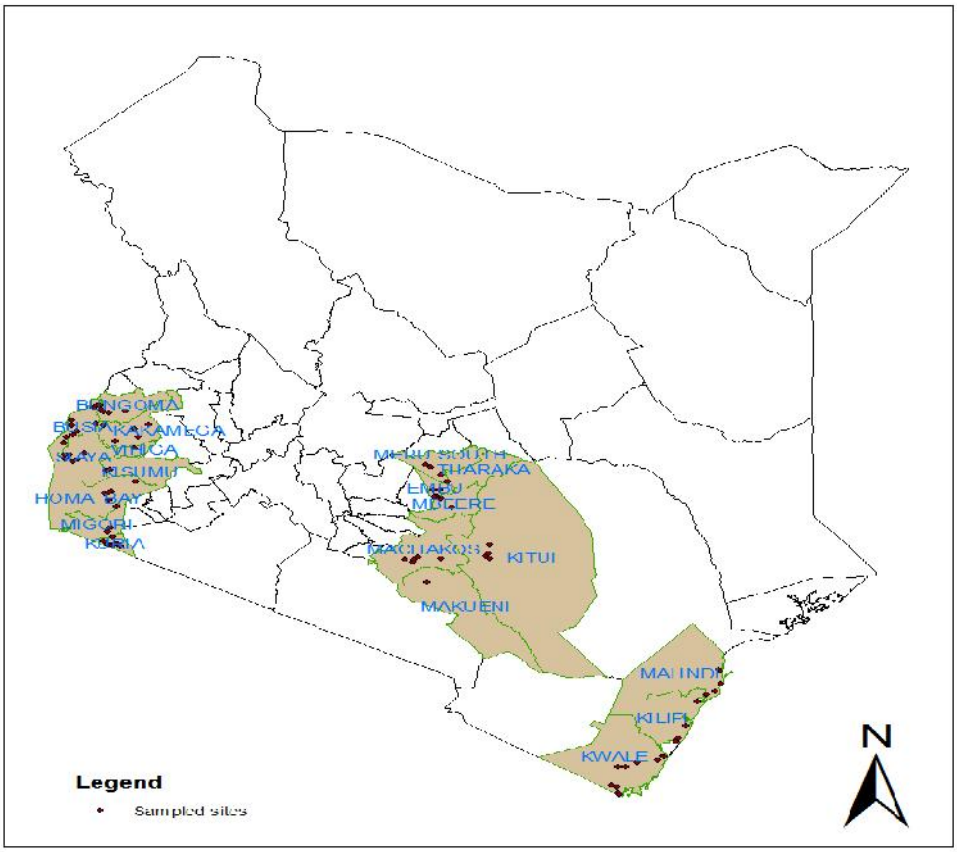

Fig 3.1. Map of Kenya showing cassava mosaic sampling sites

3.2.2 Sampling methodology

Stratified random sampling was used with the regions (Coast, Nyanza, Eastern and Western) representing the four strata. Random sampling of fields of either cassava intercropped with other crops or cassava pure stand was done. The CMD severity was

assessed in crops of between 3-12 months due to variances in planting dates in various regions.

A total of 24 fields in Nyanza, 22 in Western, 22 in Coast and 20 in Eastern were sampled. In each field, a diagonal transect was made from both ends of the field resulting into two halves within a field from which 15 plants were scored for CMD disease. In total 30 plants from each field were scored for CMD. In fields with less than 30 plants assessment was done in all the plants for comparisons amongst the fields. The following parameters were measured; disease severity, disease incidence, disease prevalence and whitefly populations. The CMD severity was determined through a visual scale (1-5) (Hahn *et al.,* 1989) while incidence was determined by calculating the percentage of visibly infected plants in relation to the total number of plants assessed (Fargette, 1985). Disease prevalence was determined as the proportion of infected fields in relation to the total number of fields sampled in each region. Direct whitefly counts were used in determining the number of adult whiteflies on individual sampled plants. Five topmost shoots were sampled for whiteflies at any sampling date (Fargette, 1985). Sampled leaves were inverted gently and adult whiteflies present recorded (Fishpool *et al.,* 1995). Infected cassava leaf samples; 68 in Nyanza, 82 in Western, 46 in Eastern and 80 in Coast were collected in silica gel-filled containers lined with cotton wool for laboratory analysis.

3.2.3 Detection of Cassava begomoviruses from collected samples

3.2.3.1 PCR for cassava geminiviruses

The cetyl trimethyl ammonium bromide (CTAB) protocol was used for extraction of total nucleic acids (Chang *et al.,* 1993) with slight modifications. Dry cassava leaf tissue of 0.04 g was placed on a mortar and crushed using a pestle with 1000 µl of CTAB extraction buffer (2% w/v, 1.4 M NaCl, 0.2% (v/v), 20 mM EDTA, 100mM Tris-HCl, pH 8.0). An aliquot of 750 µl of the samples was poured into 1.5 ml eppendorf tube and the samples incubated at 65ºC for 30 min.

Samples were then mixed with 650 µl of chroloform:isoamylalcohol (24:1) by vortexing to remove protein contaminants and then centrifuged at 13000 rpm for 10 min. Then 500 µl of the top aqueous phase was transferred into a new 1.5 ml eppendorf tube. Samples were precipitated by adding 500 µl of cold isopropanol before further centrifuging at 13000 rpm for 10 min at 4ºC and supernatants discarded. Pellets were washed in 0.5 ml 70% ethanol by vortexing and then centrifuged at 13000 rpm for 5 min. Ethanol was removed and pellets air dried for 5 min before diluting them with 50 µl of distilled water.

The integrity of DNA was determined by a sample of 2 µl run in a 2% agarose gel in TBE buffer for 30 min stained with 1 µl ethidium bromide. Negative controls were obtained from the DNA of healthy plants while DNA from symptomatic leaves was used as a positive control.

The DNA amplification was done using virus specific primers (Table 4.1) (Aloyce *et al.,* 2012) in a 14.6 µl reaction mix containing 2.5 µl 10x reaction buffer, 0.2 mM dNTPs, 0.2 mM $MgCl_2$, 0.2 µl taq polymerase, 0.25 µM forward and reverse primers, 10 µl water and 1 µl of template DNA. The primers used in this study are as shown in table 3.1 below;

Table 3.1. Primers used in DNA amplification of CMD in samples collected from cassava growing regions of Kenya

Primer	**Sequence**	**virus species**	**Target**
JSP001	ATGTCGAAGCGACCAGGAGAT	ACMV	CP
JSP002	TGTTTATTAATTGCCAATACT	ACMV	CP
EAB555F	TACATCGGCCTTTGAGTCGCATGG	EACMV	DNA-B
EAB555R	CTTATTAACGCCTATATAAACACC	EACMV	DNA-B

(Adapted from Aloyce *et al.,* 2012)

Amplification of DNA was done in a 96 well Thermocycler. The PCR cycle used for the amplification had an initial denaturation step at 95°C for 5 min, then 30 cycles of denaturation at 95°C for 0.3 min, primer annealing at 58°C for 0.3 min and elongation at 72°C for 0.45 min. This was then followed by a final elongation step at 72°C for 5 min. Ten microliter aliquots of PCR products were mixed with 2 µl of loading dye and electrophoresed on 1% agarose gel at 90 V for 1 h. Bands obtained were viewed in a gel documentation system and photos taken using ingrained camera.

3.2.3.2 Detection of DNA satellites by PCR

The cetyl trimethyl ammonium bromide (CTAB) method was used for extraction of total nucleic acids with slight modification (Chang et al., 1993). Amplification was then done using primer pair SAT III F-5'-AGGCCTCGTTACTAAAAGTGC-3', SAT III R-5'-ACCTGACGGCAGAAGGAAT-3' giving a product size of 306bp and primer pair satIIF 5'-GCCGCACCACTGGATCTC-3', satIIR 5'-CAGCAGCCAGTCAGGAAGTT-3' giving a product size of 895bp. The PCR conditions used in this reaction were, initial denaturation at 94ºC for 3min, denaturation 94ºC for 1min, annealing 55ºC for 1.5min, extension of 72ºC for 1min. and a final extension step at 72ºC for 4 min. Gel electrophoresis was done in 1% agarose gel to detect the PCR products and visualized in a bench top UV translluminator.

3.2.4 Molecular characterization of cassava begomoviruses and DNA satellites from major cassava growing regions of Kenya

3.2.4.1 Detection of CMBs

The CTAB method was used in extraction of template DNA from dry cassava leaf samples (Chang *et al.,* 1993) with 70% ethanol used in washing the DNA pellets before suspending them in distilled water.

The template was used in constituting a PCR mix with primer pair EACMKV1/EACMKV2 for the detection. Similarly, primer pair EAB555F/EAB555R was used in the detection of EACMV. The PCR products were resolved with 1.2% agarose gel using TBE buffer and bands visualized using ethidium bromide staining.

3.2.4.2 Sequencing and phylogenetic analysis of CMGs isolates

Samples that tested positive for cassava begomoviruses from the major cassava regions of Kenya were EACMV (38), EACMV-KE (24) and DNA satellites (24) were selected for sequencing. Selection for sequencing was purposive considering the geographical locations from the survey for fair representation. DNA fragments amplified by PCR were cut from the gels and purified through PCR purification kit (Qiagen) and then sequenced. The following short sequences were obtained; EACMV (38), EACMV-KE (23) and DNA satellites (23) which were then edited. To improve sequence alignment, read ends and larger gaps were deleted. The new sequences obtained from this study were first deposited in the GeneBank and aligned with currently published sequences obtained through Basic local alignment search tool (BLAST) search tool in the National Centre for Bio-technology Information database (NCBI).

3.2.4.3 Phylogenetic analysis

ClustalW. (Thompson *et al*., 1994) was used to generate phylogenetic trees using Parsimony and Maximum likelihood methods and phylogram visualized in TreeView 1.6.6. Bootstrap analysis was done with 1000 replicates. Pairwise analysis of sequences was also done to identify the percent nucleotide sequence similarity among the isolates from this study and those from the Genebank.

3.2.5 Data analysis

Before analysis of variance (ANOVA) to determine significant differences in mean CMD severity scores of the sampled plants were subjected to square root transformation. Disease incidence and prevalence were arcsine transformed before analysis. The data were subjected to a one-way analysis of variance (ANOVA) using the GenStat 12.0 statistical package. The PCR bands were scored as either present (positive) or absent (negative).

