PROSPECTIVE OF TRANSGENIC CASSAVA LINES RESISTANCE IN EFFECTIVE MANAGEMENT OF CMV AND CBSV CAUSED VIRAL DISEASES OF *Manihot esculenta* Crantz IN KENYA

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A Thesis Submitted in Partial Fulfilment for the Requirements of the Award of the degree of Doctor of Philosophy in Crop Protection of Masinde Muliro University of Science and Technology

JULY 2025

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DEDICATION

I dedicate this thesis to my children (Nasra Were and Authman Were)

ACKNOWLEDGEMENT

My heartfelt appreciation goes to my supervisors (Prof. Vitalis Ogema and Dr. Emily Wamalwa) for their continuous guidance and unwavering support throughout the journey of this work. The substabtial contributions of Prof. Wilhelm Gruissem, Dr. Mehta Devang and the entire plant pathology team at ETH Zurich; Prof. Eugenie Herbrard of IRD Montepellier France and the Biosciences Eastern and Central Africa-International Livestock Research Institute (BecA-ILRI) Hub, played a pivotal role in shaping my academic endeavour. The generous resources provided by the National Research Fund, Kenya Climate Smart Agriculture Program were instrumental in facilitating comprehensive research for this thesis. To my cherished family and friends, your constant support, keen interest and belief in my capabilities are the pillars of this academic pursuit.

ABSTRACT

Cassava *(Manihot esculenta* Crantz*)* is an essential food crop in Kenya. Despite its importance, the national average yield of 3 tonnes/hectare is among the lowest globally, primarily due to the lack of high-quality and disease-free planting materials. The most devastating viral diseases in Sub-Saharan Africa are; Cassava Mosaic Disease (CMD) and Cassava Brown Streak Disease (CBSD). The viruses are spread by whiteflies and infected planting material. Recent studies have revealed that CMD and CBSD cause up to 100% yield losses. Proposed strategies for minimizing these loses include planting resistant varieties and large-scale surveillance. This study sought to establish the sources of cassava seed among farmers, type of viruses and associated cassava disease levels in selected counties of Kenya. Further, the study evaluated the diversity of viruses, viral load, resistance and yielding efficacy levels of 27 transgenic cassava lines, engineered towards management of CMD and CBSD diseases of cassava under a confined field trial at Alupe, Busia County. A diagnostic survey was carried out in Cassava growing counties of Kenya, followed by a randomized complete block in design experiment that evaluated the transgenic lines under a confined environment. Real time Quantitative PCR, Sequencing, modelling, TAS and DAS-ELISA techniques were used to answer the objectives. To establish an infection under the confined trial; leafy and hardwood cassava stem cuttings which were CMD and CBSD-infected from growing counties were used as infectors in the confined experiment at Alupe-Busia. The diagnostic survey results showed that all the sampled counties had CMD and CBSD diseases. Out of 240 samples that had CMD symptoms at the field; 132 (55%) tested positive for CMVs, 29(12%) tested positive for CBSVs, whereas 79(32.9%) were negative of the two diseases. For the 207 samples that had CBSD symptoms; 108 (52.2%) tested positive for CBSVs, 7(3.4%) tested positive for CMVs and 92 (44.4%) were negative. The CMD incidence levels ranged from 27.6% at Kisii County to 60.34% in Busia County. Whereas, for CBSD it ranged from 26.02% in Kilifi County to 38.74% in Busia County. For phylogeny analysis, two isolates from Kenya (BUS-STR and KAK 16) clustered in Group I, while the others clustered in Group II. The Kenyan novel ACMV isolates from Busia and Kakamega were more similar to Ugandan isolates, suggesting common ancestry.CBSD resistant transgenic lines; 19, 22, 56, 398, 402, 404, 497, 498 and 500 exhibited symptoms of CBSD and CMD within the first four months after planting with varying incidence levels. For CMD resistant transgenic lines; 74, 115, 141, 145 and 157 showed CMD and CBSD symptoms within first four months after planting. Hence, were purposively not selected for viral load, resistance and yield efficacy levels evaluations in objective three and four. Among the CMD viruses detected in the transgenic lines samples; EACMV was the highest at 87.5%, with EACM-Ug most dominant strain at 85.71%. For CBSD; UCBSV was dominant at 79.1% followed by CBSV at 62.5%. The viral load varied among the CMD transgenic lines with line 167 having the highest at 4697.736, whereas line 166 had the lowest at 167.466. On the other hand, CBSD transgenic lines 407 and 506 having the highest and lowest at 8.167 and 0.2135 respectively. Focusing on resistance levels; CMD transgenic line 166 had 64.43%, with non-transformed line 60444 having the lowest at 24.44%. For CBSD transgenic lines; 501 had 82.84%, with 60444 having 59.70%. For the yielding efficacy levels; CMD transgenic lines 166 and 129 had 13.53%, with 133 having the lowest at 4.05%. For the CBSD transgenic lines; 501 had efficacy of 57.14%, whereas, 60444 had the lowest at 9.02%. These, findings confirm the significant presence of CMD and CBSD viruses in major cassava-growing regions of Kenya. The resistance of the transgenic lines varies in a continuous manner among the lines. Hence, providing an opportunity for optimization in efforts to manage CMD and CBSD effectively.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS AND ACRONYMS

ACMV	African Cassava Mosaic Virus
ASARECA	Association for Strengthening Agricultural Research in Eastern and Central Africa
CBSD	Cassava Brown Streak Disease
CBSVs	Cassava Brown Streak Viruses
CMBs	Cassava Mosaic Begomoviruses
CMD	Cassava Mosaic Disease
CMGV	Cassava Mosaic Geminivirus
CN	Cyanide
CRA	Common Region A
CRB	Common Region B
CTAB	Cetyltrimethylammonium bromide
DNA	Deoxyribonucleic Acid
EACMV	East African Cassava Mosaic Virus
EACM-ZV	East African Cassava Mosaic Zanzibar Virus
EACMV-Ug	Uganda Variant Strain of the EACMV
EACMV-Ke	Kenyan Variant of EACMV
ELISA	Enzyme-Linked Immunosorbent Assay
FAO	Food and Agriculture Organization

GPS	Global Positioning System
IITA	International Institute of Tropical Agriculture
KALRO	Kenya Agricultural and Livestock Research Organization
MAbs	Monoclonal Antibodies
NGS	Next Generation Sequencing
PCR	Polymerase Chain Reaction
CGIAR	Consultative Group on International Agricultural Research

CHAPTER ONE

INTRODUCTION

1.1: Background of the Study

World production of cassava root was estimated to be 230 million tons in 2008 rising to 274 million tons as at 2016 (FAOSTAT, 2016; Adebayor, 2023). The majority of production in 2016 was in Africa where 157.27 million tons were obtained, whereas, 89.2 and 29.8 million tons were obtained in Asia and Latin America respectively. Cassava production in Africa increased from 96.8 million tons in 2010 to 157.27 million tons in 2016, a scenario attributed partly to the release of improved varieties in most African countries (FAOSTAT, 2016; Parmar *et al*., 2017; Adebayor, 2023). Despite, the introduction of these improved varieties, most farmers have limited access to them (Hillocks & Jennings, 2003; Nakabonge *et al*., 2018; Kidasi *et al*., 2021). In most places, farmers prefer their own landraces which may be better suited to the local conditions, requirements and taste.

In Kenya, cassava production is on a steady decline. The area under cassava declined from over 70,000 ha in 2013 to 46, 535 ha in 2016 with the annual production declining from about 1.1 million tonnes in 2013 to 571848 tonnes in 2016 (FAO, 2013; FAOSTAT, 2016; Adebayor, 2023). Its cultivation encompasses the coastal, central, eastern, and western areas. Intercropping cassava with beans, maize, and bananas makes it the staple crop in several sub-counties in Western Kenya. The crop accounts for nine percent of the calories consumed by Kenyans. (GoK, 1990, FAOSTAT, 2022). The average yields in Kenya are estimated between 14.8 tons /ha and 12 tons /ha

(FAOSTAT, 2022) and on experimental plots/stations yields of up to 40 tons/ha have been recorded (Were *et al*., 2004).

However low yields of up to 3 tons/ha have been reported in western Kenya(Munguti *et al*., 2023). The poor yields are blamed on pests and diseases among other constraints. Cassava is affected by a range of bacterial, fungal and viral diseases. Viral diseases are the most important diseases of cassava in the world and in Kenya. Cassava mosaic disease (CMD) and cassava brown streak disease (CBSD) are currently considered to be the greatest global threat to cassava production (Legg *et al*., 2014).

Cassava mosaic disease is caused by nine DNA virus species in Africa , which are commonly referred to as cassava mosaic begomoviruses (CMBs); genus; Begomovirus, family; Geminiviridae namely: *African cassava mosaic virus* (ACMV), *East African cassava mosaic virus* (EACMV), *East African cassava mosaic Malawi virus* (EACMMV), *South African cassava mosaic virus* (SACMV), *East African cassava mosaic Cameroon virus* (EACMCV), *East African cassava mosaic Zanzibar virus* (EACMZV), *East African cassava mosaic Kenya virus* (EACMKV), *Cassava mosaic Madagascar virus* (CMMGV) and *African cassava mosaic Burkina Faso virus* (ACMBFV). Among them, African cassava mosaic virus (ACMV) and East African cassava mosaic virus (EACMV) and EACMV-like species such as the Uganda variant (EACMV-Ug) are widespread in the eastern parts of sub-Saharan Africa (SSA) (Mwakosya *et al*., 2022) . EACMV-Ug caused a major outbreak of CMD in Uganda in the 1990s (Munoz *et al*., 1997), which has then spread across neighbouring countries in the Great Lakes region of eastern Africa causing annual losses of 1·2–2·3 billion US$ to African cassava farmers (Legg *et al*., 2006; Legg, 2008).

In Migori County of western Kenya, yield losses of 1299.6 US$/ha have been reported (Emily *et al*., 2016).

Cassava brown streak disease is caused by two picorna-like (+)ssRNA viruses (Mbanzibwa *et al*., 2011), Cassava brown streak virus (CBSV) and Ugandan cassava brown streak virus (UCBSV) (genus Ipomovirus; family Potyviridae) (Winter *et al*., 2010). These viruses are collectively referred to as cassava brown streak viruses (CBSVs). CBSD was largely confined to the lowland areas of eastern Africa for over 70 years since its first description in the 1930s (Hillocks & Jennings, 2003). In recent years, however, severe outbreaks have been reported in inland regions of Uganda (Alicai *et al*., 2007), and areas around Lake Victoria including north western Tanzania and south western Kenya (Legg, 2008; Legg *et al*., 2011). More recently CBSD has been reported in Burundi, Rwanda, the Democratic Republic of Congo, and Madagascar at alarming incidence levels (Bigirimana *et al*., 2004; Abaca *et al*., 2012a; Mulimbi *et al*., 2012; Tomlinson *et al*., 2018). A baseline survey conducted in Kenya, Tanzania, Malawi and Uganda on the economic impact of CBSD indicated a yield loss of 3.2–7.0% in the eastern African region (Ndyetabula *et al*., 2016) , which is equivalent to an annual loss of 1.16–2.53 million tons of fresh cassava roots with a market value of approx. US$290–632 Million.

Further, the spread of CBSVs by whiteflies in a similar way as CMBs in the process of feeding during the growing season of the crop or by farmers using infected cuttings in the subsequent planting seasons has led to heightened disease levels (Ndunguru *et al*., 2016; Macfadyen *et al*., 2018). Symptoms of CBSD on cassava include, leaf chlorosis in a feathery pattern, first along the

margins of the secondary veins, later affecting tertiary veins and may develop into chlorotic blotches. Chlorosis may also appear in patches between the main veins. Initial symptoms of plants infected with UCBSV isolates appear as faint yellow spots on the affected leaves which later developed into bright yellow patches of usually irregular to occasionally circular shape (Chikoti & Tembo, 2022). However, these symptoms are more difficult to recognize in older plants as the lower leaves with prominent symptoms senesce and fall off. New leaves produced from these plants often do not show symptoms, especially at high temperatures. Also, symptoms can be transient when a period of active growth produces symptom-free tissues (Mohammed *et al*., 2012).

The cassava research community has long concentrated on CMD resistance due to the prevalence of CMD pandemics in Africa. Breeding programs have been conducted by several national programs and later at CGIAR centers to exploit natural CMD resistance using locally grown cultivars and a wild relative of cassava (*Manihot glaziovii* Muell.) (Thresh & Cooter, 2005; Vanderschuren *et al*., 2012). The molecular basis of natural resistance was investigated to generate molecular markers and ultimately to clone the dominant CMD2 resistance gene (Amoakon *et al*., 2023). CMD resistance markers also allowed the introgression of the CMD resistance trait into farmer-preferred cassava cultivars (Houngue *et al*., 2019). Cassava landraces initially identified as CMD-resistant as well as CMD-resistant breeding lines were subsequently distributed to farmers in African regions where CMD was prevalent *(* Legg *et al.,* 2006*;* Obiero *et al*., 2007).

Contrary to CMD, limited natural resistance to CBSD, in a few cassava genotypes, has been identified and demonstrated for one of the viral species (UCBSV) causing CBSD (Winter *et al*.,

2010). The deployment of CMD-resistant cassava with different levels of susceptibility to viruses causing CBSD continues to impact production and alters the occurrence of UCBSV and CBSV in the field. Because CBSD resistance has not been found in cassava genotypes traditionally grown by farmers, exploitation of genetically engineered resistant lines will be the way to go if management of CBSD in Africa is to be effective (Vanderschuren *et al*., 2012). Hence, this study sought to evaluate the host resistance potential of genetically engineered Cassava lines towards effective management of CMD and CBSD diseases in Kenya.

1.2: Problem statement

Cassava is ranked high among the top ten most significant food crops produced in the developing countries (Adebayo, 2023). Further, it is a major source of carbohydrate for human consumption throughout the tropics, but particularly in Africa (Fargette *et al*., 1990). The cassava plant gives the highest yield of food energy per cultivated area per day among crop plants, except possibly for sugarcane (Visses *et al*., 2018). Its ability to thrive in low-rainfall environments and on subpar soils makes it an invaluable agricultural tool in impoverished nations, notably in sub-Saharan Africa. Because of its long harvesting season, it can be used as a food reserve and is very helpful for organizing workers' schedules. It offers flexibility to resource-poor farmers because it serves as either a subsistence or a cash crop (Adebayo, 2023) . Cassava acts as an insurance crop due to its tolerance to drought, ability to yield under poor soil conditions and low external input requirements (MOALF&C, 2019) .

There has been a general increase in cassava production in Africa (FAOSTAT, 2016) achieved partly following the release of improved varieties in most African countries (Adebayo, 2023), and increased demand for cassava based products. However, in Kenya there has been a sharp decline from 930,922 tons in 2012, 858,461 tons in 2014, to 571,848 tons in 2016 (FAOSTAT, 2016). The yields of 3 tons/ha obtained in some parts of Western Kenya are among the poorest in the world (Githunguri & Njiru, 2021). The low yields are attributed to pests and diseases among other constraints.

Cassava Mosaic Disease (CMD) and Cassava Brown Streak Disease (CBSD) are the most important diseases of cassava with CMD reported to cause yield loss of 60-80% (Were *et al*., 2004)while cassava brown streak disease causes losses of up to 74%(Hillocks *et al*., 2001). A combination of CMD and CBSD cause yield losses of up to 100% (Abaca *et al*., 2012) . The viruses are transmitted by whiteflies and/or by farmers planting infected seed cuttings. The high population of whiteflies in Western Kenya coupled with farmers' practice of obtaining seed from their current ware crop or from neighbors, fuels the spread of the viruses in farmers' fields.

The high level of genetic diversity due to continuous recombination of the viruses makes it difficult to breed for resistant varieties. It has also been reported that CMD resistant varieties are highly susceptible to CBSD further complicating the efforts to manage the two viruses. In spite of the introduction of improved varieties with higher resistance or tolerance to the viruses, most farmers have limited access to them(Hillocks & Jennings, 2003), and the resistance breaks down after a few planting cycles. In addition, most farmers prefer their own landraces which may be better suited to the local conditions, requirements and taste but quite susceptible to viral diseases. Hence,

this study sought to evaluate resistance of genetically transformed cassava lines to determine their efficacy in managing the problem of CMD and CBSD effectively under confined field trial in selected region of Kenya towards commercialization of the same.

1.3: Justification

Cassava is an important food security crop in rural parts of Kenya and its demand for various industrial products has been on the rise. Its production however has been constrained by pests and diseases among other constraints. Use of improved varieties and clean planting material can help reduce disease incidence as reported by Obiero *et al*. (2007). However, there's lack of sustainability in producing, supplying and using clean planting material. CMD resistant varieties are highly susceptibility to CBSD. Hence, the need to screen both local and improved cassava varieties for resistance to both diseases (Abaca *et al*., 2012a).

Various stakeholders have attempted to produce and supply farmers with improved cassava varieties that combine higher disease resistance and yield, but the adoption of these varieties remains poor. To reduce these losses and stop the spread of CMD and CBSD in the area, two tactics were put forth. A preventative method that includes growing resistant cassava, ensuring the quality of planting material, and using large-scale surveillance and precise laboratory-based diagnostic for detection and monitoring (Otti *et al*., 2016). Hence, there is need to evaluate the RNAi transgenic cassava lines to determine their resistance levels to CMD and CBSD causal viruses; survey for CMD and CBSD in major cassava growing areas to determine the incidence

severity and distribution of causal viruses and to document the virus species and/or strains in the region to provide useful information in development of resistant varieties.

1.4: General objective

The general objective of this study was to evaluate transgenic cassava lines for resistance against selected diverse *Manihot esculenta* C. viruses in Kenya

1.4.1: Specific objectives

i. To identify the sources of cassava seed, the viruses and the disease levels of CMD and CBSD in major cassava growing Counties of Kenya towards focused management.
ii. To assess the disease progression, incidence and severity levels of CMD and CBSD in RNAi transgenic cassava lines under confined field trial at Alupe in Western Kenya.
iii. To determine the diversity of viruses causing CMD and CBSD coupled with selected viral loads in the RNAi transgenic cassava lines under confined field trial at Alupe in Western Kenya.
iv. To determine the resistance and yield efficacy levels of the RNAi transgenic cassava lines against CMD and CBSD causal viruses under confined field trial at Alupe in Western Kenya.

1.5: Research Questions

1. What are; the sources of cassava seed, the viruses and the disease levels of CMD and CBSD in major cassava growing areas of Kenya towards focused management?

2. What is the disease progression, incidence and severity levels of CMD and CBSD in RNAi transgenic lines under confined field trial at Alupe in Western Kenya?
3. What are the viruses and their viral load, infecting the RNAi transgenic lines in confined field trial at Alupe in Western Kenya?
4. What are the estimated resistance and yield efficacy levels of the RNAi transgenic lines against CMD and CBSD causal viruses under confined field trial at Alupe in Western Kenya?

CHAPTER TWO

LITERATURE REVIEW

2.1: The cassava plant

Cassava (*Mannihot esculenta* crantz) was domesticated in South America between 4000 and 2000 BC. It was carried to the east African coast and the Indian Ocean islands of Madagascar, Reunion, and Zanzibar, as well as presumably India and Sri Lanka, in the 18th century. (Thottappilly *et al*., 2006)

Cassava belongs to the family *Euphorbiaceae* and it generates latex and contains latifers, just like the majority of other family members. The primary source of dietary energy for a sizable population in tropical Africa is cassava, the sole member of the genus Mannihot that is grown as a food crop. It increases the food security of households and generates revenue. (Guira *et al*., 2017). It provides livelihood to about 200 million people in Africa (FAO, 2010).

There are numerous cassava varieties in each area where the crop is cultivated. They can be distinguished by morphology (leaf shape, leaf size, plant height, petiole and color, etc.), earliness to maturity, yield, and bitterness, with some having a high cyanogenic glucoside content that is distributed throughout the tuber and others having a low glucoside content that is confined primarily to the peel. The meat of the sweet cultivars is thus largely free of glucosides. (Chiwona-Karltun *et al*., 2004).Cassava grown during drought is especially high in these toxins (Shigaki, 2016) .

Cassava is ranked high among the top ten most significant food crops produced in the developing countries (Adebayo, 2023) and as a major source of carbohydrate for human consumption throughout the tropics, but particularly in Africa (Fargette *et al*., 1990). The cassava plant gives the highest yield of food energy per cultivated area per day among crop plants, except possibly for sugarcane(Visses *et al*., 2018) .

Cassava is extremely important in agriculture in poorer countries, particularly in Sub-Saharan Africa, because it thrives on poor soils and low rainfall and is a perennial crop that can be harvested as needed. Its extended harvesting window makes it an effective famine reserve as well as a tool for controlling labor schedules. It operates as both a subsistence and cash crop, giving resource-poor farmers more options.(Pinamang -Acheampong *et al*., 2021).

2.2: Uses of cassava

Cassava-based dishes are widely consumed wherever the plant is cultivated; some have regional, national, or ethnic importance (Parmar *et al*., 2017) . Cassava must be carefully boiled to cleanse it before consumption. The English explorer and naturalist Charles Waterton stated in Wanderings in South America (1836) that the people of Guyana utilized cassava to produce alcohol. (Nduka, 2014).

Harvested between three and four months of age, cassava hay has a minimum dry matter of 85% and is sun-dried for one to two days. Cassava hay has strong tannins (1.5–4% CP) and a high protein content (20–27% crude protein). For dairy or beef cattle, buffalo, goats, and sheep, it is

used as an appropriate roughage diet that can be fed directly or as a source of protein in concentrate mixes. (Chanjula *et al*., 2004). (Ravindran and Velmerugu, 1992).

The bitter leaves of cassava are used to treat hypertension, headaches, and pain. Cubans also frequently utilize cassava to treat irritable bowel syndrome; during treatment, the paste is consumed in excess. (Mohidin *et al*., 2023). As cassava is a gluten-free natural starch, there have been increasing incidences of its appearance in Western cuisine as a wheat alternative for sufferers of celiac disease (Karunarathna *et al*., 2023). Significant research has begun to evaluate the use of cassava as an ethanol biofuel feedstock (Xinglu., 2004) The efficiency of the plant's photosynthetic CO2 absorption into storage and structural compounds is one of the primary determinants of the economic feasibility of biofuel generation. Cassava can photosynthesize a lot when the conditions are right. *(*Ruiz-Vera *et al.*, 2021). For example, it has a photosynthetic rate of 40 to 50 µmol CO2 m -2 s -1 under high solar radiation, which is double that of rice which is round 20 µmol CO2 m -2 s -1 (El-Sharkawy, 2004). According to a 2012 Business Green News report, cassava (tapioca) chips have progressively emerged as a key ingredient in China's ethanol production. Built in 2007, the Beihai factory produces 200 thousand tons of cassava ethanol fuel per year, making it the largest of its kind. On average, 1.5 million tons of cassava were used by this operation. According to reports, in 2008, the Hainan Yedao Group, a Chinese corporation, put $31.8 million (or $51.5 million) into a new biofuel factory with the intention of producing 33 million gallons of bio-ethanol annually from cassava plants.

2.3: Cassava mosaic disease (CMD)

Cassava Mosaic Disease is the most severe and widespread disease limiting production of the crop in sub-Saharan Africa (Calvert & Thresh, 2001)). Mosaic, mottling, misshapen, and twisted leaflets, as well as a general diminution in leaf and plant size, are among the foliar symptoms produced by CMD. CMD-affected cassava plants produce few or no tubers depending on the severity of the disease and the age of the plant at the time of infection(Were *et al*., 2004).

Historically, the first report of CMD came from the Usambara Mountains range in northeast Tanzania in 1894. The disease was named as "Kräuselkrankheit," a German word that translates to "rippling/crinkling illness", which describes symptoms observed on affected plants (Chant, 1958). Although a virus was originally believed to be the causal agent of CMD and that its transmission occurred via whiteflies , its viral status was not proven until the 1970s when small, quasi-isometric, geminate particles, were found in symptomatic host tissues(Harrison *et al.*, 1977). However, it would not be until many years later that the virus, *African cassava mosaic virus* (ACMV), was molecularly characterized (Stanley & Gay, 1983) and Koch's postulates fulfilled (Bock, 1983) .

Additionally, molecular investigation of ACMV isolates from CMD-affected plants in Kenya subsequently identified a second virus with a genomic structure akin to ACMV, while possessing unique serological characteristics. This 'new' virus was named *East African cassava mosaic virus* (EACMV) (Hong *et al*., 1993) to reflect its geographical association, which was thought to be

restricted to East Africa. Nine different cassava mosaic viruses have been identified globally from CMD-affected cassava plants, seven of which originate from sub-Saharan Africa.

