DISTRIBUTION, MOLECULAR CHARACTERIZATION, MCMV SEED TRANSMISSION AND DIAGNOSTICS OF VIRUSES CAUSING MAIZE LETHAL NECROSIS (MLN) - IN KENYA

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Distribution, Molecular Characterization, MCMV Seed Transmission and Diagnostics of Maize Lethal Necrosis (MLN) causing Viruses in Kenya

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DECLARATION

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

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This thesis has been submitted for examination with our approval as supervisors:

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Signature……Date……

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DEDICATION

I dedicate this work to my Dad, Alexander Mwatuni Mucholwa who encouraged me to study sciences as opposed to arts subjects at my early age. He religiously mentored me by providing all the basic requirements for a good education. My sincere gratitude also goes to my mother, Susan Nakhungu who still believed I can excel in school and encouraged me to pursue higher education after High school. I also dedicate this work to my wife, Electine Sifuna who persevered my long hours on the computer for the last six years. She also encouraged me when things looked difficult and urged me not to give up but rather to press on after the example of successful people who persist and achieve their goals.

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ABBREVIATIONS

BMV	*Brome mosaic virus*
BSMV	*Barley stripe mosaic virus*
CDFA	Phytosanitary Certification Manual
CIMMYT	International Maize and Wheat Improvement Center
DNA	Deoxy Ribonucleic Acid
ELISA	Enzyme linked Immunosorbent Assay
FAO	Food and Agriculture organization
FAO - STAT	Food and Agriculture organization – Statistics
FDF	Fully-denaturing formaldehyde
FERA	Food and Environmental Research Agency
GWS	Genome Wide Selection
IITA	International Institute of Tropical Agriculture
KEPHIS	Kenya Plant Health Inspectorate Service
LAMP	Loop Mediated Isothermal Amplification
MCMV	*Maize chlorotic mottle virus*
MDMV	*Maize dwarf mosaic virus*
MLN	Maize Lethal Necrosis
MSV	*Maize streak virus*
NCBI	National Center for Biotechnology Information
NEB	New England Biolabs
NGS	Next Generation Sequencing
NMD	Non – sense mediated decay.
PMV	*Panicun Mosaic Virus*
RPA	Recombinase Polymerase Amplification
RT-LAMP	Reverse Transcriptase – LAMP
RT-RPA	Reverse Transcriptase-RPA
SCMV	*Sugarcane mosaic virus*
TAE	Tris base Acetic Acid
TBE	Tris/Borate/EDTA
TuMV	*Turnip mosaic virus*

ABSTRACT

Maize is widely grown in Kenya and ranks highly in meeting food dietary preferences of many communities in Kenya. Therefore, any factor that threatens maize production impacts negatively on food security and national economy. Maize lethal necrosis (MLN) disease is such a factor. Since the first report of MLN in Kenya in 2012, the disease has spread fast and affected up to 300,000 maize farmers in the country leading to yield losses of between 50% - 100% and an estimated financial loss of more than KShs. 2 billion (approximately $23.3 million).

This study was carried out to determine the distribution as well as the genetic diversity of *Maize chlorotic mottle virus* (MCMV) and the potyviruses that infect maize in the MLN disease complex. The study also sought to identify the component potyviruses that interact synergistically with MCMV to cause MLN in Kenya. This study analysed the rate of transmission of MCMV through seed material. The need for an effective, field-deployable MLN detection assays was addressed by developing and validating the loop mediated isothermal amplification (LAMP) and the recombinant polymerase amplification (RPA) assays for molecular the detection of MCMV.

To determine the distribution, incidence, prevalence and severity of MLN, a nationwide survey was conducted for MLN from June 2015 to May 2016 in the maize growing areas in the country. Detection of MCMV and SCMV in collected samples was performed using RT-PCR and DAS ELISA. Obtained sequences for Kenyan MCMV and SCMV isolates from Next Generation sequencing (NGS) were compared to the available sequences in the public repository (NCBI). The survey indicated that MLN incidence was 35 – 90% with a prevalence of 44 – 72% and symptoms severity of 1.7 – 4.1 on a 1-5 MLN severity scale. This study identified MCMV and SCMV as the major viruses causing MLN in Kenya through both laboratory diagnosis and NGS sequencing. The Kenyan MCMV isolates detected in this study showed 99.75% similarity to isolates previously reported in Kenya (JX286709), Ethiopia (KP798454) and Rwanda (KP851970.01). They also showed99.02% identity with MCMV isolates from Yunnan, China (KF010583.1) and 96.00 to 9700% with MCMV isolates from Kansas (X14736) and Nebraska (EU358605) in the United States of America. The SCMV genome showed high diversity within the polyprotein region ranging from (89.81 - 100%), 99.00% similarity to the genome of isolates in Rwanda (KF744392.1) and 98.00% to Ohio, US isolate (JX188385.1). They were also 99% similar to isolates from China (JX047412.1 and JX047425.1). Potential recombination events were detected in 11 SCMV genome sequences but only 3 SCMV genome sequence recombinants were realized with different possible major and minor parents.

A reverse transcription loop-mediated isothermal amplification (RT-LAMP) assay with two end point analyses was developed to detect MCMV in maize during active vegetative stages and of the seed grow out tests. Amplification was detected by colour change of Sybr green dye in the reaction tubes from orange to green and in real time on the GenieII model GEN2-02 LAMP for this RT-LAMP assay. The LAMP assay was efficient with sharp visible curves displayed between the 10^{th} and the 20^{th} minute of the reaction for MCMV positive samples. The assay discriminated against the common viruses infecting maize namely SCMV, MDMV, WSMV, PMV, and MSV.

Real-time reverse transcription recombinase polymerase amplification (RT-RPA) assay for the detection of Maize Chlorotic mottle virus (MCMV) was also developed during this study. The MCMV RT-RPA primer design targeted the MCMV genome at

2765 - 2948. The RT-RPA assay was fast in MCMV detection with amplification recorded in the (4–10 minutes). The method was also versatile on maize leaf sap samples where amplification for MCMV was clearly visible making it easy for use at field levels eliminating the tedious and expensive RNA extraction process.

Seed transmission of MCMV was evaluated in seventeen different hybrids which included the CIMMYT derived hybrids under the MLN breeding program (CMKMLN), the hybrids released under the Water Efficiency Maize for Africa (WEMA), and the popular commercial hybrids on the market in Kenya.

Out of the total 21,041seeds evaluated through planting and rigorous testing using ELISA, only 256 tested positive for MCMV. This translated to a transmission rate of 1.2%.

MLN is still widely distributed in the country with SCMV, a predominant potyvirus found to co-infect maize with MCMV to cause MLN. SCMV was found to be highly diverse while MCMV was highly conserved. The Kenya MCMV strains were highly similar to the Asian isolates. The transmission of MCMV through seed was observed to be low though it plays a major role in long distance spread of the disease.

CHAPTER ONE

INTRODUCTION

MLN was first reported in Bomet County in Kenya in 2012 but has now spread to other countries in the East and Central African regions (Wangai et al., 2012). MLN has been found to be caused by the double infection of maize plants with *Maize chlorotic mottle virus* (MCMV), a *machlomovirus* (Nutter et al., 1989; Lommel et al., 1991), and *Sugarcane mosaic virus* (SCMV), a *potyvirus* (Jones et al., 2007, 2011; Adams et al., 2013). However, MLN can also be caused by MCMV in synergy with other maize infecting viruses namely *Maize dwarf mosaic virus* (MDMV) in the genus *Potyvirus*, *Wheat streak mosaic virus* (WSMV) in the genus *Tritimovirus* (Pruss et al., 1997), or the recently described *Johnsongrass mosaic virus* (JGMV) also in the genus *Potyvirus* (Stewart et al., 2014, 2017). Any potyvirus that infects maize can potentially interact synergistically with MCMV to cause MLN (Adams et al., 2012).

Maize (*Zea mays L.*) is the third most important crop in the world, and it plays a great role in the diets of over 4.5 billion people. Maize has become an integral part of modern society: It is a staple food for humans and livestock and has a variety of other uses. In 2014, East Africa produced over 30 million tonnes of maize from over 17 million ha of cultivated land hence maize in this region remains a staple food for most households (FAO, 2016). The annual yield of maize in 2016 was 3.39 million tons, and its value exceeded $65 billion (FAO¬STAT 2016). However, since the emergence of maize lethal necrosis (MLN) disease in this region, it has been observed to cause high maize yield losses than the yield loss observed from the combination of all other biotic and abiotic factors together (Wangai et al., 2012). The United States Department of Agricultures (USDA) Foreign Agricultural Service estimated yield losses due to MLN in Kenya in the marketing year 2014/15 to be up to 10%, which translated to over 50 million US dollars (USDA 2015). A community survey assessment on the distribution and impact of MLN in Kenya in 2013 revealed that the disease affected 22% of the maize produced that year, which translated to about 187 million US dollars in losses (De Groote et al. 2016).

Kenya's total maize production for two seasons between July 2016 and June 2017 was 3.2 million MT, about 14 percent lower than the 2015/2016 when El Nino enhanced average production. About 100,000 MT of maize was imported to Kenya in the first half of 2017, due to approximately 17 percent (100,000 MT) shortfall in the estimated 3.3 million MT produced during the July 2016 to June 2017 marketing period (Food Security and Nutrition Working (FSNWG), Jan 2018).

1.1 Worldwide Maize Production and Utilization

Although maize is grown almost all over the world, there exist significant differences in yield (FAO, 2016). The world maize production estimates by 2014 was about 1,037,791,518 tons with the United States, China and Brazil harvesting over 63% of the world production. The entire Eastern Africa contributed only 30,679,856 tons, translating to just about 2.95% of the over 1bn tons produced globally. The entire African harvest was about 78,005,212 tons, translating to just 7.5% of the global harvest (Statista 2016). Table 1.1 shows the maize production figures in some countries where the crop is important.

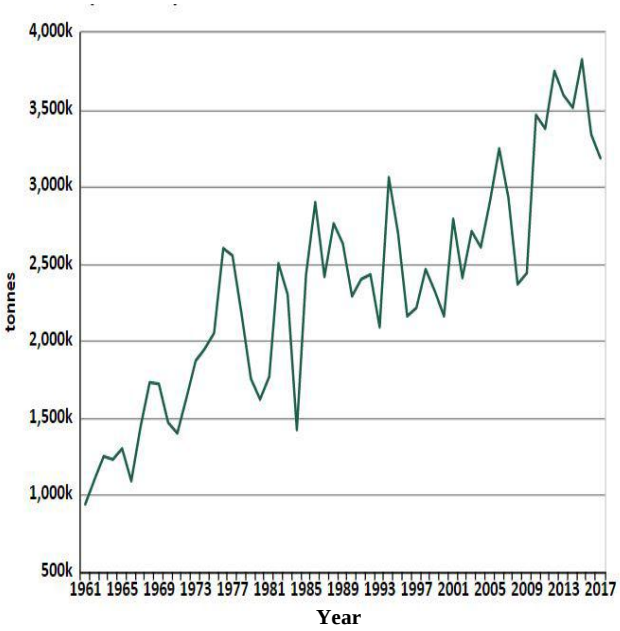
Table 1.1: Maize production by country worldwide. 2015 – 2017 (Source Statista – The statistics Portal 2018)

Country	Maize Production (tons/ha)		
	2015	**2016**	**2017**
United States	361,091,140	384,778,000	366,287,000
China, Mainland	215,646,300	219,554,290	257,350,000
Brazil	79,881,614	91,500,000	94,500,000
Argentina	33,087,165	37,500,000	46,000,000
Ukraine	28,496,810	28,280,000	35,000,000
India	23,670,000	26,070,000	26,000,000
Mexico	23,273,257	26,020,400	25,600,000
Indonesia	19,008,426	10,500,020	11,900,000
France	18,343,420	16,450,000	14,300,000
South Africa	14,250,000	14,610,000	14,210,000
Kenya	3,513,171	3,339,000	3,186,000

These production differences observed between developed and developing countries could be attributed to factors and practices in developed countries that lack in the developing countries. These include different climatic factors, land sizes, use of proper seed stocks, herbicides and pesticides, fertilizers and mechanisation, compared to traditional technologies in developing countries such as traditional breeding techniques, poor agronomic practices and multi-cropping (Izuchu *et al*., 2009). Maize is a staple food in Kenya where it is consumed directly by as "ugali" and in may other forms as roated or boiled, or mixed with beans and boiled commonly known as "Githeri". Maize is also used in animal feed formulations and other maize products, such as corn starch and corn oil.

1.2 Maize Production in Kenya

In Kenya maize is grown in over 1.6 million hectares annually, out of which 80% is owned by small holder farmers (Wambugu et al., 2012). Indeed, national food security in Kenya is pegged to availability of adequate maize supply to meet the domestic food demand of over 90% of the population (Wambugu et al., 2012). Despite continued efforts to increase maize production in Kenya by both the government and the private sector, maize demand in the country has constantly outstripped the supply. The crop is grown in all regions, but the Rift Valley region produces approximately half of the country's total production. In most of the maize growing regions however, yields without use of manure and fertilizers ranges from 1.1 to 2.5 tons per hectare (Wokabi, 2013). Production estimates by 2014 stood at about 3,513,171 tons, translating to just over 39.04 million bags. This was a significant reduction compared to the 3,592,688 tons harvested in 2013, irrespective of the increasing demand of maize in the country (FAO, 2016). Figure 1.1 below shows the production of maize trends in Kenya from 1961 to 2017.



DATE	VALUE	CHANGE, %
2017	3,186,000	-4.58 %
2016	3,339,000	-12.71 %
2015	3,825,000	8.88 %
2014	3,513,171	-2.21 %
2013	3,592,688	-4.19 %
2012	3,749,880	11.05 %
2011	3,376,862	-2.53 %
2010	3,464,541	42.05 %
2009	2,439,000	3.03 %

Figure 1.1: Maize production in Kenya from 1961 to 2017 (Source; KNOEMA portal 2019). Parentage changes in production in successive years are also shown.

1.3 Constraints to Maize Production

Maize production is affected by both biotic and abiotic conditions. Abiotic stresses adversely affect plant growth and trigger morphological, physiological, biochemical as well as molecular changes in the plant. Drought, temperature extremes and saline soils are the most common abiotic stresses in plants (Bhatnagar-Mathur et al., 2008). When a maize plant is dehydrated, the production of reactive oxygen species (ROS) such as oxygen superoxide anions, hydrogen peroxide (H_2O_2) and hydroxyl radicles increase. This eventually leads to the breakdown of vital cellular components such as proteins, lipids and nucleic acids, and subsequent cell death (Carvalho, 2008). Increasing temperatures, changing rainfall patterns and extreme weather conditions worsen the inability to meet maize demand. Soil salinity is another important abiotic threat to maize production.

Biotic stress occurs to a plant due to damage by a living organism. These stresses account for a great portion of maize yield losses in Africa where many farmers cannot afford the cost of controlling the biotic stress-causing agents. Pests and diseases are the most common biotic stresses in most crop production systems in the world (Cairns

et al., 2012). Most microbial pathogens causing maize crop diseases of economic importance are of fungal origin although viruses and bacteria also cause significant losses. Maize diseases such as the turcicum leaf blight (Northern leaf blight), gray leaf spot, crazy top disease (downy mildew), head smuts and the common rusts are all caused by fungal pathogens. The maize streak disease, maize dwarf mosaic disease and the maize lethal necrosis are caused by viruses. The Stewart's disease (Bacterial wilt) and bacterial leaf spots are caused by bacteria (Guantai et al., 2010). On the other hand, insects like moths, the African maize stalk borer and earworms, grain borers and weevils, leaf bugs, maize fleas and aphids have been reported to cause devastating losses in maize (Ortega, 1987). In 2013 alone, 25% of maize crop yield in Africa was lost to diseases. Coupled with pests, some countries have reported up to 45% crop loss (Magomba, 2013).The parasitic weed striga, (witchweed) is also a significant contributor to maize yield losses in Africa wherever it thrives. It infests over 40 million hectares in Africa and causes yield losses of 20-80% and sometimes total crop failure (Kim et al., 2002).

1.4 Problem Statement

Global crop losses caused by crop diseases have been estimated to range from 9% to 14.2% of potential yield (Orke et al. 1994). Assessment of losses made in a later investigation indicated that about 14.1% of crop may be lost due to diseases with a monetary value of $220 billion per annum, the developing countries suffering more losses compared with developed countries (Agrios 2005).

The biotic stresses on maize are primarily pathogens (fungal, bacterial, and viral), and the resultant syndromes, such as ear/stalk rot, rough dwarf disease, and northern leaf blight, are prevalent and cause heavy damage (Gong et al., 2014). Approximately 10% of the global maize yield is lost each year as a result of biotic stresses (Ahuja et al., 2010). In Kenya, abiotic and biotic stresses account for 30% yield losses in maize (Wambugu et al., 2012)

The emergence of MLN in Kenya and the steady spread in eastern Africa region has exacerbated maize yield losses. When MLN infects maize fields, its infection rates and crop damage can be very high and crop yield losses can be up to 100%. This is because

infected plants are in most cases barren and when not, ears formed are usually very small or deformed with very little or no seeds set (Kiruwa et al., 2016).

Since the first report of MLN in Kenya, the disease has spread fast and affected up to 300,000 maize farmers in the country. In the worst hit Rift valley of Kenya, at least 70% of the maize crop has been affected. The disease affected 75,000 Ha in the Long Rain season of 2012, leading to yield losses of between 50% - 100% and an estimated financial loss of KShs. 2 billion (approximately $23.3 million) to small holder farmers (Ministry of Agriculture and Livestock development, Annual report, 2014). The sharp drop of maize production later witnessed in three successive years of 2015, 2016 and 2017 is largely attributed to the recorded severe infection of maize fields by MLN. The impact of MLN for small holder farmers is significant since sometimes these farmers can lose their whole produce when affected. Maize makes up a very large contribution to food, feed and diet of many farmers in Kenya and east Africa where the disease has prevailed. Indeed, most of the staple foods in the country are based directly on maize or its bi-products. Losing harvests has had a significant effect on nutrition security of farming families in the country. As such, farmers who have depended on maize as one of their incomes earning enterprises hardly meet their financial obligations due to reduced farm income and hence have slid more into poverty. This has led to food shortages hence food insecurity.

1.5 Justification

The possibility of MCMV to combine with other native potyviruses of cereals poses a big challenge to maize production in the country. What are the genetic diversities of these viruses? What means of transmission have allowed the rapid spread of the disease in Kenya and across the Africa continent? What is the exact distribution of the disease in Kenya and neighbouring countries? And what is the mechanism(s) of the synergy between a potyvirus and MCMV?

The distribution maps of MLN will facilitate documentation of the disease status in the country and designation of the MLN-free areas for seed production. The determination of the native potyviruses that co-infects maize synergistically with MCMV will be vital in designing and deploying management options for MLN. The

studies of synergistic mechanisms may provide leads to novel control strategies for the disease since potyviruses in synergy with MCMV will have been identified (Wangai et al., 2012; Adams et al.,2013; Mahuku et al., 2015). In-depth knowledge of the causative viruses, genetic diversity and viral recombination analysis will facilitate molecular breeders to accurately design breeding programs and also the sequences generated will facilitate the design of primers and probes for the molecular based detection assays of the viruses.

The effectiveness of crop disease management systems depends heavily on the rapid, reliable and sensitive detection of microbial plant pathogens and accurate diagnosis of the diseases caused by the putative pathogen(s) (Narayanasamy 2002). Deployment of effective, affordable, field deployable molecular based diagnostic assays for MCMV will facilitate more testing for MCMV in the seed fields and in surveillance programs as an initial management strategy of MLN. Seed companies will monitor MLN easily in the field hence reduce to incur losses from entering seed fields already infected in the KEPHIS seed certification process.

The findings of the study on the transmission of MCMV through seed will be essential in crafting quarantine measures and tolerance levels of the disease in seed lots by regulatory agencies in Kenya and in other countries affected.

It is envisaged that the findings will contribute in the early warning strategies and eventually management of MLN in Kenya. This will eventually facilitate farmers to increase their yields for subsistance and income for improved livelihoods. As such the Kenyan food security situation will also be ultimately improved.