3.3 Results

3.3.1 Disease incidence, severity and geographical distribution of cassava begomoviruses and associated satellites in major cassava growing regions of Kenya

Cassava mosaic disease incidences from the survey are presented in Table 3.2. The highest CMD incidence was recorded in Western region with an average of 55.5% (Table 3.2). In the other regions surveyed the average incidences in 2013 were, 43.3% in Nyanza, 33.6% in Eastern and 47.1% in Coast. Disease prevalence was highest in Nyanza (90%), Western (89.5%) and lowest in Eastern (58.5%) region (Table 3.2).

Significant differences ($p=<0.001$) in CMD disease severity was recorded amongst the surveyed regions. The highest CMD severity was recorded in Western with a mean of 1.8 and least in Coast (1.2) region (Table 3.2). The overall mean CMD severity for the cassava growing areas was 1.6.

The mean whitefly numbers are reported in Table 3.2. There were significant differences in mean whitefly numbers amongst the regions surveyed being highest in Coast (15.6) and lowest in Western (3.2) with an overall mean of 7.7 in all the main cassava growing regions.

Table 3.2. CMD incidence, prevalence, severity and whitefly counts from major cassava growing regions of Kenya

Region	No. of fields	CMD Incidence (%)	CMD prevalence (%)	Mean CMD Severity	Mean Whitefly No/leaf
Nyanza	24	43.3	90	1.8	5.5
Western	22	55.5	89.5	1.8	3.2
Coast	22	47.1	88.9	1.2	15.6
Eastern	20	33.6	58.5	1.4	6.6
Mean		44.8	81.7	1.6	7.7

3.3.2 Detections of CMBs from infected cassava samples

Four geminiviruses namely; EACMV, EACMV-KE, ACMV and EACMV-UgV were detected in cassava growing areas. *East African cassava mosaic virus* (EACMV) was detected using primer pair EAB555F/EAB555R that amplified a product size of 556bp (Plate 3.1). The highest EACMV infection occurred in Nyanza region (70.6%) followed by Eastern (69.6%), Coast region (67.5%) and the least infections being in Western region (36.6%) (Table 3.3).

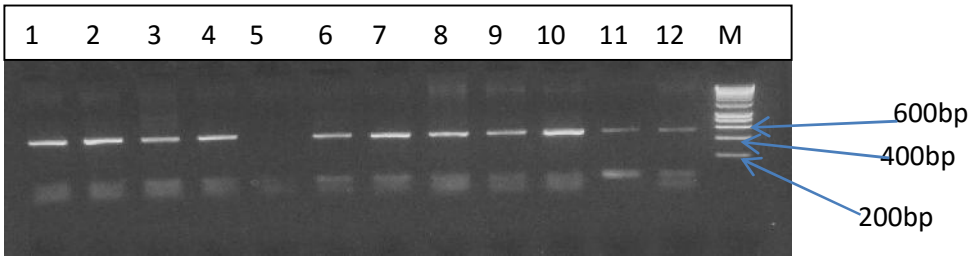

Plate 3.1. PCR product (556bp) profile showing EACMV amplification using primer pair EABF/R from infected cassava leaves; M is molecular marker of size 1kb;
Lanes 1-12: DNA extracts from field samples collected in Kenya.

The ACMV was detected through primer pair JSP001/002 giving an expected product of 774bp (Plate 3.2). The highest ACMV detections were found in Nyanza (29.4%) closely followed by Coast region with 22.5% detection and no detections of ACMV in the Eastern region of Kenya (Table 3.3).

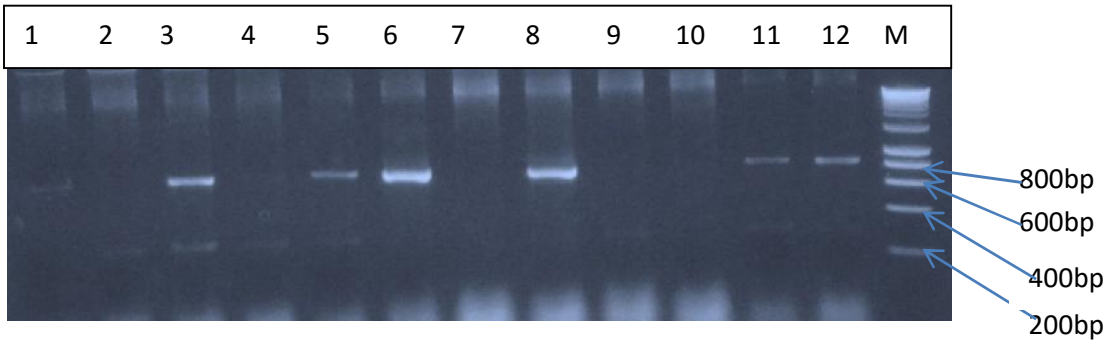

Plate 3.2. PCR product (774bp) profile showing ACMV amplification using primer pair JSP001/JSP002 from infected cassava leaves; M is molecular marker of size 1kb.

The EACMV-KE was determined through primer pair EACMKV1/EACMKV2 giving an expected fragment of 669bp size. In EACMV-KE, the highest detections were recorded in Nyanza region with 26.5%, then Coast and Eastern with 4.3% and 5%

detections, respectively. However EACMV-KE was not detected in the Western region of Kenya. In Eastern region, EACMV-KE was only detected in 2 samples while in Coast region four samples tested positive for EACMV-KE (Table 3.3).

Table 3.3 : Cassava begomoviruses detected from four major cassava growing regions of Kenya

Province	Total	EACMV	EACMV-KE	ACMV	EACMV-UGV
Nyanza	68	*48(70.6%)	18(26.5%)	20(29.4%)	28(41.2%)
Western	82	30(36.6%)	0.0	11(13.4%)	11(13.4%)
Eastern	46	32(69.6%)	2(4.3%)	0.0	11(23.9%)
Coast	80	54(67.5%)	4(5%)	18(22.5%)	12(15%)
Total	276	86(31.2%)	24(8.7%)	49(17.8%)	62(22.5%)

* Numbers outside the brackets represent actual samples testing positive for the viruses

The EACMV-UGV (Table 3.3) was recorded in all regions surveyed in this study with Nyanza province having 41.2% samples testing positive, Eastern region 23.9% samples, Coast 15% samples and the least EACMV-UGV infection occurring in the Western region of Kenya with 13.4% samples. The most widespread CMG species in the country were EACMV, followed by EACMV-UGV, ACMV, with EACMV-KE being least widespread and completely missing in the Western part of the country (Table 3.3).

3.3.3 Detection of DNA satellites

The cassava samples from the survey were positive for DNA III satellites giving a product of size 306bp (Plate 3.3). The highest number of DNA III satellites was recorded in Nyanza 39.7% followed by Western 15.9% with the lowest reported in Coast region (1%) (Table 3.4). This study did not detect the presence of DNA II satellites from the regions surveyed.

Table 3.4. Detection of DNA III satellites in CMD infected samples collected from major cassava growing regions of Kenya in 2013

Region	Total Samples	Positive samples
Nyanza	68	*27(39.7%)
Western	82	13(15.9%)
Eastern	46	6(13.0%)
Coast	80	1(1.0%)

* Numbers outside the brackets represent actual samples testing positive for the satellites

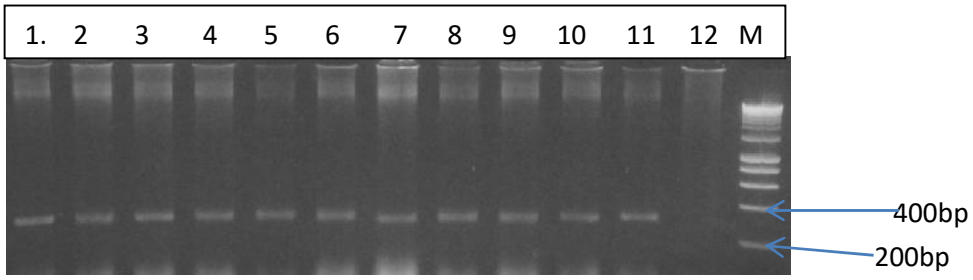

Plate 3.3. PCR product (306bp) profile showing DNA satellite amplification from symptomatic cassava leaves; M is molecular marker of size 1kb.

3.3.4 Diversity among Kenyan EACMV, EACMV-KE and DNA satellite isolates

Based on the phylogenetic analysis of the partial coat protein (CP) gene sequences for EACMV from this study, there were two clear minor and major clusters (Fig 3.2). The clustering however was not based on region of isolate origin. Isolates from the same region were found in different clusters within the phylogenetic tree. Comparison of the Kenyan EACMV isolates with those in the NCBI database revealed that the major cluster had isolates having similar sequence homology with EACMV isolates from

Uganda (HE979780.1, AJ704958.1 and AF126805.1). However isolates Odiado 1 and Odiado 2 from Western Kenya formed the second cluster and were different from other Kenyan isolates.