The International Committee on Taxonomy of Viruses (ICTV) has classified all these viruses inside the genus Begomovirus, the most extensive genus in the family *Geminiviridae*, and they are generally referred to as cassava mosaic begomoviruses (CMBs) or cassava mosaic geminiviruses (CMGs).

In the past, it was believed that CMBs displayed geographic structuring, with ICMV to India and Sri Lanka, EACMV to the eastern portion of the Rift Valley in coastal Kenya, Tanzania, Malawi, Zimbabwe, and Madagascar, and ACMV restricted to West and Central African nations toward the west of the Rift Valley and in South Africa. (Harrison *et al*., 1997)). However, subsequent reports by Patil and Fauquet (2009) have shown that most of the seven CMBs reported from sub-Saharan Africa are widespread across the sub-continent, whereas ICMV and SLCMV appear to have remained confined to cassava-growing regions of India and Sri Lanka.

It is interesting to note that South America, where cassava originated, has not yet reported any cases of CMD. This implies that cassava became a "accidental" host after being introduced to Africa, and that CMBs may be "native" to the continent, where they proliferated in native hosts. Bosque-Pérez and Buddenhagen described this kind of "new encounter phenomenon." (Bosque-Pérez & Buddenhagen, 1992), wherein an introduced plant species causes a host to "jump" to a pathogen that co-evolved with native plant species, turning it into an important pathogen, has been reported for several other crops that have been introduced to Africa (Thresh *et al*., 2003) . In order

to stop future disease outbreaks, it is crucial to identify the part that native non-cassava plant species and alternative/reservoir hosts play in the evolution and continuation of CMBs..

The epidemic that broke out in Uganda during the early to mid-1990 drew much attention to CMD (Gibson *et al*., 1996; Otim-Nape *et al*., 1997). This epidemic, characterized by a bizarre CMD symptom-type, soon spread to other countries in East Africa including Kenya and Tanzania (Legg & Thresh, 2000). The molecular characterisation of the CMB linked to this atypical type of CMD indicated that it was a unique CMB with genomic characteristics akin to both ACMV and EACMV. This novel CMB was designated an Ugandan variant of EACMV called EACMV-UG (J.P. Legg & Thresh, 2000), because its DNA-A genome was 16% and 84% similar to that of ACMV and EACMV, respectively, as a consequence of recombination between the two parental viruses(Munoz *et al*., 1997) .

The CMD pandemic caused by EACMV-UG devastated many cassava farms and forced thousands of subsistence farmers to abandon the crop and also resulted in famine-related deaths (Otim-Nape *et al*., 1997; OTIM-NAPE *et al*., 2001) . EACMV-UG has since been reported from several countries in sub-Saharan Africa including Sudan (Harrison *et al*., 1997), Rwanda (Legg *et al*., 2015), the Democratic Republic of Congo (Neuenschwander *et al*., 2002), Burundi *(Bigirimana et al*., 2004) and Gabon (Legg & Fauquet, 2004). More recently, EACMV-UG has been reported from Burkina Faso (Tiendrébéogo *et al*., 2009), and Cameroon (Akinbade *et al*., 2010). Thus, indicating the westward movement of the virus within the African continent.

The fast proliferation of this recombinant virus may result from the indiscriminate distribution of virus-infected cassava cuttings throughout sub-Saharan Africa, perhaps stemming from inadequate sanitation and ineffective quarantine measures in several nations within the sub-region.Although other recombinant CMBs, such as SACMV, EACMCV, EACMMV, EACMZV, EACMKV, SLCMV and ICMV have been identified, they appear to be localized in their distribution relative to EACMV-UG, indicating that EACMV-UG is perhaps better adapted than the other CMB strains found in sub-Saharan Africa (Harrison *et al*., 1997).

Furthermore, none of the recent disease surveys conducted in sub-Saharan African countries(Alabi *et al*., 2008; Sserubombwe *et al*., 2008; Bull *et al*., 2011) have reported the incidence of the parental EACMV virus suggesting that this 'wild-type' EACMV has been overtaken in nature by its more fit recombinant strains. It is interesting to note that many of the recombinant CMBs has an EACMV lineage, whereas no recombinant from an ACMV lineage has been reported, despite its wide distribution across Africa and its frequent existence in mixed infections with other CMBs (Pita *et al*., 2001; Ogbe *et al*., 2006; Alabi *et al*., 2008). Some of the factors that could contribute to molecular diversity among CMBs of an EACMV lineage were recently reviewed (Patil & Fauquet, 2009).

Adding to the complexity of the CMD situation is a recent report that disease resistance-breaking satellite DNA molecules have been found associated with CMD in Tanzania (Ndunguru *et al*., 2016). Many ssDNA satellites of ~1.3 kb have been found associated with several begomovirus disease complexes and they are generally of two types, the nanovirus-like DNA 1 or

alphasatellites, and the DNA B-like DNA β or betasatellites (Briddon *et al*., 2008; Nawaz-ul-Rehman & Fauquet, 2009). The alphasatellites are capable of independent replication although they depend on the helper virus-encoded proteins for their movement and encapsidation, whereas the betasatellites depend on their helper virus for replication, movement and encapsidation (Briddon *et al*., 2008; Nawaz-ul-Rehman & Fauquet, 2009).

Recent data demonstrated that several CMBs showed contrasting and differential interactions with alpha- and beta-satellites derived from other *Begomovirus* species resulting in the modulation of symptom phenotypes by these satellites in *N. benthamiana* (Patil & Fauquet, 2009). The primary focus of CMD management in sub-Saharan Africa has been the creation and implementation of disease-resistant cultivars; however, the potential existence of disease resistance-breaking satellite DNA molecules in the sub-region could exacerbate the CMD issue and significantly jeopardize cassava production.

Since cassava is a vegetatively propagated crop, CMBs and their DNA satellites can be transmitted through infected stem cuttings and by grafting infected bud wood onto healthy cassava plants. (Atiri *et al*., 2004). Experimental transmission can also occur via biolistic inoculation using a gene gun (Briddon *et al*., 1998). These approaches have been extensively employed to evaluate genotype performance in relation to CMBs during resistance breeding initiatives.

The whitefly vector, *Bemisia tabaci* (*Hemiptera*: *Aleyrodidae*), is mainly responsible for the secondary spread of CMBs(Chant, 1958; Sserubombwe *et al*., 2008) although other species of whitefly, such as *B. afer*, can also transmit CMD(Njoroge *et al*., 2017). Starvation of non-

viruliferous whiteflies prior to acquisition feeding on infected cassava accelerated the acquisition of ACMV(Dubern, 1994; Liu *et al*., 1997). Once acquired, a latent period of about 6-8 h must lapse before the whitefly is able to transmit the virus, which can thereafter be retained by an infectious whitefly for about 9 days (Dubern, 1994). Viruliferous whiteflies require 10-30 min inoculation access periods for virus inoculation into healthy cassava plants. Under experimental conditions, ten viruliferous whiteflies are needed to achieve the optimal rate of transmission when released on a cassava plant (Dubern, 1994), although a single whitefly is capable of virus transmission (Chant, 1958; Dubern, 1994). Nonetheless, ACMV is transtadially, but not transovarially transmitted (Chant, 1958; Dubern, 1994). In addition, CMBs are neither transmissible from cassava to cassava by mechanical inoculations nor through seed (Storey & Nichols, 1938).

The experimental host range of CMBs was originally believed to be restricted to members of the family *Solanaceae*, especially those belonging to the genera *Nicotiana* and *Datura* (Bock, 1983). However, SLCMV has been shown to infect *Ageratum conyzoides* L. (family *Asteraceae*) and *Arabidopsis thaliana* (L.) Heynh. (Family *Brassicaceae*) (Loebenstein & Thottappilly, 2003). ACMV and EACMV have been detected in *M. glaziovii* Müll. Arg. (a wild species of cassava), *Combretum confertum* (Benth.) Lawson (*Combretaceae*), *Senna occidentalis* (L.) Link (*Fabaceae*), and *Leucana leucocephala* (Lam.) De Witt (*Fabaceae*), while ACMV alone has been detected in *Jatropha multifida* L. (*Euphorbiaceae*), Laportea *Fleurya aestuans* (L.) Chew (*Urticaceae*), soybean (*Glycine max* L. Merr.; *Fabaceae*), *Ricinus communis* L. (*Euphorbiaceae*), and suspected to infect *Hewittia sublobata* (L.f) Kuntze (*Convolvulaceae*) in Kenya and *Laportea*

(*Fleurya*) *aestuans* in Nigeria (Ogbe *et al*., 2006; Thottappilly *et al*., 2006; Alabi *et al*., 2007; Mgbechi-Ezeri *et al*., 2008).

2.3.1: Management of Cassava Mosaic Disease (CMD)

Numerous approaches have been developed for the management of CMD and many of these approaches have been discussed in several review articles (Thresh & Cooter, 2005; Vanderschuren *et al*., 2007). Farmers in Africa have seldom employed chemical control measures against the whitefly vector due to economic considerations. Pesticides are least successful in preventing arthropod-borne viruses when the primary transmission occurs from external sources rather than within crops. (Thresh & Cooter, 2005). The detrimental effects of pesticides on the environment and the hazards they pose to beneficial creatures, including natural predators and agricultural workers' health, render pesticide use less attractive. The potential for biological control of the whitefly vector has yet to be fully investigated, however recent investigations have started in East Africa..

A considerable amount of effort and resources have been devoted to breeding for resistance to CMBs and their whitefly vector, especially by scientists at the International Institute of Tropical Agriculture (IITA, Ibadan, Nigeria), the International Centre for Tropical Agriculture (CIAT, Cali, Columbia), and their collaborators in national, regional and international laboratories. Such disease-resistant materials have been widely distributed across sub-Saharan Africa for use by local farmers.

However, one limitation of resistant varieties lies in the fact that some of them may accommodate moderate to high levels of virus (OGBE *et al*., 2003). Such kinds may be deemed tolerant rather than resistant to viral infection, as they allow for viral reproduction despite the absence of obvious symptoms. From an epidemiological perspective, these materials may act as reservoirs of viral inoculum if circumstances for vector transmission are conducive. The existence of disease resistance-breaking satellite DNA molecules in sub-Saharan Africa poses a threat to authentic resistant cassava cultivars that have been extensively disseminated throughout the area.

2.3.1.1: Transgenic cassava development for resistance against CMD

Recently, global efforts have focused on the development of transgenic cassava cultivars resistant to CMD (Vanderschuren *et al*., 2007; Patil & Fauquet, 2009). These efforts are now incorporated into the overarching objective of the Bio Cassava Plus initiative, financed by the Bill and Melinda Gates Foundation, which seeks to mitigate malnutrition by providing enhanced cassava cultivars that offer comprehensive and balanced nutrition in a highly marketable and higher-yielding food crop (http://biocassavaplus.org). Vanderschuren et al. (2007) shown that transgenic resistance to CMD has promising outcomes in controlled environments. However, their efficacy under field circumstances characterized by elevated disease pressure and a higher prevalence of vectors remains ambiguous.

Cassava mosaic disease is caused by either African cassava mosaic virus (ACMV) alone or by virulent recombinants from different cassava geminiviruses and synergisms among these geminiviruses. EACMV-UG1, a recombinant between a Ugandan isolate of ACMV and the

closely related virus East African cassava mosaic virus (EACMV), causes severe symptoms and has been linked to the recent pandemic(Gibson *et al*., 1996; Munoz *et al*., 1997; Pita *et al*., 2001). This occurrence highlights the significance of cultivating geminivirus-resistant cassava through traditional breeding or genetic engineering.

Resistance should ideally be effective against several or all ACMV strains. Despite the existence of certain tolerant cultivars, traditional breeding has proven challenging for the production of ACMV-resistant cassava due to the significant heterozygosity and pronounced inbreeding depression observed in several elite varieties and farmer-preferred landraces. The polygenic nature of CMD resistance and the fast development of geminiviruses in the field hinder traditional breeding initiatives *(*Kuon *et al*., 2019; Legg *et al*., 2004; Ntui *et al*., 2024).

Transgene-mediated approaches could offer new resistance traits/genes that currently do not exist within the cassava germplasm pool. Moderate levels of resistance against several geminivirus species have been demonstrated in transgenic tobacco and tomato expressing the AC1 gene, which encodes the replication-associated protein (Rep) (Fondong, 2017; Walsh *et al*., 2019; Loriato *et al*., 2020;). Chellappan and colleagues reported that transgenic cassava expressing the ACMV AC1 gene showed resistance to both homologous and heterologous species of cassava-infecting CMGs (Chellappan *et al*., 2004). The high levels of resistance against CMGs were correlated with posttranscriptional gene silencing (PTGS) through the production of transgene-specific siRNAs. Zhang *et al.,* (2005) demonstrated that transgenic cassava expressing antisense RNAs of ACMV Rep (AC1), TrAP (AC2), and REn (AC3) could resist ACMV infection via PTGS.

Vanderschuren *et al.* (2009), reported that the siRNAs, homologous to either the common region or AC1 in transgenic cassava, were able to suppress the replication of ACMV, leading to recovery after infection or immunity to infection by the virus (Vanderschuren *et al.*, 2007, 2009). As the common region of ACMV does not share a high degree of sequence homology with other CMGs (e.g., EACMV and SACMV), the resistance was expected to be strain specific*(*Vanderschuren *et al*., 2007). In the last two decades, RNAi-based approaches have been tested in transgenic cassava and proved to confer robust CMD resistance in the model cassava cultivar TMS 30-6044. Constructs developed in this way need to be transferred to farmer-preferred cultivars, although the stability of the engineered CMD resistance remains to be demonstrated under field conditions and over multiple cycles of propagation

Among the various strategies evaluated for CMD management, planting virus-free cuttings is the most effective for minimizing spread of the disease in Africa (Fargette, 1994; Thresh & Cooter, 2005). Nonetheless, the amalgamation of several methodologies will offer more potential for holistic treatment of CMD in the region. The possible involvement of volunteer cassava plants and alternate hosts of CMBs in the evolution of CMBs and the subsequent development of CMD have been largely neglected, potentially affecting the efficacy of any control method.

2.4: Cassava Brown Streak Disease (CBSD)

Cassava brown streak disease is caused by Cassava Brown Streak Virus (CBSV) (Monger *et al*., 2001) and Uganda Cassava Brown Streak Virus (UCBSV) (Winter *et al*., 2010) which affects all parts of the cassava plant, causing characteristic above and below ground symptoms (Hillocks &

Jennings, 2003; Rubio *et al*., 2017; Tomlinson *et al*., 2018). Leaf symptoms include blotchy yellow chlorosis or feathery necrosis, often associated with minor veins, which can appear within the first few months after planting of infected cuttings and persist in mature leaves. Brown, round or elongate streak-like lesions can occur on the young green portion of infected stems, but the main economic loss is caused by dry, brown necrotic lesions in the storage tissues of the tuberous roots of infected susceptible plants (Storey & Nichols, 1938; Legg *et al*., 2011). Root constrictions are also sometimes observed as well as brown/black lesions on green fruits, and necrotic lesions in leaf scars. In severe infections these lesions develop to kill the dormant axillary buds leading to a general shrinkage of the node and death of the intermodal tissue, so that the branch dies from the tip to cause 'dieback' (Hillocks & Jennings, 2003).

Both CBSV and UCBSV are picorna-like (+) ssRNA viruses from the genus *Ipomovirus*, family *Potyviridae* (Masinde et al., 2018; Winter et al., 2010). These viruses spread along with the infected vegetative planting material and are also transmitted in a semi-persistent manner by whitefly, *Bemisia tabaci* (Maruthi *et al*., 2004). For the first approximately 60 years that CBSD was recognized (Storey & Nichols, 1938), it occurred at relatively low levels in coastal East Africa, from Mozambique in the south to north-eastern Kenya in the north, and inland to the shores of Lake Malawi (R. Hillocks & Jennings, 2003). In the early 2000s, however, new outbreaks were reported from south-central Uganda (Alicai *et al*., 2007). Western Kenya (Were, 2016) and north-western Tanzania(Legg *et al.*, 2011). More recently CBSD has been reported from Burundi (Bigirimana et al., 2004), Rwanda (Tomlinson *et al*., 2018)and the Democratic Republic of Congo

(Mulimbi *et al*., 2012), indicating a possible spread to West Africa. The spread of CBSVs has been fuelled by 'super-abundant' whiteflies, *Bemisia tabaci* (Legg *et al*., 2014).

Furthermore, CBSD has been considered to be one of the 100 most dangerous diseases in the world(Munguti *et al*., 2023). The overall effect of CBSD is reduction of root yield by up to 74%(Herrera *et al*., 2011) and quality(Hillocks *et al*., 2001) . It causes up to 100% yield loss in susceptible varieties (Kaweesi *et al*., 2014).When combined with cassava mosaic disease, 100% yield loss can result.

2.4.1: Management of Cassava Brown Streak Disease (CBSD)

In terms of control, the most economically viable method for CBSD management is the use of host-plant resistance (Mukiibi *et al*., 2019). Thus, development of cassava varieties that are resistant to CBSD is an important component in the CBSD management. Abaca *et al*. (2012b), showed that some putative clones with some resistance to CBSD are also highly resistant to CMD, which makes them suitable genetic stocks that combine CMD and CBSD resistance.

CHAPTER THREE

MATERIALS AND METHODS

3.1: Determination of the sources of cassava seed, the viruses and the disease levels of CMD and CBSD in major cassava growing areas of Kenya towards focused management

3.1.1: Survey to determine the cassava viruses and associated diseases levels in Kenya

A diagnostic survey for CMD and CBSD was conducted in areas with intense cassava production of Kenya. The survey was carried out between May – October 2018 to take advantage of the vigorous vegetative growth of cassava during the long March – June 2018 rains in the region. Cassava fields were selected randomly along the main and feeder roads (Were *et al*., 2016). Coordinates were taken at each sampling site using a global positioning system (GPS) handset (Magellan®Triton™).

A questionnaire was used to collect information on the source of seed, varieties, and socioeconomic data. The survey was conducted by walking through cassava fields and visually inspecting cassava plants for presence of typical virus disease symptoms. In each stand, 30 cassava plants selected along two longitudinal transects were analysed (Ntawuruhunga & Legg, 2007). However, in smaller fields all the plants along the diagonals were inspected.

Disease incidence was calculated according to James (1974) and Bock *et al* (2021), as the percentage of plants showing CMD/CBSD symptoms to the total number of plants assessed in a field (James, 1974; Bock *et al*., 2021). For CMD, symptom severity was assessed as the degree of damage expression on the leaves of each sampled plant using an arbitrary scale of 1-5 according to Sseruwagi (Sseruwagi *et al*., 2004); where 1 represents no symptoms and 5 represents the most severe symptoms (Appendix viii) . For CBSD, foliar/stem and root symptom severity was

determined according to Abaca *et al*. (2012a); where a scale of 1-5 was used in both cases as per (*Appendix vi*)

At least five cassava leaf samples, showing CMD/CBSD symptoms and five without symptoms were picked per field and put into sampling bags, labelled and stored in a cool box containing ice blocks to preserve them for subsequent virus diagnosis and analysis. Virus infected cassava cuttings were taken from the plants where the leaf samples were obtained for use as infector plants in the field trial. Mean values were calculated for all factors determined both for each field, as well as for each county.

3.1.2: Sample collection for sequencing

Leaf samples for sequencing were collected from symptomatic cassava plants and immediately placed in well labelled falcon tubes containing RNA later solution to stabilize the RNA. The samples were kept in a cool box while in the field then transferred to 4^0C as described on sub-section 3.1.1 above (Rogers and Bendich, 1994; Warner, 1996).

3.1.3: DNA extraction for CMVs

Total DNA was extracted from plant tissues using CTAB method (Rogers and Bendich, 1994; Warner, 1996;Wilkie, 1996). Approximately 100 mg of leaf tissue was put into sample extraction bags, 1 ml CTAB buffer (2% CTAB, 100 mM Tris – HCl, pH 8.0, 20 mM EDTA and 1.4 M NaCl) with 0.2% β mercaptoethanol added and ground using a small hand roller. This was then be incubated at 65°C for 15 minutes after which it was centrifuged at 16000 rpm in a microfuge for

10 min at 25^0 C. The aqueous layer formed (approximately 800µl) was transferred into a clean nuclease-free 2 ml microfuge tube.

Equal volume of chloroform: isoamyl (24:1) was added, vortexed and centrifuged for 10 minutes at 25^0 C. The supernatant was transferred to a clean 2ml tube and equal volume of chloroform isoamyl (24:1) added and centrifuged at 16000 rpm for 10 min at 25^0 C. The supernatant (approximately 650 µl) was transferred to a clean 1.5 ml tube, 390 µl (0.6 vol) of cold ethanol added and kept at -80 for 30 min the centrifuged at 16000rpm for 30min at 4^0 C to precipitate the DNA.

The liquid was carefully decanted and DNA pellets washed with 500µl of 70% ethanol by centrifugation for 5 minutes at 13,000 rpm at 4^0 C twice. The final DNA pellet was air dried and re-suspended in 100µl of RNase free water. The DNA was quantified using a Nanodrop ND-1000. Samples with a ratio of ~1.8 were considered as "pure" for. Due to differences in DNA quantity, the sample extracts were normalized to a working concentration of 100 $ng\mu l^{-1}$ by addition of an appropriate amount of sterile water.

3.1.4: RNA extraction for CBSVs

Approximately 100 mg of leaf tissue was put into crushing bags, 1 ml CTAB buffer containing 2% CTAB, 100 mM Tris – HCl, pH 8.0, 20 mM EDTA and 1.4 M NaCl added and ground using a small hand roller (Rogers and Bendich; Warner, 1996; Wilkie, 1996). The mixture was thereafter incubated at 65°C for 15 minutes, after which 700 µl of chloroform: isoamyl alcohol (24:1) was introduced and centrifuged at maximum speed in a microfuge for 10 minutes at room temperature.

The generated aqueous layer was extracted and placed into a sterile, nuclease-free 1.5 ml microfuge tube, followed by the addition of an equal amount of 4 M LiCl, and incubated overnight. The solution was subsequently centrifuged for 30 minutes at a maximum speed of 13,000 g at 4°C to precipitate the nucleic acids. The pellet was re-suspended in 200 µl of TE buffer with 1% SDS. Subsequently, 100 µl of 5 M NaCl and 300 µl of ice-cold isopropanol were added, and the mixture was incubated at −20°C for 30 minutes.

Following incubation, the mixture was subjected to centrifugation for 10 minutes at 13,000 g to pellet the nucleic acid. The pellet was subsequently rinsed with 500 µl of 70% ethanol and centrifuged for 4 minutes at 4°C. The ethanol was decanted, and the pellet was dried and re-suspended in 50 µl of nuclease-free sterile water. The quality and amount of RNA were assessed using a Nanodrop ND-1000 (Nanodrop Technologies, DE, USA). The absorbance ratio at 260 nm to 280 nm was utilized to evaluate purity, with samples exhibiting a ratio of 1.8-2.0 deemed "pure" for RNA.

The samples were standardized to a working concentration of 100 ngµl−1 using sterile water due to variations in RNA amount. cDNA synthesis was performed using Moloney murine leukemia virus reverse transcriptase (M-MLV RT) in accordance with the manufacturer's guidelines (Promega, WI, USA). Oligo (dT) 15 primers were employed to synthesize the first strand cDNA, focusing on the 39-proximal end of the genome. Red-Hot Polymerase (Thermo Fisher Scientific, MA, USA) was utilized for the amplification of viral sequences using PCR.

3.1.5: Sequencing and sequence analysis

PCR products of the expected sizes were purified, and cloned into the pCR 2.1 vector using the TA cloning kit (Invitrogen, San Diego, CA). Clones containing putative viral sequences were identified by miniprep screening and confirmed positive for inserts by PCR amplification using their respective PCR primers, and inserts were subsequently sequenced in both directions. The complete and partial nucleotide sequences of CMGs were determined by the dideoxynucleotide chain termination method using an ABI 377 DNA automatic sequencer (Applied Biosystems, CA, USA). Sequence fragments of 600- 900 bp were generated. The sequence quality was checked based on the peak of the electrophoregram and trimmed using CLC main work bench v20 to remove the PCR primers.

The sequences were compiled, and any discrepancies were reconciled based on the consensus. The final consensus was subsequently exported in FASTA file format. The sequences were subjected to BLASTn analysis against the NCBI database to identify the closest match. Sequences having a nucleotide identity of at least 97% from BLAST hits were selected and then retrieved from the NCBI database. The sequences were uploaded to MEGA X software, and multiple alignment was performed with a Gap Opening Penalty of 15 and a Gap Extension Penalty of 5.5. Phylogenetic trees were generated using MEGA X. The optimal DNA model for the phylogenetic tree was derived from the Automatic Neighbor Joining Tree and the Maximum Likelihood approach of nucleotide substitution. The optimal DNA model employed for the phylogenetic tree was the Kimura 2-Parameter model with Invariant Plus Gamma. The final tree was constructed using a bootstrap value of 1000 replications.