A regional approach ensures that the causative agents of the disease are thoroughly characterized and that research outcomes will help breeders in their quest to develop resistant/tolerant varieties and farmers to manage the disease better in the country.

1.6 Objectives

1.6.1 General Objective

To determine the distribution and characterize viruses that cause maize lethal necrosis disease and their rate of transmission through seed in Kenya.

1.6.2 Specific Objectives

1. To investigate the distribution, incidence, prevalence and severity of MLN in Kenya
2. To identify and determine the genetic diversity of MLN causing viruses in the Kenya.
3. To develop rapid and effective field-deployable Isothermal based molecular detection assays for MCMV.
4. To determine seed transmission rates in both artificial and natural MLN infections

1.6.3 Null hypotheses

1. There is no elaborate data on distribution, incidence, prevalence and severity of MLN in Kenya
2. The MLN causing viruses in the Kenya are not genetically diverse.
3. Field-deployable Isothermal based molecular detection assays for MCMVare not rapid and effective
4. There is no significant difference in means of MCMV transmission rates through seed in both artificial and natural MLN infections

CHAPTER TWO

LITERATURE REVIEW

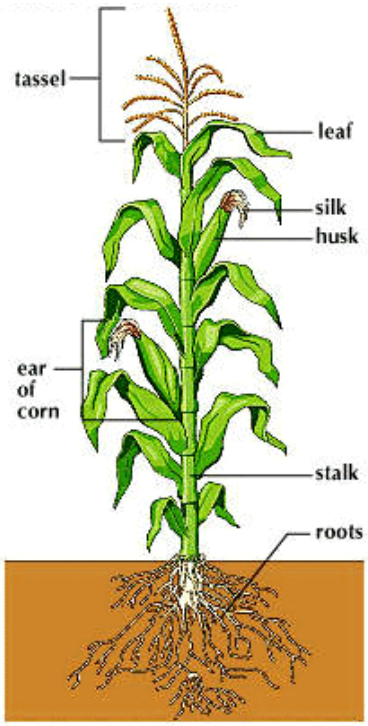
2.1. Origin and Domestication of Maize (*Zea mays*)

Maize (*Zea mays* L. ssp. *mays*), also known asteosinte corn originated from the Tehuacán Valley in the Mesoamerican region, Mexico (Galinat 1977b). From archaeological records, the domestication of maize began over 6000 years ago in South western United States, Mexico, and Central America (Mangelsdorf, 1974). It is a member of the grass family *Poaceae (Gramineae).* Together with other grasses like barley, oats, sorghum, rice and sugar cane, it is thought to have originated from a common ancestor over 55-70 million years ago (Buckler and Stevens, 2005).Several different hypotheses have emerged over time to describe the origin of maize. In the 1930s, Paul Mangelsdorf and Robert Reeves (1939) proposed the tripartite hypothesis. In this theory, maize was domesticated from some unknown wild maize relative with structures that resemble the modern maize ear (Mangelsdorf and Reeves ,1939). Specifically, the hypothesis consisted of three parts: an extinct or undiscovered South American wild maize prototype;teosinte, an offspring of maize and *Tripsacum*; contamination of sections of Tripsacum chromosomes (Buckler and Stevens, 2005). Therefore, Mangelsdorf and Reeves (1945) accounted for the extreme morphological differences between maize and teosinte by invoking a missing ancestor and relied on *Tripsacum* to explain the similarities between the two plants. In 1939, George Beadle developed his own hypothesis to describe the origin of maize, called the teosinte hypothesis. In this hypothesis, Beadle believed that small mutations with large effects transformed teosinte into maize and that the morphological differences between maize and teosinte were not so large to require an extinct ancestor (Doebley, 2004). Recently, the *Tripsacum–Z. diploperennis* hypothesis which was developed in 1995 by Eubanks emerged. In this theory, maize is a progeny of the cross between *Z. diploperennis* and *T. dactyloides* (Eubanks, 1995). However, strong evidence has refuted this claim based on the chromosome numbers of the parents and that of the present day maize (Buckler and Stevens, 2005). After the domestication of maize, many improvements have been made which led to today's maize with the desired agronomic traits for modern agriculture.

Maize was first cultivated in America by the Indians. It was taken to Europe by Christopher Columbus and has since spread to many parts of Africa and Asia (Marvin P. Miracle 1965). In Kenya it was first introduced by the Portuguese at the coast in the 15th Century (Mosley 1983; Jansen 1977). During the British occupation of Kenya, maize was introduced and cultivated in their farms as animal feed, but African workers soon used it for food by making a maize meal called 'ugali'. "Eventually the domestic demand for maize grew as Africans left their farms to work on settler farms, in mines or industrial plants, particularly in Kenya, Zambia andZimbabwe (Mosley 1983; Jansen 1977).

2.2 Botany of Maize

Maize is a tall, determinate, monoecious annual Carbon 4 (C4) plant. It has large, narrow and alternate leaves which are borne along the length of the stem. Its shoot terminates into the inflorescences which bear either staminate or pistillate flowers (Figure 2.1). The main shoot however terminates in a staminate tassel which matures earlier than the female flower. The female inflorescence usually referred to as the ear develops from one of the lateral branches that originate from the auxiliary shoot buds. Normally, maize has three types of roots: the adventitious roots which develop from the lower stem nodes below the ground, the seminal roots which grow from the radicle, and the prop roots which develop from the lower nodes above ground and are necessary for support. In favourable soils, the maize root system can grow up to 60 cm in depth and laterally. Stems of fully-grown maize plants can attain thicknesses of 3-4 cm. The lower stem internodes are fairly short and thick while the intermediate internodes become relatively longer and thicker, going up the plant, the top internodes taper. The individual maize grain is a caryopsis. This seed contains an embryo with a plumule and a radicle that give rise to a new plant (Bennetzen and Hake, 2009).



Figur 2.1: The maize plant

Source; Encyclopaedia Britannica 2018.

2.3 Maize Lethal Necrosis (MLN) Disease

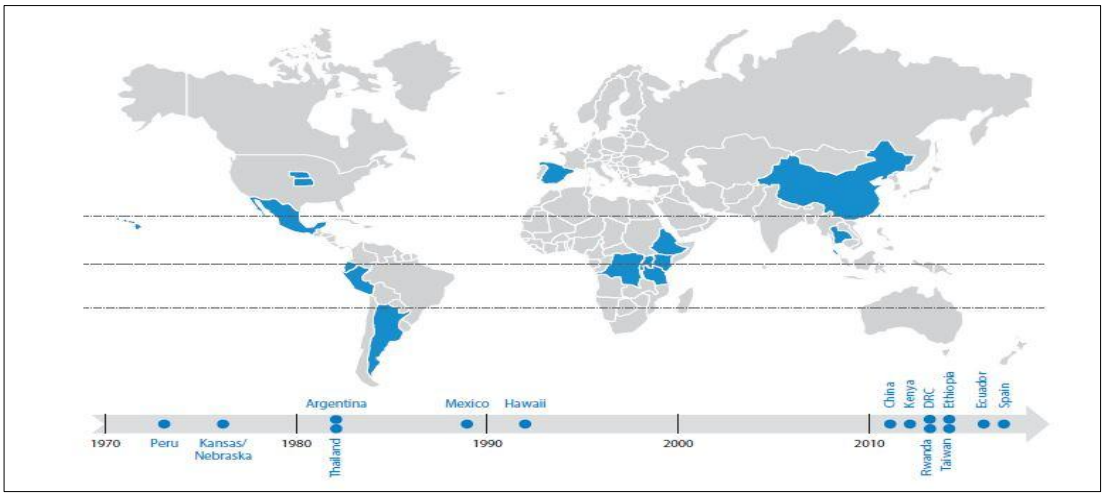
2.3.1 Global occurrence of the disease

Maize chlorotic mottle virus (MCMV) was first reported in Peru in 1973 (Hebert and Castillo, 1973) where losses in floury and sweet corn varieties were between 10 and 15%. In experimental plots, inoculated plant yields were reduced by up to 59% (Castillo-Loayza, 1977). In Kansas crop losses due to corn lethal necrosis (Synonym for MLN) have been estimated to be between 50% to 90% (Uyemoto et al., 1980; Niblett and Claflin, 1978) depending on the variety of maize and the year. In China MLN poses a major challenge to maize crop production in many regions. MCMV was also recently reported in Ecuador in 2016 (Diego et al., 2016) and in Spain the same year (Achon et al., 2016).

The occurrence of an unfamiliar maize disease was first reported in September 2011 in the low altitude zones of Bomet District, Longisa Division, 1900.m.a.s.l. affecting

200 Ha of the second season maize crop in Kenya (Wangai et al., 2012). The effects of the disease were sudden, devastating and unexplainable. The cause of the problem was unknown. Local farmers called it 'Koroito', which is the vernacular name for plague. By the end of 2011, the disease was observed to have spread to the higher altitudes of Bomet county, the neighbouring Narok South, Narok North and Naivasha sub-counties.

Between February and March 2012, samples collected from the infected maize fields were tested for viruses at the National Agricultural Research Laboratories (KALRO-NARL), Ohio State University (USA), and Food and Environment Research Agency (FERA), UK. The investigations revealed the presence of two viruses, *Sugarcane mosaic virus* (SCMV) and a new virus in Kenya, MCMV (Wangai et al., 2012). SCMV is prevalent in many parts of Kenya affecting cereal crops. The double infection of MCMV and SCMV or any of the cereal viruses in the *Potyviridae* family e.g. *Maize dwarf mosaic virus* (MDMV*)* or *Wheat streak mosaic virus* (WSMV*)*, a *Tritimovirus* (Pruss et al., 1997) may give rise to MLN. MLN occurs worldwide but it is reported in several countries in all the world continents except Australia and New Zealand (Figure 2.2 and Table 2.1).



Figur 2.2: Emergence of maize chlorotic mottle virus (MCMV). MCMV has been reported in a number of countries (blue), and in the United States primarily in Kansas, Nebraska, and Hawaii. The reported year of MCMV emergence is indicated on the timeline. Abbreviation: DRC, Democratic Republic of the Congo. (source: Stewart et al., 2018)

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Table 2.1: Chronological Year of reports of MCMV worldwide

Country	Year Reported	Associated Potyvirus
Peru	1973	NR
USA (Mainland)	1976	WSMV/MDMV
Argentina	1982	NR
Thailand	1982	NR
Mexico	1989	NR
United States, Hawaii	1992	MDMV
Colombia	1999	NR
China	2009	NR
Kenya	2012	SCMV
Rwanda	2013	SCMV
DRC	2013	SCMV
Taiwan	2014	SCMV
Ethiopia	2015	SCMV
South Sudan	2014	NR
Spain	2016	NR

NR = No potyvirus associated with MCMV reported, WSMV = *Wheat streak mosaic virus*, MDMV = *Maize dwarf mosaic virus*, SCMV = *Sugarcane mosaic virus*.

2.3.2 MLN Causative agents

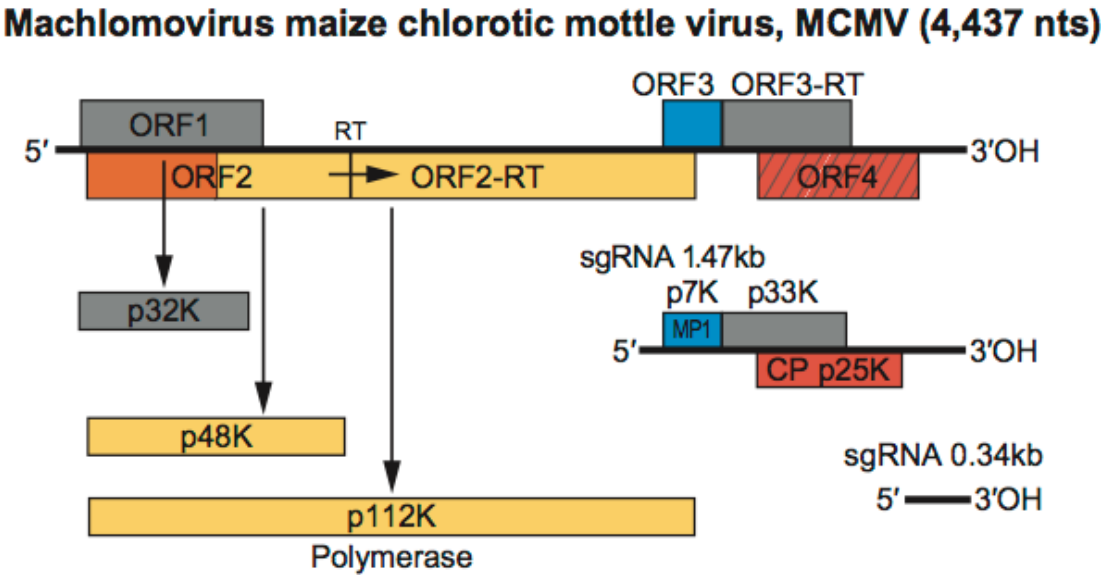
Maize (Corn) lethal necrosis is caused by the synergistic activity of MCMV with either *Maize dwarf mosaic virus* (MDMV) or the *Wheat streak mosaic virus* (WSMV) (Goldberg and Brakke, 1987). The disease was identified in China where it was attributed to the synergistic activity of the MCMV and SCMV (Doupnik, 1979). Later on, the disease was identified in Kenya, where it was attributed to be synergistically caused by the MCMV and the SCMV (Wangai et al., 2012). In Africa, the disease has continuously been associated with the potyvirus (SCMV) though there are possibilities of other potyviruses infecting maize to co-infect with MCMV causing MLN (Wangai et al., 2012; Mahuku et al., 2015a). Recent studies indicate that *Maize yellow mosaic virus* (MaYMV), a *polerovirus* infects maize synergistically with MCMV but it is not conclusive if it synergistically results in MLN (Massawe et al., 2018).

2.3.4 MCMV Genome organization

MCMV is the only member of the *Machlomovirus* genus, in the family *Tombusviridae*. Its genome is a single stranded, positive-sense RNA, with 4436 nucleotides, encapsulated in isometric icosahedral particles/virions of 30 nm wide (Lommel et al., 1991). This viral genome consists of six open reading frames (ORFs) as illustrated in

Figure 2.3. The first ORF encodes a 32-kDa hypothetic protein of unknown function while the second ORF encodes a 50-kDa protein and a 111 kDa N-terminus-overlapped protein. ORF4 encodes a 7-kDa protein which is a movement protein while ORF5 encodes a 31-kDa protein involved in cell-to-cell movement. ORF3 is thought to be involved with the expression of the coat protein of the MCMV encoded by ORF4 (Wang et al., 2017).

MCMV viral particles have icosahedral and spherical geometries with a T=3 symmetry (Niblett and Claflin, 1978) as shown in Figure 2.4. The particles have a diameter of 28-34 nm. The icosahedral virion of MCMV is composed of 180 copies of chemically identical capsid protein approximately 38 kD in size, and one copy of genomic RNA with a length of 4.4 kb (King et al., 2011).

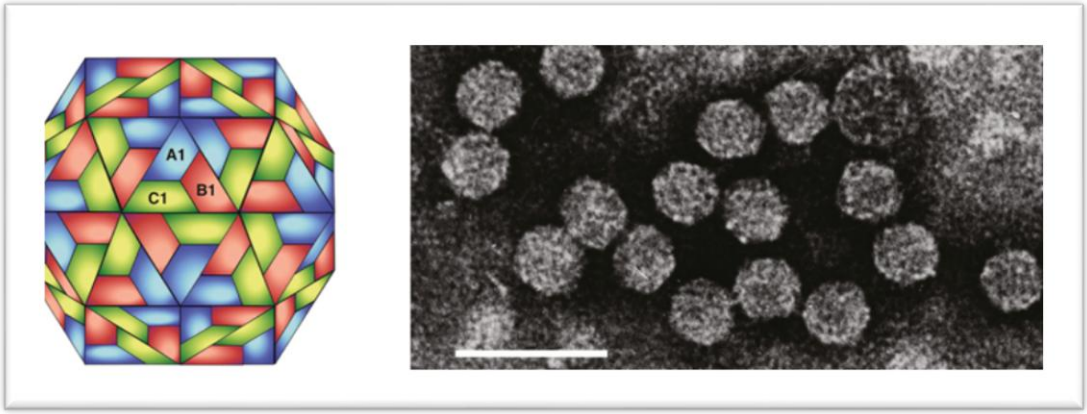


Figur 2.3: *Machlomovirus* maize chlorotic mottle virus, MCMV genome organization (adapted from International Committee for Taxonomy of Viruses (Adapted from Wang et al., 2015). Boxes represent known and predicted ORFs with the sizes of the respective proteins indicated in the bars below. Shaded ORFs indicate polymerase proteins that have a high degree of sequence conservation within the family *Tombusviridae*.

The CP that is highly conserved among those genera within the family *Tombusviridae* that lack a protruding domain. The putative cell-to-cell Movement protein (MP) that exhibits sequence conservation with similar proteins in

the carmoviruses, necroviruses and panicoviruses. The grey boxes in Figure 2.3 identify ORFs having no significant sequence similarity with other viral proteins.

Electron microscopy reveals MCMV viral particles detail as shown in Fig. 2.4 below.



Figur 2.4: Diagrammatic representation of a particle of MCMV and the electron micrograph of the MCMV viral particles. Adapted from Sit and Lommel (2015).

2.3.5 Synergistic infection by MCMV and SCMV

In plants, synergistic interactions between independent viruses in mixed infections have been well documented (Syller, 2012) but the mechanism underlying these interactions remains elusive. The expression levels of MCMV genomic RNAs and CP were shown to increase in SCMV + MCMV co-infected maize plants compared with that in MCMV infected maize plants in agreement with the results of previous reports (Goldberg and Brakke1987; Scheets, 1998). It has been demonstrated that HC-Pro, the silencing suppressor encoded by potyviruses, could enhance the pathogenicity and accumulation of other heterologous viruses (Syller, 2012). Moreover, the synergistic infection of WSMV and MCMV is independent of WSMV HC-Pro, which is not a silencing suppressor (Pruss et al., 1997). However, the effects of SCMV HC-Pro as well as WSMV P1 on the synergistic infections remain to be investigated, both of which have been proved to function as suppressors of RNA silencing (Young et al., 2012: Zhang et al., 2008). RNA silencing is a conserved surveillance mechanism in

the defense against viruses in plants, which can trigger the production of vsiRNAs in virus-infected plant cells.

In the SCMV singly or doubly (with MCMV) infected maize plants, SCMV-vsiRNAs accounts for more than half of total small RNAs, similar to the results of previous report (Xia et al., 2014). However, the accumulation level of MCMV-vsiRNAs has been found to be lower compared with endogenous small RNAs within a library, accounting for 14.75–19.49% of total small RNAs. Further analysis of SCMV + MCMV library suggested that there was a preference to SCMV RNAs for RNA silencing, which accumulates more SCMV vsiRNAs than MCMV vsiRNAs.

The nonsense-mediated decay (NMD) was reported to recognize and eliminate viral RNAs with internal termination codons and long 3′ -UTRs, but it had no effect on the potyvirus (Garcia et al., 2014). NMD, as a general virus restriction mechanism in plants, might compete for MCMV RNA substrates with RDR and decrease the accumulation of MCMV vsiRNAs in MCMV singly and doubly (with SCMV) infected maize plants. In effect, saturation of NMD by increasing amounts of viral RNAs may constitute a switch for RDR action and secondary RNA silencing during viral infection (Garcia et al., 2014). In co-infected maize plants, the increased accumulation levels of MCMV vsiRNAs might be the results of processing the increased accumulation of MCMV RNAs by RNA silencing.

For a long time, the dsRNA replication intermediates were thought to be the major origin of vsiRNAs from positive-strand RNA viruses. However, it has been reported that the vsiRNAs had a positive sense strand bias by high-throughput sequencing, suggesting that vsiRNAs originated predominantly from highly structured single-stranded viral RNAs (Szittya et al., 2010). It has been demonstrated that almost equal amount of (+) and (−)-sense vsiRNAs exists in SCMV-infected maize plants indicating that most of the S-vsiRNAs are likely generated from dsRNA precursors (Xia, Z. et al 2014). In MCMV infected maize plants, the (+)-sense MCMV vsiRNAs accumulates more than those from the (−)-sense strand suggesting that the majority of MCMV vsiRNAs are derived from MCMV genomic RNAs. By applying Fully-Denaturing Formaldehyde-Poly Acrylamide Gel Electrophoresis (FDF-PAGE), the predominant precursor of vsiRNAs was demonstrated to be a long dsRNA. However, whether this conclusion is relevant to the origin of MCMV vsiRNAs remains to be studied.