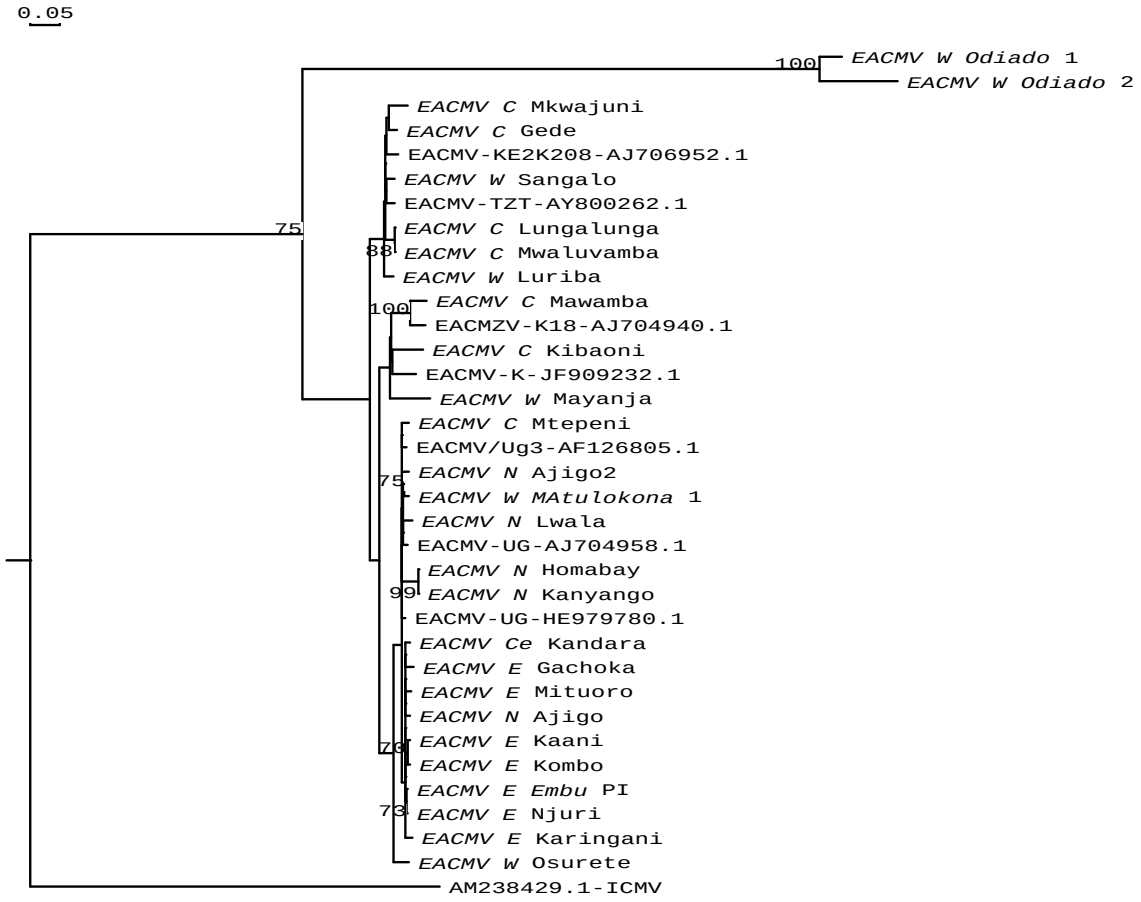

Fig 3.2. Phylogenetic tree based on EACMV partial CP nucleotide sequence of isolates collected from Nyanza, Coast, Western and Eastern regions of Kenya obtained by MEGA 5 software program.

From phylogenetic analysis, sequences from the EACMV-KE isolates from different regions of Kenya did not reveal great genetic variability within themselves (Fig 3.3). When compared with sequences in the NCBI database, the Kenyan isolates were closely related to an isolate from Tanzania (AY795988). The Kenyan EACMV-KE isolates

formed an independent clade with bootstrap value of 99%. However, sequence comparisons at the nucleotide level of EACMV-KE isolates from Kenya and those from other countries showed little correlation in the CP sequence variability with the isolates' geographical origin (Fig 3.3). Isolates from Uganda, Cameroon and Ghana formed one clade. The Kenyan isolates were more distant to those from Ghana, Uganda and Cameroon.

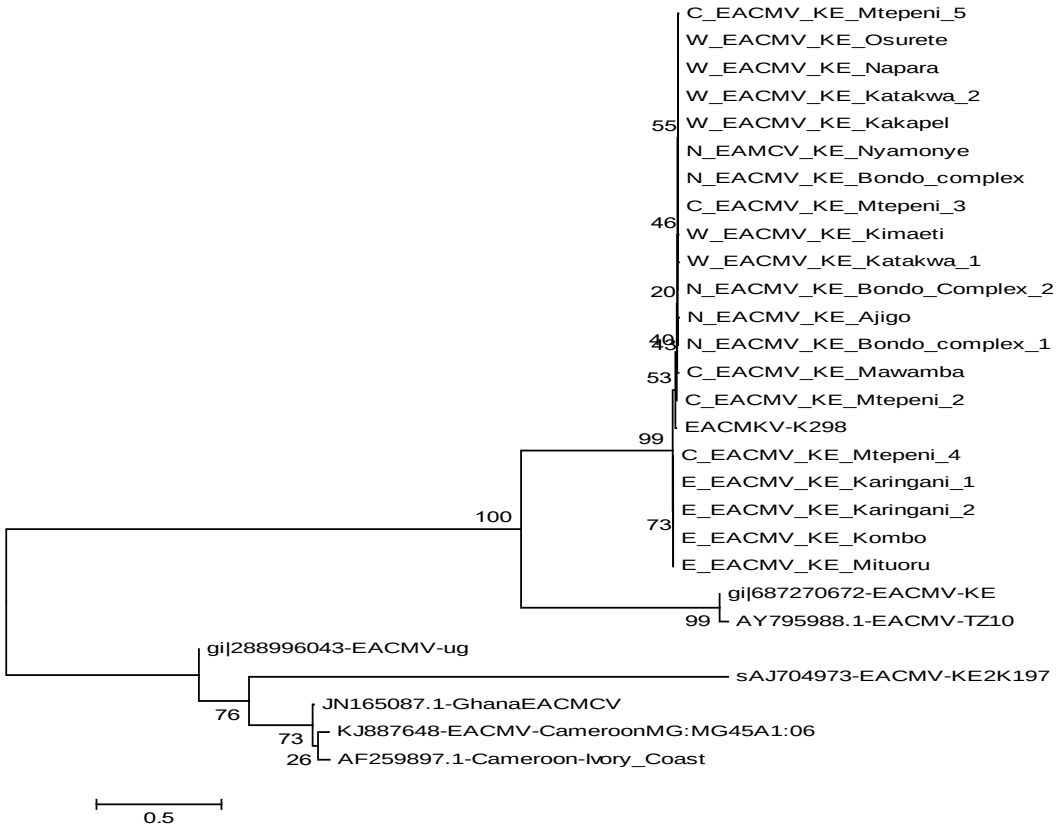

Fig 3.3. Maximum likelihood tree of base alignment of a partial CP of isolates of *East African Cassava mosaic Kenyan virus* (EACM-KE) using MEGA 5 software.

Analysis of DNA satellites showed three independent clades for the Kenyan isolates. Isolates from Kibaoni in Coast, Tagari in Nyanza, Matulokana and Luriba in Western and Lwala in Nyanza formed a single clade despite coming from different regions of Kenya (Fig 3.4). This was similar with isolates from Njuri and Kaani from Eastern, Kanyango and Opapo in Nyanza (Fig 3.4) that formed another single clade of isolates from different regions in Kenya. When compared with an isolate from the NCBI database B. DNA-III (AY836367), some isolates showed similar sequence homology (Table 3.5) forming another independent clade.

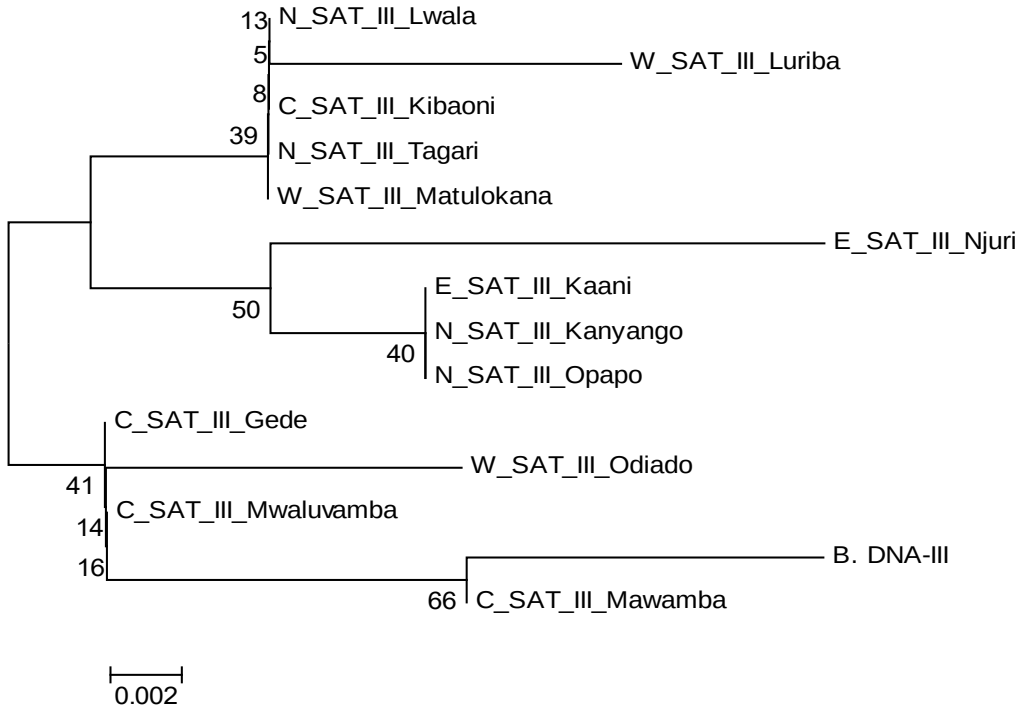

Fig 3.4. Phylogenetic tree obtained from the alignment of DNA satellites sequences from major cassava growing regions of Kenya with those available in the NCBI database using MEGA 5 software.

Table 3.5. Pairwise DNA III satellite nucleotide sequence homology matrix of 10 isolates sequences using MEGA 5 software

	Isolate	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	: B.AY836367.1	100													
2	C_SAT_III_Mawamba	99	100												
3	E_SAT_III_Njuri	96	97	100											
4	W_SAT_III_Odiado	97	98	97	100										
5	W_SAT_III_Luriba	96	97	96	97	100									
6	E_SAT_III_Kaani	96	97	98	97	98	100								
7	N_SAT_III_Kanyango	96	97	98	97	98	100	100							
8	N_SAT_III_Opapo	96	97	98	97	98	100	100	100						
9	C_SAT_III_Gede	98	99	98	99	98	98	98	98	100					
10	C_SAT_III_Mwaluvamba	98	99	98	99	98	98	98	98	100	100				
11	C_SAT_III_Kibaoni	97	98	97	98	99	99	99	99	99	99	100			
12	N_SAT_III_Lwala	97	98	97	98	99	99	99	99	99	99	100	100		
13	N_SAT_III_Tagari	97	98	97	98	99	99	99	99	99	99	100	100	100	
14	W_SAT_III_Matulokana	97	98	97	98	99	99	99	99	99	99	100	100	100	100

3.4 Discussion

The major cassava growing regions in Kenya were assessed for cassava begomoviruses. Varied disease symptoms were observed that included, leaf distortion, leaf narrowing, severe mosaic and leaf curling. Differences in disease symptoms have been linked to environmental conditions, susceptible hosts and virus species present (Legg & Thresh, 2003). This study revealed that the region most affected by CMD in terms of incidence was Western while the least (33.6%) disease incidence was recorded in Eastern region. This is in contrast with a study done in 2009 where Coast reported the highest incidence (Mwatuni *et al.,* 2015). The increase in disease incidence from (47%) in previous survey (Mwatuni *et al.,* 2015) to current (55.5%) in Western can be attributed to the

continuous use of local genotypes, high cassava density and frequent exchange of planting materials in farmers' fields especially bordering Uganda. This is in agreement with studies in Ivory Coast in which higher disease incidences were linked to recycling of planting materials from infected fields and use of two widely grown susceptible varieties (Marie *et al*., 2014).