3.2: Determination of the disease progression, incidence and severity levels of CMD and CBSD in RNAi transgenic cassava lines under confined field trials at Alupe

3.2.1: Experimental design and layout

The Experimental design was randomized complete block design (RCBD) with three replications modified from (Abaca *et al*., 2012b; Bisimwa *et al*., 2015; Mehta *et al*., 2018). Randomization of the cassava genotypes was done within the blocks using random numbers. Each block measured 12m x10m in which nine (9) experimental units measuring 3m x 2m were laid down. The units were separated by a 2m path on which the infector plants obtained from the survey were planted. Each unit had two (2) rows each of three (3) test plants spaced at 1m ×1m (6 plants).

The infector plants were sprouted by placing cassava stakes in the shade and burying the tips of the stakes in the soil before the actual planting. Plants were spaced at 1m x 1m within the blocks (along the paths). Each experimental unit (transgenic lines) was surrounded by a line of infector plants to give disease pressure to the engineered cassava lines.

3.2.2: Field evaluation

The trial was observed for above-ground symptoms during the crop growing period and for root symptoms post-harvest. Symptoms on shoots (leaves and stems) were documented for each plant at 3, 4, 5, 6, 7, and 8 months post-planting (MAP). For CMD, a severity score ranging from 1 to 5 was established, where: 1 indicates no CMD disease symptoms; 2 denotes mild chlorotic patterns on leaves; 3 reflects moderate mosaic patterns across all leaves; 4 signifies severe mosaic distribution on all leaves accompanied by a general reduction in leaf size; and 5 represents

complete (severe) chlorosis and leaf distortion, with plants exhibiting significant stunting and very short internodes.

For CBSD, a severity score of 1–5 was adopted where; 1- no apparent symptoms, 2- slight foliar chlorotic leaf mottle, no stem lesions, 3- foliar chlorotic leaf mottle and blotches with mild stem lesions, no dieback, 4- foliar chlorotic leaf mottle and blotches and pronounced stem lesions with no dieback and 5- defoliation with stem lesions and pronounced dieback (Abaca *et al*., 2012a). A mean shoot severity score was then calculated per genotype based on all individual plant scores per genotype at 8 MAP. In plants that become dually infected with CBSD and CMD, the disease severity of was assessed (Thresh & Harrison., 1998).

Severity scores for root necrosis were taken on all roots harvested per plant at 10MAP. At harvest, each root was cut across into slices approximately 5 cm apart, and the maximum severity score taken for each root (Legg & Kormawa, 2005) where 1- no necrosis, 2- mild necrotic lesions (1-10%), 3-pronounced necrotic lesions (11-25%), 4-severe necrotic lesions (26-50%) and 5- very severe necrotic lesions (>50%). The mean severity of root disease was computed for each plant and subsequently averaged to get a mean value for each genotype. The mean incidence of root necrosis was calculated as the ratio of the number of roots exhibiting symptoms to the total number of roots collected per plant. A mean value was calculated for each genotype.

In addition, at 10 MAP fresh shoot biomass (stems and leaves) and roots per plant were weighed separately (Alves, 2001).

3.2.3: Sampling

At three months after planting (3-MAP), two plants per line per replicate that show disease symptoms were tagged for leaf sampling, whereas for symptomless lines, two plants were randomly selected per line per replicate. At 3, 5, 7 and 11 MAP leaves were sampled from each tagged plant and stored in cool box and thereafter at −80°C for subsequent analysis. For CMD, young leaves 2nd or 3rd level from the top while for CBSD a mature leaf (second level from the bottom) were collected (Kaweesi *et al*., 2014). Leaves were pooled together to avoid or reduce false negative probability for detection and quantification of the viruses in cassava tissues and also to reduce the cost of analysis *(Shirima et al*., 2019).

3.3: Determination of the diversity of viruses and viral loads in the transgenic lines at Alupe confined field trials

3.3.1: Virus detection and quantification

The virus isolates used in this study were obtained from CMD/CBSD infected symptomatic cassava plants collected from the field trial experiment at Alupe Kenya. The identity of the CMD causal virus was confirmed by Triple Antibody Sandwich Enzyme-Linked Immunosorbent Assay (TAS-ELISA) and Polymerase chain reaction (PCR) (Thomas *et al*., 1986). The viruses load in the genotypes was determined by quantitative polymerase chain reaction (qPCR).

3.3.2: Sample preparation and ELISA procedure

Modified from Thomas *et al*. (1986), microtitre plates (wells) were coated with 100µl ACMV IgG diluted 1:1000 (v/v) in coating buffer and incubated for 2-4 h at 37^{0}C. The plates were washed

three times with washing buffer (*Appendix ii*), 5 min between washings, and tapped dry. The plates were blocked by adding 200µl/well of 2% skimmed milk in PBST, incubated for 30 min at 37^0C and washed three times as above. The symptomatic cassava leaf samples were ground 1:10 (w/v) in sample extraction buffer (*Appendix ii*) and the sap extracts (100µl per well) loaded on the plates, incubated overnight at 4˚C and washed three times as above. Extracts from healthy plants and of plants infected with known begomoviruses were used as negative and positive controls, respectively.

One hundred µl MABS (AS-0421/2, AS-0421/4) diluted in conjugate buffer was added to each well and incubated for 4 h at 37^0C. The plates were washed three times as above and alkaline phosphate labelled, rabbit – anti – mouse IgG (DAKO AIS, Denmark) diluted 1:1000 (v/v) in conjugate buffer (Appendix II) added (100µl / well) and incubated for 2 h at 37^0C. The plates were washed as above. The substrate was freshly prepared in substrate buffer (*Appendix ii*) and 200µl of the mixture added to each well and incubated at room temperature for up to 1 h.

The results were assessed visually and using an ELISA reader (Biotek®). Each ELISA plate contained positive and negative control samples and absorbance values (A405nm) that exceeded the negative control values by a factor of two were considered positive. Visually, samples whose wells turned yellow within this period were scored as positive.

3.3.3: DNA extraction for CMVs

Total DNA was extracted from plant tissues using CTAB method (Rogers and Bendich 1994; Warner, 1996; Wilkie, 1996). Approximately 100 mg of leaf tissue was put into sample extraction

bags, 1 ml CTAB buffer (2% CTAB, 100 mM Tris – HCl, pH 8.0, 20 mM EDTA and 1.4 M NaCl) with 0.2% β mercaptoethanol added and ground using a small hand roller. This was then be incubated at 65°C for 15 minutes after which it was centrifuged at 16000 rpm in a microfuge for 10 min at 25^0 C. The aqueous layer formed (approximately 800µl) was transferred into a clean nuclease-free 2 ml microfuge tube.

Equal volume of chloroform: isoamyl (24:1) was added, vortexed and centrifuged for 10 minutes at 25^0 C. The supernatant was transferred to a clean 2ml tube and equal volume of chloroform isoamyl (24:1) added and centrifuged at 16000 rpm for 10 min at 25^0 C. The supernatant (approximately 650 µl) was transferred to a clean 1.5 ml tube, 390 µl (0.6 vol) of cold ethanol added and kept at -80 for 30 min the centrifuged at 16000rpm for 30min at 4^0 C to precipitate the DNA.

The liquid was carefully decanted and DNA pellets washed with 500µl of 70% ethanol by centrifugation for 5 minutes at 13,000 rpm at 4^0 C twice. The final DNA pellet was air dried and re-suspended in 100µl of RNase free water. The DNA was quantified using a Nanodrop ND-1000. Samples with a ratio of ~1.8 were considered as "pure" for. Due to differences in DNA quantity, the sample extracts were normalized to a working concentration of 100 ngµl^{-1} by addition of an appropriate amount of sterile water.

3.3.4: RNA extraction for CBSVs

Approximately 100 mg of leaf tissue was put into crushing bags, 1 ml CTAB buffer containing 2% CTAB, 100 mM Tris – HCl, pH 8.0, 20 mM EDTA and 1.4 M NaCl added and ground using

a small hand roller (Rogers and Bendich, 1994; Warner, 1996; Wilkie, 1996). The mixture was thereafter incubated at 65°C for 15 minutes, after which 700 µl of chloroform: isoamyl alcohol (24:1) was introduced and centrifuged at maximum speed in a microfuge for 10 minutes at room temperature. The aqueous layer was extracted and placed into a sterile, nuclease-free 1.5 ml microfuge tube, followed by the addition of an equal amount of 4 M LiCl, and incubated overnight. The mixture was subsequently centrifuged for 30 minutes at a maximum speed of 13,000 g at 4°C to pellet the nucleic acids. The pellet was re-suspended in 200 µl of TE buffer with 1% SDS. Subsequently, 100 µl of 5 M NaCl and 300 µl of ice-cold isopropanol were added, and the mixture was incubated at −20°C for 30 minutes.

Following incubation, the mixture was subjected to centrifugation for 10 minutes at 13,000 g to pellet the nucleic acid. The pellet was subsequently rinsed with 500 µl of 70% ethanol and centrifuged for 4 minutes at 4°C. The ethanol was decanted, and the pellet was dried and re-suspended in 50 µl of nuclease-free sterile water. The quality and amount of RNA were assessed using a Nanodrop ND-1000 (Nanodrop Technologies, DE, USA). The absorbance ratio at 260 nm to 280 nm was utilized to evaluate purity, with a ratio of 1.8-2.0 being acceptable for RNA samples classified as "pure."

The samples were standardized to a working concentration of 100 ngµl−1 using sterile water due to variations in RNA amount. cDNA synthesis was performed using Moloney murine leukemia virus reverse transcriptase (M-MLV RT) in accordance with the manufacturer's guidelines (Promega, WI, USA). Oligo (dT) 15 primers were employed to synthesize the first strand cDNA,

focusing on the 39-proximal end of the genome. Red-Hot Polymerase (Thermo Fisher Scientific, MA, USA) was employed for the amplification of viral sequences using PCR.

3.3.5: Detection of cassava begomovirus species by Polymerase Chain Reaction

Samples were selected from all the varieties and replicates. Approximately 2 ng of each sample were tested using primers designed to amplify near-full length DNA-A, DNA-B and the Common Region of CMGs. (*Appendix iii*).

Agarose gel electrophoresis was performed with TAE buffer (0.04M Tris acetate pH 8.0 and 1mM EDTA). Agarose powder was incorporated into the TAE buffer (0.8% w/v) and subjected to microwave heating for 2 minutes to dissolve the powder. 0.01% ethidium bromide was included into the cooling solution, which was then put into a tray where a comb was introduced to create sample slots. The agarose gel was let to firm for 30 minutes prior to the removal of the comb, after which the tray containing the gel was submerged in the electrophoresis tank filled with TAE buffer.

To 20µl of DNA sample, 10µl of loading buffer (comprising 25% Ficoll and 25% Bromophenol blue in 5X TAE) was added, resulting in a total volume of 30µl, which was subsequently loaded onto a slot in the gel. A 1kb digested lambda phage DNA (Fermentas NBI, Germany) used as a molecular size marker, ran concurrently in a single lane of each gel. The gel was subjected to 120 volts and maximum current for 45 minutes prior to being examined under UV light and photographed..

3.3.6: Quantification of Viruses and Qt- PCR

The quantification was adapted from Adams *et al*. (2013), where primers CP3 F-CGCGTAGTGTGCCTTTCTTT and R-CTTCCTAGCCGAAGCACAAT were used. The R-T PCR assay used was based on TaqMan chemistry, using primer and probe sequences except that the CBSV probe was 5'-FAM-TAMRA-3' labeled and the UCBSV probe was 5'-VIC-TAMRA-3' labeled. In addition, COX (cytorchrome oxidase) was used as an internal control with primers PP2A-F(CGTCGCATTCCAGATTATCCA),

COX-R (CAACTACGGATATATAAGRRCCRRAACTG) and probe (FAM AGGGCATTCCATCCAGCGTAAGCA-TAMRA). COX is a widely used housekeeping gene to normalize cycle threshold (Ct) values validated for use with CBSV and UCBSV quantification.

For each RNA sample, two technical replicate reactions were prepared containing 12.5 µl of Maxima Probe qPCR Masterix (2x) Fermetas) 7.5 µM of each forward and reverse primer, 5 µM Taqman probe, 100ng of template, MMLV-Reverse transcriptase and nuclease free sterile water to a volume of 25 µl. In addition, non-template water control was included on every plate. The reactions were incubated for 60 min at 42ºC then initial denaturation step run for 10 min at 95ºC followed by 40cycles of denaturation for 15 sec at 95ºC, annealing for 30 sec at 60ºC and extension for 30 sec at 72ºC

All real-time PCR reactions were performed on an Applied Biosystems' One Step Plus® sequence detection system (Applied Biosystems, CA, USA). The generated cycle threshold (Ct) values were used to determine the fold change in expression of a target gene for both CBSV and UCBSV using

a comparative $2^{-\Delta\Delta Ct}$ method as described by Livak and Schmittgen (2001) where $\Delta\Delta Ct = (Ct_{target}\text{-}Ct_{Cox})_{\text{time x}} - (Ct_{target}\text{-}Ct_{Cox})$. An average of the Ct values was determined for each line, used to calculate delta Ct and 2^-delta Ct values that were used to generate a bar graph showing the comparison of the viral loads in the transgenic lines.

3.3.7: Sequencing and sequence analysis

PCR products of the expected sizes were purified, and cloned into the pCR 2.1 vector using the TA cloning kit (Invitrogen, San Diego, CA). Clones containing putative viral sequences were identified by miniprep screening and confirmed positive for inserts by PCR amplification using their respective PCR primers, and inserts were subsequently sequenced in both directions. The complete and partial nucleotide sequences of CMGs were determined by the dideoxynucleotide chain termination method using an ABI 377 DNA automatic sequencer (Applied Biosystems, CA, USA). Sequence fragments of 600- 900 bp were generated. The sequence quality was checked based on the peak of the electrophoregram and trimmed using CLC main work bench v20 to remove the PCR primers.

The sequences were compiled, and any discrepancies were reconciled based on the consensus. The final consensus was subsequently exported in FASTA file format. The sequences were subjected to BLASTn searches against the NCBI database to identify the closest match. Sequences having a nucleotide identity of at least 97% from BLAST hits were selected and then retrieved from the NCBI database. The sequences were uploaded to MEGA X software, and multiple alignment was performed with a Gap Opening Penalty of 15 and a Gap Extension Penalty of 5.5.

Phylogenetic trees were generated using MEGA X. The optimal DNA model for the phylogenetic tree was derived from the Automatic Neighbor Joining Tree and the Maximum Likelihood approach of nucleotide substitution. The optimal DNA model employed for the phylogenetic tree was Kimura 2-Parameter with Invariant Plus Gamma. The final tree was constructed using a bootstrap value of 1000 replications.

3.4: Determination of the resistance and yield efficacy levels of the RNAi transgenic cassava lines against CMD and CBSD causal viruses in Alupe Confined field Trial

Using a derived algorithm modified from Omayio and Ndombi (2022), was used to calculate the levels of resistance variations of the different RNAi transgenic lines. The algorithm in question incorporated two parameters; which were the quantity of the viruses and severity performance of the lines as determined from the Qt-PCR analysis and field screening described on sub-sections 3.3.6 and 3.2 respectively. The algorithm entailed calculating the natural logarithmic divergence index (DI), which calculated the level of viral load in a treatment in comparison to an ideal situation of no virus in transgenic line. The significance of utilizing natural logarithms is based on their ability to reduce exaggerated skew of values based on natural numbers and provide relative performance of subjects in an experiment (Parry, 1990; Omayio and Ndombi, 2022). The modified algorithm consisted of five levels as follows:

3.4.1: Level one

Involved getting the RNAi transgenic line's divergence index (DI) of the input data, as demonstrated by *equation 3.1* below. In order to determine relative performance levels (divergence index of treatment relative to an ideal control; a situation of no virus infection) in performance.

The generated index is an estimator of divergence from near zero along number line in mathematics;

Equation 3.1:

Divergence Index (DI) = Ln (0+0.1) - (Ln (VLT)

Where (0+0.1) = 0.1, is the constant that can give the lowest divergence absolute number from zero, to enable the equation to logarithmically determine a value that can be an estimator of divergence to almost zero (no virus load in an organism). Zero in this case stands for an ideal scenario where a transgenic line has no virus in its system (almost 100% resistance). Whereas VLT indicate the viral load of the treatment under investigation.

3.4.2: Level two

Entailed the transformation of the negative divergence index from equation 3.1 into a positive integer, to enable calculation of resistance divergence from highest levels of 100%, was modeled as per equation 3.2 below. Since a negative answer in *equation 3.1* denoted a decline in performance from the ideal control of no virus in a transgenic line. Therefore, to effectively determine percentage decline from 100% immunity, the divergence index was made positive by multiplying by (-1) as per equation 3.2 below;

Equation 3.2:

Positive Divergence Index (PDI) = (*-DI ×-1)*

-DI is the negative divergence index illustrating a decline in resistance, determined from *equation 3.1*, multiplied by negative one (-1) to change it into a positive form. The negative shows a decline

in performance caused by disease damage**.** Multiplying the negative is eliminated and remains with a positive value.

3.4.3: Level three

Involved calculating the corresponding divergence index's percentage (CDIP) modified from Omayio *et al*.(2018), since relative logarithmic efficacy indices aided the determination of the deviation of performance from the ideal control; a corresponding divergence index percentage will be ideal in estimating the performance of the transgenic lines from the ideal control (almost zero viral load) as per *equation 3.3*.

Equation 3.3:

$$CDIP\% = \left(\frac{Positive\ Divergence\ Index}{14.51}\right) \times 100$$

Where CDIP% refers to the corresponding divergence index percentage of the transgenic line. This generates the positive divergence indices corresponding percentages which are well distributed in a scale of between 0%-100%. The CDIP% of transgenic lines describes the resistance which does not factor the severity of the diseases on transgenic lines based on qualitative scores. The 14.51, is constant which describes the highest logarithmic index attained beyond which the logarithmic scale flattens as per Omayio *et al*. (2018). The 100 is used to generate the percentage of the ratio, based on the assumption that when a transgenic line is not diseased (no virus), its divergence corresponding divergence index percentage will be 0%. When infected by a disease, this divergence continues to increase as determined by levels of susceptibility of a transgenic line.

3.4.4: Level four

Entailed determination of unadjusted resistance levels of the transgenic lines based on their performance relative to the ideal control of the logarithm as per equation 3.4 below:

Equation 3.4:

URL% =100 - CDIP%

Where URL% denotes the unadjusted resistance levels of the transgenic line and CDIP% denotes the corresponding divergence index percentage determined from level three above. It's substracted from 100 (maximum percentage of highest levels of resistance) state of a plant being immune to viral attack.

3.4.5: Level five

Therefore, to determine the adjusted transgenic resistance levels of the lines. The corresponding divergence index percentage was averaged with percentages determined through modification of the qualitative severity scores of the work of Hahn *et al*. (1980) and Gondwe *et al*. (2003). These authors developed a five level severity scores for CMV and CBSV respectively, which describes the level of severity of the disease in a continuum manner. By dividing equally the five levels continuum, since resistance follows a continuum (Omayio and Ndombi, 2022), the respective percentages of the qualitative scores were derived as described on *table 3.1* below;

Table 3.1: Modification of the qualitative scores continuum key to generate corresponding resistance percentage estimates that were used to correct the unadjusted resistance levels of the transgenic lines from step three of the algorithm.

Score	Description Modified from scoring criteria of Hahn *et al*. (1980) & Gondwe *et al*. (2003)	Resistance Classification as Modified by the author	Qualitative Scores Corresponding Resistance % (QCR%)
1	No Observable Damage	Very High Resistance/Immune (81%-100%)	100%
2	Slight/Low Damage	High Resistance (61%-80%)	80%
3	Moderate Damage	Moderate Resistance (41%-60%)	60%
4	High Damage	Low Resistance (21%-40%)	40%
5	Very High Damage	Very Low Resistance (0%-20%)	20%

Therefore, the generated scores were based on the assumption that anything transgenic line with 0% qualitative resistance will not survive to be scored. Hence, generally falls under the category of very low resistance (score 5). Therefore, the adjusted resistance of transgenic lines was determined by averaging the unadjusted resistance levels of transgenic lines and their qualitative corresponding resistance percentages based on the scoring means during field evaluation as per equation 3.5 below;

Equation 3.5:

ARL% (Adjusted Resistance Levels of transgenic lines in percentage)

$$ARL\% = \frac{(CDIP\%\ of\ transgenic\ line + QCR\%\ of\ the\ transgenic\ line)}{2}$$

Where CDIP% refers to the corresponding divergence index percentage of the transgenic cassava lines, whereas QCR% refers to the qualitative corresponding resistance percentage of the transgenic lines based on their field screening as per *table 3.1* above.

3.4.6: Determination of yield efficacy levels of the transgenic lines

This was determined based on Adebayo (2023), work, where the yield efficacy index percentage describes the input of the transgenic lines towards maximum yield. The reference maximum yield being 6.65kg/6m^2. Where the figure was derived from the World's average yield per hectare of 11,080Kgs/Hectare (10,000m^2) (Adebayo, 2023). Since, the transgenic lines were planted in plots of 3m × 2m giving an area of 6m^2. The yielding was scaled downwards to give reference maximum potential yield of 6.65kg/6m^2. Therefore to calculate the yield efficacy levels of the transgenic lines equation 3.6 below was used:

$$YEL\% = \left(\frac{Actual\ Yield\ of\ transgenic\ Line\ in\ Kgs}{6.65kgs}\right) \times 100$$

Where YEL% refers to the Yield Efficacy Levels percentage. The 6.65kg represents the maximum possible yield that could have been obtained from the area of experimental units of 6m^2 used in this experiment derived from the world's average production per hectare (Adebayo, 2023).

CHAPTER FOUR

RESULTS

4.1: Source of Cassava seed, the viruses and the disease levels of CMD and CBSD in major cassava growing areas of Kenya

4.1.1: Farms sampled and mean altitudes

The coordinates of the sampled areas are captured on *appendix xiii*, and as shown in *table 4.1* below, 383 farms were visited; at least 40 farms in each county except for Kisumu which was not among the counties to be sampled; but an interesting sample was observed in a farm by the road side near Muhoroni. The sample had very bright yellow mosaic with severely disfigured spindle-like leaves but without stunting and when uprooted it had no tubers. A total of 487 samples were collected, 240 for CMD, 207 for CBSD and 40 asymptomatic. The altitudes of the sampled area ranged from 56 MASL in Kilifi to 1537 MASL in Machakos (*Table 4.1*).

Table 4.1: No of farms sampled per county and mean altitude

County	Number of farms sampled	Altitude Metres Above Sea Level (M.A.S.L)
Bungoma	50	1262
Busia	53	1220
Siaya	42	1269
Kisumu	1	1552
Kisii	47	1409
Kakamega	50	1299

Kwale	41	80
Kilifi	50	56
Machakos	50	1537
Total	**383**	**1294**

4.1.2: Source of the cassava cuttings seed by farmers in the surveyed region

Most of the farmers (67.4%) obtained their planting materials (cuttings) from their own farms while 57.3% obtained their planting material from neighbours' fields and a paltry 8.3% from other sources like CBOs, ministry of agriculture, KALRO e.t.c. Over 85% of the farmers in the surveyed area did not select clean (non-symptomatic) planting material.

Table 4.2: Farmers' source of planting material

County	**Source of planting material (%)**		
	Own seed	**Neighbor**	**Other**
Kakamega	70%	40%	5%
Busia	73%	60%	20%
Bungoma	68%	50%	10%
Kisumu	-	-	-
Kisii	64%	50%	6%
Kwale	60%	65%	8%
Kilifi	63%	70%	3%

Machakos	69%	55%	1%
Siaya	72%	68%	13%
Mean % ± S.D%	**67.4% ± 0.05%**	**57.3%± 0.10%**	**8.3%± 0.06%**

4.1.3: CMD and CBSD Symptoms observed during survey

An interview with some of the farmers in the surveyed counties established that most farmers (69%) did not know that the CMD and CBSD symptoms were as a result of viral diseases and contribute significantly to the poor yields despite their presence in the farms for over 5years.

Typical CMD and CBSD symptoms were observed in all the counties sampled (*Figure 4.1*). The CMD symptoms observed on cassava leaves ranged from pale yellow to bright yellow mosaic, mild to severe mottling and leaf deformation, stunting and reduced leaf size. The plants with a combination of CMD and CBSD symptoms had severely reduced tuber number and size.