The accumulation levels of maize *AGO2* mRNAs are induced in singly and doubly infected maize plants, which further increases the possibility that maize AGO2 participates in antiviral defense. Previous reports have shown that AGO1 plays a dominant role in the defense against RNA viruses (Qu et al., 2008: Morel et al., 2002). However, the accumulation levels of maize *AGO1a* and*AGO1b* mRNAs remains unchanged even when *AGO1c* was decreased in MCMV or SCMV + MCMV infected maize plants, although SCMV infection slightly induced the expression of *AGO1a* and *AGO1b* mRNAs. AGO1 plays a less significant role than AGO2 in the defense against SCMV and MCMV as the results obtained by recent studies (Garcia-Ruiz, H. *et al., 2010*). In addition, the presence of substantial amounts of vsiRNAs with 5′ -terminal G or C reveals that other AGOs might also be recruited to form specific RISCs involved in antiviral defense, which are reported to bind siRNAs from viruses or viroids (Takeda et al., 2008). Recent research demonstrated that AGO18, a member of a monocot-specific AGO protein clade, plays a role in antiviral defense by sequestering miR168 and is induced in virus-infected tissues (Wuet al., 2015). The expression level of maize *AGO18a* gene, a homolog of rice *AGO18*, is almost undetectable in maize leaves (Zhai, L. et al., 2014). The accumulation of *AGO18a* mRNA is significantly induced after viral infections, especially in MCMV and SCMV + MCMV co-infected maize plants (Zihao et al., 2016). The miR168 level was also found to be up-regulated in SCMV infected maize plants, in addition to the results that *AGO1a*and *AGO1b* mRNAs. This suggests that miR168 could be sequestered by AGO18a as reported previously (Wu, J. *et al.*, 2015). Interestingly, the accumulation of miR168 had no obvious change in MCMV or SCMV + MCMV infected maize plants in which the *AGO1a*, *b*, *c* mRNAs are not induced, suggesting that the significantly induced *AGO18a* might be involved in antiviral defense by other modes of action, such as influencing the function of other miRNAs associated by AGO1832 (Xia et al., 2016). However, the antiviral roles of AGO18 remain to be elucidated in maize plants, especially in MCMV and SCMV + MCMV co-infected maize plants.

2.3.6 Recombinant nature of Potyviruses

A sequence comparison reveals that positive-strand RNA viruses not only evolve by divergence from common ancestors but also by inter-viral recombination. A

considerable number of these viruses, exemplified by the family *Potyviridae*, can in fact be regarded as successful products of a number of recombination events.

Intra-species recombination is important in *Potyviridae* evolution. NGS data has demonstrated the widespread occurrence of recombination amongst RNA viruses, but it appears to be particularly common amongst the *Potyviridae* (Chare and Holmes, 2006; Sztuba-Soli´nska et al., 2011). Recombination is again thought to occur by template switching. Work on the distribution of recombination sites in the *Bromoviridae* virus, *Brome mosaic virus*(BMV), showed that recombination clustered in areas of alternating GC-rich and AU-rich sequences, which was suggested to promote dissociation of the RdRP from the initial template due to the weaker AU base pairing (Nagy and Bujarski, 1998; Sztuba-Soli´nskaet al., 2011).*Potyviridae* recombination has not been experimentally proved but analyzing the sequences around breakpoints in the potyvirus *Turnip mosaic virus*(TuMV) showed that recombination sites typically also had GC-rich regions upstream and AU-rich regions downstream (Ohshima et al., 2007). In addition to extensive intra-specific recombination, inter-specific recombination may have also played a role in *Potyviridae* evolution, for example in duplication and diversification of *Potyviridae* P1 proteins (Valli et al., 2007).

2.3.7 Transmission of the viruses causing MLN

The maize plant is susceptible to MLN from seedling to mature plants. Transmission of this disease is largely mechanical, or by either insect vectors and/or seed-borne. Mechanical transmission includes the movement of people, machinery, and animals through an infected field. Although seed transmission exists, it has been shown to be very low, with that of MCMV being 0.04% and that of the potyviruses e.g. MDMV being 0.03% (Mikel et al., 1987). Therefore there is need for implementation of phytosanitary regulations (Flett and Mashingaidze, 2016) to limit the spread of the virus. However, there is no data on the rate of transmission of these MLN viruses in the tropical regions of the world. The rapid spread of MCMV across the East African region points to higher rates of transmission through seed.

The vectors which transmit MCMV and any of the associated potyviruses are all associated with MLN (Nyasani et al., 2014). Some of the vectors associated with the transmission of MCMV are thrips such as the maize thrips (*Franklinealla williamsi*), onion thrips (*Franklinealla schultezei*), the common blossom thrips (*Franklinealla tabaci*), maize flea beetle (*Chaetocnema pulicaria*), southern maize rootworm (*Diabrotica undecimpunctata*), northern maize rootworm (*D. lonimaizeis*), western maize rootworm (*D. virgifera*), flea beetle (*Systena frontalis*) and cereal leaf beetle (*Oulema melanopa*). The Potyviruses are primarily spread by aphids. Other insect pests thought to transmit potyviruses include; *Peregrinus maidis, Sardia pluto, Empoasca solana,Adoretus sinicus* and *Tetranychus sp* (Flett and Mashingaidze, 2016; Mahuku et al., 2015a).

The disease is also thought to be transmitted through the soil and infected plant debris because the virus can survive in infected plant residues hence continuous planting of maize in the same field increases chances of occurrence of both the disease and the vector (Scheets 2004).

2.3.8 MLN symptoms

MLN infected plants show an array of symptoms characteristic of a viral disease (Jardine, 1998; Doupnik, 1994). A chlorotic bright green-yellow mottle on young whorl leaves (Plate.2.1A) which begins from the base to the tips, either mild or severe mottling of leaves, stunting and premature aging of plants, necrosis on the leaf margins which progresses to the midrib and eventually to the whole leaf, necrosis on young leaves in the whorl before expansion which leads to the "dead heart" symptom. Plate 2.1B shows healthy maize plants while Plate 2.2 shows symptoms of MDMV, MCMV, SCMV and MLN.

Plate 2.1A: Leaf mosaic with fine, chlorotic, longitudinal yellow streaks parallel to leaf veins. Plate 2.1B: Clean maize plants with no symptoms of MLN. Photo LM Suresh – CIMMYT, 2017.

Plate 2.2: Symptoms of MDMV, MCMV, SCMV and MLN. Photo LM Suresh – CIMMYT, 2017.

2.3.9 MCMV Host range

MCMV is known to be restricted to the *Poaceae (Graminae)* family with maize as the main natural host (Gordon et al., 1984). There are some unpublished reports which suggest that MCMV is found in other grasses namely *Cyperus rotundus* in the *Cyperaceae* family. *Brachiaria brizantha* harbours either MCMV or SCMV separately (Kusa et al., 2015). SCMV is also restricted to the *Poaceae* family. Louie (1980) found SCMV in *Digitaria abyssinica, Digitaria velutina, Cynodon dactylon and Setaria verticillata* However, samples of *Setaria verticillata* collected in Makueni County tested negative for both MCMV and SCMV. *Panicum maximum* tested positive for

MCMV in Hawaii (Nelson et al., 2011). Recently, sugarcane has been found to host MCMV naturally (Wang et al., 2014). SCMV has also been reported in Napier grass in Kenya (Louie, 1980) Sorghum *(Sorghum bicolor)* and finger millet *(Eleusine coracana)* constitute cereal crops that serve as alternative hosts of both MCMV and SCMV either dually or singly. *Sorghum bicolor* was susceptible to MCMV-K by mechanical inoculation (Bockleman, 1982). Sorghum is also a host of SCMV (Louie, 1980). Table 2.2 below shows MCMV host plants documented in the last 33 years.

Table 2.2: Host Plants of MCMV.

Common name	**Botanical name**	**Country of identification**	**Natural infection**	**Reference**
Johnsongrass	*Sorghum halepense*	Spain	Yes	Achon et al., (2017)
Finger millet	*Eleusine coracana*	Kenya	Yes	Kusia et al., (2016)
Sorghum	*Sorghum bicolor*	Kenya	Yes	Mahuku et al., (2015)
Kikuyu grass	*Panicum clandestinium*	Kenya	Yes	Mahuku et al., (2015)
Sugarcane	*Saccharum spp*	Kenya	Yes	Mahuku et al., (2015)
Napier grass	*Pennisetum purpureum*	Kenya	Yes	Mahuku et al., (2015)
Proso millet	*Panicum miliaceum*	NA	No	Mahuku et al., (2015)
Foxtail millet	*Setaria italica*	NA	No	Mahuku et al., (2015)
Beard grass	*Andropogon scoparius)*	NA	No	Bockelman (1982)
Cheat grass	*Bromus japonicus*	NA	No	Bockelman (1982)
Cheat grass	*Bromus secalinus*	NA	No	Bockelman (1982)
Cheat grass	*Bromus tectorum*	NA	No	Bockelman (1982)
Blue grama	*Bouteloua gracilis*	NA	No	Bockelman (1982)
Prairie sandreed	*Calamovilfa longifolia*	NA	No	Bockelman (1982)
Purple crabgrass	*Digitaria sanguinalis*	NA	No	Bockelman (1982)

Sand lovegrass	*Eragrostis trichodes*	NA	No	Bockelman (1982)
Little barley	*Hordeum pusillum*	NA	No	Bockelman (1982)
Autumn millet	*Panicum dichotomiflorum*	NA	No	Bockelman (1982)
Proso millet	*Panicum miliaceum*	NA	No	Bockelman (1982)
Japanese bristlegrass	*Setaria faberi*	NA	No	Bockelman (1982)
Green foxtail	*Setaria viridis*	NA	No	Bockelman (1982)
Prairie cordgrass	*Spartina pectinata*	NA	No	Bockelman (1982)
Bread wheat	*Triticum aestivum*	NA	No	Bockelman (1982)

Key: Yes – infection under natural conditions No- not infected under natural conditions (in which case natural=no), NA- Not known where the host was first identified.

Source: Plantwise Knowledge Bank

2.3.10 Plant Resistance for MLN

Maize has been the only known natural host plants of MCMV until the virus was identified in sugarcane and even more recently in sorghum (Huang et al., 2016; Wang et al., 2014). However, the host range for this disease is restricted to the family *Poaceae*, with maize as the main host. Since there is no known effective control and cure for this disease yet, the use of tolerant varieties/cultivars remains the most effective means of managing MLN. Indeed, there exists tropical maize line that are tolerant to MLN. Research and screening programs by the International Maize and Wheat Improvement Centre (CYMMIT) and the Kenya Agricultural Livestock and Research Organisation (KALRO) have identified several inbred lines which have shown tolerance to this disease (CGIAR Research Program (CRP) on Maize, 2013). Further research has led to the development of highly resistant single cross hybrids which have the potential to be used as female parent in breeding programs in Kenya (Beyene et al., 2017). The use of Genome wide selection (GWS) as a breeding tool in maize has enabled the identification of quantitative trait loci (QTLs) which could be associated with resistance to MLN (Gowda et al., 2015). Using GWAS, 615 tropical and subtropical maize lines were evaluated to develop the genetic architecture towards

resistance to MLN, from which 24 Single Nucleotide Polymorphisms (SNPs) were identified and associated with resistance to MLN (Gowda et al., 2015).

2.3.11 Diagnostics Methods for MLN Causing Viruses

There have been many methods developed to detect and identify plant viruses. Although single diagnostic techniques could sometimes provide adequate information to identify a certain viral disease, a combination of techniques is always necessary if a detailed diagnosis is required. In Africa, most cases of MLN have been reported to be the synergistic activity of both MCMV and SCMV, most diagnostic techniques and protocols for MLN have focused on these two viruses. In order to prevent introduction of MCMV through international exchange of maize seeds and MCMV transmission, there is an urgent need for a reliable and sensitive assay for detection of MCMV and SCMV. Many kinds of assays have been reported for detection of MCMV, such as biological indexing (Uyemoto, 1983), ELISA (Wu et al., 2013), electron microscopy (Moraleset al., 1999), surface plasmon resonance (Zeng et al., 2013) and biosensor based on a quartz crystal microbalance (Huang et al., 2014). In many countries where MLN is prevalent and where there is possibility of MCMV introduction, continuous monitoring of the virus through testing seed is vital. The two major techniques have been employed by Sanitary and Phytosanitary regulators are the serological technique enzyme linked immunosorbent assay (ELISA) and the molecular technique reverse transcription-polymerase chain reaction (RT-PCR). DAS-ELISA for MCMV has been developed by Bioreba and Agdia which are two of the leading pathogen diagnostics companies (Wu et al., 2013). DAS ELISA for MCMV is currently being used for monitoring MCMV in farms, seed fields and in seed lots for countries with MLN like China, Taiwan (Quwen et al., 2013) and all the eastern Africa countries that have MLN (KEPHIS Annual reports 2013 and 2014). Conventional RT-PCR has been used for a while in the successful detection of MCMV in maize seeds. A real-time TaqMan RT-PCR procedure for efficient detection of MCMV was developed by Zhang et al. (2010). The sensitivity of the method was 25 copies of RNA transcripts, which was approximately ten-fold higher than conventional RT-PCR gel electrophoresis method. The choice of the technique depends on a number of factors, including the availability of financial resources, access to laboratory facilities and reagents, the availability of expertise and skills, the level of specificity/sensitivity required, the number of samples

to be tested, availability of information on the virus to be tested, and the time required to complete the test (CIMMYT, 2015).

ELISA technique is the most commonly used when one wants to test large numbers of samples in a short time because it's relatively simple, economical with reagents, fast, adaptive and sensitive. The use of this technique is based on the availability of the antibodies that recognise and bind to specific antigens (Tonuttia et al., 2004). The sensitivity and reliability of an ELISA assay depends on the quality of antibodies, the handling, preparation and storage of reagents, the incubation period and temperatures, the type of sample selected and the use of suitable extraction buffers (Schrijver and Kramps, 1998). There are several commercially available ELISA kits for the detection of SCMV and MCMV, most of which use the double antibody sandwich (DAS) technique (Clark and Adams, 1977).

The more sensitive RT-PCR involves the amplification of the viral RNA. In this technique, the RNA genome of the virus (either MCMV or SCMV) is reverse transcribed into DNA using a reverse transcriptase. Thereafter, primers hybridize to the target sequences and Taq DNA polymerase amplifies segments during repeated PCR cycles. The resulting PCR products are fractionated and visualised on an agarose gel. For MCMV, the forward primer 5′-ATGAGAGCAGTT GGGGAATGCG-3' and reverse primer 5′-CGAATCTACACACACACACTCCAGC -3' have been used and produce an amplicon of about 550bp. To detect SCMV, the forward primer 5′-GCAATGTCGAAGAAAATGCG-3' and reverse primer 5′-GTCT CTCACCAAGAGACTCGCAGC-3' have been used produce an amplicon of about 900bp. Although both techniques are accurate and reliable, the RT-PCR technique is better but care must be taken when using this technique with seed samples since seeds contains factors that may reduce the sensitivity of RT-PCR assays (Mahuku et al., 2015).

2.3.12 Control and Management of MLN

To appropriately manage and control MLN, many approaches can be combined and employed together simultaneously. For example, there is need to adequately control the insect vectors using the appropriate insecticides, employ proper cultural practises like crop rotation to eliminate alternative hosts for these vectors and at the same time

identify and plant maize varieties that are tolerant to this disease. These approach has proven to be quite effective in the control of MLN in Hawaii and central United States (Nelson, Brewbaker, and Hu, 2011). It is however not yet known the best combination of factors involving vector control strategies and cultural/agronomic management practices that would result in the best management strategy for MLN in Africa. It is thought that an intensive vector management system as well as an intensive application of proper cultural practices could play a great role towards achieving seeds free from MLN. The challenge to this hypothesis however especially in Eastern Africa is that small holder farmers in this region interplant maize with other crops and there is a shortage of resources and knowledge on vector control as well as cultural management practises (Mahuku et al., 2015).

General management control mechanisms for other viral diseases in maize and other crops could be employed to assess their impact towards controlling MLN. These include the following: certain agronomic practices including early planting, introducing a maize free period in the planting cycle and crop rotation, enactment of proper phytosanitary practises to control seed production. and movement, treatment of seeds, and ensuring maize fields properly weeded to eliminate alternative hosts for insect vectors that transmit the disease.

2.3.13 Molecular characterization of viruses

Molecular characterization involves analysis of genetic, hereditary and molecular differences, predominately in DNA/RNA sequences, to gain information on an organism's evolutionary relationships (Fleckenstein, 2004). From these analyses, it is possible to determine the processes by which diversity among species has been achieved. The result of a molecular phylogenetic analysis is expressed in a phylogenetic tree. Molecular phylogenetics is one aspect of molecular systematics, a broader term that also includes the use of molecular data in taxonomy and biogeography.

Molecular evolution is the process of selective changes (mutations) at a molecular level (genes, proteins, etc.) throughout various branches in the tree of life (evolution). Molecular phylogenetics makes inferences of the evolutionary relationships that arise

due to molecular evolution and results in the construction of a phylogenetic tree (Hillis and Moritz, 1996).

Hundreds of plant viral diseases have been described and apparent new ones continue to be reported. Often the causal agents are described as new viruses on totally inadequate grounds. There are 2284 virus and viroid species distributed amongst 349 genera, 19 sub families, 87 families and 6 orders worldwide (International Committee for Taxonomy of Viruses (ICTV) 9thReport ,2011). There are also unassigned viruses which are probably representatives of new genera and/or families. When an unknown virus disease is being investigated, it has to be determined if the virus (or viruses) concerned is identifiable with any that are already known. This 'diagnostic phase', in some instances may not need to go beyond determination of particle morphology to determine the group to which the virus belongs, followed by an appropriate serological test to identify the virus. Molecular characterization has become an important tool for the analysis of viruses including their classification.

Using sequence analysis of complete nucleotides of coat protein gene, it is possible to differentiate plant viruses into isolates, species and strains (Padidam *et al*., 1995). The criteria utilized to distinguish between plant viruses as per International Committee on Taxonomy of Viruses (ICTV) are 90-100% for isolates, 80-90% for strains and at 89% for species demarcation for DNA/RNA component (Fauquet et al., 2003). The complete sequences obtained can be used to develop specific primers for molecular based diagnostics protocols for the virus in question.

CHAPTER THREE

OCCURANCE AND DISTRIBUTION OF MAIZE LETHAL NECROSIS DISEASE CAUSING VIRUSES IN KENYA

3.0 Abstract

Maize Lethal Necrosis occurs in maize growing counties of Kenya. However, information of the counties where it occurs, their incidences, prevalence and symptom severity is not adequate. In order to assess the occurrence and distribution of viruses that cause MLN in Kenya, a countrywide survey was carried out in 2015 and 2016. Farms with maize crop were visited where the presence of MLN was determined based on the symptom's identification. The farms Global Positioning System (GPS) coordinates were recorded, MLN incidence assessed as the percentage of infected plants in a farm and MLN prevalence as the percentage of farms in each county that had MLN infected maize plants. MLN symptom severity was assessed based on the MLN severity scale. The component causative viruses for MLN were determined by RT-PCR using virus-specific and group-conserved primers and DAS-ELISA. The MLN distribution map was generated from the RT-PCR and DAS-ELISA testing based on the GPS coordinates. From the survey conducted in 2016/2017, MLN incidence was found between 35 – 90%, prevalence between 44 – 72% and symptoms severity between 1.7 – 4.1 countrywide. Laboratory diagnosis of the samples collected during the survey identified MCMV and SCMV as the major viruses causing MLN in Kenya. There were no other potyviruses identified that co-infects maize with MCMV in the country as suggested by some previous studies. The two MLN viruses occur in all the maize growing regions at varying levels of incidence, prevalence and severity.