Nyanza and Western regions reported the highest CMD prevalence while the least disease prevalence was in the Eastern region. Another study in 2009 (Mwaituni *et al.*, 2015) reported similar findings that Nyanza region had the highest prevalence while the Eastern recorded the least disease prevalence. This study also recorded an overall countrywide CMD severity of 1.6, a reduction from that recorded in a previous study of 1.8 (Mwaituni *et al.,* 2015).

The highest severity was 1.8 in Western region and lowest in Coast with 1.2. In terms of CMD severity, South Africa has shown to have lower values (1.6-1.7) in comparison with 3.0 recorded in East Africa in times of higher epidemics (Legg *et al.,* 2001).

The highest reported whitefly average counts were recorded in Coast region (15.6) and the lowest being in Western (3.2). In terms of disease incidence and prevalence however, Western had the highest showing a lack of correlation between whitefly counts and disease incidence. A latent period between inoculation of the virus by whiteflies and symptom development has been linked to lack of a relationship (Fauquet & Fargette, 1990). In surveys done in South Africa, variations in whitefly numbers were linked to differing climatic conditions. Areas with high rainfall had vigorous plant growth and consequently high whitefly numbers. According to Doughty (1958) high CMD incidences are reported in wet regions. Legg *et al.* (1994) linked whitefly infestation with altitude reporting higher whitefly activity at low altitude of 170m above sea level (a.s.l) as compared to higher altitude areas of 900m.

This study determined that ACMV, EACMV, EACMV-UV, EACMV-KE and DNA satellites are present in the major cassava growing regions of Kenya agreeing with a

study done that showed an overlap in geographical distribution of EACMV and ACMV (Were *et al.,* 2004; Mwatuni *et al.,* 2015). The most widespread CMB species from this study were EACMV and EACMV-UG. Highest EACMV and EACMV-UG detections occurred in Nyanza and lowest in Western. Similar studies in Ivory Coast and Togo determined that EACMV and ACMV were the most common begomoviruses (Adjata *et al.,* 2008; Marie *et al.,* 2014).

In Madagascar, cassava mosaic begomoviruses showed variations in geographical distributions influenced by altitude. *East Africa cassava mosaic Kenyan variant* (EACMV-KE) and EACMV were reported to occur in Coastal areas and lowlands, whereas ACMV was most prevalent in Central highlands (Cours *et al*., 1997; Harimalala *et al*., 2015). Ogbe *et al*. (2001) reported that in Nigeria, EACMV was found prominently in southern Guinea savannas, coastal areas and humid forests. In this study, the highest EACMV-KE recorded was in Nyanza, Coast and Eastern regions respectively with no detections reported in Western region. Similar results were recorded for ACMV with Nyanza recording highest virus infections and lowest in Western region. However, ACMV was not detected in samples from Eastern region of Kenya in contrast with a previous study in 2009 where one sample tested positive for ACMV in Eastern Kenya (Mwaituni *et al.,* 2015). These results have shown that Nyanza region is a CMBs hotspot as indicated by the higher number of detections for all the studied begomoviruses.

Satellites, which are molecules derived from helper virus DNA-A or DNA-B, have been reported in Tanzania and Kenya (Ndunguru *et al.,* 2006; Mwatuni *et al.,* 2015). They have been linked to symptom modulation, increasing or reducing symptom severity and have broken resistance of some tolerant genotypes like TME3 (Ndunguru *et al.,* 2005). In this study, DNA III satellites were reported from all the surveyed regions in Kenya, being highest in Nyanza and lowest in the Coast region. Results of DNA III satellites from this study showed a reduction in detection rate from the cassava growing regions of Kenya compared to a previous study that had higher detections (Mwatuni *et al.,*

2015). This was however not the case for DNA II satellites which were not detected from this study. This is in contrast to a report by Mollel *et al.* (2014) that reported their presence in Kenya.

From the partial analysis of CP gene sequences of EACMV isolates in this study, there were two clear clusters. However, the EACMV isolates from Western Kenya (Odiado 1 and Odiado 2) were significantly different from the other isolates. A similar study in Togo identified two clusters of EACMV isolates with one cluster having isolates similar to a Kenyan isolate and three isolates from Uganda (Adjata *et al.,* 2008b). The EACMV-KE isolates from this study did not show great genetic variability within themselves. They grouped together in one cluster with bootstrap values of 99%. This indicates that these isolates are strains of the same virus (EACMV-KE) based on recommendations from International committee of taxonomy of viruses that considers begomoviruses with values between 90-99% similar (Bull *et al*., 2006). The Kenyan EACMV-KE isolates grouped together with a Tanzanian isolate but when compared with isolates of the same size from Uganda, Cameroon and Ghana, no genetic similarity was observed.

DNA III satellites were widely distributed in the major cassava growing regions of Kenya. The DNA III satellites from different regions (Kibaoni from Coast, Tagari from Nyanza, Luriba and Matulokana in Western and Lwala from Nyanza) grouped together forming an independent clade. However, when the Kenyan DNA III satellites were compared with an isolate from the NCBI database (AY836367), an isolate from Mawamba showed close sequence homology. This is in agreement with a study that compared some DNA-III satellites from Kenya, Tanzania and Uganda that showed the sequences having the GC region being similar to episomal DNA-III (AY836367) (Ndunguru *et al.,* 2008).

In conclusion, there was a slight increase in disease incidence in Western region with other regions like Coast, Eastern and Nyanza reporting a decline in incidence compared

to a previous survey (Mwatuni *et al.,* 2015). However, the study showed high cassava mosaic begomoviruses infections in Nyanza, a cause for concern for policy makers. All the detected begomoviruses in this study were found in this region. Amongst the cassava begomoviruses, EACMV-KE had the lowest number of detections being absent in Western region. Similarly, there were no detections of ACMV in Eastern region. This study did not also detect the presence of DNA II satellites despite being present in a previous survey.

The EACMV isolates and DNA satellite sequences from Kenya were heterogeneous based on phylogenetic analysis as they formed independent clusters. This demonstrates a large genetic diversity within the cassava mosaic begomoviruses and their associated satellites. Two EAMCV isolates from Western region were significantly different from all the others as they formed an independent cluster. Clustering of isolates was not based on region of origin but rather random. Isolates from the same region were found in different clusters within the phylogenetic tree.

CHAPTER FOUR

4.0 Reaction of elite cassava genotypes to infection by Cassava mosaic and Cassava brown streak diseases in Kenya

Abstract

A study was done to determine the reaction of elite cassava genotypes to infection by cassava mosaic and cassava brown streak diseases under field conditions at high disease pressure. Five elite cassava genotypes (08/363, F10-30-R2, Nl, Tajirika, Ex-mariakani), susceptible genotype (Kibanda Meno) and a local check/control (Shibe) were grown under natural disease. The populations of the whitefly vector, *Bemisia tabaci* (Gennadis), severity and incidence of cassava mosaic virus were recorded during the growth period at 3, 6 and 9 months after planting (MAP). Infection was determined through disease severity, incidence, Enzyme linked immunosorbent assay (ELISA) and Polymerase Chain Reaction (PCR) assays. The Cassava mosaic and Cassava brown streak diseases incidence was highest in Kibandameno (>70%) and lowest in Shibe and Tajirika. Mean CMD/CBSD severities in the tested genotypes was highest in Kibanda meno (>1.5). Both ELISA and PCR detected CMD and CBSD in asymptomatic and symptomatic cassava samples. Genotypes 08/363 and F10-30-R2 had greater resistance to cassava mosaic and cassava brown streak disease while Kibandameno and Ex-mariakani were highly susceptible.

4.1 Introduction

Cassava is considered a perennial woody herb mostly grown as an annual in tropical lands. It is used as a food crop and an industrial raw material for both the rural and urban populations (Cleanstar, 2012). In Africa, it's estimated that the annual cassava production is 141 million tons (FAOSTAT, 2012). The crop however faces many

challenges during growth of abiotic and biotic nature (Nduguru *et al.,* 2005). Key diseases include cassava begomoviruses and Cassava brown streak disease (CBSD) (Legg et al., 2011).

Cassava begomoviruses have been described as the most significant cassava viruses in sub-Saharan Africa (Legg *et al.,* 2006). The viruses lead to stunted plant growth and highly reduced yield (Otim-Nape *et al.,* 2000). The increase in incidence and severity of begomoviruses has been linked to increased whiteflies (*Bemisia tabaci)* populations (Morales, 2006). Cassava brown streak disease (CBSD) also endangers cassava production across East Africa and is second to cassava mosaic virus in crop losses (Hillocks and Jennings, 2003; Rwegasira *et al.,* 2011). The disease is caused by two distinct but related *Ipomoviruses, Cassava brown streak virus* (CBSV) and *Ugandan cassava brown streak virus* (UCBSV) (Mbanzibwa *et al.,* 2011).

According to Fehr (1987) there have been gains in productivity of crops in the twentieth century driven by crop breeding. Cassava has greatly gained from breeding efforts (Kawano, 2003). Among the most important achievements in cassava is breeding for resistance to viruses. However, viruses undergo frequent genetic mutations which may break resistance bred into cassava varieties and therefore breeding efforts must always stay ahead of diseases (Nagib & Ortiz, 2010).

Most cassava breeding programmes use symptom expression for selection and detection of viruses. In these programmes absence of virus infection is shown by absence of visual symptoms. Visual disease estimation besides being subjective has been determined to underestimate disease severity in times of low infection levels (Kwack *et al.,* 2005). Another challenge with visual observation is that some symptoms have similarities with other crop constraints such as senescence and plant mineral deficiencies hence inconsistent expression. This makes use of visual assessment difficult in guiding the mapping of disease progress rates, control measures and the

prediction of yield loss. Breeding against cassava viruses can be enhanced through the use of robust, reliable and sensitive detection tools, which include serological and nucleic acid based diagnostic techniques.