Cassava brown streak disease symptoms included, veinal and interveinal chlorosis, pale to bright yellow patches, chlorotic spots and blotches, upward leaf curl and brown patches on the lower leaf surfaces (*Figure 4.1*). The stems had necrotic spots and scratches and the tubers had constrictions, necrotic areas and in some cases they were corky covering > 80% of the tuber. Interestingly some plants lacked foliar CBSD symptoms but their tubers had pronounced symptoms and vice versa.

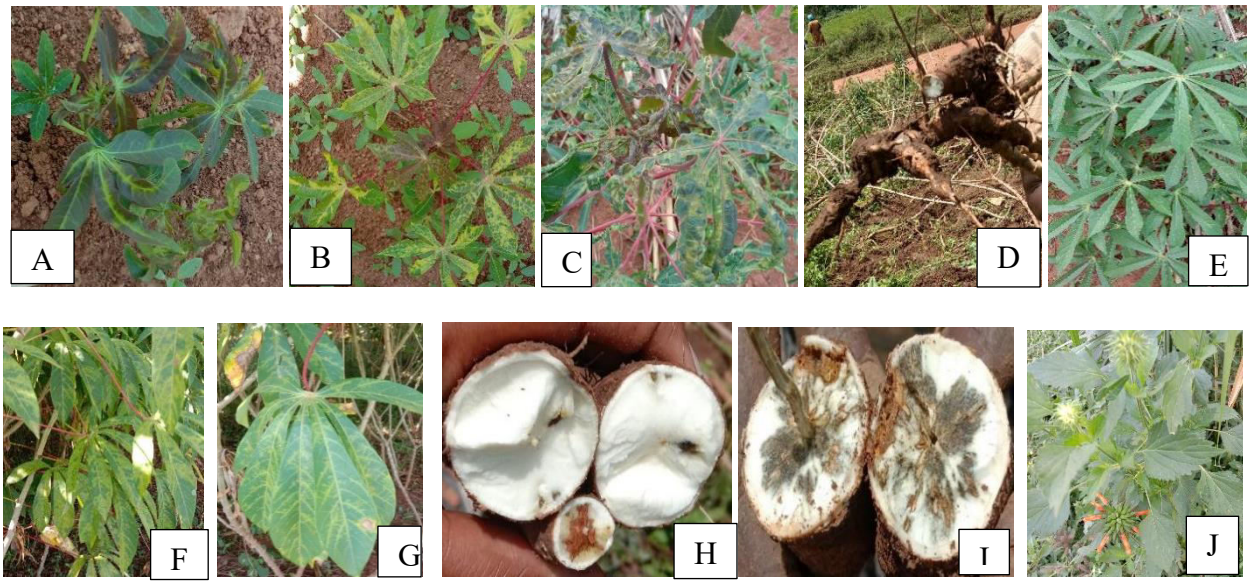


Figure 4.1: CMD and CBSD symptoms observed during the survey. A: Young plant with mosaic, leaf deformation and mottling; B: Bright mosaic, mottling and deformation; C: Severe leaf deformation, mosaic and mottling; D: Tuber loss and necrotic areas; E: Healthy; F: Chlorotic blotches; G: feathery chlorosis along the secondary and tertiary veins; H & I dark brown necrotic areas on root; J: *Leonotis leonarus.*

The symptoms were more severe in some varieties than others as shown in *Figure 4.2* below. In this farm located in Munami area at the border of Kakamega and Busia County, most of the TME plants labelled B lacked CMD but had pronounced CBSD symptoms. In the few plants with CMD, the symptoms were only present in the young leaves but absent in older leaves suggesting more recent infection. All the plants of the variety labelled A had very severe CMD symptoms in all the leaves but no visible CBSD foliar symptoms. In addition the plants had 95% tuber loss and the small tubers present had CBSD necrotic spots with a severity of 2.

Figure 4.2: Varietal differences in CMD expression

4.1.4: Incidence and Severity of CMD and CBSD in the surveyed area

Cassava Mosaic Disease (CMD), was more prevalent in the surveyed farms than CBSD with incidences ranging from 27.64% in Kisii County to 60.34% in Busia (*Table 4.3*). For CBSD mean incidence ranged from 26.02% in Kilifi County to 38.74% in Busia County (*Table 4.3*). There was significant difference in both CM and CBSD incidence among the counties surveyed ($p = 0.001$) but did not vary significantly between the counties ($p \geq 0.05$). The CMD severity ranged between 1 to 5 while CBSD severity was between 1 and 4. There was significant difference in severity among the counties surveyed (P=0.001). In cassava growing counties of Kenya; all the farms visited had both CMD and CBSD (*Table 4.3*). Worst case was in a farm located at Amaase in Busia County where there was 100% CMD incidence mixed with 40% CBSD. The CMD severity score for the farm was 5 while CBSD severity was 4. Each plant uprooted from this farm had 100%

tuber loss and yet the farmer was still keeping the crop hoping for some produce. In some instances, the CMD symptoms were so severe that it was difficult to recognize any CBSD symptoms. In another farm in Ejinja Kakamega County, CBSD incidence was 90% while CMD incidence was 13.5%. The CBSD infected plants in this farm produced very large tubers but with CBSD root severity of 4 hence the tubers could not be consumed. In another farm in Maondo Kakamega County, CMD incidence was 100% with a severity score of 5. There was severe tuber loss where the plants had very few thin tubers and in some cases total tuber loss. No foliar CBSD symptoms were observed in this field but the tubers had slight necrotic areas with a severity of 2. In Kilifi, and Kwale counties, the situation was similar since CMD was more prevalent than CBSD with incidences of up to 100% in individual farms. Busia had the highest mean CBSD severity of 3.8 followed by Kwale 3.7 (*Table 4.3*). The sampled field in Kisumu had no CBSD symptoms hence severity not scored. Kilifi County had the lowest mean CBSD severity of 2.9. In individual farms however, severity scores of 5 were reported in at least one farm in all the counties sampled. In some cases, root necrosis covered more than 80% (*Figure 4.1*) with severe constrictions rendering them non-consumable.

Table 4.3: Mean CMD and CBSD incidence and severity per county

County	**Mean Incidence% ± S.E (CMD)**	**Mean Incidence% ± S.E (CBSD)**
Bungoma	59.74 ± 2.855	33.32 ± 1.747
Busia	60.34 ± 2.898	38.74 ± 2.716

Kakamega	53.76 ± 3.587	33.50 ± 1.674
Kilifi	41.80 ± 2.584	26.02 ± 1.420
Kisii	27.64 ± 1.860	33.77 ± 2.990
Kwale	50.00 ± 4.193	37.15 ± 2.309
Machakos	37.78 ± 3.411	27.30 ± 1.490
Siaya	53.40 ± 3.388	32.80 ± 1.732

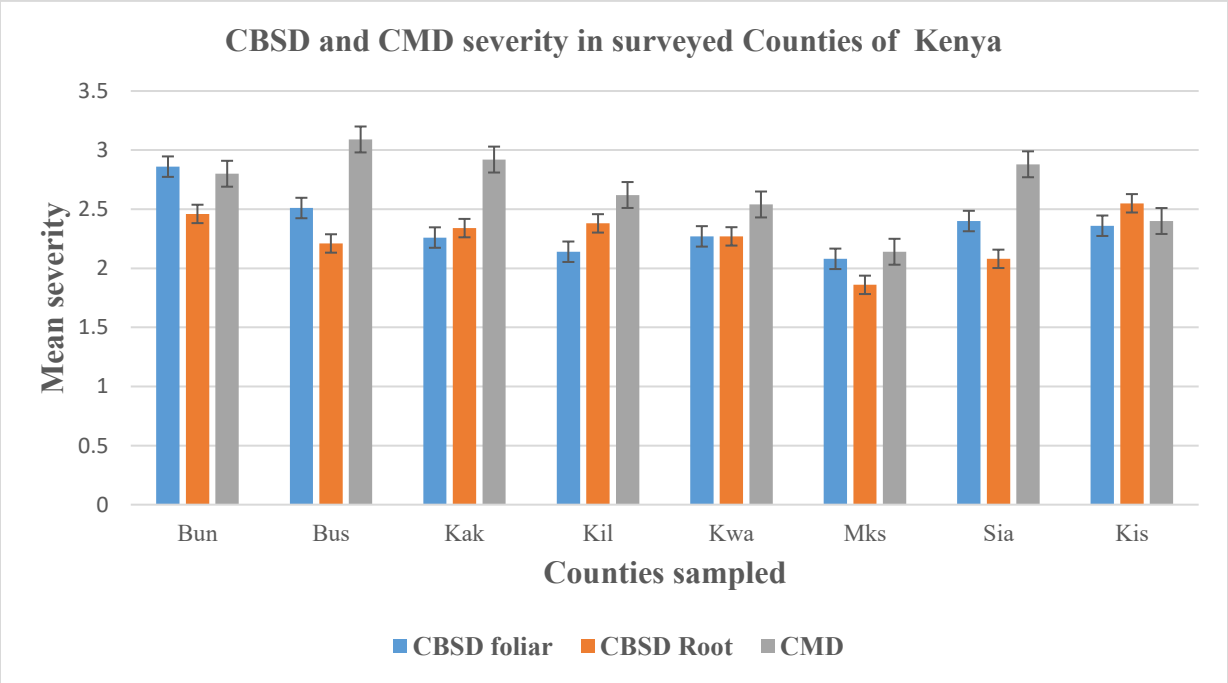


Figure 4.3: Mean CMD and CBSD foliar and root severity per county

4.1.5: Enzyme linked immunosorbent assay for detection of CMVs and CBSVs

All the samples collected were subjected to TAS-ELISA for detection of ACMV and EACMV, and DAS-ELISA for detection of CBSV and UCBSV (*Tables 4.4 & 4.5*). All the counties sampled

had the two diseases (CMD and CBSD) caused by CMVs and CBSVs with varying prevalence levels (*Tables 4.4 & 4.5*). Based on the 240 samples collected that had CMD symptoms; 79 (32.9%) tested negative of the viruses while 132 (55%) tested positive for CMVs, whereas 29 (12%), tested positive for CBSVs (*Table 4.4*). For the 207 collected samples that exhibited CBSD symptoms; 92 (44.4%) tested negative for CBSVs, while 108 (52.2%) tested positive for CBSVs, whereas 7 (3.4%) tested positive for CMVs (*Table 4.5*).

Table 4.4: ELISA results for CMD symptomatic samples

County	No. of samples	Samples Tested ACMV/EACMV +ve	Samples Tested CBSV +ve
Kakamega	24	18 (75%)	1 (4%)
Busia	42	24 (57%)	10 (24%)
Bungoma	36	20 (56%)	3 (8%)
Siaya	29	20 (69%)	6 (21%)
Kisumu	1	1 (100%)	1 (100%)
Kisii	27	18 (67%)	4 (15%)
Machakos	27	11 (41%)	0 (0%)
Kwale	25	18 (72%)	3 (12%)
Kilifi	29	20 (69%)	1(3%)
Total	**240**	**132 (55%)**	**29 (12%)**

Table 4.5: ELISA results for CBSD symptomatic samples

County	No. of samples	Samples Tested CBSVs +ve	Samples Tested ACMV/EACMV +ve
Kakamega	22	9 (41%)	2 (9%)
Busia	40	17 (43%)	1 (3%)
Bungoma	30	11 (37%)	1 (3%)
Siaya	25	13 (52%)	0 (0%)
Kisii	24	12 (50%)	1 (4%)
Machakos	25	10 (40%)	1 (4%)
Kwale	20	19 (95%)	0 (0%)
Kilifi	21	17 (81%)	1 (5%)
Total	**207**	**108 (52.2%)**	**7 (3.4%)**

4.1.6: PCR to detect viruses in the samples collected

From the PCR results, 62.1% of the samples tested by PCR were positive for EACMV while 31.8% were positive for ACMV. Mixed ACMV/EACMV infections were reported in 28.8% of the samples tested. Both ACMV and EACMV were detected in samples from all the counties surveyed. However all the samples from Busia County had both ACMV and EACMV. In samples from Bungoma County it was reported that ACMV was detected more than EACMV. Samples from Kwale, Kilifi, Siaya, Kakamega and Machakos counties had more EACMV than ACMV.

Thirty one percent of the samples tested positive for CBSV while 25.5% were positive for UCBSV. Surprisingly, using primers for CP to detect CBSVs the results were slightly different with 38% of the samples testing positive for CBSV and 16% positive for UCBSV. Mixed CBSV/UCBSV infection was detected in 15.9% of the samples tested and 39% of the CBSV positive samples.
In the weed samples analysed, EACMV was detected in only Leonotis leonarus and no ACMV was detected.

4.1.7: Diversity of viruses infecting cassava in Kenya based on samples collected from the survey

The genomic characterization of CMD associated viruses was determined by analysis of the sequence reads obtained by Sanger Sequencing. Three ACMV AV1 gene sequences of the novel Kenyan isolates were assembled and compared with ACMV AV1 sequences available in the GenBank (*Figure 4.4*). Seven EACMV DNA-B sequences of the Kenyan isolates obtained in this study were also assembled and compared with those available in the GenBank (*Figure 4.5*). Two isolates from Kenya (BUS-STR and KAK 16) clustered in Group I, while the others clustered in Group II (*Figure 4.4*). The Kenyan novel ACMV isolates from Busia and Kakamega were more similar to Ugandan isolates, suggesting common ancestry (*Figures 4.4 & 4.5*).

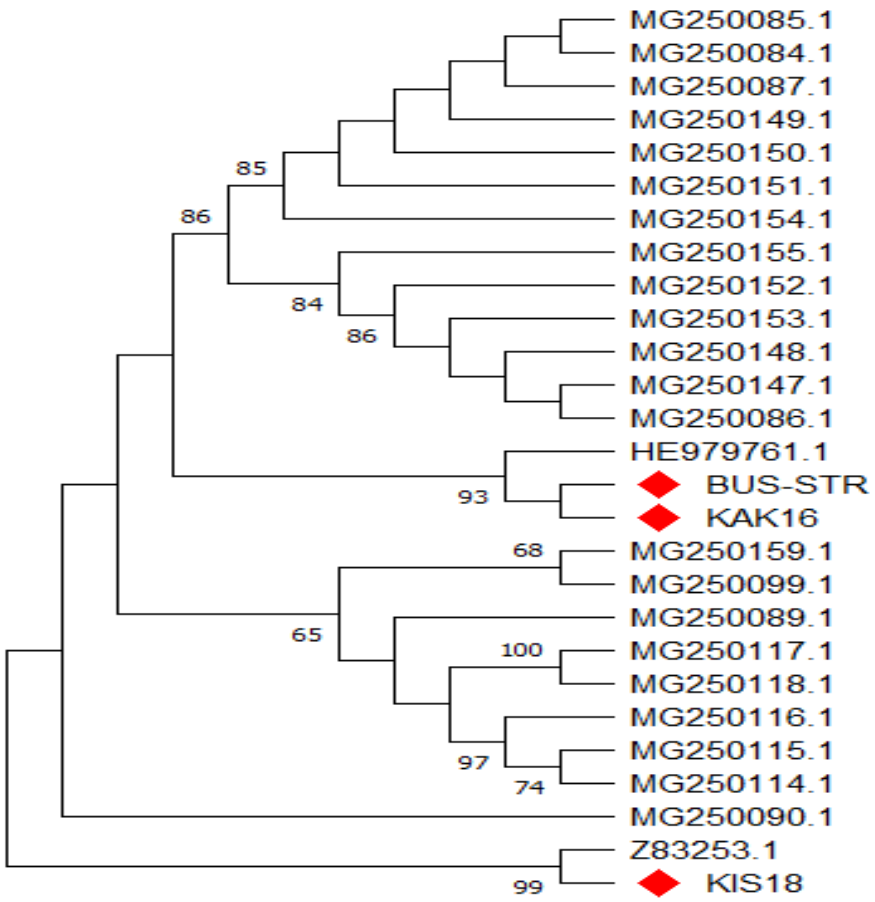


Figure 4.4: Phylogenetic tree of Kenyan ACMV isolates AC1 region and GenBank isolates.

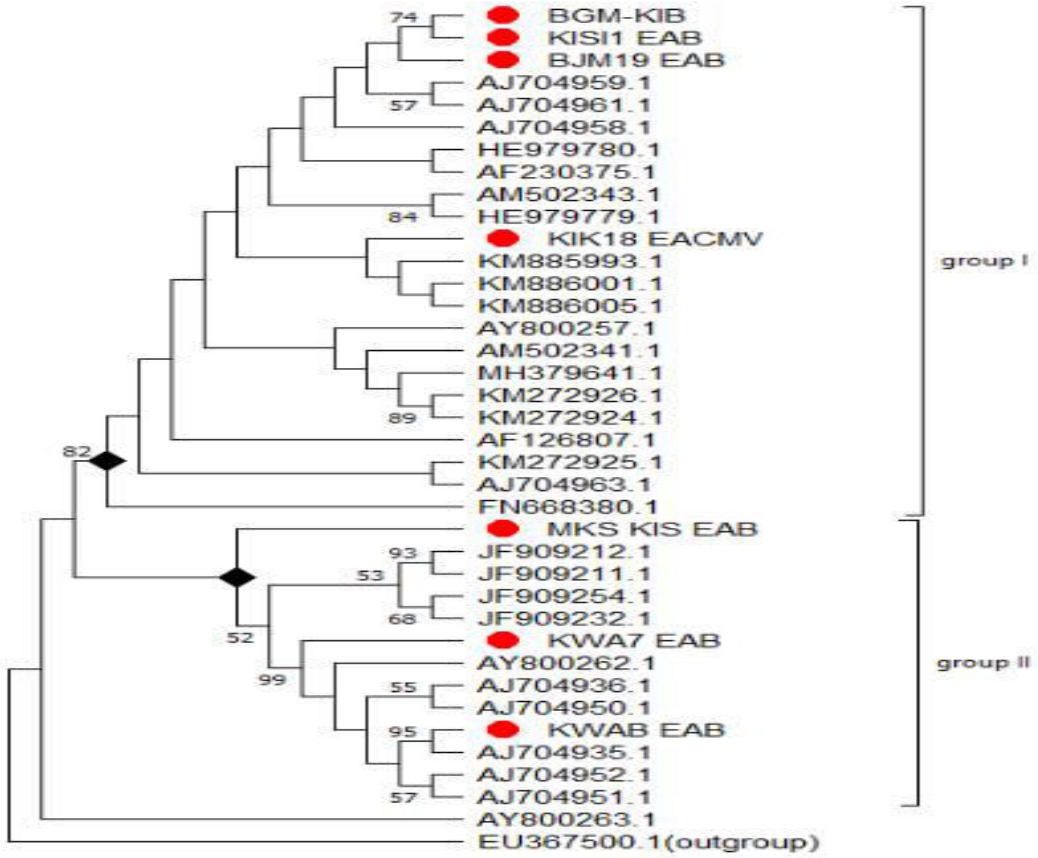


Figure 4.5: Phylogenetic tree of Kenyan EACMV isolates BC1 DNA B region and GenBank isolates.

4.2: Disease progression, incidence and severity levels of CMD and CBSD in RNAi transgenic lines

Both CMD and CBSD symptoms were observed in the transgenic lines (*Table 4.6*).

In lines engineered for CMD resistance, high CMD incidences were reported in some cases. For instance, line 166 had mean CMD incidence of 71 ± 5.7% but with the highest mean tuber weight

of 0.9 ± 0.6 kg (*Table 4.6*). Line 145 had the lowest CMD incidence of 20 ± 6.3% with many small tubers; that had a mean tuber number of 27 ± 7.4, with a mean weight 0.7± 0.4 kg and the severity was 2 ± 0.5. Line 74 had high CMD incidence of 93 ± 8.1% with almost total tuber loss having a mean tuber number of 2 ± 1.1 and mean tuber weight of 0.1 ± 0.1 kg. This line was badly degenerated by the viruses even worse than the non- transformed control 60444. Line 157 had the highest CMD incidence of 94 ± 2.9% with mean tuber number of 9 ± 2.2 and 0.5 ± 0.1 kg mean weight. It was also observed that lines 74, 137, 166 and 348 had no CBSD symptoms on the leaves (*Table 4.6*). For line74, the leaves had severe CMD symptoms hence making it difficult to notice any CBSD symptoms.

Table 4.6: Incidence, severity and yield data for transgenic lines at Alupe CFT

Line/entry	Description of recombination event	Mean±S.D % CMD incidence	Mean±S.D CMD Severity	Mean±S.D Tuber No.	Mean±S.D Tuber weight	CBSD symptoms
60444	60444	82±3.5	5 ± 0.9	6 ± 2.1	0.4±0.3	+
74	ds CR-2	93±8.1	5 ± 0.6	2 ± 1.1	0.1±0.1	-
115	ds AC1-2	75±6.1	3 ± 0.5	7 ± 4.4	0.5±0.5	+
129	ds ACI-101	54±3.5	2 ± 0.6	5 ± 1.9	0.9±0.1	+
133	ds AC1-152	76±4.8	4 ± 0.5	3 ± 2.1	0.3±1.3	+
137	ds AC1 ds AV1-59	76±10.5	2 ± 0.7	5 ± 2.6	0.5±1.4	-
141	ds AC1 ds AV1-55	79±5.0	2 ± 0.00	5 ± 2.3	0.5±0.3	+
145	ds AC1 ds AV1-59	20±6.3	2 ± 0.5	27 ± 7.4	0.7±0.4	+

157	ds AC1 ds AVI-113	94±2.9	3 ± 0.6	9 ± 2.2	0.5±0.1	+
166	Double single 52	71±5.7	2 ± 0.5	7 ± 0.8	0.9±0.6	-
167	Double single 53	59±8.9	2 ± 0.5	8 ± 0.6	0.8±0.2	+
348	Pc 1301 -2k	85±6.4	2 ± 0.6	5 ± 1.1	0.6±2.6	-

For lines engineered for CBSD resistance, six lines (19, 402, 407, 501, and 506) had both foliar and root CBSD symptoms inclusive of non-genetically engineered line 60444 (*Table 4.7*). Nine lines (22, 56, 398, 401, 404, 406, 497, 498 and 499) had only root symptom with a severity score ranging between 2 and 3. Lines 405 and 500 had neither root nor foliar symptoms thus promising for CBSD resistance. It is worth noting that line 405 showed highest CMD symptom while 500 had moderate mean CMD symptoms. It is however important to note that the non-genetically engineered line (line 60444) had more severe root damage with a severity score of 4 ± 2.1 implying that the transformation events provided some level of resistance/tolerance to the viruses.

Table 4.7: CBSD symptoms and severity in the CBSD transgenic lines at Alupe CFT

Line/Entry	**CBSD symptoms on leaf**	**CBSD Mean± S.D severity score on leaf**	**CBSD symptoms on root**	**CBSD root Mean±S.D severity score**	**CMD symptoms on leaf**	**Grand Mean±S.D Severity Score**	**Mean±S.D Tuber Weight Kgs**
60444	+	2±0.4	+	5±1.3	+	4±2.1	0.6±4.4
19	+	2±1.1	+	3±0.8	+	3±0.7	1.1± 2.8
22	-	1±0.8	+	3±0.6	+	2±1.4	0.7±3.6
56	-	1±0.2	+	3±0.4	+	2±1.4	0.9±2.8

398	-	1±0.5	+	3±1.4	-	2±1.4	1.3±2.6
401	-	1±0.4	+	2±1.8	+	2±0.7	2.6±4.2
402	+	2±0.1	+	3±2.0	+	3±0.7	0.8±1.7
404	-	1±0.3	+	2±0.8	+	2±0.7	1.0±2.8
405	-	1±0.6	-	1±0.6	-	1±0.0	0.9 ±2.4
406	-	1±1.0	+	2±1.4	+	2±0.7	0.7±3.6
407	+	2±0.8	+	3±0.6	+	3±0.7	0.8±1.8
497	-	1±0.2	+	2±1.9	+	2±1.4	2.4±3.1
498	-	1±0.5	+	3±0.8	+	2±0.7	1.9±2.1
499	-	1±0.8	+	2±0.2	+	2±0.7	1.6±3.3
500	-	1±1.1	-	1±1.3	+	1±0.0	2.6±1.9
501	+	2±0.4	+	2±1.8	+	2±0.0	3.8±2.7
506	+	2±0.2	+	3±0.2	-	3±0.7	0.8±1.2
TOTAL	6		15		14		

+CBSD/CMD present -CBSD/CMD absent

4.2.1: Monthly expression of CMD and CBSD symptoms in the transgenic lines

The CBSD resistant transgenic lines; the lines 499, 401, 406 and 407 had the lowest plants showing CBSD and CMD symptoms at 0 plants and 0.0% incidence levels, (0(0.0%)); on the eight month after planting (8-MAP) (*Table 4.8*) On the other hand, line 501 had more plants showing symptoms of CBSD and CMD at 32 plants with an estimated incidence of 57.1%, (32(57.1%)); followed by

non-transformed line 60444 which had 14 plants with an estimated incidence of 25.0%, (14(25.0%)) (*Table 4.8*). Lines 60444, 19, 22, 56, 398, 402, 404, 497, 498 and 500 exhibited symptoms of CBSD and CMD within the 4 months after planting (4-MAP) (*Table 4.8*). Therefore, lines 401, 406, 407, 499, 501 and 506 were purposively selected for evaluation of their viral loads, resistance and yield efficacy levels in specific objectives three and four.