3.1 Introduction

Maize is one of the most important crops in Eastern Africa. It is a staple food crop as well as a commercial enterprise in Kenya and a staple food crop in Tanzania and Rwanda. About 90% of the Kenyan population depends on this crop directly or indirectly in terms of food, labour, and income. The national maize consumption per capita is 98 kg-100kg (Nyoro et al., 2004) with a production of 25,000 tons annually

against a national annual demand of 35,000 tons (Ministry of Agriculture and Livestock Development, 2012).

The recent occurrence of MLN, however, has exacerbated maize yield losses. Since the first report of MLN in Kenya (Wangai et al., 2012), the disease has spread fast and affected up to 300,000 maize farmers in the country (Ministry of Agriculture and Livestock Development, 2012). In western Kenya, more than half of the farmers affected, experienced yield losses of 50%, and models suggested an overall loss of 500,000 metric tons (MT) or about 22% of total maize production in 2016 (De Groote et al., 2016).

MLN incidence has however reduced in the country as per yearly surveys by the Kenyan National Plant Protection Organization, Kenya Plant Health Inspectorate Service (MLN survey reports 2016 and 2017). This is due to sustained implementation of interventions on management strategies to farmers and seed growers countrywide.

MLN is caused by double infection of maize plants with *Maize chlorotic mottle virus* (MCMV), a *machlomovirus* (Nutter et al., 1989; Lommel et al., 1991), and *Sugarcane mosaic virus* (SCMV), a *potyvirus* (Jones et al., 2007, 2011; Adams et al., 2012). However, MLN can also be caused by MCMV in synergy with other potyviruses, namely *Maize dwarf mosaic virus* (MDMV) or *Tritimoviruses* like *Wheat streak mosaic virus* (WSMV) (Pruss et al., 1997). Any potyvirus that infects maize can potentially interact synergistically with MCMV to cause MLN (Adams et al., 2012).

Maize Lethal Necrosis has spread fast in the eastern and central Africa region. For instance, in Tanzania, the disease was first reported in 2012 in regions around Lake Victoria and Arusha (CIMMYT, 2012). The government of Tanzania and The Maize and Wheat Improvement Centre (CIMMYT) carried out surveys and tests that revealed the presence of MCMV and SCMV (CIMMYT Periodic Newsletter, Dec 2012). MLN was also reported in Uganda in 2012 in the Kenya border districts of Busia and Tororo (CIMMYT, 2012) and has been detected in eastern Uganda districts Iganga and Mbale (Kagoda et al., 2016). Currently, the disease is present in the western, south western and recently in the northern parts of Uganda (MLN Surveillance report, NACRRI Uganda Nov. 2017, 2018). The disease was officially reported in the Democratic Republic of Congo (DRC) in 2014 (Lukanda et al., 2014). There are reports of MLN

in Southern Sudan (G. Mahuku and A. Wangai, 2015, unpublished results) and Burundi (Ministry of Agriculture, 2017). Burundi's borders Rwanda, Uganda and DRC that are MLN endemic.

Due to the destructive nature of MLN, the rapid spread of the disease continues to negatively affect negatively the livelihoods of many smallholder farmers in Kenya and in the Eastern Africa region and beyond. MLN is a relatively new disease in Africa and indeed MCMV is a new virus to infect maize in Africa (Wangai et al., 2012). The possibility of MCMV to combine with other native potyviruses of cereals poses a big challenge to maize production in this region.

Despite its devastating effects, the precise geographical distribution of the MLN causing viruses, their incidence, prevalence and severity countrywide are not known. Previous MLN distribution studies done in Kenya focused on very few counties mostly for MLN viruses diversity and other viral metagenomics studies. Samples were collected in 17 counties (Mahuku et al., 2015a) but no detailed evaluation on the disease and causative viruses prevalence, incidence and symptoms severity was done. In another study (Mwathi et al., 2018), samples were collected from sixteen counties for Maize viruses metagenomic analysis but no details on the parameters aforementioned. This study therefore focused on detailed evaluation of the geographical distribution of MLN and its causative viruses, the prevalence, incidence and MLN symptoms severity in 27 counties in the country. This includes most counties in the eastern, South rift and coastal areas that are not covered in previous studies. The disease incidences and distribution will allow the MLN-free areas identified for growing of MLN free seed. It is also paramount to determine the evolutionary path of the MLN causing viruses in Kenya.

3.2 Materials and Methods

3.2.1 Survey

A survey was carried out in 27 counties during the maize growing seasons in Kenya in 2015 and 2016. The counties which were surveyed are shown in Figure. 3.1. In each county, individual farms were visited and sampled. A total of 118 farms were visited during the survey. Disease incidence and disease prevalence were determined by the

percentage of the plants showing MLN symptoms in a farm and the percentage of the farms with symptoms of MLN respectively. A disease severity score was recorded using the 1-5 MLN symptom severity scale (CIMMYT, 2013) as illustrated in Figure 3.2. Fields having a maize crop as a pure stand or intercropped with other crops were selected and surveyed along selected routes in each county. The sasme was done for the survey in seed fields in the proposed 11 counties.

An MLN survey protocol was developed and used during the survey activity in the field (Appendix 1). This was administered to farmers during the survey and included questions to address the specific objectives of the study.

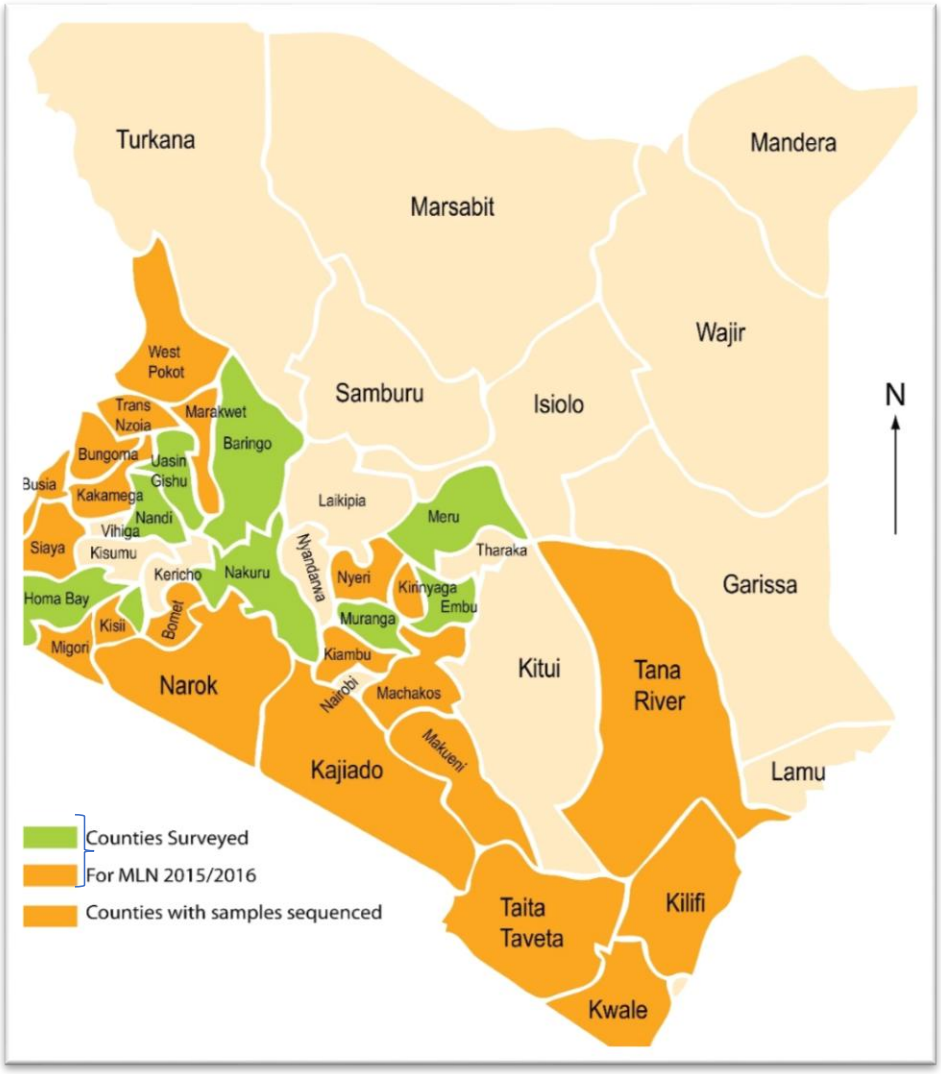


Figure 3.1: Counties surveyed for MLN during 2015-2016 and the counties where sequenced samples were obtained.

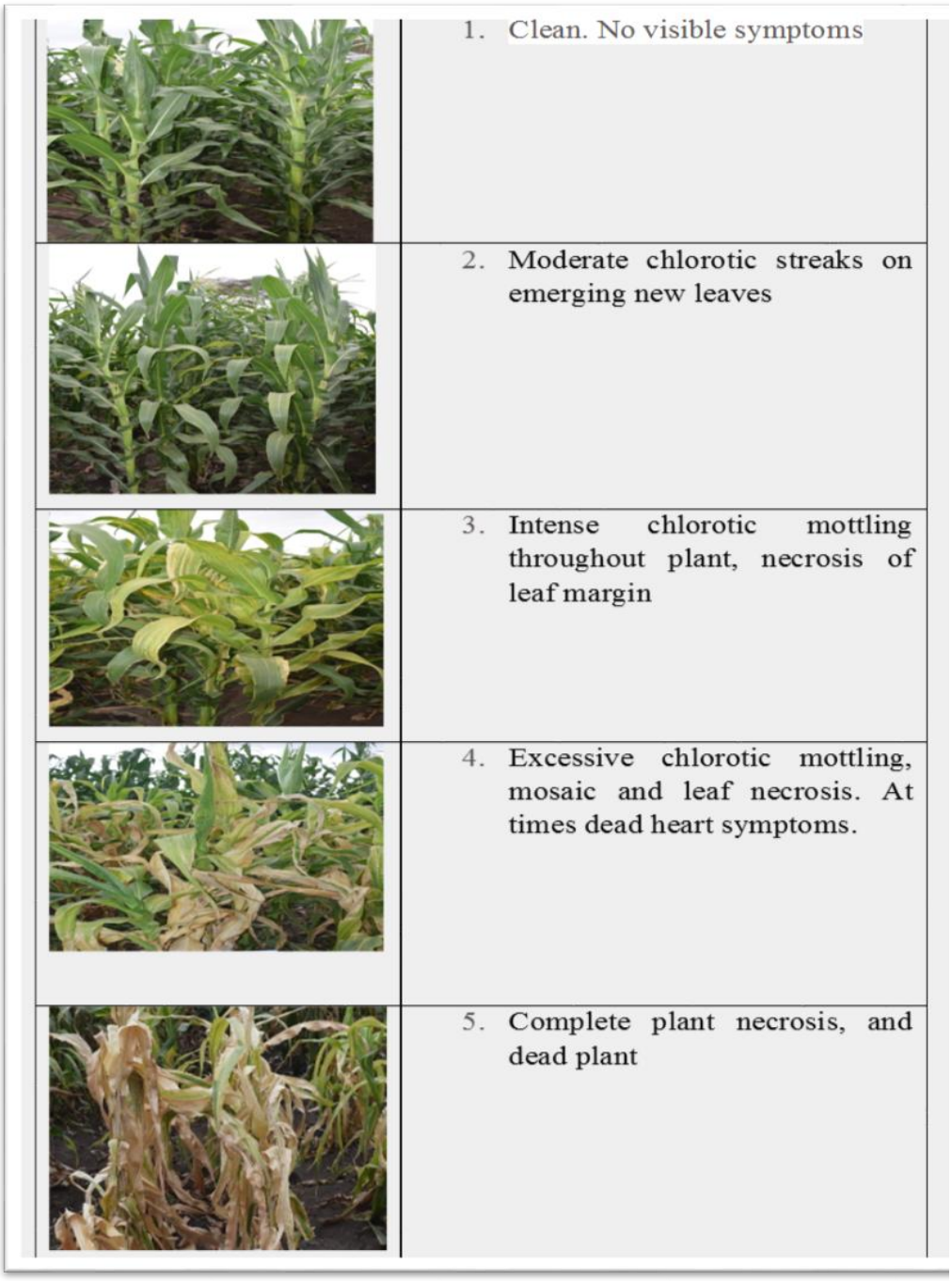


Figure 3.2: MLN severity scale 1-5

3.2.2 Sampling Protocol

The staggered "X" pattern (Fig. 3.2), recommended for most maize fields' inspection and surveys (CDFA Phytosanitary Certification Manual, 1985), was adopted. Maize plants were examined for MLN symptoms along one side of the field then diagonally in a staggered pattern across rows to the far maize plants, and across the far side of the

field and diagonally back to starting maize plants. Five young symptomatic leaves from maize plants were sampled along the transect in each farm. Plants with MLN like symptoms spotted away from the chosen pattern were also included in the five samples collected in each farmer's field. The five samples from each farm were put together to make a composite sample representing that farm. This field inspection pattern ensured that all parts of the farm were adequately and proportionately represented in the plants inspected and sampled.

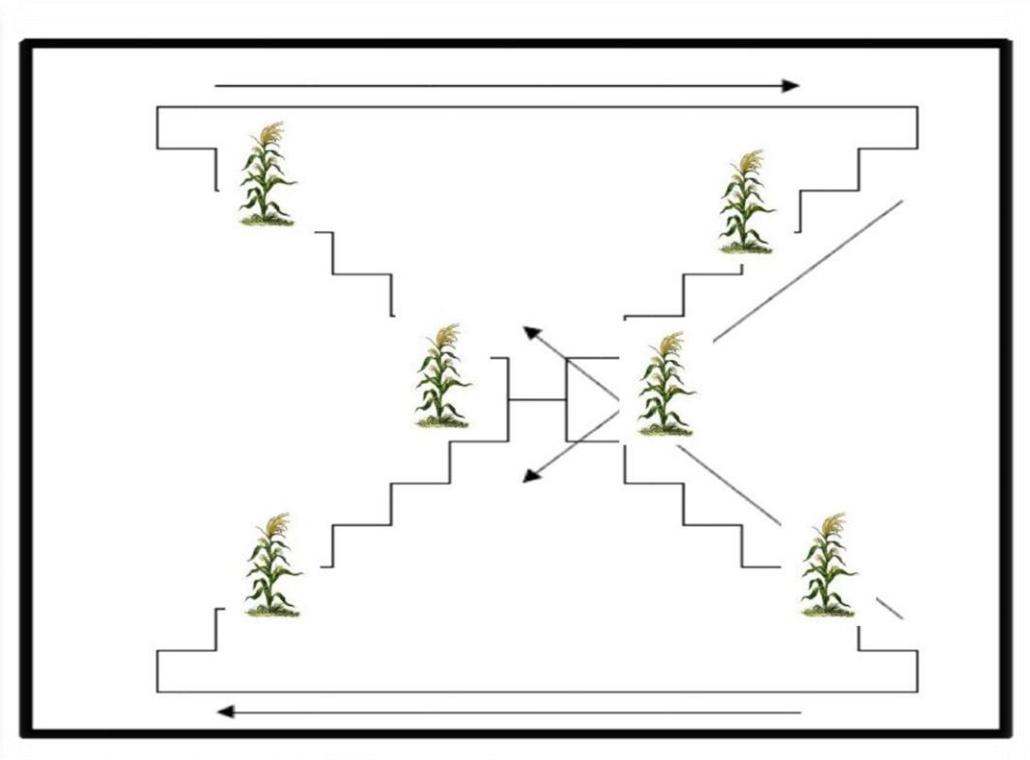


Figure 3.3: Staggered "X" pattern of field inspection and sampling for plants with MLN symptoms with a total of 5 plants sampled in each farm to form a composite sample per farm.

Disease incidence and severity were recorded in each farm surveyed. The incidence was determined by calculating the percentage of the plants in a farm that have clear MLN symptoms. One hundred maize plants were examined along the transect and the number that showed MLN symptoms were determined to calculate the incidence on that farm. The county MLN incidence was derived by the mean of all the farm incidences surveyed in the respective county. The same was used for determining the county severity. MLN symptoms severity was scored using the MLN severity scale of

1-5. The MLN disease severity scale was developed by the International Maize and Wheat Improvement Center (CIMMYT) based on the diseased areas in leaves, the number of diseased leaves in a plant, and plant height was used (Figure 3.2). This allows uniform reporting of the MLN severity across the MLN endemic counties in the country.

The samples were labelled, put in khaki bags, placed in a cool box containing dry ice and transported quickly to the laboratory for storage at -80^0C pending laboratory analysis.

The survey was done for seed fields in 11 counties under this study (Baringo, Nakuru, Taita Taveta, Tana River, Trans Nzoia, Elgeyo Marakwet, Machakos, Makueni, Meru, Embu and Uasin Gishu). The sampling protocol was the same as for farmers' fields outlined.

3.2.3 Survey Data Analysis

The data collected in the field as per the questionnaire during the survey of the farmers' fields and subsequently analysed. The data included the diseases incidence, prevalence and symptom severity (Table 1). The average of the figures for incidence, prevalence and severity from farms surveyed in each county were used to represent the county. Statistical analysis (Analysis of Variance) was done on all the parameters i.e. incidence, prevalence and severity under investigation for MLN in the survey using Statistical Package for the Social Sciences (SPSS), a statistical data analysis software. Mean separation was done using the t-Test.

3.3 Detection of MLN viruses in collected samples

3.3.1 Ribonucleic acid extraction

RNA was extracted from the 118 composite samples using the ZR RNA MiniPrep™ kit (ZYMO RESEARCH CORPORATION, Irvine, CA, USA) following manufacturer's recommendations.

Concentration of RNA in the samples was checked by the Qubit quantification platform (Thermo Fischer Scientific, Wilmington, USA).

The overall quality of RNA extracted was assessed by electrophoresis on a denaturing agarose gel. The denaturing gel electrophoresis was prepared as follows; Tris-acetate-EDTA (TAE) 1X buffer was diluted from the 10X stock in sterile distilled water. The 10X TAE(IL) contains 900 mls dH_2O, 48.4 g Tris base (Triz), 11.4 mls glacial acetic acid and 3.72 g EDTA topped up to 1L with dH_2O. To make a 1.2% agarose gel, 1.2 g of the agarose powder was added to 100 mls of 1X TAE buffer and heated gently in the microwave to dissolve. The gel was then cooled to 50^oC. Thereafter 3 µl of the gel red staining dye (Biotium, Inc. Fremont, CA 94538) was added and stirred to mix. The gel was then casted on the trays with the combs inside to make the wells.

RNA was prepared by taking 5µl of RNA, 4µl of formaldehyde and 1ul of the RNA loading dye. Each sample had 10µl of the reaction volume. Care was taken when using formaldehyde for it is toxic through skin contact and inhalation of vapours. The mixture was incubated in a thermocycler at 65^oC for 5 minutes. The samples were then removed and quickly placed on ice to stop the reaction.

The samples were then loaded in the prepared 1.2% agarose gel and run for 60 mins. The characteristic RNA 18S and 28S bands on the RNA gels were evaluated to ascertain the RNA quality prior to subsequent analyses. DNASe digestion was done on all the samples to ensure that the RNA was not contaminated by traces of DNA.

3.3.2 RT - PCR for MCMV and DAS-ELISA for SCMV

cDNA synthesis

cDNA was synthetized using the Maxima First Strand cDNA Synthesis kit (Thermo Fischer Scientific, Wilmington, USA). The kit contains oligo(dT)18 and random hexamer primers to prime synthesis of first strand cDNA. This primer mixture ensures high sensitivity in low copy number transcript detection assays. The reaction mixture contained 4 µL of 5X reaction Mix, 2 µL Maxima Enzyme Mix, 1 µL of the RNA template and 13 µL nuclease-free water. The reaction solution was mixed gently and centrifuged. The reaction mixture was then incubated in the thermocycler for 10 mins at 25^oC followed by 15 mins at 50^oC. The reaction was terminated by heating at 85^oC for 15 mins. The cDNA product was then stored at - 20^oC for further use in the PCR for virus detection.