In Kenya no studies have been done on the status of CMD and CBSD infestation of cassava germplasm in breeding nurseries using sensitive diagnostic tools. The aim of this study therefore was to determine the infection level of cassava germplasm in breeding nurseries at Kenya Agricultural and Livestock research Organization (KALRO)-Mtwapa.

4.2 Materials and methods

4.2.1 Study site and experiment set up

Cassava materials used in this study were obtained from Kenya Agricultural and Livestock research Organization (KALRO)-Mtwapa in Mombasa Kenya (latitude 3° 50'S, longitude 39° 44'E). Laboratory work was done in the Department of Horticulture of the Jomo Kenyatta University of Agriculture and Technology. To determine the incidence and severity of cassava mosaic and cassava brown streak diseases in cassava elite genotypes, an experiment was set up at (KALRO)-Mtwapa under natural disease infection. The climatic conditions of the site are; an altitude of 5m above sea level, rainfall of 1000mm per annum, average daily temperature of 32ºC and sandy soil type.
Five cassava genotypes (08/363, F10-30-R2, Nl, Tajirika and Ex-mariakani), a local CMD/CBSD susceptible genotype (Kibandameno) and a local improved genotype against CMD/CBSD (Shibe) (check/control varieties) were used. Before planting, all cassava planting materials were confirmed as being free of CBSV and CMBs. This was done through screening of the mother plants by PCR/RT-PCR using standard protocols for the absence of the viruses. The test genotypes were grown in a randomized complete block design (RCBD) replicated four times. The plots measured 6 m × 6 m and had 7 rows with 6 plants each with the separation from successive plot being At a distance of

2 m. The experiment was done under rain fed conditions without application of fertilizer and pesticides.

4.2.2. CMBs severity and incidence

Four plants of each variety were randomly selected within the net plot of the elite genotypes and tagged for data collection. The CMBs severity and incidence was recorded at 6 and 9 months after planting (MAP). The severity ratings were based on visual scale of (1-5) with 1 indicating healthy plants and 5 denoting very severely infected plants (Table 4.1) (Hahn *et al.,* 1989).

Table 4.1: Disease rating and corresponding symptom expression for cassava mosaic disease (CMD)

Rating	
1	No symptoms observed
2	Mild chlorotic pattern on entire leaflets or mild distortion at base of leaflets
3	Strong mosaic pattern on entire leaf, and narrowing cum distortion of lower one-third of leaflets
4	Severe mosaic distortion of two-thirds of leaflets and general reduction in leaf size
5	Severe mosaic distortion of four fifths or more leaflets, twisted and misshapen leaves

Disease incidence was calculated as;

$$\text{Disease incidence} = \frac{(N - n)}{N} \times 100$$

Where;

N = Total number of observations
n = Total number of plants with no disease symptoms.

4.2.3. Serological detection of CMBs

Symptomatic and asymptomatic leaves were sampled from each of the four tagged plants and preserved using silica gel-filled containers lined with a layer of cotton wool. Triple antibody sandwich-enzyme linked immunosorbent assay (TAS-ELISA) using polyclonal antibody immunoglobulin (IgG) and monoclonal antibody (Mab) was used to detect the presence of CMBs. Wells were initially coated with immunoglobin G(IgG) prepared by dissolving 10 µl IgG in 10 ml buffer and 100 µl of this solution added to each microtitre plate and incubated at 37°C for 3 h. The plates were then washed with phosphate buffered saline tween (PBS-T) using a wash bottle and soaked for a few minutes and rinsed twice. The plates were blotted by tapping them upside down on a paper towel. Addition of 100 µl blocking solution of 2% skimmed milk in PBS-T was done in each well and incubated at 37°C for 30 min.

The blocking solution was removed by tap-drying before 100 µl test sample was added to duplicate wells. Cassava leaf extracts from healthy and infected plants were used as negative and positive controls respectively while a buffer/water was used as a blank to enhance the test's validity. This was incubated overnight at 4°C and washed three times using PBS-T then 100 µl of MAb in conjugate buffer was added to each well and

incubated at 37°C for 2 hours before washing thrice using PBS-T. Addition of 100µl of RAM-AP in conjugate buffer was done in each well and incubated for 2 h at 37°C. Washing was then done three times before addition of 200 µl freshly prepared substrate to each well and incubated for 60 min at 37°C. Incubation of plates was done for one hour after substrate addition at room temperature. Measurement of enzymatic reactions was done using an ELISA micro plate reader. Leaf samples with mean absorbance values at 405 nm (A405) twice higher than the negative control were considered virus infected.

4.2.4. Molecular detection of cassava mosaic disease

The cetyl trimethyl ammonium bromide (CTAB) protocol was used for extraction of total nucleic acids with slight modification (Chang *et al*., 1993). Dry Cassava leaf tissue of 0.04 g was placed on a mortar and crushed using a pestle with 1000 µl of CTAB extraction buffer (2% w/v, 1.4 M NaCl, 0.2% (v/v), 20 mM EDTA, 100mM Tris-HCl, pH 8.0). An aliquot of 750 µl of the samples were poured into 1.5 ml eppendorf tube and the samples incubated at 65ºC for 30 min.

Samples were then mixed with 650 µl of chroloform: isoamylalcohol (24:1) by vortexing to remove protein contaminants and then centrifuged at 13000 rpm for 10 min. Then 500 µl of the top aqueous phase was transferred into new 1.5 ml eppendorf tube. Samples were precipitated by adding 500 µl of cold isopropanol before further centrifuging at 13000 rpm for 10 min at 4ºC and supernatants discarded. Pellets were washed in 0.5 ml 70% ethanol by vortexing and then centrifuged at 13000 rpm for 5 min. Ethanol was removed and pellets air dried for 5 min and dried pellets diluted each with 50 µl of distilled water. The integrity of DNA was determined by a sample of 2 µl run in a 2% agarose gel in TBE buffer for 30 min stained with 1 µl ethidium bromide.

For CMBs, DNA amplification was done using virus degenerate primer EAB555/F, EAB555/R giving a product size of 556bp (Fondong et al., 2000) in a 14.6 µl reaction mix containing 2.5 µl 10x reaction buffer, 0.2 mM dNTPs, 0.2 mM $MgCl_2$, 0.2 µl taq polymerase, 0.25 µM (Promega) forward and reverse primers, 10 µl water and 1 µl of template DNA. The DNA from virus free and symptomatic cassava plants was used as negative control and positive control respectively. Amplification reaction was done in a 96 well thermal cycler Gene Amp® PCR System 9700 (Applied Biosystems, USA). The PCR cycle used for the amplification had an initial denaturation step at 95°C for 5 min then 30 cycles of denaturation at 95°C for 30s, primer annealing at 58°C for 30s and elongation at 72°C for 45s. This was then followed by a final elongation step at 72°C for 5 min. Ten microliters aliquot of PCR products were mixed with 2 µl of loading dye and electrophoresed on 2% agarose gel at 90 V for 1 h. Bands obtained were viewed in a gel documentation system.

4.2.5 CBSD symptom assessment

Symptoms were assessed on four plants in the net plots of the cassava genotypes tagged for CBSD as was done for CMD in section 4.2.2. Severity was determined at 6 and 9 months after planting. Severity of CBSD was done using a scale of 1-5 where 1 represents healthy plants and 5 severely infected plants (Hillocks& Thresh, 1998).

4.2.6 Molecular detection of Cassava brown streak disease causing viruses

From the CBSD tagged plants, 2-3 leaves were collected from the net plot of the evaluated varieties and preserved in silica gel-filled containers lined with cotton wool for laboratory analysis.

The cetyl trimethyl ammonium bromide (CTAB) method was used for extraction of total nucleic acids (DNA) with slight modification as described in section 3.2.4 (Chang et al., 1993). Integrity of DNA was determined by a sample of 2 µl run in a 2% agarose gel in TBE buffer for 30 min stained with 1 µl ethidium bromide. The samples were then used to prepare cDNA which was the template for CBSV amplification. For cDNA synthesis of virus RNA, a kit was used following the manufacturer's instructions (Promega, UK). Synthesis was done as master mix one (MM1) 7.25 µl and master mix two (MM2) 3 µl in a total volume of 10.25 µl. Master mix one was incubated at 65ºC for 5 min and quickly chilled with ice for 2 min. Then cDNAs were prepared by mixing MM1 and MM2 by placing aliquots in 0.5 ml microfuge tubes. Components of MM1 included random primers, RNA, dNTPS and distilled water in different concentrations. MM2 component contained first strand buffer, DTT, and reverse transcriptase enzyme. The mixtures were then incubated at 25 ºC for 5 min, 55 ºC for 60 min and 70 ºC for 15 min. Generated cDNAs were ready for use in PCR. Amplification was done in a thermal cycler Gene Amp® PCR System 9700 (Applied Biosystems, USA).

The primer pair CBSDDF2/ CBSDDR (Mbanzibwa *et al.,* 2011a) was used for amplification of DNA. Amplification was done in a thermal cycler Gene Amp® PCR System 9700 (Applied Biosystems, USA) to detect the viruses using various temperature profiles with initial denaturation occurring at 94 ºC for 1 min, final denaturation at 94 ºC for 30 seconds, annealing at 52 ºC for 30 s, initial extension at 72 ºC for 45 s and final extension at 72 ºC for 5 min. Resolution of the RT-PCR products was done with 2% agarose gel using TBE buffer and bands visualized using ethidium staining (Abashi et al., 2010). Scoring was done for either presence (CBSV/UCBSV positive) or absence (CBSV/UCBSV negative) of the amplified products.

4.2.7 Determination of whitefly population for the evaluated varieties

Direct counts of adult whitefly populations on the five topmost leaves of the four tagged plants was done (Ariyo *et al.,* 2005). Counting was done early in the morning (0800-0900h) when conditions are relatively cool and the whiteflies are immobile (Fauquet *et al.,* 1987). Cassava leaves were turned upside down carefully and the number of whiteflies on the lower surface counted and recorded (Asare *et al.,* 2014). The insect counts were done at 3 months (September 2013; **dry season**), at 6 months (December 2013; **Wet season**) and 9th month (February 2014; **dry season**).