Table 4.8: Monthly CBSD/CMD infected plants and incidence levels on CBSD resistant transgenic lines. Key: MAP- Months After Planting.

Line	**CBSD/CMD infected plants and incidence levels (%), based on a total (N=56) Plants for each line**					
	3 -MAP	4 -MAP	5- MAP	6- MAP	7- MAP	8 -MAP
60444	0(0.0%)	10(17.9%)	12 (21.4%)	12 (21.4%)	13 (23.2%)	14 (25.0%)
19	0(0.0%)	7(12.5%)	8(14.3%)	8(14.3%)	8(14.3%)	8(14.3%)
22	0(0.0%)	8(14.3%)	8(14.3%)	10(17.9%)	10(17.9%)	11(19.6%)
56	0(0.0%)	9(16.1%)	9(16.1%)	10(17.9%)	13(23.2%)	13(23.2%)
398	0(0.0%)	5(8.9%)	5(8.9%)	6(10.7%)	6(10.7%)	10(17.9%)
401	0(0.0%)	0(0.0%)	0(0.0%)	0(0.0%)	0(0.0%)	0(0.0%)
402	0(0.0%)	2(3.6%)	2(3.6%)	3(5.4%)	6(10.7%)	10(17.9%)
404	0(0.0%)	1(1.8%)	4(7.1%)	4(7.1%)	8(14.3%)	8(14.3%)
405	0(0.0%)	3(5.4%)	6(10.7%)	6(10.7%)	6(10.7%)	7(12.5%)
406	0(0.0%)	0(0.0%)	0(0.0%)	0(0.0%)	0(0.0%)	0(0.0%)
407	0(0.0%)	0(0.0%)	0(0.0%)	0(0.0%)	0(0.0%)	0(0.0%)
497	0(0.0%)	2(3.6%)	2(3.6%)	4(7.1%)	4(7.1%)	6(10.7%)
498	0(0.0%)	4(7.1%)	4(7.1%)	6(10.7%)	8(14.3%)	8(14.3%)
499	0(0.0%)	0(0.0%)	0(0.0%)	0(0.0%)	0(0.0%)	0(0.0%)

500	0(0.0%)	2(3.6%)	2(3.6%)	2(3.6%)	3(5.4%)	3(5.4%)
501	0(0.0%)	0(0.0%)	0(0.0%)	0(0.0%)	30(53.6%)	32(57.1%)
506	0(0.0%)	0(0.0%)	0(0.0%)	0(0.0%)	11(19.6%)	11(19.6%)

For the CMD resistant transgenic lines; the lines 166, 137 and 167 had the lowest plants exhibiting CMD and CBSD symptoms at 3 plants and incidence levels of 5.4%, (3(5.4%); at the eight month after planting (8-MAP) (*Table 4.9*) On the other hand, line 133 had more plants showing symptoms of CMD and CBSD at 23 plants at an estimated incidence of 41.1%, (23(41.1%), followed by line 115 which had 22 plants at an estimated incidence of 39.3% (22(39.3%) (*Table 4.9*). Lines 74, 115, 141, 145 and 157 showed symptoms of CBSD and CMD within the first four months after planting (4-MAP) (*Table 4.9*). Therefore, lines 129, 133, 137, 166, 167 and 348 were purposively selected for evaluation of their viral loads, resistance and yield efficacy levels in specific objectives three and four.

Table 4.9: Monthly CBSD/CMD infected plants and incidence levels on CMD resistant transgenic lines. Key: MAP- Months After Planting.

Line	**CBSD/CMD infected plants and incidence levels (%), based on a total (N=56) Plants for each line**					
	3 MAP	4 MAP	5 MAP	6 MAP	7 MAP	8 MAP
74	0(0.0%)	10(17.9%)	18(32.1%)	19(33.9%)	19(33.9%)	19(33.9%)
115	0(0.0%)	16(28.6%)	20(35.7%)	20(35.7%)	22(39.3%)	22(39.3%)
129	0(0.0%)	0(0.0%)	0(0.0%)	0(0.0%)	0(0.0%)	8(14.3%)
133	0(0.0%)	0(0.0%)	16(28.6%)	19(33.9%)	23(41.1%)	23(41.1%)

137	0(0.0%)	0(0.0%)	0(0.0%)	0(0.0%)	3(5.4%)	3(5.4%)
141	0(0.0%)	3(5.4%)	10(17.9%)	10(17.9%)	14(25.0%)	14(25.0%)
145	0(0.0%)	5(8.9%)	5(8.9%)	8(14.3%)	11(19.6%)	13(23.2%)
157	0(0.0%)	12(21.4%)	12(21.4%)	14(25.0%)	14(25.0%)	16(28.6%)
166	0(0.0%)	0(0.0%)	0(0.0%)	0(0.0%)	2(3.6%)	3(5.4%)
167	0(0.0%)	0(0.0%)	0(0.0%)	3(5.4%)	3(5.4%)	3(5.4%)
348	0(0.0%)	0(0.0%)	1(1.8%)	1(1.8%)	4(7.1%)	4(7.1%)

4.3: Diversity of viruses and selected viral loads in the transgenic cassava lines at Alupe confined field trial

4.3.1: Detection of Geminiviruses in the transgenic lines by PCR

Appendix Vii shows the results of CTAB DNA extraction. Samples with absorbance value 260/280 of 1.8 to 2.0 were selected and normalized to a working concentration of 20ng using DEPC water.

4.3.2: Polymerase Chain reaction for detection of geminiviruses

The PCR results shown in *table 4.10*, *Figures 4.6* and *4.7* revealed that EACMV was more prevalent in the transgenic lines than ACMV. EACMV was detected in 87.5 % of the samples tested and in all lines. EACMV-Ug was the most dominant strain detected in 85.71 % of the EACMV positive samples. EACMV-KV, EACMV-TZ, EACMV-ZV and EACMV-KE were not detected in the samples tested. ACMV was detected in all the lines tested with 66.7 % of the

samples testing positive. Of the ACMV positive samples, 93.75% were positive for ACMV-UG 34 strain while 81.25% tested positive for ACMV –UG and ACMV-SEV strains (*Table 4.10*). It is worth mentioning that in ACMV-UG SEV was occurring in mixed infection with ACMV-UG34 in all cases. ACMV KE was not detected in the samples tested. Mixed ACMV/ EACMV infections were reported in 65, 5% of the samples tested. All the samples tested for line 133 and 137 were positive for both ACMV and EACMV implying that they were quite susceptible to the viruses (*Table 4.10*). Lines 129 and 167 however showed better results, for instance in line 129, no virus was detected in the second pool tested.

Table 4.10: Detection of Cassava mosaic viruses in the transgenic cassava lines at Alupe CFT in Western Kenya

Line	ACMV	EACMV	EACMV -UG	ACMV -UG34	ACMV -SEV	EACMV -TZ	ACMV -TZ	EACMV -KE	EACMV -ZV	EACMV -KV
60444	+	_	_	+	+	_	_	_	_	_
60444	+	+	+	+	+	_	_	_	_	_
60444	+	+	+	+	+	_	_	_	_	_
129	_	+	+	_	_	_	_	_	_	_
129	_	_	_	_	_	_	_	_	_	_
129	+	+	+	+	_	_	_	_	_	_
133	+	+	+	+	_	_	_	_	_	_
133	+	+	+	_	_	_	_	_	_	_
133	+	+	+	+	+	_		_	_	_
137	+	+	+	+	+	_	_	_	_	_
137	+	+	+	+	+	_	_	_	_	_

137	+	+	+	+	+	_	_	_	_	_
141	_	+	+	_	_	_	_	_	_	_
141	+	+	+	+	+	_	_	_	_	_
141	+	+	+	+	+	_	_	_	_	_
145	+	+	+	+	+	_	_	_	_	_
145	+	+	+	+	+	_	_	_	_	_
145	-	-	-	_	_	_	_	_	_	_
166	+	+	-	+	+	_	_	_	_	+
166	_	+	+	_	_	_	_	_	_	_
166	_	+	+	_	_	_	_	_	_	_
167	_	+	_	_	_	_	_	_	_	_
167	_	+	_	_	_	_	_	_	_	_
167	+	+	+	+	+	_	_	_	_	_
+ve	**16**	**21**	**18**	**15**	**13**	**0**	**0**	**0**	**0**	**1**
Total	**24**	**24**	**24**	**24**	**24**	**24**	**24**	**24**	**24**	**24**
% +	**66.67**	**87.5**	**75**	**62.5**	**54.17**	**0**	**0**	**0**	**0**	**4.2**

4.3.3: Detection of CBSVs by RT-PCR

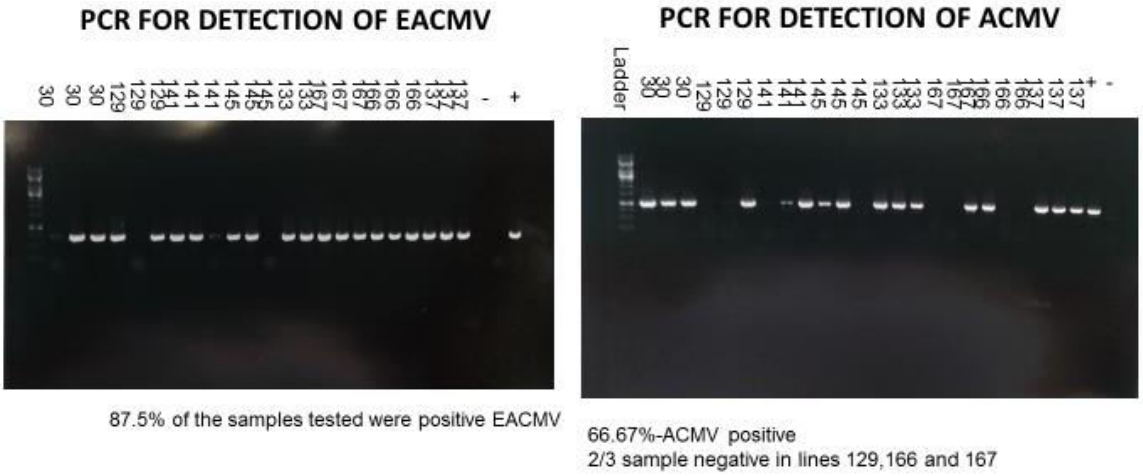
For CBSVs; UCBSV was more prevalent (79.1%) than CBSV (62.5%). Most samples had mixed infections of CBSV and UCBSV (58.3%) as per table 4.11 below:

Table 4.11: Detection of Cassava brown streak viruses in the transgenic cassava lines at Alupe CFT in Western Kenya

LINE	UCBSV	CBSV
56	+	_

56	+	–
56	–	–
401	+	+
401	+	+
401	+	+
402	+	+
402	+	+
402	+	–
406	–	+
406	+	+
406	–	–
407	+	+
407	+	+
407	+	+
499	–	–
499	+	+
499	+	+
506	+	–
506	–	–
506	+	+

6044	+	+
6044	+	–
6044	+	+
TOTAL POSITIVE	**19**	**15**
Total tested	**24**	**24**
% positive	**79.17%**	**62.5%**



A B

Figure 4.6: Gel electrophoresis of PCR amplified DNA using primers specific for all ACMV and EACMV viruses respectively. Expected band size 927 and 600bp respectively

Figure 4.7: Gel electrophoresis of RT-PCR amplified RNA using primers for CBSV and UCBSV viruses respectively. Expected band size 330 and 440bp respectively

4.3.4: Virus quantification in the transgenic lines by qPCR

4.3.4.1: Quantification of geminiviruses

As shown in *Figure 4.8* below qPCR, the results showed that line 167 had the highest viral load, higher than the susceptible control 60444 with mixed ACMV/EACMV infection. However, this line had very low symptom expression in the CFT as well as virus detection by PCR. Line 166 on the other hand, had the lowest viral load and low symptom expression but with mixed ACMV/EACMV infection. This could imply that this line is able to suppress the virus. Lines 348 and 129 also had relatively low viral loads (*Figure 4.8*).

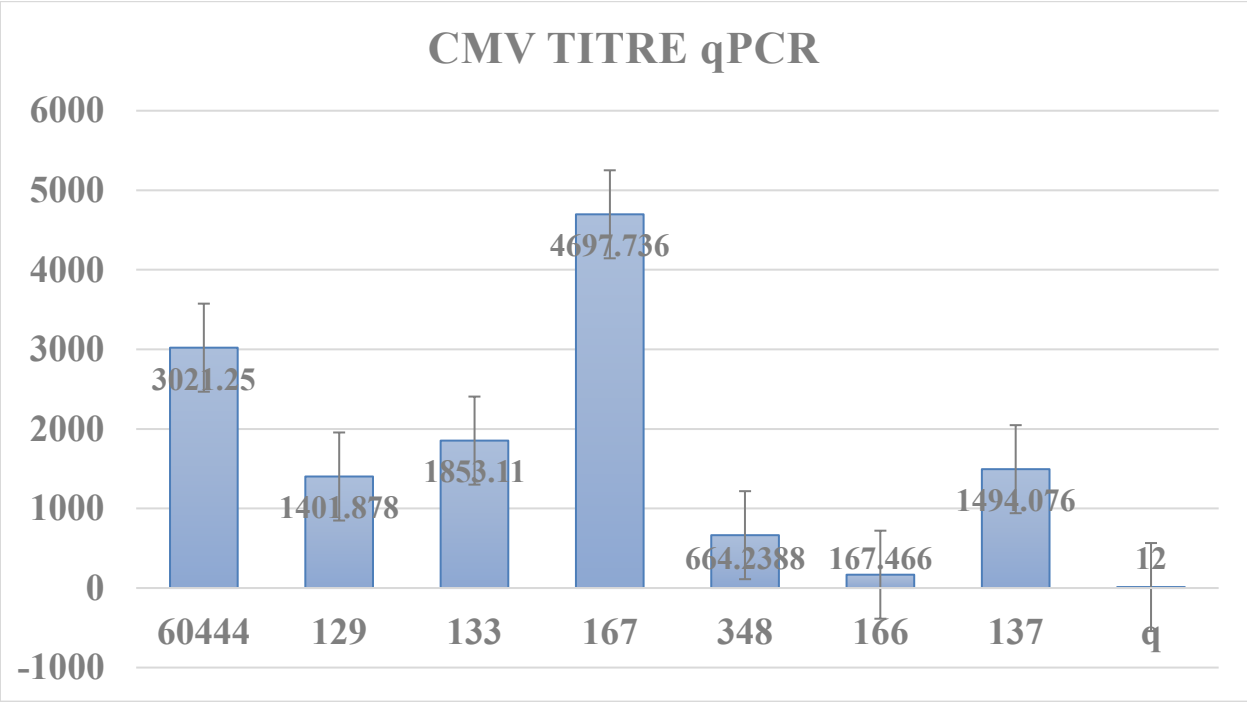


Figure 4.8: qPCR results for cassava mosaic begomoviruses in transgenic CFT lines

4.3.4.2: Quantification of CBSVs by qPCR

As shown in *Figure 4.9* below, it was noted that line 407 had the highest viral load higher than the susceptible host 60444 sample obtained from the CFT. In addition all the samples analyzed had mixed CBSV/UCBSV infection. It also had a severity score of 3 root symptom. It however tested negative for cassava mosaic Begomoviruses. Line 506, 501 and 401 had the lowest viral loads that correlated well with the symptoms in the field. The samples from these lines also tested negative for cassava mosaic Begomoviruses (ACMV and EACMV) and UCBSV. Samples from line 501 had moderately low viral load

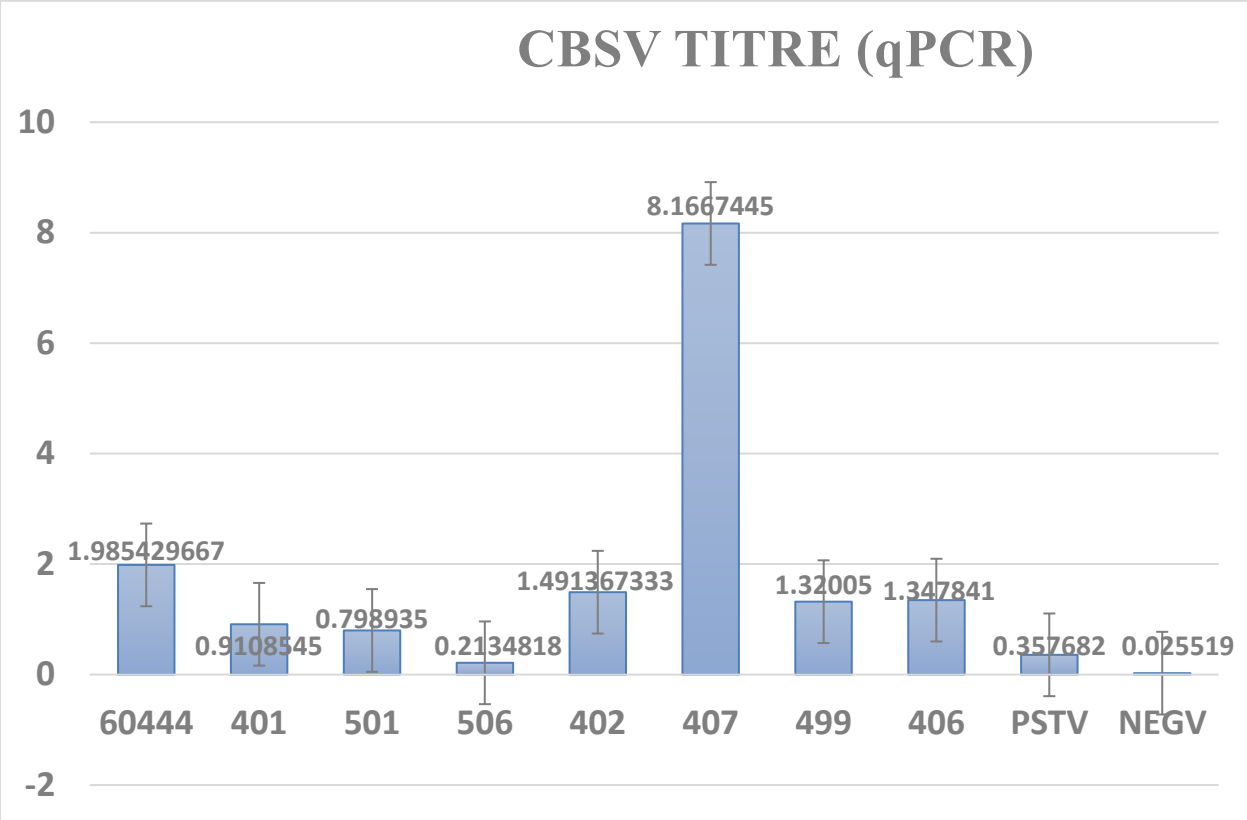


Figure 4.9: qPCR results for CBSVs in transgenic cassava CFT lines

4.4: Resistance and yield efficacy levels of the RNAi transgenic cassava lines against CMD and CBSD viral diseases based on evaluations at Alupe confined field trial

4.4.1: Resistance levels of the transgenic lines against CMD viral disease

The resistance levels against CMD were determined and based on the analysis; the transgenic line 166 had the highest levels at 64.43% with a classification of high resistance based on the algorithm, followed by transgenic line 348 at 59.68% with a classification of moderate resistance (*Table 4.12* and *Table 3.1*) and (*Figure 4.10*). In terms of low performance non-transformed line 6044 had

24.44% with a classification of low resistance, followed by transgenic cassava line 133 which had resistance levels of 36.13% with a classification of low resistance (*table 3.1*), based on resistance estimation algorithm (*Table 4.12 & Figure 4.10*).

Table 4.12: Shows the adjusted resistance levels of the lines against CMD (Cassava Mosaic Disease) based on viral load and severity scores during screening at the field. The mean severity score used to arrive at the resistance levels by the algorithm are captured in table 4.6

LINES RESISTANCE LEVELS BASED ON CMV Qt-PCR TITRES AND MEAN SEVERITY QUALITATIVE SCORES IN THE FIELD

Cassava Lines	Viral Load	Divergence Index	Positive Divergence Index	CDIP%	URL%	ARL%
60444	3021.25	-10.32	10.32	71.12%	28.88%	24.44%
129	1401.88	-9.55	9.55	65.82%	34.18%	57.09%
133	1853.11	-9.83	9.83	67.75%	32.25%	36.13%
167	4697.74	-10.76	10.76	74.16%	25.84%	52.92%
348	664.24	-8.80	8.80	60.65%	39.35%	59.68%
166	167.47	-7.42	7.42	51.14%	48.86%	64.43%
137	1494.08	-9.61	9.61	66.23%	33.77%	56.89%
Negative Control	12	-4.79	4.79	33.01%	66.99%	N/A
ICA	0	0	0	0.00%	100.00%	100.00%

KEY: **URL**- Unadjusted Resistance Levels of the transgenic lines, **ARL**-Adjusted Resistance Levels of transgenic lines, **CDIP**- Corresponding Divergence Index Percentage of the lines, **ICA**-Ideal Control of the Algorithm (Zero Infection/No virus)

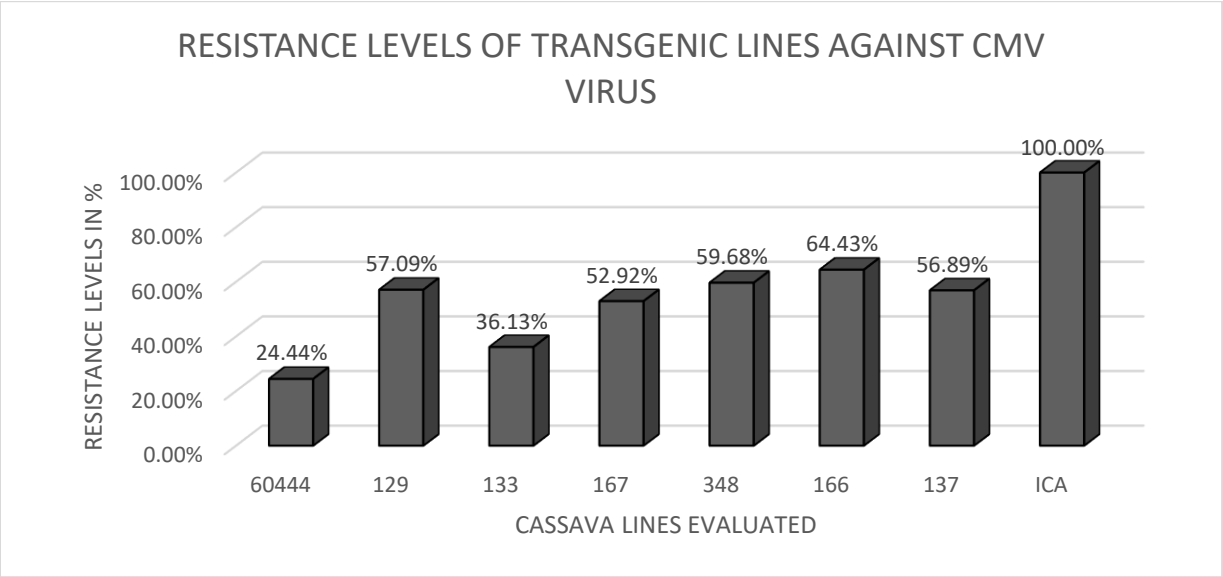


Figure 4.10: Showing the resistance levels of the transgenic lines against CMD (Cassava Mosaic Disease). Non-transformed line 6044 had the lowest levels at 24.44%, whereas transgenic line 166 had the highest resistance levels at 64.43% .The ideal control of the algorithm (ICA) generated the highest levels at 100.00% as predicted.

4.4.1.1: Yield efficacy levels of the transgenic lines against CMD viral disease

The yield efficacy levels against CMD were equally determined and based on the analysis; the transgenic lines 166 and 129 had the highest levels at 13.53% each. The transgenic line 348 which had second highest levels of resistance (*Figure 4.10*) had yield efficacy levels of 9.02% (*Table 4.13*).) In terms of low performance transgenic line 133 had the lowest yield efficacy levels at 4.05%, followed by non-transformed line 6044 that had 6.02% (*Table 4.13*).