3.3.3 RT - PCR for MCMV

Reverse transcription-polymerase chain reaction (RT-PCR) was conducted as described by Chen and colleagues (Chen et al. 2005). A two-step RT-PCR for MCMV was done on all the samples to ascertain the presence of MCMV. The MCMV primers used were (MCMV F 5' – CCG GTC TAC CCG AGG TAG AAA – 3' and MCMV R 5' – TGG CTC GAA TAG CTC TGG ATT T – 3') designed by USDA – Research unit at OARDC, Ohio State University (OSU) The product amplicon is195bp. Detection of SCMV was done by Double Antibody Sandwich – Enzyme Linked Immunosorbent Assay (DAS-ELISA) using the commercial SCMV antiserum (Agdia, Elkhart, IN, USA) according to manufacturer's instructions.

3.4 Results

3.4.1 Distribution of MLN and its causative viruses in Kenya

The distribution of the MLN causative viruses was noted in all the 27 surveyed counties, the major maize growing counties in the country. MCMV was present as indicated in Figure 3.4 in all the counties surveyed. The same was noted with SCMV, which was evident in all the counties growing maize as illustrated in Figure 3.5. MLN is distributed in all maize growing areas of the country. This was noted in all samples that tested positive for both MCMV and SCMV and mapped out as shown in Table 3.1 and Figure 3.6. However, several samples had either MCMV or SCMV alone and no visible symptoms of MLN.

Table 3.1: MLN surveillance points, incidence, Prevalence and symptom severity 2015-2016

Farm	County	GPS coordinates	Incidence (%)	Prevalence (%)	Symptom Severity (0-5)	MCMV	SCMV
1	Kiambu	S-01.12654 E03980.564	49	54	2.1	+Ve	+Ve
2	Kiambu	S-01.13799 E03637.864	35	45	1.8	+Ve	+Ve
3	Kiambu	S-0128008 E03781575	48	45	2.1	+Ve	+Ve
4	Kiambu	S-0088420 E-033.4532	-	-	-	-Ve	-Ve
5	Kirinyaga	S- 0072643 E-03727301	56	62	2.2	+Ve	+Ve
6	Kirinyaga	S.56816 38 E037.317439	68	72	2.8	+Ve	-Ve
7	Kirinyaga	S-00659924 E037.25740	52	62	1.8	+Ve	+Ve
8	Kirinyaga	S-046.5526 E0347.3577	65	68	2.3	+Ve	+Ve
9	Meru	S-0049803 E-037.56648	34	42	2.3	+Ve	-Ve
10	Meru	S-00.02188 E-037.57830	45	51	1.8	+Ve	+Ve
11	Meru	N-005.809 E-037°39.77	56	58	1.7	+Ve	+Ve
12	Meru	N00.13356 E37.66201	24	45	2.2	+Ve	+Ve
14	Embu	S29.405 29 E037.364630	25	34	1.7	+Ve	-Ve
15	Embu	S-00.46804 E037.53638	-	-	-	-Ve	+Ve
16	Embu	S-00.49464 E037.46553	45	54	2.4	+Ve	+Ve
17	Embu	S-00.62358 E037.53022	44	55	2.2	+Ve	+Ve

18	Machakos	S-01.14748 E037.52370	-	-	-	-Ve	-Ve
19	Machakos	S-01.23277 E037.46314	55	48	1.6	+Ve	+Ve
21	Machakos	S-01.34111 E037.44740	58	56	2.1	+Ve	-Ve
22	Machakos	S-01.45982 E037.42378	-	-	-	-Ve	+Ve
23	Machakos	S-01.70483 E037.35184	-	-	-	-Ve	-Ve
24	Machakos	S-01.25644 E037.56790	56	35	1.8	+Ve	+Ve
25	Makueni	S-01.78288 E037.28856	48	54	2.2	-Ve	-Ve
26	Makueni	S-01.76670 E037.26326	68	45	1.9	+Ve	+Ve
27	Makueni	S-01.94253 E037.53283	58	44	1.8	+Ve	+Ve
28	Makueni	S-02.14564 E037.58229	52	58	2.4	+Ve	+Ve
29	Makueni	S-02.25091 E037.78274	-	-	-	+Ve	+Ve
30	Makueni	S-02.44998 E037.97371	-	-	-	-Ve	-Ve
31	Kajiado	S-01.09909 E-035.84049	75	68	2.8	+Ve	+Ve
32	Kajiado	S-01.04835 E-035.84034	88	66	2.4	+Ve	+Ve
33	Kajiado	S-02.93353 E037.53585	-		-	-Ve	-Ve
34	Kajiado	S-02.96304 E037.57605	85	63	3.3	+Ve	+Ve
35	Kajiado	S-02.96304E037.57605	90	68	4.2	+Ve	-Ve
36	Kajiado	S-02.96304 E037.57605	-	-	-	-Ve	+Ve
37	Kajiado	S-02.98787 E037.56916	95	85	4.8	+Ve	+Ve

38	Bomet	S-00.10712 E-034.52663	78	67	4.2	+Ve	+Ve
39	Bomet	S-00.59410 E-035.23077	86	85	3.9	+Ve	+Ve
40	Bomet	S-00.68370 E-035.26849	68	58	3.5	+Ve	-Ve
41	Bomet	S-00.71089 E-035.25032	77	67	3.2	+Ve	+Ve
42	Bomet	S-00.76239 E-035.2311	95	76	4.5	+Ve	+Ve
43	Bomet	S-00.79677 E-035.19434	-	-	-	-Ve	-Ve
44	Bomet	S-00.77725 E-035.38800	78	68	3.9	+Ve	+Ve
45	Bomet	S-00.77734 E-035.28803	85	79	3.4	+Ve	+Ve
46	Bomet	S-00.69646 E-035.33325	68	60	2.9	+Ve	+Ve
47	Bomet	S-00.64187 E-035.28678	76	65	2.8	+Ve	+Ve
48	Bomet	S-00.64242 E-035.28725	-	-	-	-Ve	-Ve
49	Bomet	S-00.83663 E-035.35748	85	76	3.3	+Ve	+Ve
50	Narok	S-00.95766 E-035.41281	80	72	3.8	+Ve	+Ve
51	Narok	S-00.95800 E-035.41262	78	68	3.1	+Ve	-Ve
52	Narok	S-00.99056 E-035.58024	-	-	-	-Ve	-Ve
53	Narok	S-01.02521 E-035.68428	69	62	2.6	+Ve	+Ve
54	Narok	S-01.02524 E-035.68423	76	74	3.1	+Ve	+Ve
55	Narok	S-01.17893 E-034.65644	52	45	2.1	+Ve	+Ve
56	Narok	S-01.09905 E-035.84048	86	65	2.7	+Ve	+Ve
57	Narok	S-01.09909 E-035.84049	-	-	-	-Ve	-Ve
58	Narok	S-01.04835 E-035.84034	62	72	3.2	+Ve	+Ve

59	Narok	S-01.16078 E-034.64253	55	48	2.3	+Ve	+Ve
60	Nakuru	S-00.16596 E-036.18067	55	48	2.3	+Ve	+Ve
61	Nakuru	N00.03903 E 036.22986	53	43	2.1	-Ve	-Ve
62	Nakuru	N00.00568 E 036.22449	68	55	2.7	+Ve	+Ve
63	Nakuru	S0029.5346 E036.67343	35	48	1.2	+Ve	-Ve
64	Nakuru	S00.62992 E 035.6324	45	51	1.9	+Ve	+Ve
65	Nakuru	S0019.671 E 03603.650	45	42	1.5	+Ve	+Ve
66	Nakuru	S0029.546 E03624.608	35	45	1.8	+Ve	-Ve
67	Taita -Taveta	S03.24696 E037.76369	65	65	2.7	-Ve	+Ve
68	Taita -Taveta	S03.25243 E037.76385	75	70	3.3	+Ve	+Ve
69	Taita -Taveta	S03.19484 E037.71234	-	-	-	-Ve	-Ve
70	Taita -Taveta	S03.19484 E037.71234	-	-	-	-Ve	-Ve
71	Taita -Taveta	S03.19484 E037.71234	78	59	3.1	+Ve	+Ve
72	Taita -Taveta	S03.26732 E037.73499	72	70	3.1	+Ve	+Ve
73	Kilifi	S03.68076 E039.81532	-	-	-	-Ve	+Ve
74	Kilifi	S03.68076 E039.81532	65	58	2.5	+Ve	+Ve
75	Kilifi	S03.33798 E039.94253	69	59	3.6	+Ve	-Ve
76	Kilifi	S03.69873 E039.85644	63	55	2.5	+Ve	+Ve
77	Kwale	S04.18793 E039.46605	55	62	2.7	+Ve	+Ve
78	Kwale	S04.50467 E039.26941	-	-	-	-Ve	-Ve

79	Kwale	S04.52759 E039.17626	58	65	2.9	+Ve	+Ve
80	Baringo	S01.15481 E036.63848	65	55	2.9	+Ve	+Ve
81	Baringo	N00.42905 E 036.00695	78	67	3.1	+Ve	-Ve
82	Baringo	N00.47466 E 036.01105	76	66	3.1	+Ve	+Ve
83	Baringo	N00.48327 E036.00901	85	72	3.8	+Ve	+Ve
84	Baringo	N00.46709 E 036.08966	73	62	3.2	+Ve	+Ve
85	Nandi	N0.313039 E35.352276	-	-	-	-Ve	-Ve
86	Nandi	N0.16667 E035.09	75	68	2.9	+Ve	+Ve
87	Nandi	N0.1000 E035.09	45	44	2.4	+Ve	+Ve
88	Uasin Gishu	N0.516678 E035.2833	63	56	2.2	+Ve	-Ve
89	Uasin Gishu	N0.824566 E035.38	-	-	-	-Ve	+Ve
90	Uasin Gishu	N0.3900 E035.25	65	55	2.5	+Ve	+Ve
91	Trans-Nzoia	N1.1090 E034.95	-	-	-	-Ve	-Ve
92	Trans-Nzoia	N1.063445 E034.57	68	55	2.5	+Ve	+Ve
93	Trans-Nzoia	N00.96739 E034.84672	86	78	3.8	+Ve	+Ve
94	Trans-Nzoia	N01.03447 E034.82410	77	68	3.3	+Ve	-Ve
95	Trans-Nzoia	N01.03447 E034.82410	73	66	3.3	+Ve	+Ve
96	Trans-Nzoia	N01.03447 E034.82410	69	59	2.7	+Ve	-Ve
97	Siaya	N01.05183 E034.85102	-	-	-	-Ve	+Ve
98	Siaya	N01.05183 E034.85102	45	46	1.8	+Ve	+Ve

99	Siaya	N01.05183 E034.85102	55	52	2.3	+Ve	-Ve
100	Siaya	N01.04621 E034.85925	-	-	-	-Ve	+Ve
101	Siaya	N01.04621 E034.85925	65	55	2.6	+Ve	+Ve
102	Migori	S-01.10855 E-034.47896	56	48	2.1	+Ve	+Ve
103	Migori	S-01.17045 E-034.58256	72	64	3.4	+Ve	+Ve
104	Kisii	S-00.81004 E 034.93539	68	54	2.2	+Ve	+Ve
105	Kisii	S-00.8746 E 034.89989	56	48	2.3	+Ve	+Ve
106	Kisii	S-00.92828 E-034.80853	65	58	2.8	+Ve	-Ve
107	Kisii	S-00.75763 E-034.91269	56	51	2.1	-Ve	+Ve
108	Homa Bay	S-00.37780 E-034.63345	35	33	1.5	+Ve	+Ve
109	Homa Bay	S-00.39365E-034.61610	45	40	1.9	+Ve	+Ve
110	Kakamega	N00.22219 E034.62167	45	31	1.8	+Ve	-Ve
111	Kakamega	N00.22240 E034.62675	65	56	2.9	+Ve	+Ve
112	Kakamega	N00.21878E034.63065	45	38	2.4	+Ve	+Ve
113	Bungoma	N01.06674 E034.83498	65	56	2.1	+Ve	-Ve
114	Bungoma	N01.07150 E034.87053	78	67	3.2	+Ve	+Ve
115	Bungoma	N01.07376 E034.85079	65	60	2.9	+Ve	+Ve
116	Bungoma	N01.07376 E034.85079	46	56	2.0	+Ve	-Ve
117	Bungoma	N01.06533 E034.84172	-	-	-	-Ve	+Ve
118	Bungoma	N01.06533 E034.84172	68	58	2.7	+Ve	-Ve

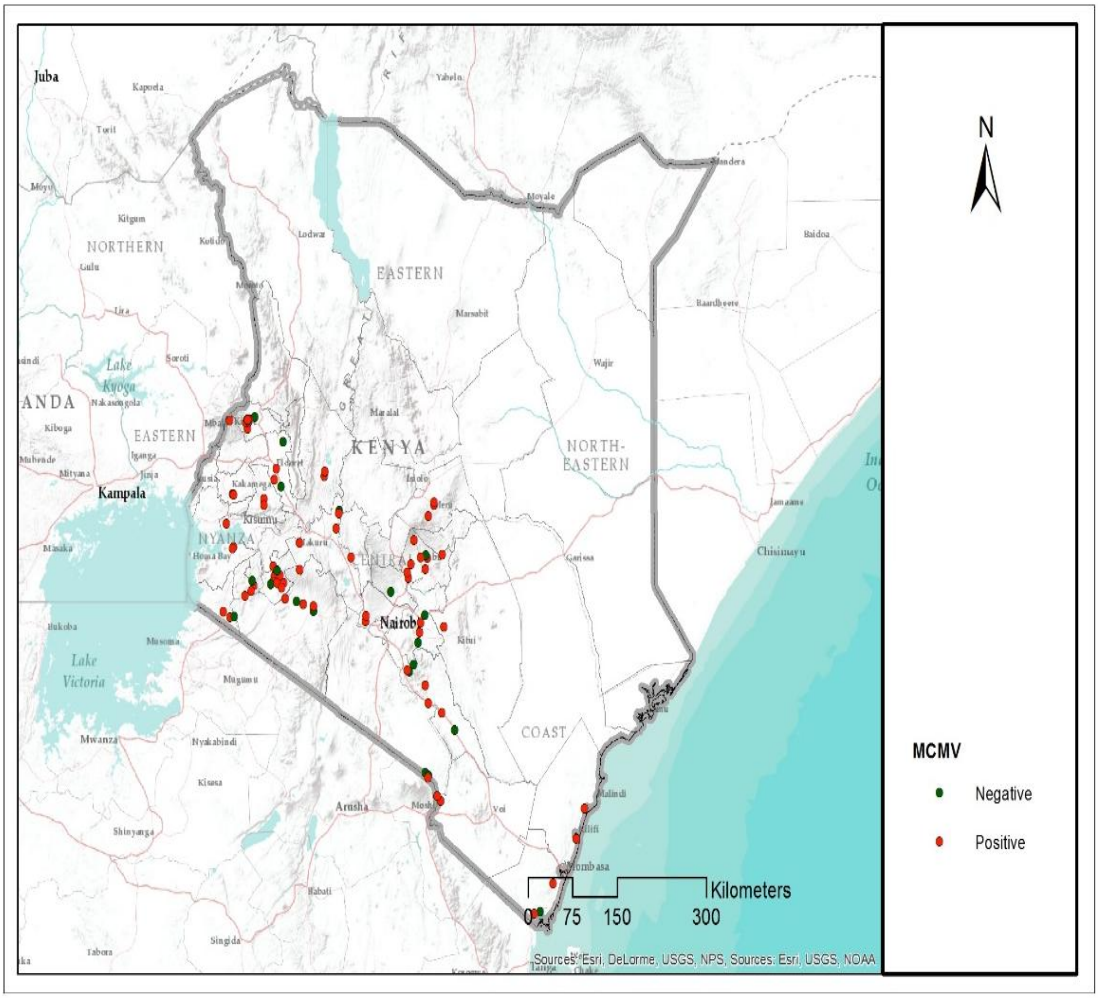


Figure 3.4: Map of Kenya showing the distribution of MCMV; The red points indicate the samples that tested positive while the green those that tested negative for MCMV.

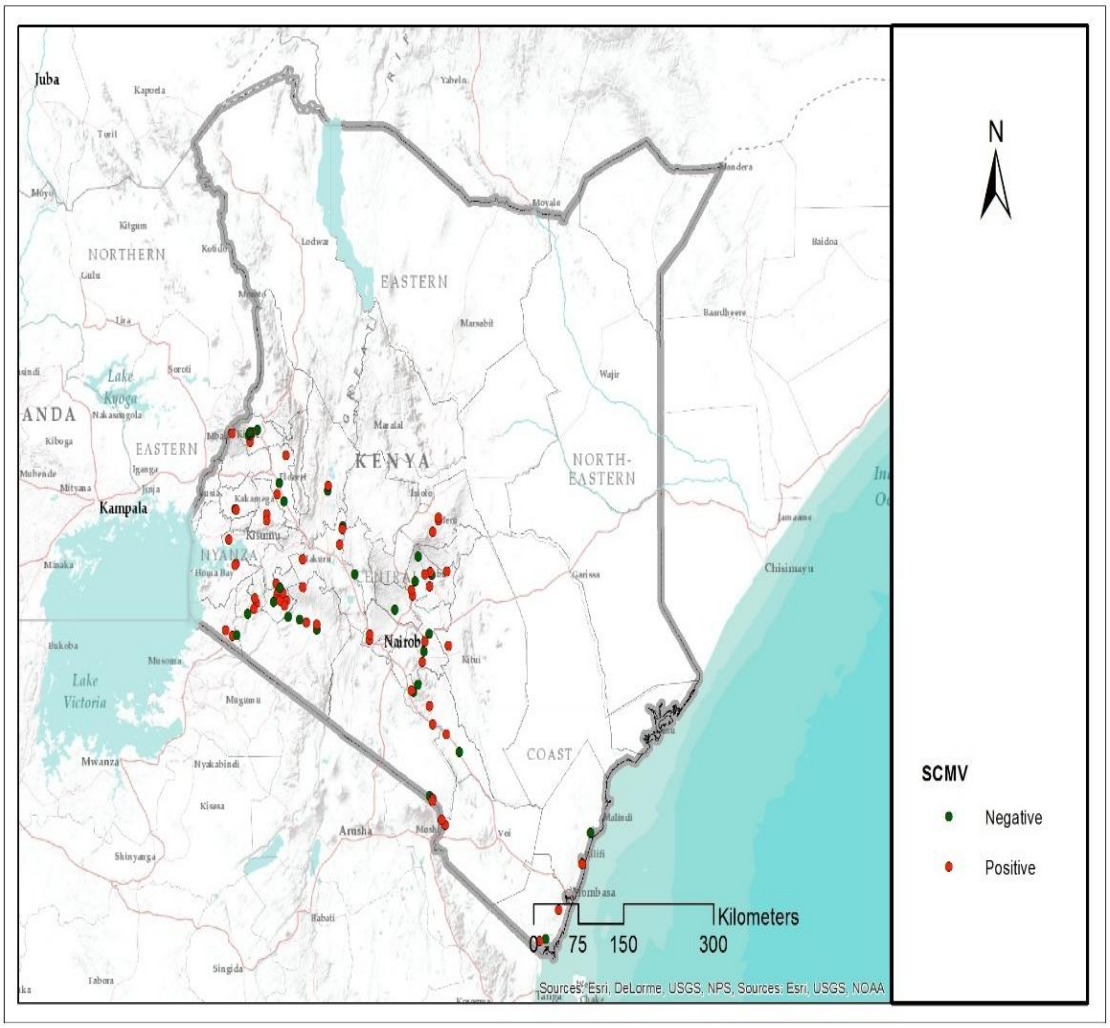


Figure 3.5: Map of Kenya showing the distribution of SCMV; Red points show the positive samples while green points show negative samples for SCMV.