4.2.8 Data analysis

Before analysis of variance (ANOVA), severity scores for all plants were subjected to square root transformation. Disease incidence values were subjected to arcsine transformation before analysis of variance (ANOVA). Data were analyzed by Genstat Statistical software, Version 12 and treatment means compared by the least significance difference test.

4.3 Results

4.3.1 Incidence and symptom severity of Cassava mosaic disease

A range of CMD symptoms were observed on infected cassava plants. The most common symptoms recorded included mottling, mosaic and vein banding. The other symptoms recorded included leaf chlorosis, leaf curling, shoe string and distortion. At 3 MAP, there was on observable symptoms on all plants sampled. The mean CMD incidence varied significantly ($p=<0.001$) among the genotypes in the range of 0 to 74.9 at 6 MAP and 0 to 82.5 at 9 MAP (Table 4.2). At 6 MAP, Kibandameno recorded the highest mean incidence at 74.9 followed by Ex-mariakani with a mean incidence of 26.2. The least mean incidence was recorded in genotypes Tajirika, Shibe, Nl and F10-30-R2 with zero each. At 9 MAP, highest mean disease incidence was recorded still in Kibanda meno at 82.5. There was no disease incidence in genotypes Nl, Shibe and Tajirika (Table 4.2).

Table 4.2; Cassava mosaic disease incidence recorded on cassava genotypes at 6 and 9 months after planting (MAP)

Genotypes	6 MAP	9 MAP
F10-30-R2	0a	29.9b
Nl	0a	0a
Shibe	0a	0a
Tajirika	0a	0a
08/363	7.5ab	26.2b
Ex-mariakani	26.2b	26.2b
Kibandameno	74.9c	82.5c

Means with the same letter in the same column are not significantly different based on Turkey test at (p=<0.001).

At 6 MAP, Kibandameno had the highest mean CMD severity of 1.6. This was followed by variety Ex-mariakani mean CMD severities of 1.2. Varieties Nl, 08/363, Shibe, Tajirika and F10-30-R2 recorded the least mean CMD severity of one (Table 4.3).

At 9 MAP, the highest CMD severity was recorded in variety Kibandameno with a mean of 1.7. This was followed by variety 08/363 with a mean CMD severity of 1.1. The lowest mean CMD severity of 1 was recorded in varieties Nl, Shibe and Tajirika (Table 4.3)

Table 4.3. Cassava mosaic disease severity of 7 cassava genotypes evaluated at 6 and 9 MAP

Cassava Genotypes	6 MAP	9 MAP
Nl	1a	1a
Shibe	1a	1a
Tajirika	1a	1a
F1O-30-R2	1a	1.1a
08/363	1a	1.1a
Ex-mariakani	1.2a	1.2a
Kibandameno	1.6b	1.7b

Means followed by the same letter in each column are not significantly different ($p<0.001$) according to Turkey test.

4.3.2 Serological detection of CMD from evaluated cassava genotypes

Enzyme linked immunosorbent assay (ELISA) detected CMD in both symptomless and symptomatic samples (Table 4.4). Highest symptomatic and infected samples were recorded in Kibandameno 5 (31%) and the least in 08/363, Ex-mariakani and F10-30-R2 with 1 (6%) each. Of the 16 Kibandameno samples, 11 (68%) of them did not test positive in ELISA though initially symptomatic while 6 out of 16 (37%) Shibe leaf samples were CMD infected but asymptomatic. It was also determined that 10 out of 16 (62%) leaf samples from genotypes 08/363 and Shibe were symptomless and CMD tested negative.

Table 4.4. Detection of Cassava begomoviruses by ELISA in symptomatic and asymptomatic cassava leaf samples

Genotype	Asymptomatic and Negative	Asymptomatic and positive	Symptomatic and negative	Symptomatic and positive
08/363	*10(62%)	*3(18%)	*2(12%)	*1(6%)
Ex-mariakani	9(56%)	2(12%)	4(25%)	1(6%)
F10-30-R2	9(56%)	4(25%)	2(12%)	1(6%)
Kibandameno	0	0	11(68%)	5(31%)
Nl	7(43%)	3(16%)	4(25%)	4(25%)
Shibe	10(62%)	6(37%)	0	0
Tajirika	13(81%)	3(18%)	0	0

*Numbers outside the brackets represent actual samples testing positive for the viruses

3.3.3 Relationship between foliage CMD symptoms and molecular detection

Cassava mosaic disease detection by PCR (Plate 4.1) showed virus presence in both asymptomatic and symptomatic samples (Table 4.5). The highest asymptomatic and infected leaf samples 10 (62%) were recorded in Shibe while the lowest were in genotype F10-30-R2 with 2 (12%) (Table 4.5). Genotype F10-30-R2 had the highest symptomless and negative samples at 11 (68%). The highest symptomatic and CMD infected samples were recorded in Kibandameno 12 (75%) and the lowest being in 08/363 and F10-30-R2 with 2 (12%) each. Results also showed 4 (25%) of Kibanda meno samples being symptomatic and CMD negative (Table 4.5).

Table 4.5. Detection of Cassava begomoviruses in asymptomatic and symptomatic cassava genotypes by PCR

Genotype	Asymptomatic and Negative	Asymptomatic and positive	Symptomatic and negative	Symptomatic and positive
08/363	*9(56%)	*4(25%)	*1(6%)	*2(12%)
Ex-mariakani	7(43%)	4(25%)	2(12%)	3(18%)
F10-30-R2	11(68%)	2(12%)	1(6%)	2(12%)
Kibandameno	None	None	4(25%)	12(75%)
Nl	5(31%)	3(18%)	2(12%)	6(37%)
Shibe	6(37%)	10(62%)	None	None
Tajirika	7(43%)	9(56%)	None	None

*Numbers outside the brackets represent actual samples testing positive for the viruses

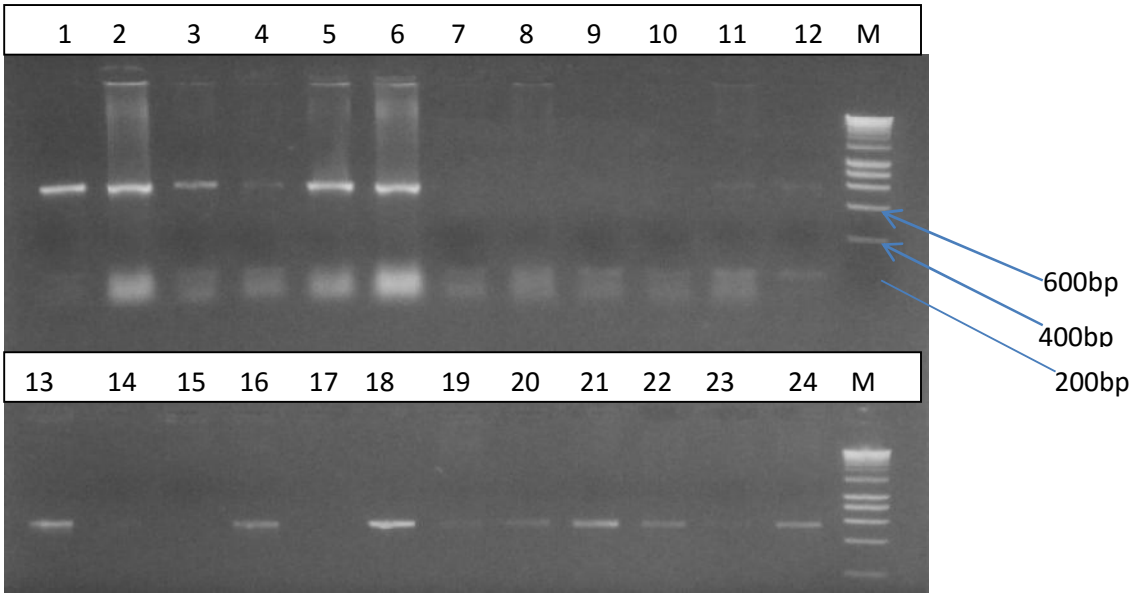

Plate 4.1. PCR profile for samples **(1-24)** using universal primers EABB555-F/EAB555-R (Fondong *et al*., 2000) to detect Cassava begomoviruses **Lane 3:** negative control from healthy plant; **lane 12**: positive control

4.3.4. Incidence of CBSD in elite genotypes

Genotypes Shibe, Tajirika and Nl did not show any shoot symptoms during this experiment. However, in those genotypes with symptoms, disease incidence was significantly different (p=<0.001) ranging from 7.5 to 74.9 at 6 MAP and 26.2 to 89.9 at 9 MAP respectively (Table 4.6). At 6 MAP, the highest incidence was recorded in Kibandameno (74.9) followed by Ex-mariakani (26.2). The same trend was observed at 9 MAP where Kibandameno recorded an incidence of 89.9 (Table 4.6).

Table 4.6. Cassava brown streak disease foliage symptom incidence at 6 and 9 MAP for 7 cassava genotypes

Genotypes	6 MAP	9 MAP
Shibe	0a	0a
Tajirika	0a	0a
08/363	7.5a	26.2b
F10-30-R2	0a	29.9b
Nl	0a	0a
Ex-mariakani	26.2b	26.2b
Kibandameno	74.9c	89.9c

Means followed by the same letter in the incidence column are not significantly different at 1% significant level

4.3.5. Severity of CBSD in cassava genotypes

The CBSD symptoms observed in this study were; feathery chlorosis, brown streaks on stems and die back in severely infected plants. Significant ($p=<0.001$) differences in CBSD severity were recorded in the test genotypes with Kibandameno recording the highest severity of 1.5 at 3 MAP and 1.6 at 9 MAP. The lowest severities were recorded in varieties Tajirika, Shibe and 08/363 at 1 (Table 4.7).

Table 4.7. CBSD shoot severity for seven cassava genotypes at 6 and 9 months after planting (MAP)

Cassava genotypes	6 MAP	9 MAP
08/363	1a	1a
Shibe	1a	1a
Tajirika	1a	1a
F1O-30-R2	1.2b	1.2b
Ex-mariakani	1.3bc	1.3bc
Nl	1.4c	1.4cd
Kibandameno	1.5c	1.6d

Means followed by the same letter in the columns are not significantly different at 1% significant level; severity was square root transformed for data normalization.