Table 4.13: Shows the yield efficacy levels of the CMD resistant transgenic lines in percentage

YIELD EFFICACY LEVELS OF THE EVALUATED CMD TRANSGENIC LINES		
Cassava Lines	Mean ± S.D Actual Yield (Kgs)	Yield Efficacy in %
60444	0.4 ± 0.3	6.02%
129	0.9 ± 0.1	13.53%
133	0.3 ± 1.3	4.50%
167	0.8 ± 0.2	12.03%
348	0.6 ± 2.6	9.02%
166	0.9 ± 0.6	13.53%
137	0.5 ± 1.4	7.50%
The Maximum Potential Yield = 6.65Kgs/6m^2		

4.4.2: Resistance levels of the transgenic lines against CBSD viral disease

The resistance levels against CBSD were determined and based on the analysis the transgenic line 501 had the highest levels at 82.84% with a classification of very high resistance based on the algorithm, followed by transgenic line 401 at 82.39% with a classification of very high resistance (*Table 3.1*), (*Table 4.14*) and (*Figure 4.11*). In terms of low performance non-transformed line 60444 had the lowest at 59.70% with a classification of moderate resistance, followed by transgenic cassava line 407 which had resistance levels of 64.84% with a classification of high resistance based on resistance estimation algorithm (*Table 3.1*), (*Table 4.14 & Figure 4.11*).

Table 4.14: Shows the adjusted resistance levels of the transgenic lines against CBSD (Cassava Brown Streak Disease) based on viral load and severity scores during screening at the field. The mean severity score used to arrive at the resistance levels by the algorithm are captured in table 4.7

LINES RESISTANCE LEVELS BASED ON CBSV Qt-PCR TITRES AND MEAN SEVERITY SCORES IN THE FIELD						
Cassava Lines	**Viral Load**	**Divergence Index**	**Positive Divergence Index**	**CDIP%**	**URL%**	**ARL%**
60444	1.985	-2.9882	2.988204	20.61%	79.39%	59.70%
401	0.911	-2.20937	2.209373	15.23%	84.77%	82.39%
501	0.799	-2.07819	2.078191	14.33%	85.67%	82.84%
506	0.213	-0.75612	0.756122	5.24%	94.76%	77.38%
402	1.491	-2.70203	2.702032	18.61%	81.39%	70.70%
407	8.167	-4.40269	4.402687	30.32%	69.68%	64.84%
499	1.32	-2.58022	2.580217	17.78%	82.22%	81.11%
406	1.348	-2.60121	2.601207	17.92%	82.08%	81.04%
ICA	0	0	0	0.00%	100.00%	100.00%

KEY: **URL**- Unadjusted Resistance Levels of the transgenic lines, **ARL**-Adjusted Resistance Levels of transgenic lines, **CDIP**- Corresponding Divergence Index Percentage of the lines, **ICA**-Ideal Control of the Algorithm (Zero Infection/No virus)

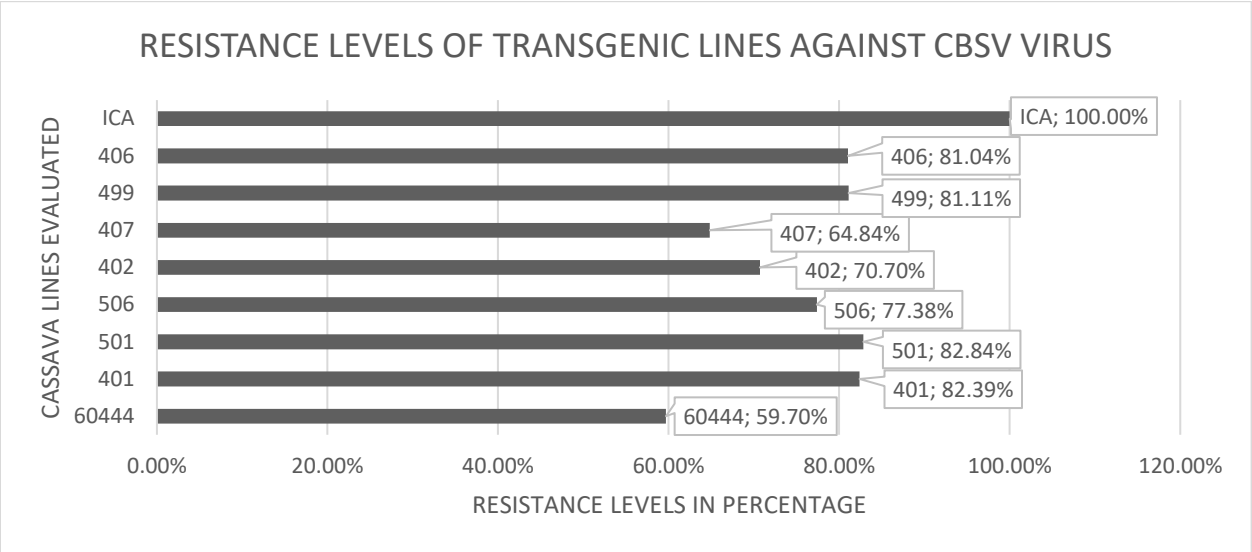


Figure 4.11: Showing the resistance levels of the transgenic lines against CBSD. Non-transformed line 60444 had the lowest levels at 59.70%, whereas transgenic line 501 had the highest resistance levels at 82.84%. The ideal control of the algorithm (ICA) generated the highest levels at 100.00% as predicted.

4.4.2.1: Yield efficacy levels of the CBSD resistant transgenic lines

The yield efficacy levels of the CBSD resistant transgenic lines were determined and based on the analysis; the transgenic line 501 had the highest levels at 57.14%, followed by transgenic line 401 at 39.10% (*Table 4.15*). In terms of low performance non-transformed line 6044 had 9.02%, followed by transgenic line 406 at 10.53% (*Table 4.15*).

Table 4.15: Shows the yield efficacy levels of the CBSD resistant transgenic lines in percentage

YIELD EFFICACY LEVELS OF THE EVALUATED CBSD TRANSGENIC LINES		
Cassava Lines	Mean ± S.D Actual Yield (Kgs)	Yield Efficacy in %
60444	0.6 ± 4.4	9.02%
401	2.6 ± 4.2	39.10%
402	0.8 ± 1.7	12.03%
406	0.7 ± 3.6	10.53%
407	0.8 ± 1.8	12.03%
499	1.6 ± 3.3	24.06%
501	3.8 ± 2.7	57.14%
506	0.8 ± 1.2	12.03%
The Maximum Potential Yield = 6.65Kgs/6m^2		

CHAPTER FIVE

DISCUSSION

This study was conducted in regions with high cassava production and where cassava is a vital crop, aligning with the recommendations by Sseruwagi *et al*. (2004). These regions include low-altitude areas, such as Kwale and Kilifi, and medium-altitude areas, including Machakos, Kisii, Siaya, Busia, Bungoma, and Kakamega. This selection of study sites reflects the integral role of cassava in diverse farming systems across varying environmental conditions. Kwale and Kilifi, located along Kenya's coastal region, experience a tropical climate conducive to cassava growth, making it a staple food crop and an essential part of the local agricultural economy (Obare *et al*., 2024).

The warm, humid conditions in these low-altitude areas favor vector proliferation, highlighting the prevalence of CMD and CBSD (Sseruwagi *et al*., 2004). In the Eastern region, Machakos County represents a medium-altitude area with a semi-arid climate where cassava's drought resistance is crucial for sustaining agricultural productivity and food security (Thiongo & Ngaira, 2019). In the Western region, Kisii County features a highland equatorial climate with abundant rainfall, supporting extensive cassava cultivation for subsistence and as a cash crop.

The moderate altitude and favorable climatic conditions in Kisii make it essential to understand CMD and CBSD distribution and severity (SPCSL, 2022). Siaya, Busia, Bungoma, and Kakamega counties in the Lake Victoria Basin and Western region represent medium-altitude areas with diverse agroecological zones. These regions support extensive cassava cultivation, where common mixed farming practices contribute to food security and income generation(Obiero *et al*., 2007).

The comprehensive study across these regions provided an overview of CMD and CBSD prevalence and distribution, capturing the multifaceted challenges posed by these viral diseases. The findings underscore the necessity for targeted interventions, including the development of disease-resistant cassava varieties and the implementation of effective disease management strategies tailored to the specific conditions of each region (Obiero *et al*., 2007; Were, 2016). This holistic approach ensures that the research findings are relevant and applicable to the diverse agricultural landscapes where cassava is a critical component of food security and livelihoods.

5.1: Diversity of the viruses causing CMD and CBSD in major cassava growing areas of Western Kenya

5.1.1: Prevalence and distribution of CMD and CBSD

The study confirmed earlier findings that CMD and CBSD are prevalent in all cassava-growing regions in Kenya (Were, 2016; Osogo *et al*., 2014). Symptoms were pronounced in all surveyed fields despite ongoing efforts by various stakeholders to develop and distribute resistant varieties (Obiero *et al*., 2007; Nyirakanani *et al*., 2021). This work demonstrated that CMD and CBSD occur in low and high-altitude areas, contradicting Nichols' earlier report that CBSD was confined to low-altitude areas (Nichols, 1950).

Subsequent reports have shown CBSD in higher altitude areas (>1000 masl) in Kenya, Uganda, Tanzania, Rwanda, Burundi, and DRC (Alicai *et al*., 2007; Mware *et al*., 2009; Mulimbi *et al*., 2012; Ndunguru *et al*., 2016; Nyirakanani *et al*., 2021). The occurrence of mixed infections of CMD and CBSD in all surveyed regions aligns with the findings of (Alabi, 2011; Abacaa, *et al*; 2012; Chikoti & Tembo, 2022), who reported that such infections lead to up to 100% yield loss.

This poses a significant threat to cassava production in Kenya, as CMD and CBSD mixed symptoms were observed widely. The limited access to virus testing and the masking effect of CMD symptoms on CBSD complicate the management of these diseases. CBSD symptoms may go unreported until harvest, exacerbating the impact on yield.

In the low-altitude areas such as Kwale and Kilifi, CMD and CBSD were highly prevalent, reflecting the conducive environment for whitefly vectors that transmit these viruses. The tropical climate of these coastal regions promotes the proliferation of whiteflies, thereby increasing the spread of CMD and CBSD (Munguti *et al*., 2021). In the medium-altitude areas like Machakos, Kisii, Siaya, Busia, Bungoma, and Kakamega, the prevalence of these diseases was similarly high. The varied agro-ecological zones in these regions support extensive cassava cultivation, making it essential to address the disease challenges effectively.

The study also highlighted the challenge of managing CMD and CBSD in the context of mixed infections. Mixed infections complicate diagnosis and control measures, as the symptoms of one disease can mask the presence of the other. For instance, CMD symptoms can mask CBSD, leading to the latter going undetected until the root necrosis is evident at harvest. This delayed detection exacerbates the impact on yield, as CBSD can cause significant root rot, leading to substantial post-harvest losses.

Furthermore, the study's findings underscore the need for comprehensive disease management strategies that consider the prevalence and distribution of CMD and CBSD across different altitudes and ecological zones. Recent advancements in disease management, including the development of molecular diagnostic tools and resistant cassava varieties, offer promising avenues

for mitigating the impact of these diseases (Sheat & Winter, 2023; Devi *et al.*, 2024; Ntui *et al*., 2024). Enhancing access to virus testing facilities and implementing integrated disease management approaches, including resistant varieties, timely diagnosis, and effective vector control measures, are critical steps in managing CMD and CBSD.

The study's insights into the epidemiology of CMD and CBSD provide a foundation for developing targeted interventions to mitigate the impact of these devastating diseases on cassava production in Kenya. Continuous monitoring and adaptation of disease management strategies can reduce the incidence and severity of CMD and CBSD, thereby improving cassava yields and food security in the affected regions (Tomlinson *et al*., 2018).

5.1.2: Farmers' practices and disease spread

The study revealed that most farmers still rely on their seeds or seeds obtained from neighbors, with little to no selection for disease-free planting material. For example, in Kwale County, no selection was practiced, indicating a lack of awareness or resources to effectively implement disease management strategies. This practice contributes significantly to the spread of CMD and CBSD, as infected planting materials perpetuate the disease cycle. In such scenarios, the lack of clean seed systems is a critical bottleneck in controlling CMD and CBSD spread (Legg *et al*., 2017; Rey & Vanderschuren, 2017).

In some regions like Busia and Siaya counties, farmers reported obtaining seeds from the Ministry of Agriculture, Kenya Agricultural and Livestock Research Organization (KALRO), and non-governmental organizations (NGOs) through community-based organizations (CBOs). However, the sustainability of providing clean seeds remains a challenge. Despite these efforts, the early

onset of disease symptoms in these areas underscores the limitations of these interventions. Early infection is particularly devastating, leading to higher yield losses compared to late infection (Bisimwa *et al*., 2015). The persistent issue of early disease onset suggests that even formal seed systems need improvement in maintaining seed health and disease resistance (Chikoti & Tembo, 2022).

The use of non-selected seed increases disease pressure and should be discouraged (Osogo *et al*., 2014; Were, 2016;). Practices such as rigorous selection of planting material for disease resistance and the establishment of decentralized, farmer-managed seed multiplication sites can help improve the quality of planting material. Research has shown that community-based seed systems can effectively enhance the availability and use of clean planting materials, provided there is adequate training and support for farmers (Legg *et al*., 2022).

Additionally, whitefly populations, a known vector for these viruses, exacerbate the spread in cassava fields (Munguti *et al*., 2021).Whiteflies are highly efficient at transmitting CMD and CBSD, and their populations can quickly increase under favorable climatic conditions. Integrated pest management (IPM) strategies that include biological control agents, resistant varieties, and cultural practices such as intercropping and timely planting can reduce whitefly populations and virus transmission (Navas-Castillo *et al*., 2011; Omongo *et al.*, 2022).

The study also highlights the importance of education and awareness among farmers regarding disease identification and management practices. Training programs that focus on the identification of CMD and CBSD symptoms, the importance of using clean planting material, and effective vector control measures are crucial. Extension services and farmer field schools can play

a vital role in disseminating this knowledge and supporting the adoption of best practices (Waddington *et al*., 2014).

Furthermore, collaboration between research institutions, governmental agencies, and NGOs is essential to continuously supply clean, disease-resistant planting materials. Strengthening the capacity of local institutions to produce and distribute certified planting material can help sustain the efforts to combat CMD and CBSD (Yadav *et al*., 2020). Policymakers must also support these initiatives through funding and the development of policies that promote the adoption of disease management practices at the grassroots level (Barbosa, 2024). The reliance on non-selected, potentially infected planting materials remains a significant challenge in managing CMD and CBSD. Effective disease management strategies require a combination of clean seed systems, IPM practices, farmer education, and institutional support to reduce the prevalence and impact of these diseases on cassava production (Chipeta *et al*., 2016).

5.1.3: Symptom observation and virus detection

The symptoms observed were typical of CMD and CBSD, consistent with previous reports (Fargette, 1994; Were, 2016). CMD symptoms such as mosaic patterns, leaf curling, and stunted growth were widespread across surveyed regions. CBSD symptoms, characterized by root necrosis, constricted stems, and chlorotic blotches on leaves, were also prevalent. However, unusual CMD symptoms were noted in Funyula, Busia County, with bright yellow mosaic on leaves but no deformation, mottling, or stunting. This is a typical presentation of CMD symptoms might indicate the presence of mixed virus infections or novel virus strains, complicating diagnosis and management(Patil & Fauquet, 2009; Ndunguru *et al*., 2016).

Additionally, some cassava plants appeared healthy above ground but exhibited severe CBSD symptoms upon uprooting, corroborating findings by (Beyene *et al*., 2016; Were, 2016). This phenomenon, where plants display no foliar symptoms but have substantial root necrosis, underscores the insidious nature of CBSD. Asymptomatic above-ground expression can lead to underestimation of disease prevalence and severity, further complicating efforts to control the disease (Alicai *et al*., 2007; Winter *et al*., 2010).

The discrepancy in symptom expression complicates field-based selection of disease-free planting material. Asymptomatic plants can still harbor the viruses, acting as reservoirs and perpetuating disease spread through infected cuttings (Bisimwa *et al*., 2015). Routine virus testing is crucial to identify and eliminate infected plants, ensuring that only disease-free materials are propagated. Techniques such as polymerase chain reaction (PCR) and enzyme-linked immunosorbent assay (ELISA) are essential for accurate virus detection. They should be integrated into seed certification protocols (Munguti *et al*., 2024).

Stakeholder involvement is paramount in maintaining clean seed systems. This includes farmers, extension workers, researchers, and policymakers working collaboratively to monitor, manage, and mitigate disease spread. Training programs on disease identification and management, supported by regular field inspections and virus testing, can significantly reduce the incidence of CMD and CBSD(Yadav *et al*., 2020). Moreover, strengthening local capacity for virus diagnostics and clean seed production can empower communities to manage cassava diseases sustainably (Maruthi *et al*., 2017).

Recent advancements in genomic and biotechnological approaches offer promising avenues for improving disease resistance in cassava. For instance, developing transgenic cassava lines with enhanced resistance to CMD and CBSD has shown potential in reducing disease impact (Masinde *et al*., 2021; Sheat & Winter, 2023). CRISPR/Cas9 technology is also being explored to create virus-resistant cassava varieties, providing a sustainable long-term solution to these viral diseases (Ndudzo *et al.*, 2024). Observing CMD and CBSD symptoms in the field highlights the complexity of managing these diseases. Routine virus testing, coupled with effective stakeholder collaboration and the adoption of advanced biotechnological tools, is essential for maintaining clean seed systems and mitigating the impact of CMD and CBSD on cassava production (Yadav *et al*., 2020).

5.1.4: Phylogenetic analysis

Sequencing of PCR products revealed that the ACMV and EACMV isolates from various regions clustered with known sequences, indicating common ancestry and widespread distribution. This finding aligns with earlier studies suggesting that CMD-causing viruses in Kenya share a significant genetic relationship with isolates from neighboring countries (Badamasi *et al*., 2020; Maruthi, 2020; Mkamilo *et al*., 2024). The phylogenetic analysis further confirmed the presence of diverse virus strains across different regions, demonstrating these pathogens' extensive distribution and genetic variability.

The detection of EACMV in non-cassava hosts such as *Leonotis leonarus* suggests a broader epidemiological role of non-cassava plants in CMD transmission. This finding supports previous research indicating that various plant species can act as reservoirs for cassava mosaic geminiviruses, facilitating their spread across different ecological zones (Badamasi *et al*., 2020).

Studies by Briddon *et al*.(2008) and Alabi *et al*.(2008), have also highlighted the role of alternate hosts in maintaining virus populations during non-cassava growing seasons, which can significantly impact the epidemiology of CMD (Alabi *et al*., 2008; Briddon *et al*., 2008).

The broader implication of these findings is that comprehensive CMD management strategies must consider the role of alternate hosts in disease spread. As CMD viruses can persist in non-cassava hosts, eradication efforts solely focused on cassava plants may be insufficient. Integrated pest and disease management approaches should include monitoring and controlling alternate hosts to reduce the overall virus load in the environment (Deguine *et al*., 2021).

Recent studies have emphasized the need for landscape-level management practices targeting cassava and non-cassava plants to curb CMD transmission effectively. For example, Igwe recommends incorporating the management of alternative hosts into existing cassava disease control programs to disrupt the virus life cycle (Igwe, 2023). Additionally, the use of molecular tools to track virus movement between cassava and non-cassava hosts can provide valuable insights for designing targeted interventions (Alabi *et al*., 2008; Peng *et al*., 2022).

Moreover, enhancing farmer awareness and capacity-building initiatives to manage non-cassava hosts could reduce CMD spread. Training programs should emphasize identifying and eradicating potential virus reservoirs within and around cassava fields. Collaborative efforts involving researchers, extension services, and farmers are essential to implement these strategies effectively (Maruthi, 2020; Yadav *et al*., 2020). The phylogenetic analysis underscores Kenya's genetic diversity and widespread distribution of CMD-causing viruses.

The detection of EACMV in non-cassava hosts highlights these plants' significant role in CMD epidemiology. To achieve sustainable CMD control, integrated management strategies that address cassava and non-cassava hosts are imperative. Leveraging recent advances in molecular diagnostics and landscape-level disease management practices can enhance these strategies' effectiveness and contribute to cassava production systems' long-term resilience (Badamasi *et al*., 2020; Maruthi, 2020).

5.2: Disease incidence and severity levels of the RNAi transgenic cassava lines to CMD and CBSD causal viruses

Transgenic cassava lines engineered for CMD resistance exhibited CMD symptoms in the field, with severity scores ranging from 2 to 5, contrary to the resistance observed in controlled screen house conditions *Table 4.6* (Vanderschuren *et al*., 2012). This indicates a potential loss of resistance under field conditions, possibly due to environmental stress factors or virus strain variability not present in the screen house. The higher CMD severity in these lines correlated with lower tuber yield, aligning with the findings of Bisimwa and the team that severe CMD infections significantly reduce cassava yields(Bisimwa *et al*., 2015).

Moreover, all CMD-resistant transgenic lines showed CBSD symptoms, indicating susceptibility to both viruses. This dual susceptibility complicates breeding efforts to develop cassava varieties resistant to CMD and CBSD. Interestingly, lines 129 and 166 showed no detection of ACMV-UG Severe, a strain known to cause significant yield loss (OWOR *et al*., 2004). These lines also had the highest tuber yields, suggesting potential resistance or tolerance to this virulent strain. Line

166, in particular, had the lowest viral load in qPCR tests, making it a promising candidate for further development and breeding programs.

For CBSD-resistant lines, several exhibited no foliar CBSD symptoms but showed root severity scores, rendering them unsuitable for selection based solely on foliar symptoms. This finding is critical as it highlights the importance of evaluating root health and foliar symptoms for accurate disease resistance screening(Ndunguru *et al*., 2015). Lines 56, 405, and 506 demonstrated dual resistance potential, with no CMD symptoms and low CBSD viral loads. These lines represent promising candidates for further research and development into robust, dual-resistant cassava varieties.

Recent advancements in molecular breeding and genetic engineering offer new avenues for enhancing cassava resistance to CMD and CBSD. CRISPR/Cas9 technology, for instance, has shown potential in creating virus-resistant cassava varieties by targeting and editing specific viral genomes (Gomez *et al*., 2019; Tripathi *et al*., 2022; Otun *et al*., 2023). Additionally, integrating genomic selection with traditional breeding methods can accelerate the development of cassava varieties with durable resistance to multiple viral pathogens (Masinde *et al*., 2021; Ntui *et al*., 2024). The molecular detection of CMD and CBSD and the performance of transgenic cassava lines highlight the ongoing challenges and opportunities in cassava disease management. The use of sensitive diagnostic tools like PCR and advanced genetic engineering techniques holds promise for developing cassava varieties that can withstand the dual threats of CMD and CBSD. Collaborative efforts among researchers, breeders, and farmers are essential to translate these

scientific advancements into practical solutions that ensure sustainable cassava production (Maruthi, 2020; Yadav *et al*., 2020).

5.3: Diversity of viruses in the transgenic line

An array of diverse viruses were tested positive in the transgenic line (*Table 4.9 & 4.10*). EACMV was more prevalent in the transgenic lines than ACMV. Further, in all lines. EACMV-UG was the most dominant strain detected. Whereas some strains were not detected in the samples tested (*Table 4.9*). The findings confirm previous studies that highlighted the prevalence of these viruses in East Africa(Were *et al*., 2004; Aloyce *et al*., 2013; Were, 2016). The discrepancy between ELISA and PCR results, where symptomatic samples tested negative on ELISA but positive on PCR, underscores the limitations of ELISA in detecting low virus titers. This aligns with observations by Aloyce and colleagues, indicating that PCR is a more sensitive diagnostic tool for detecting CMD pathogens (Aloyce *et al*., 2013).

Surprisingly, some symptomatic plants tested negative by both ELISA and PCR, suggesting the potential presence of new or divergent virus strains that escape detection by conventional methods. This possibility has been noted by Bull and colleagues, who reported on the high variability and recombination events within cassava mosaic geminiviruses(S. E. Bull et al., 2003). The high recombination rates in both CBSD (Ndunguru *et al*., 2015) and CMD (Tiendrébéogo *et al*., 2012) further support the hypothesis that new virus strains may be emerging, complicating disease management strategies.