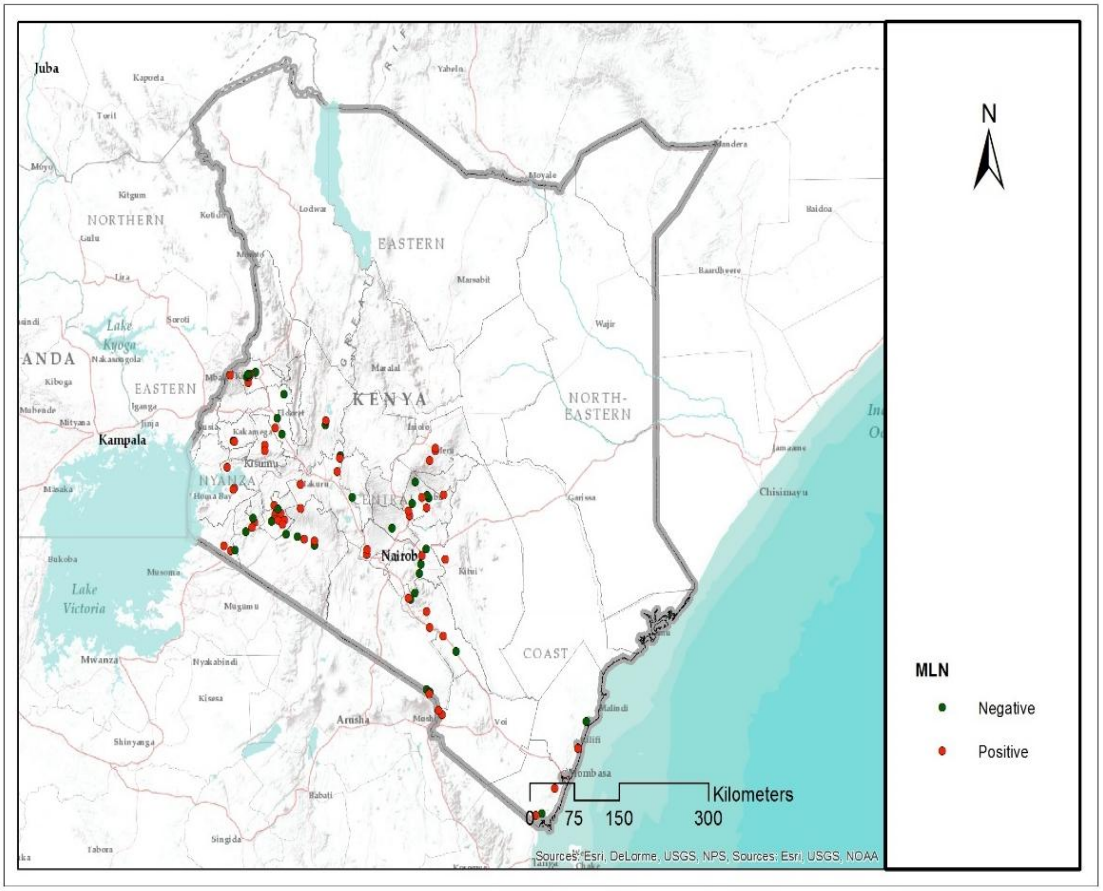


Figure 3.6: Map showing MLN distribution. Red points show the MLN positive samples while the green points show MLN negative samples.

3.4.3 Distribution, incidences, prevalence and severity of MLN in surveyed areas

Maize Lethal Necrosis disease occurs in maize growing regions of Kenya (Figure 3.6). From all the surveyed areas, it was observed that the counties in the south rift region had the highest incidences and symptom severity of MLN as illustrated in Table 3.2, and 3.3 respectively These were Kajiado, Bomet, Narok and Baringo. Disease incidences of between 75% - 90% were recorded in these counties with Kajiado county registering the highest MLN incidence in the country.

The central Kenya counties of Kiambu and Kirinyaga also registered presence of MLN though with lower disease incidence of between 44% - 60% as illustrated in Table 3.2 below.

Table 3.2: Incidences of MLN by counties in farmers' fields for 2015/2016. Data which was in proportions (percentages) was arcsine transformed to meet model assumptions. The difference in the mean level of incidence among the different counties was statistically significant at α=0.05 (F-Value=8.654, P-value=0.00000000000312).

County	Mean	groups
Kajiado	67.88±2.34	a
Bomet	63.64±2.04	ab
Baringo	60.88±2.78	abc
Narok	59.75±2.57	abc
Trans-Nzoia	59.59±2.89	abcd
Taita -Taveta	58.92±1.8	abcde
West Pokot	68.32±2.12	abcde
Bungoma	56.24±1.97	abcdef
Tana River	55.68±1.32	abcdef
Kilifi	54.93±1.22	abcdefg
Uasin Gishu	53.11±0.60	abcdefg
Kisii	52.55±2.13	abcdefg
Marakwet	48.60±0.61	abcdefg
Busia	57.21±1.72	abcdefg
Migori	51.63±3.20	bcdefg
Nandi	51.04±8.93	bcdefg
Kirinyaga	50.95±2.2	bcdefg
Makueni	48.93±1.98	cdefg
Kwale	48.72±0.87	cdefg
Machakos	48.72±0.5	cdefg
Siaya	47.91±5.8	cdefg
Kakamega	45.98±3.86	cdefg
Nakuru	44.77±3.25	defg
Meru	42.26±3.69	efg
Kitui	41.86±2.85	efg
Kiambu	41.50±2.63	fg
Homa Bay	38.80±1.73	g
Embu	37.88±3.95	g

Table 3.3: MLN severity analysis. The difference in the mean level of severity among the different counties was statistically significant at α=0.05 (F-Value=5.271, P-value=0.0000000592)

County	Mean	Groups
Bomet	3.56±0.55	a
Kajiado	3.24±0.93	ab
Baringo	3.23±0.39	abc
Taita -Taveta	3.10±0.28	abcd
Trans-Nzoia	3.08±0.59	abcde
West Pokot	3.06±0.45	abcde
Tana River	3.05±0.78	abcde
Kilifi	2.98±0.54	abcde
Narok	2.94±0.46	abcde
Kwale	2.80±0.14	abcde
Bungoma	2.73±0.46	abcde
Nandi	2.65±0.35	abcde
Migori	2.53±0.75	abcde
Kisii	2.43±0.32	abcde
Marakwet	2.62±0.33	abcde
Busia	2.91±0.43	abcde
Kakamega	2.37±0.55	abcde
Uasin Gishu	2.35±0.21	abcde
Kirinyaga	2.28±0.41	bcde
Siaya	2.20±0.57	bcde
Embu	2.10±0.36	bcde
Nakuru	2.05±0.42	cde
Makueni	2.02±0.27	cde
Kiambu	2.00±0.17	cde
Meru	1.93±0.32	cde
Kitui	1.89±0.31	de
Machakos	1.85±0.29	de
Homa Bay	1.63±0.23	e

Table 3.4: Incidence and severity of MLN in maize seed production fields in the counties surveyed.

County	Incidence	Severity
Baringo	100.0a	3.5a
Nakuru	100.0a	3.1b
Taita Taveta	75.0b	3.1b
Tana River	68.0c	2.8c
Trans Nzoia	63.0d	2.5d
Elgeyo-Marakwet	58.0e	2.3e
Machakos	45.0f	2.2ef
Makueni	35.0g	2.1f
Meru	25.0h	1.7gh
Embu	18.0i	1.6h
Uasin Gishu	16.0j	1.8g
Mean	54.8	2.42
LSD (P≤0.05)	1.64	0.17
CV	1.8	4.3

Means denoted by the same letter are not significantly different at P≤0.05

The county bordering Kajiado county on the south, Taita Taveta also had high incidences, prevalence and symptom severity of MLN. Overall, the coastal counties of Taita Taveta, Kwale, Kilifi and Tana River had high incidences of the disease ranging from 60% - 78% (Table 3.2). The highest incidence was recorded in Tana River followed by Taita Taveta county. The North Rift counties of Trans Nzoia, West Pokot, Marakwet and Uasin Gishu had medium to relatively high incidence of MLN in the country. W. Pokot had the highest incidence of 68% with Marakwet recording the lowest incidence of 48%.

The central Kenya counties of Kiambu and Kirinyaga also registered presence of MLN though with lower disease incidence of between 41% and 51%respectively.

Counties in the western Kenya region registered average disease incidences. Busia county had the highest incidences of 57% while Homa-Bay County had the lowest incidence of 38%. However, counties in Eastern region namely Embu, Meru, Machakos and Makueni had the lowest disease incidence and prevalence in the country during the survey. Embu county had the lowest i.e. 37% while Machakos recorded

55%. The same trend was observed for disease prevalence where Embu county had 44% but Machakos had 48%. Counties.

Bomet county registered the highest mean disease symptoms severity of 3.6 on the 1-5 MLN disease symptoms severity scale (Table 3.3). Counties in the north rift and south rift on average recorded the highest MLN symptoms severity ranging from 3.1 for West Pokot to 3.6 in Bomet as earlier indicated. Homa Bay county had the lowest MLN symptoms severity in the country recorded at 1.6 on average. The counties in coast region namely Taita Taveta, Kwale and Kilifi had moderate symptom severity posting figures between 2.80 in Kwale and 3.1 in Taita Taveta. The same was observed in counties in the western Kenya regions like Kakamega, Bungoma, Busia, Siaya and Migori as illustrated in Table 3.3.

The highest MLN incidence in seed fields was recorded in Baringo County and the neighboring Nakuru County where all the seed fields visited had MLN incidences of 100% (Table 3.4). Baringo county also recorded the highest disease symptoms severity of 3.5 followed by Nakuru and Taita-Taveta counties with severity of 3.1 in seed fields. In general, seed fields in the counties of the North Rift, South Rift and the coast had higher disease incidence and symptoms severity (Table 3.4). However, counties in the eastern part of the country recorded the lowest incidences and symptom severity in seed fields. These include Machakos, Makueni, Meru and Embu with incidence range of 18% – 45% and disease severity scales of 1.6 – 2.2 (Table 3.4).

3.4.3 RT PCR and ELISA Testing results

MCMV was detected by RT-PCR in 95 pooled leaf samples out of the total 118 pooled samples collected during the survey (Table3.5). For SCMV DAS ELISA analysis, 85 samples tested positive out of the 118 samples. The percentage MCMV and SCMV positive samples in the total samples collected were high hence correlated well to symptomatic plants sampled.

Table 3.5: Analysis results for MLN viruses testing using RT-PCR for MCMV and DAS-ELISA for SCMV.

No of samples tested		**Positive tests (+ve)**	**% Positive**
MCMV (RT-PCR)	118	95	87
SCMV (ELISA)	118	85	72
MCMV+SCMV	118	73	62
MCMV (RT-PCR) Seed	44	44	100

3.5 Discussion

Maize Lethal Necrosis disease remains a major threat to maize production in Kenya and in the entire Eastern Africa region where it is endemic (Miano et al., 2016). The survey results from this study indicate that MCMV which is the major component causative virus for MLN is still widely distributed in Kenya, with most maize growing regions testing positive for the virus (Fig. 3.5 and 3.6). SCMV which co-infects maize plants with MCMV to cause MLN was also widely distributed in the country. Previous studies indicated the presence of MLN causing viruses (Mahuku et al., 2015 and Wamaitha et al., 2018). It was evident that the counties in the South rift region had the highest prevalence, incidences and symptom severity of MLN (Table 1). There was significant difference in disease prevalence and incidences in the counties in the north rift regions. This was also noted in other regions in the country.

It was also evident that the disease is severe in the regions where there is continuous growing of maize especially in counties with supplementary irrigation like in Baringo, Kajiado and Taita Taveta. This is more prominent in areas like Loitoktok, Kajiado county in the South Rift and in Pekera irrigation scheme, Baringo county where maize is grown round the year due to availability of irrigation facilities. This further shows that the viral inoculum is higher in areas where continuous maize cropping is practiced compared to areas with distinct maize growing seasons. However, the incidences were higher in counties with continuous growing in the two-yearly seasons and low in counties with a single yearly season like Makueni and Embu.

Counties in the north rift region like, West Pokot, Marakwet and Uasin Gishu and counties in western Kenya had relatively high to moderate incidence and prevalence of MLN. This is partly due to the cropping seasons where there is one major maize cropping season that is rain-fed. Farmers in these areas also have a higher adoption rates in using certified seed compared to farmers from other areas due to higher levels of awareness and proximity to local stockists of maize seed (Ouma et al., 2014). As such, there is a possibility of these farmers using seed with low levels of infection and contamination by MLN causing viruses.

MCMV was more prevalent than SCMV in all the areas under the survey in both farmers' fields. Among the 118 composite leaf samples collected, 92 tested positive for MCMV by RT PCR while 80 tested positive for SCMV through DAS-ELISA (Table 3.5). Co infection of the two viruses was witnessed in 73 samples evenly distributed across the country. Previous studies on co-infection of MCMV and SCMV demonstrated increased accumulation of MCMV (Zihao Xia et al., 2016). However, there were samples where only MCMV was present but posted high incidences and severity of the disease. This indicates that MCMV alone can lead to severe infections like those for plants with coinfection on the aforementioned viruses.

Higher incidences, prevalence and the severity of the disease symptoms were recorded in Baringo, Taita Taveta and Tana River counties. These counties have many farms and seed fields under irrigation hence partly explains why the incidence, prevalence and severity is high. However, relatively high levels of the same disease parameters were noticed in other counties like and Elgeyo Marakwet. The counties in the eastern part of the country recorded the lowest incidences and symptom severity. These include Machakos, Makueni, Meru and Embu with incidence range of 18% – 45% and disease severity scales of 1.6 – 2.2. Monitoring MLN incidences in seed fields is very important for the seed produced by several seed companies may fuel the spread of MLN. As such, the status of MLN in these fields gives an indication of the disease situation and the probability of the seed playing a role on the new infections in subsequent planting seasons. The rate of transmission through seed from previous studies show low rate of transmission (Jensen et al.,1991) but the presence of MCMV in commercial seed lots may lead to amplified spread by insect vectors endemic in the maize growing areas. As indicated in the Table 3.1 high incidences in Uasin Gishu and

Baringo counties must be addressed by relevant authorities since most seed production fields are located in these counties.

Efforts by International Maize and Wheat Improvement Center (CIMMYT) and other National Research institutions have fast tracked release of MLN tolerant hybrids, but they are yet to be deployed on to the market. Optimized diagnostics and MLN free seed production checklists have been developed and currently being used by National Plant protection organizations (NPPOs) and seed companies to check the spread of MCMV through seed. It is envisaged that with synchronized implementation of multiple MLN management approaches, the incidences of MLN hence its devastating effect on yields and farm income will be greatly reduced redeeming small holder livelihoods in Kenya in the near future.

3.6 Conclusion

MLN is widely distributed in all maize growing counties in Kenya. The two causative viruses for MLN were confirmed i.e. MCMV and SCMV hence confirming that they are the prevalent in Kenya. Commercial seed fields were also infected with two MLN causative viruses posing danger for distribution of seed contaminated with MCMV which is seed borne. It is worth noting that further studies should be done to ascertain the sum effect of other viruses, or abiotic factors that complicate the etiology of MLN in Kenya and by extension in eastern Africa.

CHAPTER FOUR

MOLECULAR CHARACTERIZATION AND VIRAL RECOMBINATION ANALYSISOF VIRUSES CAUSING MLN IN KENYA

4.0 Abstract

The emergence of MLN in 2011 has had a devastating effect on maize yields with affected areas recording yield losses of 20 –100%. In order to assess the MLN causing viruses in Kenya, their genetic diversity and recombination, a countrywide survey was carried out where MLN symptomatic plants were sampled. The component causative viruses for MLN were determined by testing leaf samples with RT-PCR using virus-specific and group-conserved primers and DAS-ELISA. Next generation sequencing (NGS) data was generated, the viral sequences identified and the genetic diversity of the synergistic potyviruses and MCMV were determined by BLAST, phylogenetic analysis and nucleotide and amino acid identity comparison. Recombination analysis was evaluated for the MLN viruses determined through NGS.

Laboratory testing of the maize leaf samples and NGS sequencing identified MCMV and SCMV as the major viruses causing MLN in Kenya. The Kenyan MCMV isolates detected in this study showed 99.75% identity to isolates previously reported in Kenya (JX286709), Ethiopia (KP798454) and Rwanda (KP851970). They were 99.02% identical to MCMV isolates from China (KF010583.1) and 96 to 97% with MCMV isolates from Kansas and Nebraska in the United States of America. The SCMV genomes showed high diversity within the polyprotein region ranging from (89.81 - 100%). They exhibited high levels of similarity to isolates from Rwanda, USA and China. Potential recombination events were detected in 11 SCMV genome sequences but only 3 SCMV genome sequence recombinants with different possible major and minor parents were strongly supported. Potential viral recombination was not detected in the MCMV genomes. Results indicated that the MCMV isolates in Kenya are highly identical to the China isolates. The SCMV isolates derived in the study are similar to those in the eastern Africa region, China and those from Ohio state in the US. The high genetic recombination activity in SCMV partly explains the elevated level of diversity of this virus in Kenya and in the region.

4.1 Introduction

The rapid spread of the disease has affected very many maize growing farmers with yield losses of 20%– 100% (De Groote et al., 2016). MLN still remains a threat to the livelihoods of many smallholder farm families in the Eastern Africa region and beyond if it is not curbed. MLN is a relatively new disease in Africa and indeed MCMV is a new virus to infect maize in Africa (Wangai et al., 2012). The possibility of MCMV to combine with other native potyviruses of cereals poses a big challenge to maize production in this region. Previous work by Adams et al., 2012 and Mahuku et al., 2015 have pointed out MCMV and SCMV to be the major causative agents but concludes that there may be other potyviruses that can synergize with MCMV to cause MLN. What are the genetic diversities of these viruses? What means of transmission have allowed the rapid spread of the disease across the Africa continent. Insect transmission of MLN Causing viruses has been documented (Mahuku et al., 2015b and Nyasani and Sevgan, 2012).

This study focused on determining potyviruses that synergize with MCMV to cause MLN, to determine the genetic diversity of these viruses using Next generation sequencing (NGS) technology (Illumina MiSeq) and to access the genetic recombination of these viruses.

It has been demonstrated that maize-infecting viruses in the family *potyviridae* can co-infect maize with MCMV to cause MLN (Niblett and Claflin 1978; Stewart et al., 2017). These potyviruses include MDMV, SCMV and recently *Johnsongrass mosaic virus* (JGMV) which are in the genus *potyvirus* and WSMV in the genus *Tritimovirus*. Amongst these potyviruses, SCMV is more dominant in East Africa, but MDMV and JGMV have been found also to be present (Wangai et al., 2012; Mahuku et al, 2015 and Stewart et al., 2017). WSMV is not known to occur in Africa but found in North America, south America, Europe and Australia (Hadi et al., 2011). However, in most studies of MLN emergence in eastern Africa, SCMV has been found to co-infect maize with MCMV though these studies conclude that there is still a possibility of the other potyviruses to co-infect maize with MCMV to cause MLN (Mahuku et al., 2015, Adams et al, 2014 and Wamaitha et al., 2018.

A national approach ensures that the causative agents of the disease are thoroughly characterized and that research outcomes will help molecular breeders in their quest to develop resistant/tolerant varieties. Currently, 15 MLN tolerant hybrids have been officially released in eastern Africa and two are already commercialized. CIMMYT is coordinating many initiatives like the MLN Diagnostics and management project (CIMMYT, 2017). One of the objectives in this initiative is strengthening the National Plant Protection Organizations (NPPOs) capacity to test for MLN viruses especially MCMV in seed lots for seed certification. Another one is adoption of MLN free seed production protocols developed by partners and seed companies in eastern African countries where MLN is endemic. There is also an initiative in studying various factors in the MLN viruses' epidemiology (CIMMYT, 2018). Several studies are being pursued in understanding the mode of MCMV transmission through commercial seed within endemic areas allowing more effective control. Of importance is generating knowledge about relationship between seed infestation and seed transmission of MCMV (CIMMYT, 2018).

It is also paramount to determine the evolutionary path of the MLN causing viruses through Virus Recombination analysis. Recombination is a pervasive process generating diversity in most viruses (Martin et al., 2015). It joins variants that arise independently within the same molecule, creating new opportunities for viruses to overcome selective pressures and to adapt to new environments and hosts. As such, full genome sequences of the causative viruses were analysed for genetic viral recombination using the RDP4 recombination analysis program (Martin et al., 2015).

RDP4 simultaneously uses a range of different recombination detection methods to both detect and characterize the recombination events that are evident within a sequence alignment without any prior user indication of a non-recombinant set of reference sequences. Unlike the original RDP method, it includes the BOOTSCANNING method (Salminen *et al*., 1995; Martin *et al*., 2005b), the GENECONV method (Padidam *et al*., 1999), the Maximum Chi Square method MAXCHI; (Maynard Smith, 1992; Posada and Crandall, 2001), the CHIMAERA method (Posada and Crandall, 2001), the Sister Scanning method (SISCAN; Gibbs *et al*., 2000), the 3SEQ method (Boni *et al*., 2007), the VisRD method (Lemey et al., 2009) and the BURT method.