4.3.6 Molecular detection of CBSD

Both CBSV and UCBSV were detected by RT-PCR from the cassava genotypes used in this study (Plate 4.2). The highest (44%) CBSV detections were recorded in genotypes, Kibandameno, Ex-mariakani and Nl and least (13%) in Shibe. Similarly, UCBSV was highest (38%) in Nl, Kibandameno (31%) and Ex-mariakani (25%) with zero detection in variety Shibe (Table 4.8).

Table 4.8. Detection of Cassava brown streak virus (CBSV) and Cassava brown streak virus Ugandan variant (UCBSV) in 7 asymptomatic and symptomatic elite cassava genotypes

Genotype	Total	Symptomatic	Asymptomatic	Positive UCBSV	Positive CBSV
08/363	16	5(31%)	11(69%)	2(13%)	4(25%)
Ex-mariakani	16	16(100%)	0	4(25%)	7(44%)
F10-30-R2	16	5(31%)	11(69%)	1(6%)	2(13%)
Kibandameno	16	16(100%)	0	5(31%)	7(44%)
Nl	16	12(75%)	4(25%)	6(38%)	7(44%)
Shibe	16	0	16(100%)	0	2(13%)
Tajirika	16	0	16(100%)	1(6%)	4(25%)
Total	112	54	58	19	33

Numbers outside the brackets represent actual samples testing positive for the viruses

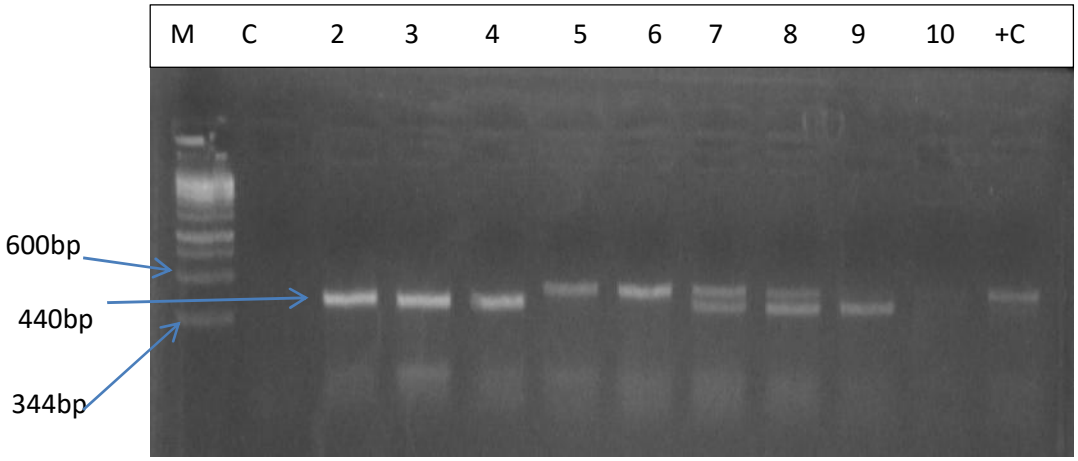

Plate 4.2. Gel electrophoresis amplification showing fragments 344bp (CBSV) and 440bp (UCBSV) from cassava leaves using the specific primer pair CBSDDF2 and CBSDDR. M is the ladder; C-Negative control and +C-Positive control. Lane 2-10 are the cassava test samples

4.3.7 Whitefly population

Varying counts of whiteflies were observed with respect to time of sampling and genotype. Higher mean counts of whiteflies were recorded in the dry season (3MAP) as compared to the wet season (6 MAP) and at 9 MAP. Significant differences (P=<0.001) in whitefly infestation were also recorded amongst the elite genotypes (Table 4.9)

At 3MAP, Nl recorded the highest number of whiteflies with a mean of 4.6 followed by genotype Kibandameno with a mean whitefly number of 3.9. Tajirika had the least mean number of whiteflies of 0.9 (Table 4.9).

At 6 MAP, the highest whitefly numbers were recorded in genotype Nl with a mean of 2.6. This was followed by Kibandameno with a mean number of whiteflies of 2.4 and F10-30-R2 with a mean number of whiteflies of 1.8. Ex-mariakani recorded the least mean number of 0.7 per the five top most leaves (Plate 4.3).

Plate 4.3. Field cassava leaves infested with whiteflies

At 9 MAP, Nl still recorded the highest mean number of whiteflies of 2.7. This was followed by Kibandameno and F10-30-R2 with a mean number of 2.3 and 1.5

whiteflies per five top most leaves respectively. E-mariakani exhibited the least number of whiteflies with a mean of 0.7 (Table 4.9)

Table 4.9: Population of whiteflies (*Bemisia tabaci)* recorded on the seven elite cassava genotypes at 3, 6 and 9 months after planting (MAP)

Genotype	3MAP	6MAP	9MAP
Ex-mariakani	2.7^{ab}	0.7^{a}	0.7^{a}
Shibe	1.5^{a}	1.1^{ab}	1.1a
08/363	1.6^{a}	1.3^{abc}	1.3^{ab}
Tajirika	0.9^{a}	1.6^{abcd}	0.9^{a}
F1O-30-R2	2.5^{ab}	1.8^{bcd}	1.5^{ab}
Kibandameno	3.9^{bc}	2.4^{cd}	2.3^{bc}
Nl	4.6^{c}	2.6^{d}	2.7^{c}

Means with the same letter along columns are not significantly different ($P<0.001$) according to Turkey test.

Cassava mosaic and cassava brown streak diseases are propagated by infected stem cuttings and vectors such as whiteflies. To determine the relationship between cassava virus severity and whitefly *Bemisia tabaci* vector, the correlation determination R^2 was calculated. At 6 and 9 MAP there was a small regression coefficient (R^2=0.1086 and 0.1896) between CMBs severity and whitefly numbers depicting a positive weak association (Fig 4.1). Slightly higher regression coefficients (R^2=0.3507 and 0.481) and a slightly stronger positive association was observed between CBSD severity and whitefly numbers at both 6 and 9MAP (Fig 4.2).

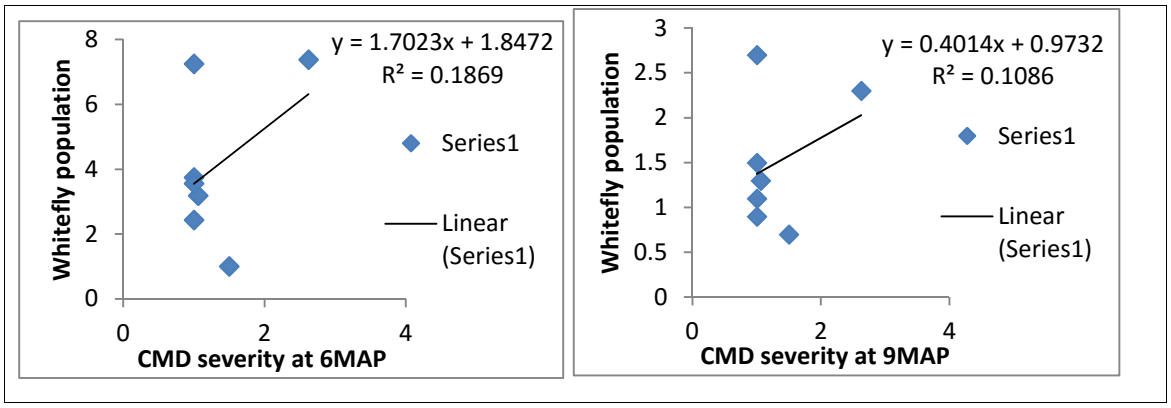

Fig 4.1 Relationship between CMBs severity and whitefly *Bemisia tabaci* numbers in cassava test genotypes at 6 and 9 MAP

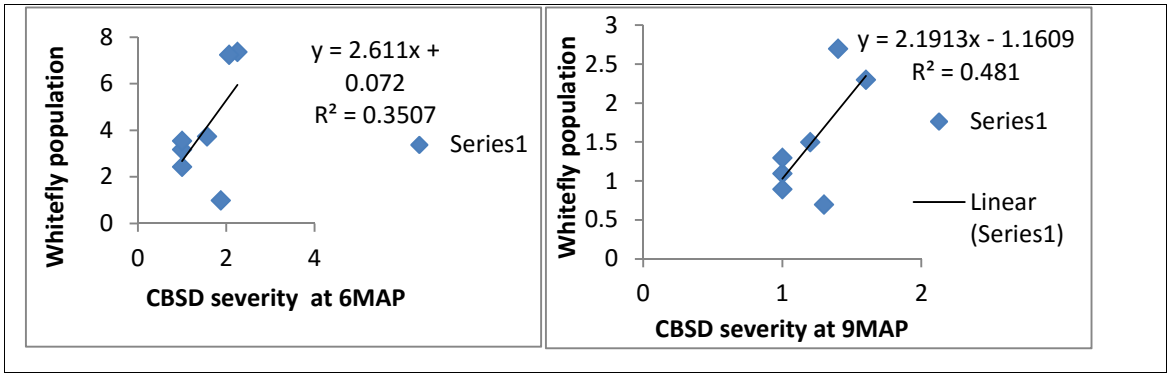

Fig 4.2 Association between CBSD severity and whitefly (*Bemisia tabaci)* numbers in cassava test genotypes at 6 and 9 MAP

3.4 Discussion

Production of cassava in Africa is constrained by CMD and CBSD with a number of strategies developed to control them. Current control mechanisms include the use of phytosanitation, disease surveillance and diagnostics and the use of virus resistant genotypes. Most cassava breeding programmes use symptom expression for selection

against virus diseases. In these programmes, absence of virus infection is shown by absence of visual symptoms. This study evaluated the cassava elite genotypes for resistance to CMD and CBSD infection under conditions of high disease and whitefly pressure.

From the test results, there were considerable variations in CMD incidence and severity in the cassava genotypes. In most of the genotypes such as 08/363, F10-30-R2 and Ex-mariakani, it took up to 6 months for the development of severe symptoms like leaf narrowing, distortion of leaves and mosaic pattern. Thresh (1994) reported that CMD severity is severe in susceptible genotypes than in resistant varieties. There were no observable symptoms in genotypes Shibe, Nl and Tajirika but higher disease incidences were recorded in Kibandameno (82.5%) and Ex-mariakani (26.2%) which also recorded mean severities of 1.7 and 1.2, respectively.