5.4: Resistance levels of the RNAi transgenic cassava lines against CMD and CBSD causal viruses

The respective RNAi transgenic cassava lines exhibited variation of their resistance against CMD and CBSD viral caused diseases (*Table 4.11 & 4.12*). This demonstrates the continuum nature of resistance where it varies based on the interaction of the respective genotypes with the environment leading to the observed differences especially with horizontal resistance in comparison to vertical resistance (Omayio and Ndombi, 2022). Ability of the algorithm to predict the resistance levels demonstrated consistency in determination of the variable based on viral load and mean severity scores at the field. Thus, the generation of an output quantitatively that can be relied on in estimating the levels of an occurrence characterizes the qualities of an objective and unbiased scientific approach (Li, 2019; Hunt *et al*., 2002). In the study the ideal control of the algorithm had very high levels of resistance (100.00%), whereas non-transformed line 60444 had the lowest consistent with the primary data recorded at the field *Table 4.11* and *4.12*. This prediction was possible, because the algorithm took into consideration the main variables (viral load and severity) which are significantly affected when plants mount resistance against pathogens (Parry, 1990; Omayio, 2020)

CHAPTER SIX

CONCLUSIONS, RECOMMENDATIONS AND SUGGESTIONS FOR FURTHER RESEARCH

6.1: Conclusions

1) Cassava Mosaic Disease (CMD) and Cassava Brown Streak Disease (CBSD) are the predominant diseases affecting cassava in Kenya, exhibiting widespread distribution across low and high-altitude regions. The symptoms of these diseases were highly pronounced in all surveyed cassava fields, occurring in both single and mixed infections. Mixed infections were particularly detrimental, leading to total tuber loss. The study also detected the presence of CMD and CBSD viruses in both symptomatic and asymptomatic samples, indicating that the viruses can exist in a latent form. The research found that East African cassava mosaic virus (EACMV) is more prevalent than African cassava mosaic virus (ACMV) and is distributed across all cassava-growing areas in Kenya. Mixed ACMV/EACMV infections resulted in more severe symptoms than single infections. EACMV-infected plants consistently showed typical CMD symptoms, whereas ACMV-infected plants did not always exhibit these symptoms. There were also instances where symptomatic plants tested negative for ACMV and EACMV, highlighting potential limitations in the current diagnostic methods. The geographical distribution of ACMV showed its presence in the western and coastal regions but not in the eastern region of Kenya. The ACMV isolates obtained in this study were similar to those previously reported in Kenya and Uganda. EACMV isolates from the coast clustered together with sequences from earlier reports, suggesting the long-term presence of similar viruses in the region. The detection of EACMV-UG in both weed species and cassava implies natural interspecific transmission in the field.

2) All transgenic lines evaluated in the study displayed symptoms of CMD and CBSD, with the causal viruses detected in all lines. The incidence and severity varied across the different transgenic lines evaluated.

3) Diverse viruses were detected in the transgenic lines with varied viral load levels. Line 166 exhibited the lowest viral load and low symptom expression despite mixed ACMV/EACMV infection, suggesting that this line can suppress the virus and may be promising for developing CMD-tolerant varieties. ACMV-UG Severe and ACMV-Ug 34 strains of ACMV were identified in the transgenic lines, while ACMV-KE and ACMV-TZ were not detected. EACMV-Ug was the only strain of EACMV found.
4) Resistance and yield efficacy levels of the RNAi transgenic lines varied in a continuum manner. For CMD lines 166 and 348 demonstrated significantly very high resistance levels (*Table 4.11*), whereas for CBSD lines 501 and 401 had very high resistance levels (*Table 4.12*). Indicating their potential for further investigation as CMD and CBSD-resistant lines respectively.

6.2: Recommendations

1) CMD and CBSD are widespread across both low and high altitude regions in Kenya, with mixed infections causing significant yield losses. Therefore, need for integrated strategies to manage the significant problem. Ranging from host plant resistance to phytosanitray approaches.
2) The presence of CMD and CBSD in the transgenic lines indicates how aggressively these diseases are spread. Hence, the need to enhance surveillance levels and implement urgently breeding programs that can take advantage of the established resistance in the transgenic lines to develop highly resistance hybrids of cassava.
3) The diverse viruses in the transgenic lines supports the aggression of the viruses in spread. Hence, need for development of a stringent management protocol that integrates management approaches ranging from; host plant resistance, phytosanitary legislation on

transfer of cassava planting materials, certified seeds programs initiation and vector control involved in the spread of the viruses.

4) Finally, the varying resistance levels in the RNAi transgenic lines be taken advantage of by breeders to develop very high resistance hybrids, which can effectively help in management of CMD and CBSD. Further, extensive field trials of promising transgenic lines such as 166, 348, 501, and 401, should be conducted across different ecological zones to validate their resistance and yield performance under natural conditions.

6.3: Suggestions for Further Research

1) Future, research should focus on developing more comprehensive and sensitive primers to detect all strains of CMD and CBSD viruses, including those present in asymptomatic plants.
2) The presence of CMD and CBSD viruses in asymptomatic samples and the inability of current primers to detect all symptomatic cases highlight the need for more robust diagnostic tools.
3) Develop and implement integrated disease management strategies that incorporate the control of alternate hosts and resistant varieties. Emphasis should be placed on farmer education and the establishment of sustainable clean seed systems.
4) Investigate the mechanisms of virus latency and the role of asymptomatic plants in the epidemiology of CMD and CBSD. Additionally, explore the interspecific transmission dynamics involving non-cassava hosts.

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APPENDICES

Appendix i: Survey questionnaire

SURVEY OF VIRUSES CAUSING CASSAVA MOSAIC DISEASE IN WESTERN KENYA

General Farm characteristics

Enumerator's name ……

Date of interview:……

Questionnaire No…….

Location

1. District……Division…..……Location…….

Sub- location……Village……..

GPS reading: Altitude…….Longitude……Latitude (N/S)……..

Household Characteristics

2. Farmer's name……

3. Gender: Male [ ] Female [ ]……

4. Household head……..

5. Age of farmer (15-25) [ ] 26-35 [ ] 36-45 [ ] 46-55 [ ]> 56yrs……

6. Education level: Primary [ ] Secondary [ ] Informal [ ] others [ ]

7. Land ownership: Hired [ ], Owned [ ], Communal [ ], Family [ ]

8. Farm size (ha/acres)……How much is under cassava?…… What was the yield of cassava last season (dry)?……bags/ha. Of this, how much was sold?….. … Price per bag (KShs.)…….…

9. How often do you feed family/children with cassava meal?... . . . . days/Week

10. Method of harvesting (Once or piecemeal?) and why do you prefer it?…….

11. Culture (language if applicable)…….……..

Cassava characteristics

12a. Varieties grown……..…….

12b. Age of the plant (crop) . . . . ……..... . . . . . …

13. Which Field generation are they?. . … .. . . ……... .. . .. ….

14. Up to how many generations do you grow? ……..

15. Is it seed or ware crop? . . .. …….

16. Source of cassava cuttings for planting……if own seed, do you select clean cuttings?……

17. What is your view with regard to seed availability……

If available, comment on the price .. ……..…

Practices and constraints

18. Do you know of any cassava diseases and or pests? yes [ ] No [ ]

19. If yes, which one (s)?

20. When did you start noticing them?

21. Other constraints to cassava production?

22. Are you a member of any farmer organization? Yes [ ], No [ ] If yes, specify. …….. . .

.

23. Did you use any information from MMUST or KARI on cassava production? Yes [ ]

No [ ]

27. Any diseases/Pests that you have heard about but not in your farm.

Diseases/Pest type ;	1.Aware (which one)
Virus:	2. Not aware
Bacterial blight of cassava:	
Anthracnose:	
Whiteflies:	
Rodents:	
Other (Specify)	

26. What is your view with regard to clean cassava seed production?

Plan for sample collection

i. In the field divide it into 4 quadrants; in the quadrant:

- Calculate the CMD incidence as number of plants infected/total number of plants

ii. Collect leaves from cassava plants or suspicious plants that could habour the virus eg. Volunteer cassava plant etc.

iii. Bring leaf material to the lab for ELISA test (for virus)

iv. Store the soil at 4^{o}C and uprooted plants at -20^{o}

Appendix ii: Elisa buffers

Coating buffer (pH 9.6)
1.59 g sodium carbonate (Na_2CO_3)
2.93 g sodium bicarbonate ($NaHCO_3$)
0.20 g sodium azide (NaN_3)
Dissolve in 900 ml H_2O, adjust pH to 9.6 with HCL and make up to 1l.

PBS (pH 7.4) phosphate buffer saline
8.0 g sodium chloride (NaCl)
0.2 g monobasic potassium phosphate (KH_2PO_4)
1.15 g dibasic sodium phosphate (Na_2HPO_4)
0.2 g potassium chloride (KCl)
0.2 g sodium azide (NaN_3)
Dissolve in 900 ml H_2O, adjust pH to 7.4 with NaOH or HCI and make up to 1 l.

PBS-Tween (PBST)
PBS + 0.5 ml Tween 20 per liter

Sample extraction buffer (pH 7.4)
PBST + 2% PVP (Serva PVP-1S polyvinyl pyrrolidone)

Conjugate buffer
PBST + 2% PVP + 0.2% egg albumin (Sigma A-S253)
Substrate buffer
97 ml diethanolamine
600 ml H_2O
0.2 g sodium azide (NaN_3)
Adjust to pH 9.8 with HCI and make up to I liter with H_2O
Buffers can be stored at 4°C for at least 2 months. Warm to room temperature before use

Appendix iii: List of primers for RT-PCR

Virus species/strain	Primer name	Sequence	Product size
ACMV	VD590-ACMV-A	CCTTGGTATCTGTAAGGT	820
	C1410-ACMV-A	CAACAACGACCATTCCTGCT	
EACMV	A24669	TGGAGATGAGGCACCCCATC	722
		TCCTCCGCACCTTGGATACG	
EACMCV	F1197-EACMCV-A	ACATGATGCACTGCATCT	522
	R1719-EACMCV-A	GAGGACAAGAATTCCAATC	
EACMKV	F316-EACMV	GGTTCTACGGTGTAAGAG	1956
	R2272-EACMKV	CAACAATCCGCCAATGAC	
EACMV-UG	V590	CCTTGGTATCTGTAAGGTGA	1681
	R2271-EACMV	CCAGCATTTAGCTCAGGT	

ACMV- UG Sev	ACMV UG Sev	ACCGGTTGGCCCCGCCCCCCTTTAAA
		AATATTAAACGGTTGGCCCCT
ACMV-UG 34	ACMV UG 34	ATAACCGGTTGGCCCCGCCCCCCTTTA
		AATATTATACGGTTGGCCCCTTGGGT
CBSV/UCBSV	CBSVF2	GCTMGAAATGCYGGRTAYACAA
	CBSVDR	GGATATGGAGAAAGRKCTCC
ACMV-[TZ]	AT-F	GTGACGAAGATTGCATTCT
ACMV-[TZ]	AT-R	AATAGTATTGTCATAGAAG
EACMV-KE-[TZT]	UGT-F	TCGTCTAGAACAATACTGATCGGTCTCC
EACMV-KE-[TZT]	UGT-R	CGGTCTAGAAGGTGATAGCCGAACCGGGA

Primer name	**Nucleotide sequence (5'→3')**	**Begomovirus isolate**	**DNA component**
			DNA-A fl[a]
			DNA-A fl

Primer name	Nucleotide sequence (5'→3')	Begomovirus isolate	DNA component
3T-F	ACGTCTAGAACAATACTGATCGGTCTC	EACMV-TZ-[YV]	DNA-A fl
3T-R	GTGCTCTAGAAGGTGATAGCCGAACCGGGA	EACMV-TZ-[YV]	DNA-A fl
TZ1B-F	GCGCGGAATCACTTGTGAAGCAGTCGT	EACMCV-[TZ1]	DNA-B fl
TZ1B-R	GCCGGGATTCGGTGAGTGGTTTACATCAC	EACMCV-[TZ1]	DNA-B fl
EAB555/F	TACATCGGCCTTTGAGTCGCATGG	CMGs	BC1/CR
EAB555/R	CTTATTAACGCCTATATAAACACC	CMGs	BC1/CR
UNI/F	KSGGGTCGACGTCATCAATGACGTTRTAC	CMGs	DNA-A nfl
UNI/R	AARGAATTCATKGGGGCCCARARRGACTGGC	CMGs	DNA-A nfl
AT-F	GTGACGAAGATTGCATTCT	ACMV-[TZ]	DNA-A ps
AT-R	AATAGTATTGTCATAGAAG	ACMV-[TZ]	DNA-A ps
ATZ1-F	TAAGAAGATGGTGGGAATCC	EACMCV-[TZ1]	DNA-A ps

Primer name	Nucleotide sequence (5'→3')	Begomovirus isolate	DNA component
ATZ-R	CGATCAGTATTGTTCTGGAAC	EACMCV-[TZ1]	DNA-A ps
TZ7-F	TGGTGGGAATCCCACCTT	EACMCV-[TZ7]	DNA-A ps
TZ7-R	GTATTGTTATGGAAGGTGATA	EACMCV-[TZ7]	DNA-A ps
TZM-F	TATATGATGATGTTGGTC	EACMV-UG2Svr-[TZ10]	DNA-A ps
TZ10-R	TAGAAGGTGATAGCCGTA	EACMV-UG2Svr-[TZ10]	DNA-A ps
TZM-F	TATATGATGATGTTGGTC	EACMV-KE-[TZM]	DNA-A ps
TZM-R	TAGAAGGTGATAGCCGAAC	EACMV-KE-TZM]	DNA-A ps

Appendix iv: DNA extraction results

REP	PLOT NO	PLANT NO	LINE NO	No	DNA ng/ul
2	4	6	129	246B	1241
2	5	1	30	251A	582
2	5	4	30	254A	1488
2	5	6	30	256A	3045
2	8	5	133	285E	621
2	8	1	133	281E	657
2	8	4	133	284E	2668
2	8	2	133	282E	677
2	2	1	167	221F	792
2	2	6	167	226F	2573
2	2	4	167	224F	967
2	2	2	167	222F	4179
2	4	4	167	244F	2849
2	4	2	348	242G	1842
2	4	5	348	245G	2180
2	4	3	348	243G	2367
2	1	3	348	213G	5665
2	1	1	348	211G	4733
2	1	4	348	214G	143
2	1	6	166	216H	1148

2	1	5	166	215H	1227
2	1	2	166	212H	3665
2	7	3	137	273K	3338
2	7	4	137	274K	2204
2	5	5	167	255F	2420
1	3	1	129	231B	4100
2	3	4	129	234B	1621
2	3	3	129	233B	2437
2	3	2	129	232B	3464
2	6	1	141	261C	1267
2	6	2	141	262C	5295
2	6	4	141	264C	2512
2	6	3	141	263C	61
2	9	6	145	296D	1336
2	9	3	145	293D	2623
2	9	1	145	291D	9514
2	9	4	145	294D	6286
2	9	5	145	295D	3916
2	5	3	30	253A	935
1	9	3	129	193B	6368
1	8	6	30	186A	1861
3	5	1	141	351C	708
3	4	1	167	341F	1803

3	4	3	167	343F	5460
3	4	2	167	342F	159
3	4	4	167	344F	837
3	4	6	167	346F	4943
3	3	5	166	335H	3272
3	2	3	166	323H	4237
3	2	2	166	322H	738
3	2	4	166	324H	1573
3	3	1	166	331H	78
3	3	6	166	336H	4968
3	2	1	145	321D	4994
3	2	1	145	321D2	3670
3	2	3	145	323D	8747
3	2	4	145	324D	6120
3	1	1	30	311A	1715
3	8	4	30	384A	4733
3	8	5	348	385G	2697
3	8	2	348	382G	4685
3	7	4	137	374K	590
3	7	3	137	373K	3532
3	1	3	30	313A	2620
3	1	1	30	311A2	3899
3	1	6	30	316A	1385

3	1	5	30	315A	5218
3	1	4	30	314A	3973
3	8	1	129	381B	4107
3	9	5	129	395B	5207
3	9	1	129	391B	4896
3	8	6	129	386B	1233
3	9	4	129	394B	364
3	6	1	133	361E	603
3	6	5	133	365E	640
3	6	6	133	366E	301
3	6	3	133	363E	811
3	6	2	133	362E	1931
3	5	3	141	353C	1372
3	5	6	141	356C	1346
3	4	2	167	342F	1024
1	4	6	141	146C	453
2	6	3	141	263C	230

Appendix v: RNA Quality check of the samples

SAMPLE IN	RNA conc in ng/µl	A260	A280	260/280
KK1607	278.9	2.286	1.128	2.03
PS1	75.5	1.886	0.937	2.01
KK1620	635.9	15.898	7.667	2.07
P5	311.4	15.898	7.667	2.07
BS1590	270.9	6.772	3.413	1.98
BUS 1010	58.7	1.092	0.587	1.86
BGMT90	144.2	3.605	1.767	2.04
MUN 2	129	3.225	1.627	1.98
KK1639	379.7	9.491	4.651	2.04
SG1	241.1	1.053	0.583	1.81
KISII	466.4	2.286	1.128	2.03
SB1	190.1	1.886	0.937	2.01

Appendix vi: Severity scoring keys used in evaluation of the transgenic lines

The cassava mosaic disease symptom scale of 1-5 (Hahn *et al*., 1980)

Scale	Symptom Description
1	Unaffected shoots, no symptoms
2	Mild chlorosis, mild distortions at bases of most leaves, while the remaining parts of the leaves and leaflets appear green and healthy
3	Pronounced mosaic pattern on most leaves, narrowing and distortion of the lower one-third of the leaflets
4	Severe mosaic distortion of two thirds of most leaves and general reduction of leaf size and stunting of shoots
5	Very severe mosaic symptoms on all leaves, distortion, twisting, mis-shapen and severe leaf reductions of most leaves accompanied by severe stunting of plants

CMD severity scoring for transgenic plants

Score description scale 0-4 for aerial symptoms (Hahn et al., 1980)

Scale	Description
0	Apparent field resistance, no symptoms observed
1	A mild chlorotic pattern over entire leaflets, or mild distortion only at the base of the leaflets with the remainder of the leaflets appearing green and healthy

2	Strong mosaic pattern throughout the leaf, narrowing and distortion of the lower one third of leaflet
3	Severe mosaic, distortion of two thirds of the leaflets and general reduction of leaf size
4	Severe mosaic, distortion of all leaflets, twisted and misshapen leaves

CBSD severity symptoms in leaves and stems scale of 1 to 5 (Gondwe *et al*., 2003)

1 = no apparent symptoms

2 = slight leaf feathery chlorosis with no stem lesions

3 = pronounced leaf feathery chlorosis, mild stem lesions and no dieback

4 = severe leaf feathery chlorosis, severe stem lesions and no dieback

5 = defoliation, severe stem lesions and dieback.

CBSD root symptom severity scores scale of 1 to 5 (Gondwe *et al*., 2003)

1 = no apparent necrosis

2 = <5% root necrosis

3 = 5–10% root necrosis

4 = 11–25% root necrosis with mild root constriction

5 = >25% root necrosis with severe root constriction.

Appendix vii: Results of the viruses detection of the samples from the surveyed areas of Kenya

	County	Location	Symptom	PCR				PCR	
				ACMV	EACMV	CBSV	UCBSV	CBSV-cp	UCBSV-cp
1.	Busia	Matayos (Busia)	CMD/CBSD	-	+	+	+	+	+
2.	Busia	Buriya(Busia)	CMD/CBSD	+		+	+	+	+
3.	Busia	Sidende(Busia)	CMD/CBSD	-	+	-	+	-	+
4.	Busia	Funyula (Busia)	CBSD	-	-	+	+	+	+
5.	Kilifi	Kilifi	Gnut	-	-	-	-	-	-
6.	Kilifi	Kilifi-1	CBSD	-	+	+	-	+	-
7.	Kilifi	Kilifi-2	CMD	-	+	-	-	-	-
8.	Kilifi	Kilifi-3	CMD	-	+	-	-	-	-
9.	Kwale	Kwale-1	CMD	-	+	-	-	-	-
10.	Kwale	Kwale-5	CMD/CBSD	-	+			-	-
11.	Busia	Buhuyi (Busia)	CMD	-	+	+	+	+	+
12.	Kwale	Kwale-1	CBSD	-	-	+	+	+	+
13.	Kwale	Kwale-2	CMD		+	-	-	-	-
14.	Kwale	Kwale-2	CBSD	-	+	+	-	+	-
15.	Busia	Ardap(Busia)	CMD/CBSD	+	-	+	+	+	+
16.	Kwale	Kwale-3	CBSD	-	-	+	-	+	-
17.	Kwale	Kwale-4	CLEAN	-	-	+	-	+	-
18.	Kwale	Kwale-7	CMD	-	+	-	-	-	-
19.	Kwale	Kwale-8	CMD	-	+	+	-	-	-
20.	Kwale	Kwale-8	CBSD	-	-	+	-	+	-
21.	Busia	Bugeng'i (Busia)	CMD	+	-	-	+	-	+
22.	Bungoma	Bungoma	CMD	-	+	-	-	-	-

23.	Bungoma	Kapsokwonyi (Bungoma near mt.Elgon	CBSD	-	_	-	+	-	+
24.	Bungoma	Bungoma-Kimilili		-	+	-	-	-	-
25.	Bungoma	Chwele-1(Bungoma)	CMD	-	+				
26.	Bungoma	Kibinge Near Chwele–iii (Bungoma)	CMD	-	+	-	-	-	-
27.	Bungoma	Kibinge-ii (Bungoma)	CMD	-	+	-	-	-	-
28.	Bungoma	Kibinge-ii (Bungoma)	CMD	-	+	-	-	-	-
29.	Bungoma	Chwele-iii (Bungoma)	CMD	-	+	+	-	+	-
30.	Kakamega	Kakamega, origin Busia	CBSD	-	-	-	-	-	-
31.	Bungoma	Kimilili-2 (Bungoma)	CMD	-	+	-	-	-	-
32.	Kakamega	Makunga (Kakamega)	CMD	+	-	-	-	-	-
33.	Busia	Butula-2 (Busia)	CMD	+	-	+	-	+	-
34.	Busia	Butula (Busia)	CMD/CBSD	+	-	+	-	+	-
35.	Busia	Mundika (Busia)	CMD	+	-	-	-	-	-
36.	Busia	Bumala (Busia)	CMD/Strange	+	-	-	-	-	-
37.	Busia	Butunyi (Busia)	CMD	-	+	-	-	-	-
38.	Busia	Nangina (Busia)	CMD	+	-	+	+	+	+
39.	Busia	Busire (Busia)	CMD	+	-	-	-	-	-
40.	Busia	Otimong (Busia)	CBSD	-	-	+	-	+	-
41.	Busia	Olepito (Busia)	CBSD	-	+	+	-	+	-
42.	Busia	Kemodo (Busia)	CBSD	-	-	+	+		
43.	Busia	Munami (Busia)	CMD/CBSD	-	+	+		-	-

44.	Busia	Bumala/Funyula (Busia)	CMD/CBSD	+	-	+	+	-	-
45.	Busia	Otimong (Busia)	CMD	-	+	-	-	-	-
46.	Busia	Kemodo (Busia)	CMD/CBSD	-	+	-	+	-	+
47.	Kakamega	Makunga (kakamega)	CMD/CBSD	+	+	+	-	+	-
48.	Kakamega	Ejinja/Butula (Kakamega)	CMD/CBSD	-	+	+	-	+	-
49.	Busia	Esidende-2 (Busia)	CMD/CBSD	+	+	+	-	+	-
50.	Busia	Butula (Busia)	CMD/CBSD	-	+	-	+	-	+
51.	Busia	Otimong (Busia)	CMD	-	+	-	+	-	+
52.	Busia	Bumala (Busia)	CMD/CBSD	-	+	+	+	+	+
53.	Machakos	Machakos	CMD/CBSD	-	+	-	-	-	-
54.	Machakos	Machakos	CMD/CBSD	-	+	+	-	+	-
55.	Machakos	Machakos	CMD/CBSD	-	+	-	-	-	-
56.	Kisii	Kisii-1	CMD	-	+	-	-	-	-
57.	Kisii	Kisii-Suneka	CMD	-	+	-	-	-	-
58.	Kisii	Kisii-chungo	CMD	-	+	-	-	-	-
59.	Kisii	Kisii-4	CMD	-	+	-	-	-	-
60.	Kisii	Kisii-2	CMD	-	+	-	-	-	-
61.	Kisii	Kisii-3	CMD	-	+	-	-	-	-
62.	Kisii	Kisii-3	CMD	-	+	-	-	-	-
63.	Kisii	Kisii-3	CMD	-	+	-	-	-	-
64.	Kakamega	KALRO 319-Kakamega		+	-	+	+	+	+
65.	Kakamega	USA-EJUMULA KALRO KAKAMEGA		-	-	+	+	+	+

66.	Kakamega	Maondo-KAKAMEGA(from BUSIA)		-	-	+	-	-	-
67.	Busia	Bumala Matayos (Busia)	CMD/CBSD	-	+	+	-	+	-
68.	Kisumu	Kisumu Muhoroni	CMD	+	+	+	-	+	-
69.		TOTAL		15	44	31	18	26	16
70.		%POSITIVE		22	68	46	26	38	24

The survey to determine the occurrence, incidence, severity of CMD and CBSD causal viruses was carried out in 2018.