4.2 Materials and Methods

4.2.1 Detection of MLN viruses in collected samples

4.2.1.1 Ribonucleic acid extraction

RNA was extracted from the 118 composite samples collected during the survey as illustrated in the survey and sampling section in chapter three, section 3.3. The ZR RNA MiniPrep™ kit (ZYMO RESEARCH CORPORATION, Irvine, CA, USA) was used for RNA extraction following the manufacturer's instructions.

4.2.1.2 RNA quality determination using denaturing Gel electrophoresis

Concentration of RNA in the samples was checked by the Qubit quantification platform (Thermo Fischer Scientific, Wilmington, USA).

The overall quality of RNA extracted was assessed by electrophoresis on a denaturing agarose gel. The denaturing gel electrophoresis was prepared as follows:

Tris-acetate-EDTA (TAE) 1X buffer was diluted from the 10X stock in sterile distilled water. The 10X TAE (IL) contains 900 mls dH_2O, 48.4 g Tris base (Triz), 11.4 mls glacial acetic acid and 3.72 g EDTA topped up to 1L with dH_2O. To make a 1.2% agarose gel, 1.2 g of the agarose powder was added to 100mls of 1X TAE buffer and heated gently in the microwave to dissolve. The gel was then cooled to 50^{o}C. Thereafter 3µl of the gel red staining dye (Biotium, Inc. Fremont, CA 94538) was added and stirred to mix. The gel was then casted on the trays with the combs inside to make the wells.

RNA was prepared by taking 5 µl of RNA, 4ul of formaldehyde and 1 µl of the RNA loading dye. Each sample had 10ul of the reaction volume. Care was taken when using formaldehyde for it is toxic through skin contact and inhalation of vapours. The mixture was incubated in a thermocycler at 65 ^{o}C for 5 minutes. The samples were then removed and quickly placed on ice to stop the reaction.

The samples were then loaded in the prepared 1.2% agarose gel and run for 60 mins. The characteristic RNA 18s and 28s bands on the RNA gels were evaluated to ascertain the RNA quality prior to subsequent analyses. Additionally, quality of RNA extracted was also assessed by testing using a NanoDrop ND-1000 Spectrophotometer (Thermo Fischer Scientific, Wilmington USA).

Figure 4.1 below shows the gel pictures of RNA after RNA denaturing electrophoresis. The quality samples were evaluated as per the presence of clear 28S and 18S bands as illustrated in Figure 4.1 below. Completely degraded RNA will appear as a very low molecular weight smear. Note: Poly (A) selected samples will not contain strong rRNA bands and will appear as a smear from approximately 6 kb to 0.5 kb (resulting from the population of mRNAs, and depending on exposure times and conditions), with the area between 1.5 and 2 kb being the most intense (this smear is sometimes apparent in total RNA samples as well).

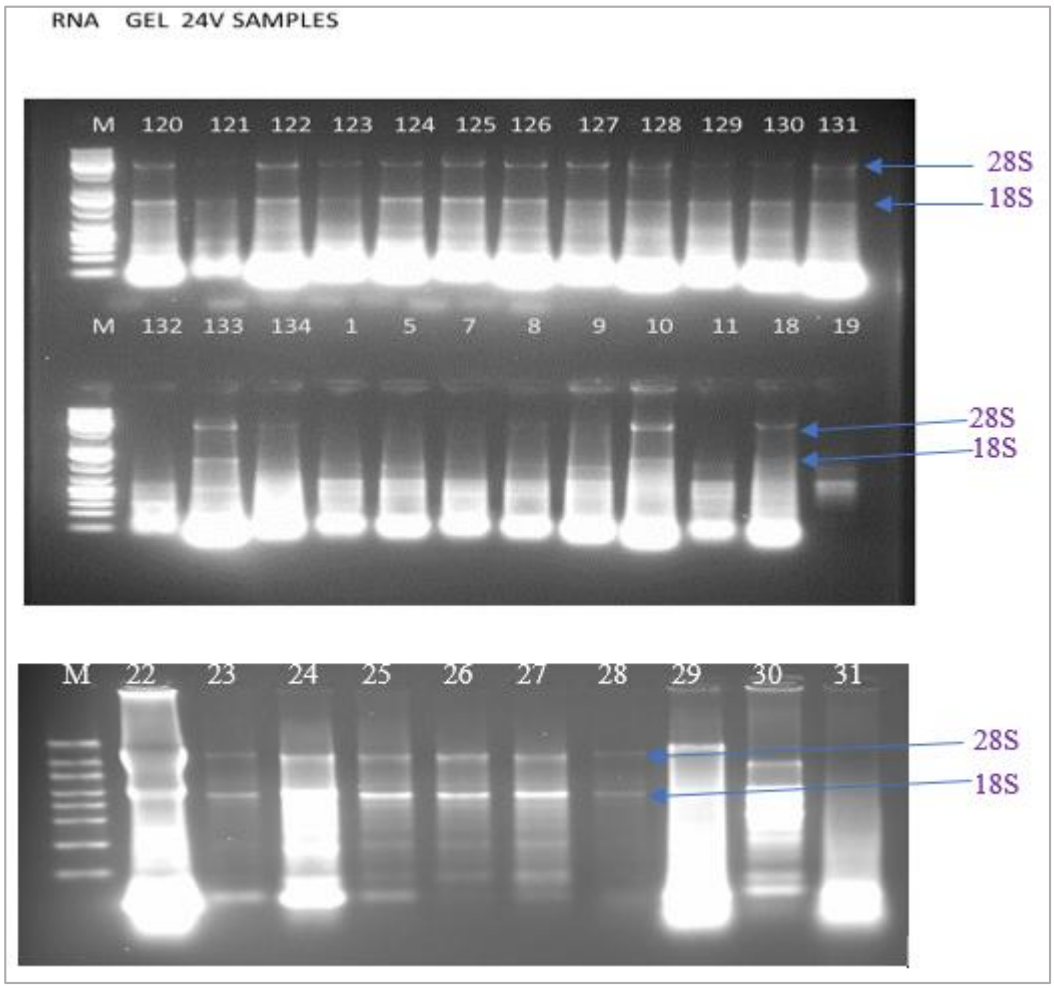


Figure 4.1: RNA gel picture for some samples for RNA quality evaluation. The distinct 25S and 18S bands indicated good quality RNA. The RNA samples without the two bands i.e., 25S and 18S are not suitable for further analysis. The Millennium RNA marker 0.5 – 9kb was used.

Intact total RNA run on a denaturing gel usually has a sharp 28S and 18S rRNA bands (eukaryotic samples). The 28S rRNA band is approximately twice as intense as the 18S rRNA band. This 2:1 ratio (28S:18S) is a good indication that the RNA is intact. Partially degraded RNA has a smeared appearance, lack the sharp rRNA bands, and does not exhibit a 2:1 ratio.

MCMV and SCMV cDNA were synthesised as earlier explained in Chapter three, section 3.3.2 where the detailed procedures for testing MCMV and SCMV using RT-PCR were outlined. The primers for testing the *potyviridae* family were also used on some samples to detect if potyviruses were present in our samples.

4.2.1.3 Detection of MCMV and SCMV by DAS-ELISA

Detection of MCMV and SCMV was carried out using Double Antibody Sandwich – Enzyme Linked Immunosorbent Assay (DAS-ELISA). Manufacturer's instructions were followed in carrying out the procedure for detection of both MCMV and SCMV (Agdia, Elkhart, IN, USA). This procedure was performed on 118 samples. The double antibody sandwich ELISA semi-quantifies the virus between two layers of virus specific antibodies (a capture and a detection antibody).

4.2.2 Miseq Next Generation Sequencing

4.2.2.1 Sample selected for sequencing

Forty-eight samples were selected representing the regions in Kenya. The representative samples had good quality libraries for sequencing on the Miseq Illumina platform (Table 4.1). The most crucial factor was the county locations where the samples were obtained in the field. Many diversity studies require comprehensive comparison of the genetic variability of the pathogen under study across several county regions within a country or a region. In this case, samples from all the major maize growing counties were selected to represent these areas for the reasons earlier mentioned. The other consideration in the selection process as indicated earlier was the quality of the libraries synthesized e.g. the concentration, purity, and the total yield.

Table 4.1: Sample selected for sequencing

Sample No.	Lab No.	County	Location collected	GPS Coordinates
1	S1	Bungoma	Kapsokwany	N01.07150 E034.87053
2	S2	Bungoma	Kaptama	N01.07376 E034.85079
3	S5	Bungoma	Miyanga	N01.07376 E034.85079
4	S2-1	Bungoma	Bumula	N01.07376 E034.85079
5	S2-2	Bungoma	Chwele	N01.06533 E034.84172
6	S2-3	Bungoma	Sirisia	N01.06674 E034.83498
7	S7	Marakwet	Sergoit	N01.82102 E035.38884
8	S6	Uasin Gishu	Endebes	N01.03447 E034.82410
9	S8	Uasin Gishu	Ziwa	N01.51667 E035.28433
10	S9	Bomet	Kapmgoso	S00.68370 E035.26849
11	S10	Narok	Olerai	S01.09909 E035.84049
12	S11	Kajiado	Esikuta	S02.96304 E037.57605
13	S12	Kajiado	Ndarara	S02.98787 E037.56916
14	S30	Kajiado	Rombo	S02.96304 E037.57605
15	S31	Kajiado	Isineti	S02.93353 E037.53585
16	S32	Kajiado	Matepes	S02.96304 E037.57605
17	S13	Makueni	Mavinye	S01.78288 E037.28856
18	S14	Makueni	Ivauli	S01.76670 E037.26326
19	S15	Makueni	Matiliku	S01.94253 E037.53283
20	S16	Machakos	Athi River	S01.23277 E037.46314
21	S17	Machakos	Kangundo	S01.34111 E037.44740
22	S18	Machakos	Katumani	S01.45982 E037.42378
23	S19	Kilifi	Mavueni	S03.68076 E039.81532
24	S20	Kilifi	Mida	S03.33798 E039.94253
25	S21	Tana River	Bura Irrigation	S03.76321 E039.57803
26	S22	Tana River	Hola Irrigation	S03.68076 E039.81532
27	S23	Kwale	Kikoneni	S04.18793E039.46605
28	S24	Kwale	Mwangwei	S04.50467 E039.26941
29	S25	Kwale	Tanga	S04.50467 E039.26941
30	S26	TaitaTaveta	Ziwani	S03.24696 E037.76369
31	S27	TaitaTaveta	Challa	S03.25243 E037.76385
32	S28	TaitaTaveta	Njukini	S03.19484 E037.71234
33	S29	TaitaTaveta	Njukini	S03.19484 E037.71234
34	S36	Taita Taveta	Jipe	S03.25243 E037.68247
35	S33	Kiambu	Muguga	S01.12799 E03637.864
36	S34	Kirinyaga	Mwea	S00.65992 E037.25740
37	S35	Nyeri	Wambugu	S04.60552 E037.47365
38	S3	W. Pokot	Kapenguria	N01.03447 E034.82410
39	S4	W. Pokot	Sigor	N01.03447 E034.82410
40	S2-4	Busia	Kolanya	N01.05500 E034.84150
41	S2-5	Busia	Amukura	N01.05457 E034.78327
42	S2-6	Busia	Adungosi	N01.05598 E034.79346
43	S2-7	Kakamega	Sigalagala	N00.22240 E034.62675
44	S2-8	Kakamega	Munami	N00.21878 E034.63065
45	S2-9	Siaya	Yala	N01.05183 E034.85102
46	S2-10	Siaya	Bondo	N01.06234 E034.78994
47	S2-11	Migori	Suna	S01.17045 E034.58256
48	S2-12	Kisii	Gesusu	S00.87462 E034.89989

The sample location including the county and sub countyare documented. The actual area and GPS coordinates from where the selected samples were collected are indicated below.

4.2.2.2 DNase Digestion

The samples selected for Illumina sequencing were treated first with the Thermo-Scientific DNase reagents following the manufacturer's protocol (Life Technologies Inc., 5791 Van Allen Way, Carlsbad, California 92008). The procedure was carried out to ensure that trace DNA was eliminated so as not to interfere with subsequent processes in the analysis.

4.2.2.3 Libraries preparation

The RNA extracted from the maize leaf samples from the survey was verified for quality and concentration. A total of 48 samples were selected as explained in section 4.2.2.1 for sequencing on the Illumina MiSeq platform. The sequencing was done in 2 runs with 24 samples in each run as described below.

The TruSeq RNA Sample Preparation v2 Guide was used to prepare RNA before the synthesis of libraries for NGS. The first step involved the removal of ribosomal RNA (rRNA) using biotinylated, target-specific oligos combined with Ribo-Zero rRNA removal beads. Following purification, the RNA was fragmented into small pieces using divalent cations under elevated temperature. The cleaved RNA fragments were copied into first strand cDNA using reverse transcriptase and random primers. This was followed by second strand cDNA synthesis using DNA polymerase and RNase H. Single 'A' base was added to the cDNA fragments synthesized and subsequently ligated to the adapter. The products were purified and enriched with PCR to create the final cDNA library.

Quantitative PCR was used to determine the optimal amplification of the ~200bp cDNA fragments and 33 PCR cycles were used for subsequent cDNA amplification.

4.2.2.4 Libraries quality check by gel electrophoresis, Qubit and Bioanalyzer (HS Screen Tape analyser).

The libraries synthesized in the above-mentioned procedures were checked for quality by gel electrophoresis. The 48 samples were again evaluated for their quality and concentration on the 2200 TapeStation in the Bioanalyzer Pat No. G2940CA (Agilent Technologies - Santa Clara, California, United States).

The Agilent Tape D1000 Station system is an automated platform for simpler, faster and more reliable electrophoresis. It is made up of three elements namely 2200 TapeStation Nucleic Acid system (G2965AA), D1000 ScreenTape (5067- 5582) with D1000 Reagents (5067- 5583) and the Agilent Software packages (2200 TapeStation Controller Software, and TapeStation Analysis Software). The D1000 ScreenTape system is designed for analyzing DNA molecules from 35 -1000 bp.

The D100 screen tape procedure was used as described in the Agilent D1000 ScreenTape System Quick Guide. The Agilent reagents were brought to equilibrium with room temperatures for 30mins while the library samples were thawed on ice. The reaction mixture was constituted by mixing 3ul of the buffer and 2µl of the ladder and 2µl of the libraries. An aliquot of 2µl of the prepared mixture for each sample was loaded in the gel and ran for 45 mins at 100V. Sample analysis was done by loading the reaction mixtures for all the 48 library samples.

The Invitrogen Qubit 4 Fluorimeter was also used to quantify the DNA libraries prepared as a quality control tool. The Quibit Broad Range (BR) protocol for DNA quantification was used where 199 µl of the buffer mix was mixed with 1 µl of the dimethyl sulfoxide (DMSO) to make 200µl of the reaction solution. The two standard solutions were made by mixing 190ul of the buffer mix to 10µl of standard solution 1 and 2. Samples were prepared by taking 198µl of the buffer mix with 2ul of the library DNA samples, mixed and vortexed.

The concentrations (ng/µl) were analyzed by the Qubit equipment by loading 2 µl to the reader plate and running the fluorimeter. The readings were recorded.

4.2.2.5 Libraries synthesised and their quality analysis

Good quality DNA libraries were obtained from the library preparation procedure as per the Illumina TruSeq protocol for MiSeq sequencing. The gel picture in Figure 4.2 visualised under UV 100V shows a cluster of DNA fragments between 200-300 bp as expected of samples 25 - 36.

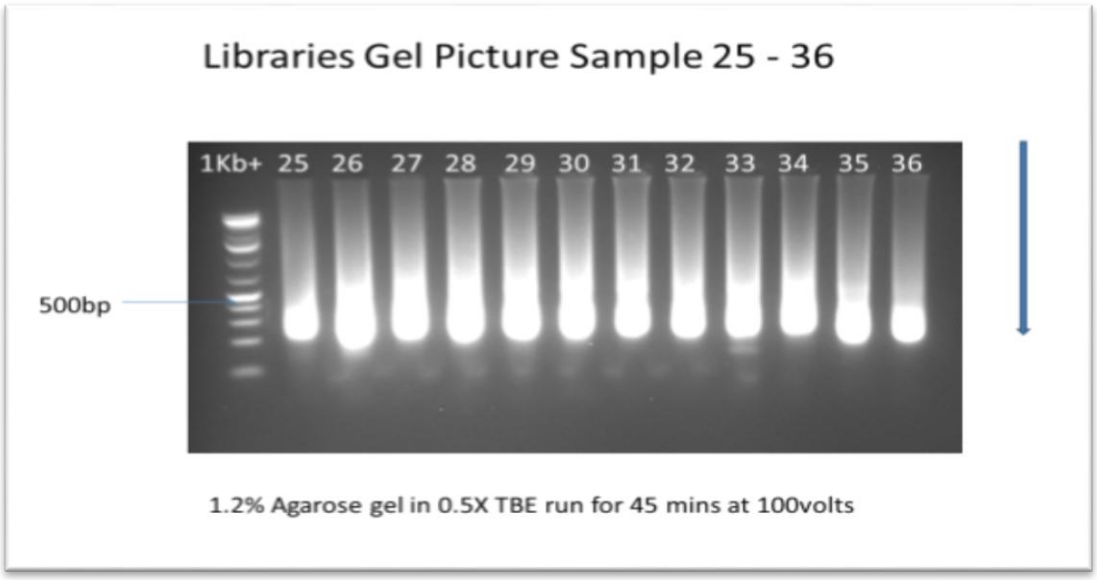


Figure 4.2: Agarose gel picture for the libraries prepared for NGS sequencing. The gel picture shows the 500bp concentration of the DNA libraries generated. 1kb marker was used.

Analysis of the libraries by the Agilent TapeStation 2100 through the Bioanalyzer also gave consistent results for the DNA libraries derived. Figure 4.3 shows the Gel image for High Sensitivity D1000 Screen tape for analysed samples with most samples giving quality libraries band sizes of between 250 - 500bp.

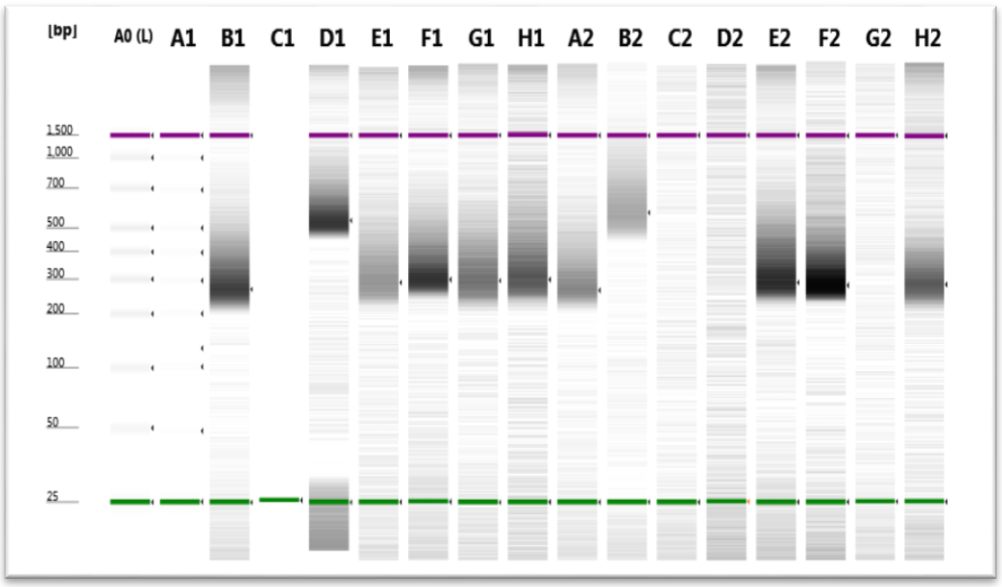


Figure 4.3: Gel Image for High Sensitivity D1000 Screen tape for analysed samples. There are two limits of the markers both the lower and upper limits. The darker colours show the concentration of the DNA libraries in the samples

being analysed. Samples C1 and C2 showed very poor yields of the libraries and were discarded for the onward process of sequencing. The libraries concentrations are always between 250bp – 700bp with peaks at the 500bp.