Variations in CBSD severity and incidence were observed among the genotypes which is in agreement with studies done by Hillocks and Jennings (2003). The highest CBSD incidence and severity were reported in Kibandameno with 89.9 and a mean severity of 1.6, respectively. However, there were no observable CBSD symptoms in genotypes Shibe and Tajirika. This indicates that these genotypes have properties useful in CBSD resistance breeding. Kaweesi *et al.* (2014) suggested that to completely ascertain the resistance potential of such genotypes, further diagnostics are necessary to eliminate disease 'escapes'. This information is important in the development of cassava seed certification system.

For sound disease control strategies, there is need for adequate disease diagnosis and understanding of disease development. Serological tests detected CMBs in both symptomatic and asymptomatic cassava samples in agreement with studies done on yam where TAS-ELISA detected yam mosaic virus (YMV) in initially symptomless plants (Toualy *et al.,* 2013). The highest detection of CMBs in symptomatic cassava samples through ELISA was in Kibandameno (31%) and the lowest in genotypes, Ex-mariakani, F10-30-R2 and 08/363 with 6% each. In PCR, higher numbers of

symptomless samples in Shibe and Tajirika tested positive for CMBs. Some samples that were symptomatic in Kibandameno also tested negative in PCR. This study also detected CBSD species (CBSV and UCBSV) through RT-PCR in both infected and healthy cassava leaves. Mathews (1981) reported that non-detection of disease in symptomatic plants suggests that symptoms could be caused by abiotic factors like nutrition deficiency or another pathogen. Yellowing and necrosis in sugar beet characteristic of beet yellow virus (BYV) has been caused by iron and magnesium deficiency (Uchida, 2000). Non detection of the virus in symptomatic materials could also be caused by failure of PCR due to faulty primers or presence of contaminants.

Whiteflies (*Bemisia tabaci)* are widely distributed and important in agriculture. They can be involved in direct feeding and transmission of plant viruses (Perrings, 2001). This pests have been highly associated with the spread of cassava viruses (Fargette *et al*., 1985). This study revealed a weak association between CMBs and whitefly vector but slightly stronger positive association between CBSD and the vector. Similarly, the study reported variations in whitefly infestations in varieties and at different times of the year with higher numbers in the dry season (3MAP) as compared to the wet season (6MAP). This is in agreement with Appiah *et al.* (2012) who reported increased numbers of whiteflies on jatropha accessions during the dry season than in the wet season. This could be due to the death of first instar larvae and suppression of oviposition caused by higher rainfall during the wet season. Mware *et al.* (2009) also reported higher whitefly populations at young plant age of 3 months showing whitefly preference for young leaves. Fishpool et al. (1987) reported that the activity of whiteflies is dependent on climatic conditions, host nutritional quality and the populations of natural enemies.

In conclusion, the use of visual symptoms in detection of viruses is not conclusive as initially asymptomatic samples tested positive for cassava viruses using sensitive diagnostic tools. The use of multiple detection tools by cassava breeders screening genotypes for resistance is recommended. Similarly, the whitefly vector that transmits

cassava viruses was prolific during the dry season as compared to the wet season. Vector control strategies should be deployed at times of higher populations for effective control.

CHAPTER FIVE

5.0 GENERAL DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 General discussion

Emergence of virus strains in both cassava begomoviruses and cassava brown streak disease has made distinction of symptoms for the individual strains difficult. This gap can be filled by detecting viruses in healthy and infected cassava materials through sensitive diagnostic tools complementing symptom based detection. This study tested the presence of viruses in elite genotypes through multiple detection methods namely; visual observation, serological and molecular. Varying results were obtained using the methods with higher disease detections occurring through molecular methods especially in varieties Shibe and Tajirika. This agrees with findings of Kaweesi *et al.* (2014) who reported that to completely ascertain resistance potential of genotypes, multiple diagnostic techniques are essential to eliminate disease 'escapes.' Multi-locational studies across different agro-ecological zones are also needed to determine the overall response of these elite genotypes to virus infections. This is because disease surveys have linked presence of virus strains and virulence to climatic and geographical considerations (Ariyo *et al.,* 2009).

Multiple CMGs were present in all cassava growing regions. There were variations in CMD incidence and severity from region to region in agreement with other studies (Legg & Thresh, 2004). In Kenya, CMD severity has been generally high, in contrast to those recorded in South Africa. Eastern Africa is considered a high epidemic area in regard to CMD severity. Highest CMD scores in this study were recorded in Western and Nyanza regions of Kenya respectively. In CMD epidemic areas, severity scores of up to 3.0 have been recorded (Legg *et al.,* 2001). Cassava mosaic disease severity has been found to be influenced by soil quality, cultivars, virus species and amount of rainfall. Higher disease severity in the regions has also been linked to co-infection of

ACMV and EACMV and possible recombinations (Fondong *et al*., 2001; Pita *et al.,* 2001). Similarly, severity of CMD has also been linked to different eco zones growing different cassava varieties (Ariyo *et al.,* 2009).

The challenge of comparisons of diseases amongst regions however is the fact that there are varying climatic conditions amongst the surveyed regions. The highest whitefly numbers were recorded in the Coast region during the 2013 survey with lowest being in the Western region. Climatic conditions have been shown to have an influence on whitefly numbers. High numbers have been linked with dry weather as determined in this study. This is in agreement with studies done on Jatropha accessions where highest whitefly numbers were reported during the dry season (Appiah *et al.,* 2012).

In this study four begomoviruses species were found in farmers' fields namely; EACMV, EACMV-KE, ACMV, EACMV-UG. This shows a high species diversity of begomoviruses infecting cassava in Kenya similar to observation made by (Mwaituni *et al.,* 2015). East African cassava mosaic virus (EACMV) was the dominant species amongst the cassava growing regions. Fondong *et al.* (2000) reported that EACMV induces more severe symptoms as compared to ACMV.

This study also determined that EACMV isolates from Kenya are not homogenous as they were in different clusters showing greater diversity whereas EACMV-KE isolates formed one clade with bootstrap value of 99% suggesting single introduction of EACMV-KE to Kenya. This reduces the risk of introduction of a new virus in the areas covered by this study through movement of planting materials. However, for in depth understanding of CMBs diversity, full length characterization of CP sequences should be done. Crossing of virus species across boundaries occurs through trafficking of cassava cuttings like the reporting of EACMV into Cameroon where it was not present (Fondong *et al*., 2000).

The fact that some isolates from some regions did not cluster with samples from their regions shows that there are no barriers to long distance spread of CMGs in Kenya. This

is in agreement with diversity studies done in Egypt on papaya ringspot virus (Omar *et al*., 2011). Wide divergence of isolates of papaya ringspot virus were linked to the different cultural practices and different cropping systems within the surveyed regions leading to differing selection pressure from the virus.

In comparison with known EACMV isolates from other regions, the Kenyan isolates showed limited variability. This can be due to low selection pressure by the virus. Generally, the Kenya EACMV isolates showed close relationship amongst themselves in phylogenetic trees. Sequence variability can enhance the use of CP genes in development of transgenic plants resistant to viruses. In designing transgenes for potyvirus resistance it has been determined that selection of regions with at least 90% sequence identity between strains can enhance wider disease resistance (Moreno *et al.,* 1998).

The sequence homology amongst the EACMV-KE isolates was found to be homogeneous with most isolates clustering together. The phylogenetic analysis of DNA satellites showed close similarity of isolates from other regions which are unexpected This is in agreement with a study done on plum pox virus (PPV) where isolates from different regions of Egypt clustered together (Matic *et al.,* 2011).

5.2 Conclusions

Genotypes 08/363 and F10-30-R2 from this study showed greater resistance to both CBSD and CMD while Kibandameno and Ex-mariakani were highly susceptible. The study showed that the absence of virus infection cannot be confirmed by the absence of foliar symptoms on the leaves. Healthy and phenotypically mild cassava isolates were determined to harbor cassava viruses. Absence of disease symptoms or presence of latent virus infections in plants has an impact on the spread as this will act as disease reservoirs. Therefore, newly developed varieties should be able to restrict virus

accumulation or be completely immune to eliminate sources of inoculum hence contain virus spread. The threshold at which viruses overcome plant defense mechanism can also be studied in order to determine resistance durability which will help in development of cassava planting materials seed systems.

Controlling plant pathogens especially viruses requires adequate understanding of their molecular characteristics like evolution, diversity and genetic variability and from this then a realistic breeding plan can be developed. The fact that most CMGs species were recorded in most of the cassava growing regions implies that there is risk of new virus introductions to the regions. Worth noting however was the non-detection of ACMV and EACMV-KE in Eastern and Western regions respectively. The non-detection of ACMV and EACMV-KE in Eastern and Western regions could perhaps be due to altitude. This study also revealed genetic variability in EACMV isolates with 2 isolates clustering independently. This is cause for alarm as they can potentially be new strains which further complicate disease management strategies. In EACMV-KE, the Kenyan isolates clustered together implying a single introduction. This reduces the risk of introduction of a new virus in the areas covered by this study through movement of planting materials.

5.3 Recommendations

This research has determined that molecular techniques (PCR and ELISA) can detect cassava viruses from both symptomatic and asymptomatic cassava breeders' materials. Further tests can also be done focusing on the virus load in order to develop a correlation between symptom severity and virus load in the cassava genotypes. Newly released resistant varieties should have either, low virus load or can restrict virus accumulation reducing source of inoculum, hence limiting virus spread. Further studies

can also be done focusing on thresholds at which plant defence systems are overcome through degeneration trials which will inform resistance durability.

Previous and present studies have shown higher levels of infection of cassava plants in Nyanza region with most of the cassava begomoviruses. Urgent strategies focusing on this area can be deployed to contain the viruses. In this case strategies such as phytosanitation, breeding and promotion of disease resistant genotypes should be implemented to control crop losses. Similarly, strict quarantine measures should be applied especially at border points to restrict entry of infected planting materials to the country. The study also determined that two isolates from Western region formed an independent cluster. This implies that they could be new strains of cassava begomoviruses. However, for clear understanding of CMBs diversity, full length screening of DNA sequences should be done. The study also determined the presence of DNA satellites from the major cassava growing regions of Kenya. The satellites have been shown to modulate disease symptoms. It is recommended that further studies be done to determine the effect of the satellites co-infection on virus load of cassava infected plants.

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