Appendix viii: Phytosanitary certificate from KEPHIS on handling of transgenic lines

No. 0057262

PHYTOSANITARY CERTIFICATE No. KEPHIS/6712/2015

MINISTRY OF AGRICULTURE
KENYA PLANT HEALTH INSPECTORATE SERVICE

PHYTOSANITARY CERTIFICATE

FOR SOIL, ORGANIC MANURES, ROOTING AND/OR POTTING MIXTURES AND REGULATED ARTICLES
(Agricultural Produce (Export) Act Cap. 319)

1. Name and Address of Exporter **PROF. HASSAN K. WERE PHD** **PROFESSOR OF MOLECULAR PLANT VIROLOGY** **DEPARTMENT OF BIOLOGICAL SCIENCES** **MASINDE MULIRO UNIVERSITY OF SCIENCE AND TECHNOLOGY** **P.O. BOX 190 -50100, KAKAMEGA, KENYA**	2. Dealer's name and address of Consignee **PROF. WILHELM GRUISSEM** **PLANT BIOTECHNOLOGY ETH ZURICH** **UNIVERSITAETSTRASSE 2 8092 ZURICH** **SWITZERLAND**
3. Place of origin: **Kenya**	4. To Plant Protection Organization of **SWITZERLAND** 5. Declared means of Conveyance AIR
6. Declared point of Entry **ZURICH**	7. Distinguishing marks **Research samples**
8. Number and description of packages ***10 KGS OF LEAFY AND STEM SAMPLES OF MANIHOT ESCULENTA (CRANTZ) FOR RESEARCH PURPOSES***	9. Name of regulated Article(s) ***Manihot esculenta for Research Purpose Only***
10. Scientific identity of Regulated Article(s) ***Manihot esculenta***	11. Quantity declared **10 Kgs**
12. This is to certify that the Article(s) described above has/have been inspected according to appropriate procedures and are considered to be free from quarantine pests and practically free from other injurious pests in conformity with the current phytosanitary regulations of the importing country.	
13. Additional declaration: **For Scientific Research Only.**	
14. Disinfestation and/or disinfection treatment: None 15. Chemical (Active ingredient): None 16. Duration and temperature: None 17. Concentration None 18. Date 19. Any additional information **Research samples**	Place of issue **KEPHIS - HEADQUARTERS** Date: **09/09/ 2015** Official stamp Name of Inspector: **Mathew Obonyo** 09/SEP 2015 Signature ..... of Authorizing officer PLANT INSPECTOR

MINISTRY OF AGRICULTURE KENYA PLANT HEALTH INSPECTORATE SERVICE KEPHIS

Note: The certificate shall be completed in typescript or in block capitals

No financial liability with respect to this certificate shall attach to Kenya Plant Health Inspectorate Service or to any of its officers or representatives.

Appendix ix: Permit to import the materials for experimentation from KEPHIS

No. 1428668

PERMIT No. KEPHIS/14623/2013

REPUBLIC OF KENYA

MINISTRY OF AGRICULTURE & RURAL DEVELOPMENT

KENYA PLANT HEALTH INSPECTORATE SERVICE (KEPHIS)

PLANT IMPORTATION PERMIT

(Plant Protection Act Cap 324) Date 27 November, 2013

The importer must furnish the supplier with a copy of this import permit before plant material is despatched.

*Permission is hereby granted to DR. HASSAN K. WERE C/O MASINDE MULIRO UNIVERSITY

of P. O. 190-5000 KAKAMEGA, KENYA

to import from PROF. WILHELM GRUISSEM ETH ZURICH DEPARTMENT OF BIOLOGY PLANT BIOTECHNOLOGY UNIVERSITAETSTRASSE 2 8092 ZURICH SWITZERLAND.

the following Plantlets

60 Plantlets Manihot Esculenta

subject to the following conditions

1) All Plantlets to be the produce of and grown in Switzerland

2) The consignment to be inspected on arrival and the importing authority reserves the right to treat, destroy or refuse the importation.

3) Plants or plantparts must be entirely free from soil, chaff and/or leaf mould.

4) Each consignment shall be accompanied by an original copy of this import permit and Phytosanitary Certificate (International Model or its equivalent) from country of origin;

Additional Declarations:

i) Fix Q label on the package.
ii) The plantlets should be free from any pests & diseases associated with Manihot spp
iii) Nature of research to be declared on request by KEPHIS Plant Inspector.
iv) To be used only at the approved quarantine facility.
NB: Details to be stated on Phytosanitary Certificate.

Failure to furnish the required certificates may result in prohibition of entry of the plant materials.

5) Packaging The following materials must **not** be used: banana leaves, maize, rice, sorghum, palm, wheat straw soil or leaf mould. If any other plant residue is used as packaging material, the consignment must be accompanied by a certificate stating: all seeds, pathogens and insects have been killed before use of the material either by heating to 180°F / 83°C for ten minutes or by chemical treatment (N.B:- Details to be stated on Phytosanitary Certificate).

This permit is valid for six months from date of issue, but may be cancelled at any time by the Director of Agriculture or by the officer issuing the permit on his behalf

Official Stamp

Mathew Obonyo

(Signed) .....

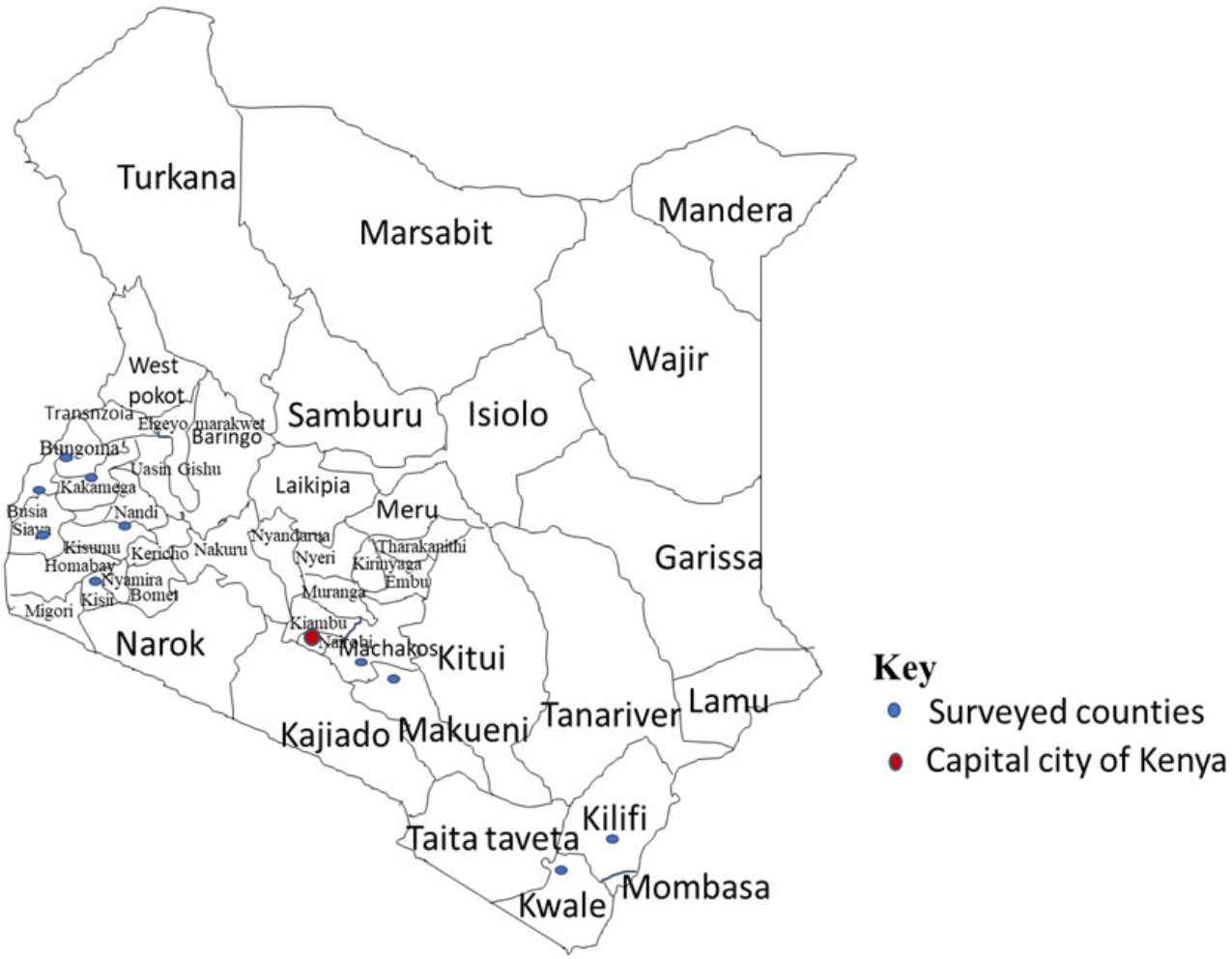
for Director of Agriculture

MINISTRY OF AGRICULTURE KENYA PLANT HEALTH INSPECTORATE SERVICE 27 NOV 2013 PLANT INSPECTOR KEPHIS

"Import of genetically modified material will require clearance from the National Biosafety Authority in compliance with the Biosafety Act"

The permission hereby granted is additional to any permission or licence required under any other law.
Full name and address of supplier to be stated

Appendix x: Mapped areas of Kenya showing surveyed areas denoted by blue dots from where samples with CMD and CBSD symptoms were collected



Appendix xi: Publication from this work Were *et al*. (2021)

E. Afri. Agri. For. J (2021,Volume 85, 1-4, Pg. 423-430)

DIVERSITY OF CASSAVA MOSAIC DISEASE CAUSAL VIRUSES IN KENYA

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Masinde Muliro University of Science and Technology, P. O. BOX 190-50100, Kakamega, Kenya.

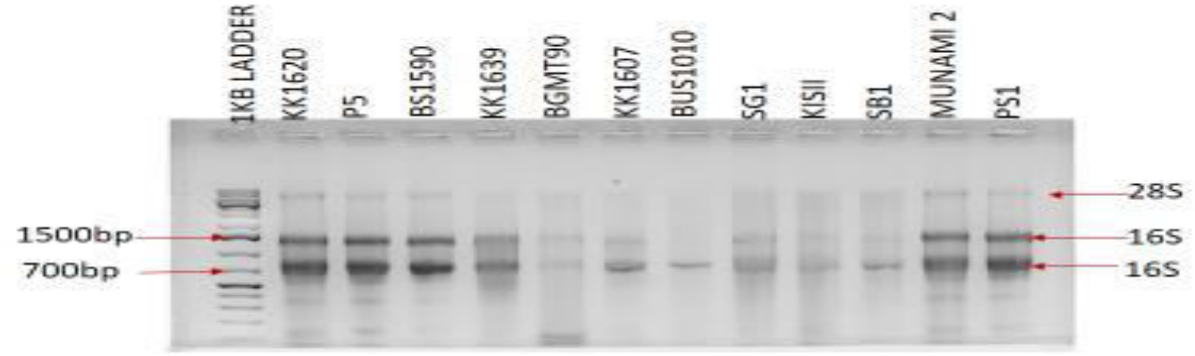
ABSTRACT

Cassava Mosaic Disease (CMD) caused by Cassava Mosaic Begomoviruses (CMBs) causes poor Cassava (*Mannihot esculenta* Crantz.) production in Kenya. Lack of genomic characterization of CMBs hampers control and management of the disease. This study was conducted to determine the diversity and distribution of begomoviruses causing CMD in Kenya. A survey of CMD was conducted in the counties of Bungoma, Busia, Siaya, Kakamega, Kilifi, Kwale, Machakos and Kisii. The DNA was extracted from 60 leaf samples using Cetyltrimethlammonium bromide protocol. Specific primers targeting all EACMV and ACMV viruses were then used to amplify DNA fragments of CMD pathogens. PCR products were prepared and sequenced using Sanger sequencing platform. The sequences were assembled BLAST searched against NCBI database using BLASTn. Phylogenetic trees were constructed based on automatic Neighbor Joining Tree and Maximum Likelihood method of nucleotides substitution by Kimura 2 Parameter with Invariant Plus Gamma. ACMV isolates from Busia and Kakamega clustered together in group I while the rest clustered in group II. They were more similar to each other and to Ugandan isolate implying common ancestry than with the other isolate from Kisii. The EACMV isolate from Kisii

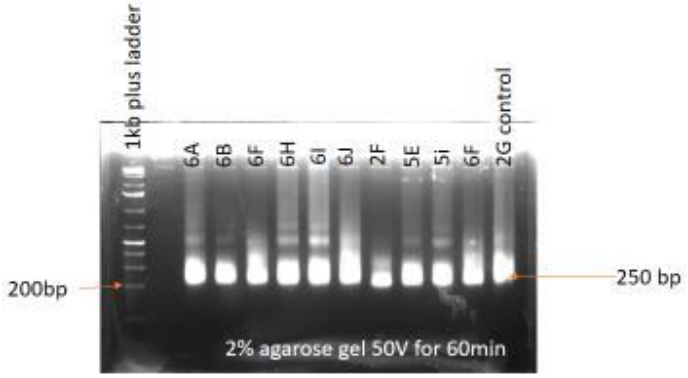
INTRODUCTION

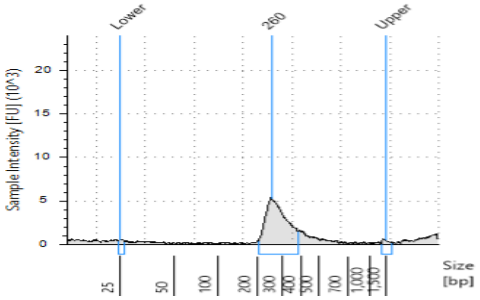
Cassava Mosaic Disease (CMD) is the most severe and widespread disease limiting production of Cassava in sub-Saharan Africa (Calvert 2002). CMD produces a variety of foliar symptoms that include mosaic, mottling, misshapen and twisted leaflets, and an overall reduction in size of leaves and plants. CMD-affected cassava plants produce few or no tubers depending on the severity of the disease and the age of the plant at the time of infection (Were *et al*., 2004). CMD is caused by 9 viruses in the genus *Begomovirus*, the largest genus in the family *Geminiviridae*, and collectively, they are also called the cassava mosaic begomoviruses (CMBs) or cassava mosaic geminiviruses (CMGs). The epidemic that broke out in Uganda and Kenya during early to mid1990 drew much attention to CMD (Gibson *et al.,* 1996 and Otim-Nape *et al*., 1998). Molecular characterization of the CMB associated with this unusual form of CMD revealed that it was a novel CMB that shared genomic properties with both ACMV and EACMV. This novel CMB was designated as Ugandan variant of EACMV and named EACMV-UG (Deng *et al*., 1997), because its DNA-A genome was 16% and 84% similar to that of ACMV and EACMV, respectively, as a consequence of recombination between the two parental viruses (Xhou *et al*., 1997).

Appendix xii: RNA Quality check on 2% agarose gel and quality control of cDNA libraries; on 2% agarose gel and graphical display from 2200 tape station using HSD1000 screen tape



Further, the libraries validation on a gel are shown below:





Appendix xiii: Sampled areas from the survey showing their coordinates and altitude

S/NO.	Survey Form Number	County	GPS Coordinates Latitude	GPS Coordinates Longitude	Altitude
1.	1	Kilifi	-3.8494	39.5273	179.30
2.	3	Kilifi	-3.8859	39.5550	169.70
3.	2	Kilifi	-3.8394	39.5405	176.40
4.	4	Kilifi	-3.8582	39.5469	181.30
5.	5	Kilifi	-3.8311	39.5842	210.40
6.	6	Kilifi	-3.9308	39.7253	26.70
7.	7	Kilifi	-3.9292	39.7251	25.30
8.	8	Kilifi	-3.5573	39.8240	84.10
9.	9	Kilifi	-3.9330	39.7380	25.90
10.	10	Kilifi	-3.5427	39.8464	32.50
11.	11	Kilifi	-3.5629	39.8570	26.30
12.	12	Kilifi	-3.5723	39.8525	21.40
13.	13	Kilifi	-3.5720	39.8536	16.70
14.	14	Kilifi	-3.5739	39.8442	34.40
15.	15	Kilifi	-3.5732	39.8620	20.10
16.	16	Kilifi	-3.3968	39.9193	23.40
17.	17	Kilifi	-3.2498	40.0433	44.80
18.	18	Kilifi	-3.2480	40.0430	28.90
19.	19	Kilifi	-3.2470	40.0448	28.00

20.	20	Kilifi	-3.2478	40.0452	34.10
21.	21	Kilifi	-3.2739	40.0340	12.00
22.	22	Kilifi	-3.2957	40.0287	34.60
23.	23	Kwale	-4.1339	39.6230	22.40
24.	24	Kwale	-4.1371	39.6177	29.60
25.	25	Kwale	-4.1432	39.6085	23.90
26.	26	Kwale	-4.1428	39.6098	25.30
27.	27	Kwale	-4.1841	39.5599	116.50
28.	28	Kwale	-4.1865	39.5216	90.80
29.	29	Kwale	-4.1859	39.5214	94.60
30.	30	Kwale	-4.1880	39.4928	290.20
31.	31	Kwale	-4.1562	39.4561	357.90
32.	32	Kwale	-4.2019	39.3986	182.60
33.	33	Kwale	-4.1855	39.5960	16.60
34.	35	Kwale	-4.3639	39.5317	23.10
35.	36	Kwale	-4.3633	39.5310	30.10
36.	48	Kwale	-4.4707	39.2956	84.50
37.	49	Kwale	-4.4992	39.3205	48.40
38.	34	Kwale	-4.2087	39.5861	28.10
39.	38	Kwale	-4.4800	39.4544	4.30
40.	37	Kwale	-4.4387	39.4883	23.40

41.	39	Kwale	-4.4871	39.4487	12.90
42.	40	Kwale	-4.5658	39.1462	34.10
43.	41	Kwale	-4.5331	39.1460	62.60
44.	42	Kwale	-4.4947	39.2109	73.50
45.	43	Kwale	-4.4934	39.2129	76.70
46.	44	Kwale	-4.4887	39.2481	62.80
47.	45	Kwale	-4.4727	39.2634	83.10
48.	46	Kwale	-4.4695	39.2880	55.90
49.	47	Kwale	-4.4692	39.2922	65.00
50.	50	Kwale	-3.7332	39.0460	384.40
51.	12	machakos	-1.6736	37.3362	1622.28
52.	13	Machakos	-1.6668	37.3406	1614.72
53.	14	Machakos	-1.6604	37.3400	1590.42
54.	15	machakos	-1.6403	37.3383	1573.58
55.	16	Machakos	-1.6058	37.3231	1525.22
56.	17	machakos	-1.5835	37.2998	1486.21
57.	18	machakos	-1.4879	37.2659	1674.32
58.	19	machakos	-1.4504	37.2488	1679.96
59.	20	machakos	-1.4215	37.2326	1873.99
60.	21	Machakos	-1.4057	37.5384	1260.91
61.	22	machakos	-1.4238	37.5588	1254.29

62.	4	Kakamega	0.2992	34.4981	1291.50
63.	5	Kakamega	0.2992	34.4981	1292.90
64.	6	Kakamega	0.1634	34.6013	1468.20
65.	7	Kakamega	0.1616	34.6139	1447.90
66.	8	Kakamega	0.1568	34.6260	1434.70
67.	9	Kakamega	0.1577	34.6261	1429.60
68.	10	Kakamega	0.2223	34.5048	1371.50
69.	13	Kakamega	0.4253	34.5056	1349.60
70.	14	Bungoma	0.6168	34.5262	1498.80
71.	15	Bungoma	0.5923	34.5938	1461.30
72.	16	Bungoma	0.5931	34.6122	1489.20
73.	17	Bungoma	0.6003	34.6256	1500.90
74.	18	Bungoma	0.6047	34.6360	1468.30
75.	19	Bungoma	0.6526	34.5177	1558.20
76.	20	Bungoma	0.6909	34.5557	1558.10
77.	21	Bungoma	0.7135	34.5464	1510.90
78.	23	Bungoma	0.7673	34.5363	1644.30
79.	24	Bungoma	0.7489	34.6098	1614.80
80.	25	Bungoma	0.7509	34.6351	1569.20
81.	26	Bungoma	0.7533	34.6545	1554.90
82.	27	Bungoma	0.7815	34.7268	1655.90

83.	29	Bungoma	0.8060	34.7632	1670.70
84.	30	Bungoma	0.8089	34.7427	1694.20
85.	28	Bungoma	0.7884	34.7480	1681.90
86.	1	Kakamega	0.2824	34.7506	1575.40
87.	1	Kakamega	0.2826	34.6418	1392.40
88.	W002	Kakamega	0.3238	34.5266	1351.60
89.	W003	Kakamega	0.3223	34.5143	1338.90
90.	11	Kakamega	0.3975	34.4913	1308.60
91.	22	Bungoma	0.7683	34.5369	1652.00
92.	12	Kakamega	0.3961	34.4910	1295.70
93.	O35	Busia	0.6230	34.3405	1164.40
94.	36	Busia	0.6277	34.3090	1165.90
95.	38	Busia	0.6277	34.2606	1156.00
96.	39	Busia	0.6258	34.2551	1127.00
97.	31	Bungomba	0.5943	34.5295	1432.50
98.	32	Bungoma	0.5905	34.5087	1366.10
99.	33	Bungoma	0.5905	34.5048	1354.30
100.	34	Busia	0.6095	34.3543	1229.10
101.	40	Busia	0.6255	34.2544	1136.50
102.	41	Busia	0.6049	34.2357	1156.50
103.	42	Busia	0.5972	34.2252	1134.90

104.	43	Busia	0.5953	34.2223	1154.50
105.	44	Busia	0.5940	34.2223	1150.90
106.	45	Busia	0.5917	34.2198	1138.10
107.	46	Busia	0.5812	34.2074	1165.00
108.	47	Busia	0.5809	34.2089	1163.90
109.	48	Busia	0.5661	34.1896	1167.10
110.	49	Busia	0.5311	34.1636	1213.50
111.	50	Busia	0.4735	34.2577	1191.70
112.	51	Busia	0.4738	34.2571	1217.20
113.	53	Busia	0.4651	34.2513	1190.10
114.	54	Busia	0.4602	34.2518	1166.30
115.	E16	Machakos	-1.3252	37.4458	1262.50
116.	E017	Machakos	-1.2566	37.4636	1194.70
117.	23	Machakos	-0.9645	37.6864	1222.30
118.	E01	Machakos	-1.5849	37.2397	1618.30
119.	E02	Machakos	-1.5876	37.2385	1617.40
120.	23	Machakos	-0.9645	37.6864	1222.30
121.	WW01	Busia	0.6396	34.2928	1158.20
122.	W02	Busia	0.6648	34.2992	1226.70
123.	WW03	Busia	0.6912	34.3215	1322.60
124.	W05	Busia	0.6500	34.2913	1189.00

125.	W06	Busia	0.5890	34.2153	1095.70
126.	W07	Busia	0.5335	34.1645	1147.80
127.	W08	Busia	0.4745	34.1286	1191.40
128.	W09	Busia	0.4574	34.1648	1195.40
129.	W10	Busia	0.4755	34.1976	1208.60
130.	W11	Kisumu	-0.2925	34.9461	1156.30
131.	W23	Kisii	-0.7143	34.6577	1486.10
132.	20	Kisii	-0.5703	34.7243	1435.20
133.	21	Kisii	-0.5688	34.7246	1448.10
134.	22	Kisii	-0.5635	34.7281	1451.70
135.	22	Kisii	-0.5639	34.7276	1438.60
136.	24	Kisii	-0.5655	34.7340	1493.90
137.	20	Kisii	-0.5703	34.7243	1435.20
138.	21	Kisii	-0.5688	34.7246	1448.10
139.	22	Kisii	-0.5635	34.7281	1451.70
140.	22	Kisii	-0.5639	34.7276	1438.60
141.	24	Kisii	-0.5655	34.7340	1493.90
142.	27	Kisii	-0.5630	34.7408	1503.90
143.	28	kisii	-0.6215	34.8846	2013.00
144.	29	Kisii	-0.5929	34.8598	1910.10
145.	30	Kisii	-0.5942	34.8749	1909.10

146.	27	Kisii	-0.5630	34.7408	1503.90
147.	28	Kisii	-0.6215	34.8846	2013.00
148.	29	Kisii	-0.5929	34.8598	1910.10
149.	30	Kisii	-0.5942	34.8749	1909.10