The libraries also gave good concentrations required for sequencing after Qubit analysis in Table 4.2 below.

Table 4.2: Qubit results for some DNA libraries synthesised for sequencing.

Samples	Broad Range (BR) Readings		High Sensitivity (HS) Readings	
	1st Reading ug/µl	2nd Reading ug/µl	1st Reading ug/µl	2nd Reading ug/µl
26	28.3	27.0	20.3	20.2
27	32.9	32.2	23.6	23.4
28	21.6	21.3	14.5	14.2
29	35.6	35.2	27.9	28.0
30	28.9	28.0	19.3	19.4

All the samples were within the concentrations range required for both Broad range (BR) and High Sensitivity (HS) test hence suitable for the illumine Miseq Illumina sequencing procedure.

4.2.2.6 Sequencing on the Illumina Miseq platform

The library samples were prepared for loading and sequencing using the Illumina Preparing Libraries for Sequencing on MiSeq protocol, 2014. Sequencing by synthesis was performed using the Illumina Genome Analyzer II with 51 cycles. The sequencing was done in 2 runs with 24 samples each giving a total of 48 samples sequenced on the Illumina MiSeq platform.

4.2.3 Sequence data analysis

The data was checked for quality using Fastqc (FastQC, A. (2018). Low quality bases and adapters were trimmed off using Trimmomatic V 0.33 (Bolger et al., 2014). *Denovo* assembly was performed on the remaining reads using metaSPAdes V 3.10. (Nurk et al., 2017). The resulting contigs were blasted against a local download of NCBI nucleotide plant virus database (Johnson et al., 2008). Reference mapping of the assembled contigs to the most similar viral genomes was performed using CLC

Genomics Workbench 5.5.1 (CLC bio, Cambridge, MA, USA). All the viral contigs were aligned to their homologous sequences from the NCBI nucleotide database and these alignments were used to construct phylogenetics trees in MEGA V6 (Tamura et al., 2013).

Blastn through Krona and conventional sequence alignment was carried out on the assembled contigs with a script designed to blast only plant viral sequences. Krona allows hierarchical data to be explored with zooming, multi-layered pie charts. Krona charts are created by Krona Tools (Ondov et al., 2011) which includes support for several bioinformatics tools and raw data formats. The interactive charts are self-contained and can be viewed with any modern web browser. The output of the Blast results was viewed under the Krona from a krona designed script and through a tabular method with accessions aligned to query sequences.

4.2.4 Viral Recombination Analysis

The recombination detection program RDP4 v4.84 (Martin et al., 2015) was used to analyse both MCMV and SCMV samples sequences.

The saved SCMV and MCMV sequence alignment file was opened in RDP4. All the aligned sequences were well displayed in the RDP4 display panel. This confirmed the sequences selected for recombination analysis. The default selection that employs the use of all the detection methods was utilized to complete automated analysis. There were two major phases in the automated analysis where the first involved the detection of recombination signals in the alignment and the second involved inference of the number and characteristics of unique recombination events that had been generated by these signals.

4.3 Results

4.3.1 RT- PCR and ELISA MCMV and SCMV testing results

MCMV was detected by RT-PCR in 95 pooled leaf samples out of the 118 samples while SCMV was detected by DAS-ELISA in 85 samples out of the same 118 (Table 3.1 in Chapter three). Seventy-three (73) samples showed double infection (MCMV + SCMV). The high percentage of positive samples reflected the high number of symptomatic plants sampled. MCMV and SCMV were detected in all the 33 samples

collected from the seed fields during the survey. The disease was prevalent in all the areas surveyed as per the RT-PCR results. Primers for SCMV did not capture the virus in the samples. However, it was possible to amplify MCMV and Potyviruses using RT PCR as shown for some samples in Figure 4.4.

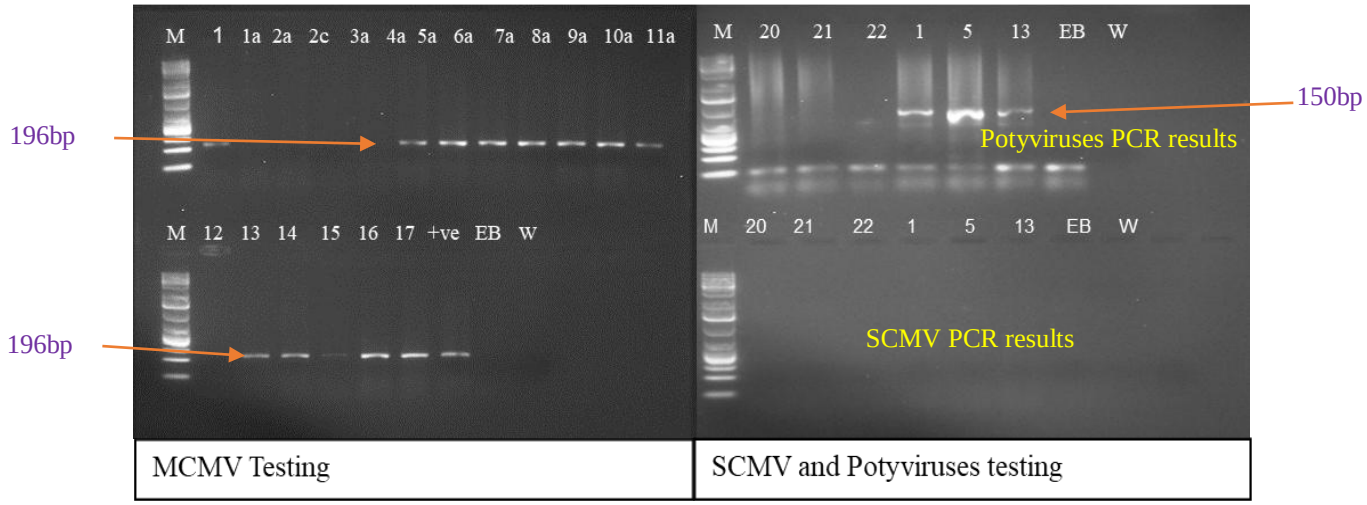


Figure 4.4: RT- PCR for MCMV and SCMV.

MCMV amplification recorded with amplicons of 196bp. Water and buffer (EB) samples show no amplification as controls. The conserved primers of potyviruses showed amplification with 150bp amplicons (Top row) but the SCMV primers showed no amplifications (Bottom row). The marker used was 1kb.

All the 118 samples screened for by DAS-ELISA had 85 samples testing positive for SCMV. Table 4.3 below shows one of the ELISA results plate representation diagrams depicting the ELISA plate after reading through the ELISA reader. From these samples, twenty-two tested positive (in pink) for SCMV as shown in the Table 4.3. The positive samples had twice or more OD values than those of the negative controls and buffer alone.

Table 4.3: ELISA reader results for SCMV. Samples highlighted in blue were positive and the remaining are negative for SCMV. All samples were in duplicates.

	1	2	3	4	5	6	7	8	9	10	11	12
A	1	1	2	2	3	3	4	4	5	5	6	6
B	7	7	8	8	9	9	10	10	11	11	12	12
C	13	13	14	14	15	15	16	16	17	17	18	18
D	19	19	20	20	21	21	23	23	24	24	25	25
E	26	26	27	27	28	28	29	29	30	30	31	31
F	32	32	33	33	34	34	35	35	36	36	Buffer	Buffer
G	+ve	+ve	-ve	-ve								
H												

4.3.2 Sequence data generated

Approximately 13 million clusters were obtained on a single sequencing lane of the Illumina flow cell. Approximately 74% of these clusters were analysed and yielded quality sequence data. The paired-end sequencing yielded 102,169,109 reads (35-151bp). After quality control 53,297,590 good quality reads were obtained (17-122bp) (Table 4.4). Following contigs assembly and BLAST identification of the contigs, most samples strongly showed the presence of MCMV and SCMV.

Table 4.4: Sequence data generated from NGS before and after trimming for the 2 runs.

Reads Parameters	Before Trimming	After Trimming
No. of reads	102,169,109	53,297,590
%GC Content	49	50
Reads length	35-151	17-122

4.3.3 Blast results by Krona and Blastn analysis

MCMV was present in all the samples sequenced based on blast analysis which showed percentages of 45-80% of samples having the virus. SCMV was also detected amongst the potyviruses in the samples analysed through blast by Krona (Fig 4.5) and by sequence alignment.

Luteoviruses were detected in the preliminary blast results in particular, *Maize Yellow dwarf virus* (MYDV) and *Barley Yellow dwarf virus* (BYDV) albeit in small percentage (Figure 4.5). The same was observed with potyviruses namely, *Sugarcane mosaic virus* (SCMV) and *Papaya ringspot virus* (PRV). Other viruses also showed up in this Krona Blstn analysis namely the *Grapevine leaf roll virus* associated virus 1 and viroids like the Avocado sunblotch viroid and the Citrus exocortis viroid. However, only MCMV and SCMV sequences were obtained with *Denovo* assembly.

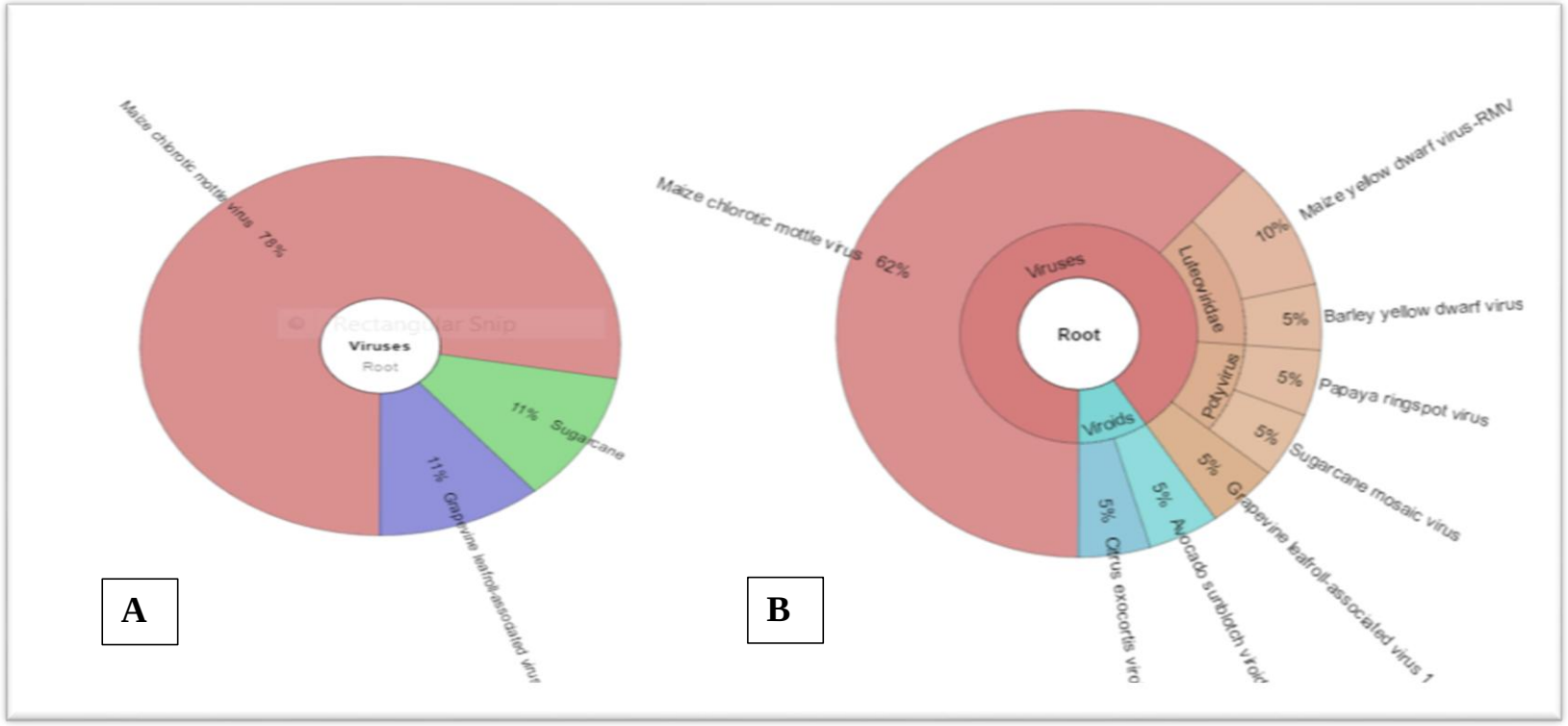


Figure 4.5: Blastn results by Krona representation. A shows the viruses that were identified in sample S2 while B the viruses identified in Sample S12

Full genome and partial sequences of MCMV and SCMV were obtained with the *Denovo* assembly tool as shown in Table 4.5 below.

Table 4.5: Summary of the BLAST results of assembled contigs. The plant viruses detected are; MCMV, SCMV, Maize Yellow Mosaic Virus (MaYMV). The percentage similarity, genome coverage and the accessions of the blasted sequences are illustrated. Isolates S9, S12, S16 and S18 with the Asterix sign (*) have only MCMV infection without SCMV or MaYMV.

Sample ID	Virus	Strain	% Similarity	E-Value	Coverage	Accessions
S1	MCMV	TF1F6S1	99	0	25158.9	MF510250.1
S1	SCMV	R1	99	0	59.8	KF744392.1
S2	MCMV	Isolate 1	99	0	44554.5	KP851970.3
S2	SCMV	R1	98	0	153.85	KF744392.1
S3	MCMV	TF1F6S1	99	0	31978	MF510250.1
S3	SCMV	R1	99	0	82	KF744392.1
S4	MCMV	TF1F6S1	99	0	9766.32	MF510250.1
S4	SCMV	R1	99	0	31.9	KF744392.1
S5	MCMV	TF1F6S1	99	0	14678.4	MF510250.1
S5	SCMV	R1	98	0	31.6	KF744392.1
S6	MCMV	Isolate 1	99	0	16979.2	KP851970.3
S6	SCMV	R1	95	0	36.7	KF744392.1
S7	MCMV	Isolate B3_S3	99	0	14705.9	MF510251.1
S7	SCMV	R1	95	0	36.28	KF744392.1
S9*	MCMV	Isolate 1	99	0	11220.3	KP851970.3
S10	MCMV	Isolate B3_S3	99	0	5043.9	MF510251.1
S10	SCMV	HZ8	99	0	12.25	JX047392.1
S11	MCMV	Isolate B3_S3	99	0	9440.7	MF510251.1
S11	SCMV	HZ8	99	0	22.14	JX047424.1
S12*	MCMV	Isolate 1	99	0	1763.4	KP851970.3
S13	MCMV	Isolate 1	99	0	956.56	KP851970.3
S13	SCMV	SCMV-M5	97	0	4.1	KP772216.1
S14	MCMV	Isolate B3_S3	99	0	5684.63	MF510251.1
S14	SCMV	HZ8	99	0	20.27	JX047424.1
S15	MCMV	Isolate 1	99	0	4302.56	KP851970.3
S15	SCMV	CD1	99	0	15.98	JX047392.1
S16*	MCMV	TF1F6S1	99	0	9230.35	MF510250.1
S18*	MCMV	Isolate 1	99	0	271.96	KP851970.3
S19	SCMV	HZ7	99	0	19.79	JX047424.1
S20	MCMV	Isolate 1	99	0	3223.81	KP851970.3
S20	SCMV	R1	99	0	4.8	KF744392.1
S21	SCMV	HZ7	99	0	70.5	JX047424.1
S22	MCMV	TF1F6S1	99	0	6.8	MF510250.1
S23	MCMV	Isolate 1	99	0	281.82	KP851970.3
S24	MCMV	Isolate B3_S3	99	0	332.1	MF510251.1
S25	MCMV	Isolate B3	99	0	6651.55	MF510251.1
S25	SCMV	HZ7	99	0	535.2	JX047424.1
S25	MaYMV	T2F3S4	99	0	3.8	MF425876.1
S25	MaYMV	T2F3S4	99	0	5.1	MF425876.1
S25	MaYMV	T2F3S4	99	0	2.2	MF425876.1
S25	MaYMV	T2F3S4	99	0	4.8	MF425876.1
S25	MaYMV	T2F3S4	99	0	2.2	MF425876.1
S26	MCMV	T1F6S3	99	0	10538.1	MF510244.1
S26	MaYMV	T2F3S4	99	0	1.2	MF425876.1
S26	MaYMV	T2F5S2	99	0	2.6	MF425878.1
S26	SCMV	HZ7	99	0	68.8	JX047424.1
S26	SCMV		98	0	12.3	KF744391.1
S27	MCMV	Isolate 1	99	0	31241.1	KP851970.3
S27	MaYMV	T2F5S1	99	0	1.6	MF425877.1

S27	MaYMV	T2F5S1	99	0	3.5	MF425877.1
S27	MaYMV	T2F3S4	99	0	1.6	MF425874.1
S27	SCMV	R2	96	0	141.1	KF744391.1
S28	MCMV	Isolate B3	99	0	12466.4	MF510251.1
S28	MaYMV	T2F5S1	99	0	1.5	MF425877.1
S28	SCMV	R2	96	0	91	KF744391.1
S29	MCMV	Isolate B3	99	0	5008.95	MF510251.1
S29	SCMV	HZ7	99	0	186.164	JX047424.1
S30	MCMV	Isolate 1	99	0	6724.5	KP851970.3
S31	MCMV	Isolate 1	99	0	11782.5	KP851970.3
S32	MCMV	Isolate B3	99	0	8510.1	MF510251.1
S32	MaYMV	T2F5S2	99	0	1.1	MF425878.1
S32	SCMV	HZ7	99	0	131.1	JX047424.1
S33	MCMV	T1F6S3	99	0	11680.6	MF510244.1
S33	MaYMV	T1F8S2	99	4e-154	1.35	MF425872.1
S33	SCMV	R2	96	0	63.9	KF744391.1
S34	MCMV	T1F6S3	99	0	11897.4	MF510244.1
S34	MaYMV	T2F5S2	99	0	1.9	MF425878.1
S34	SCMV	R2	95	0	111.9	KF744391.1
S34	MCMV	T1F6S1	99	0	5709	MF510250.1
S35	SCMV	R2	95	0	74.5	KF744391.1
S36	MCMV	Isolate 1	99	0	705.2	KP851970.3

Denovo assembly confirmed the presence of MCMV and SCMV with full and partial genome sequences assembled. Most samples showed presence of MCMV and SCMV from blast results visualized in Krona (Ondov et al., 2011).

All the assembled MCMV sequences aligned with several MCMV sequence accessions in the NCBI database. However, MCMV sequences in different samples aligned the best with different strains of MCMV in the NCBI plant viruses' nucleotide database, indicating the presence of several strains of the virus within our samples as outlined in Table 4.6. Some MYMV contigs were also detected in samples S25, S26, S27, S28, S29, S32, S33 and S34. However, these MYMV contigs were very short and had such low coverages to conclude their presence in the samples. MCMV sequences had exceptionally good coverage from the samples hence were present in these samples. Some samples though with MCMV had very low coverages. SCMV was prominent in all my samples with moderate coverage. This showed the presence of this virus in the samples.

Most samples had the full MCMV genome while in some samples we only got fragments (1000-3000 bp) with some of the MCMV ORF's present. A total of 15 MCMV full genomes were assembled with genome lengths of 4403 – 4437 bp (Appendix 2). Seven MCMV sequences were deposited in the NCBI nucleotide database (accession numbers: MH238449- MH238455). The isolate S6 with the

accession number MH238454 was selected as a representative for further nucleotide and amino acid comparison with other MCMV isolates available in the NCBI nucleotide database.

The sequence identity (%) of the complete genome sequences of MCMV in the samples ranged from 99.28% - 99.97% through pairwise identity matrix analysis (Table 4.6). This close similarity is indicative of common recent ancestor among Kenyan isolates. This is furtherer support by the MCMV phylogeny analysis in Figure 4.6.

Table 4.6: Identity matrix of the full length MCMV isolates obtained from the sequenced samples

S20_Kilifi	**100%**													
S12_Kajiado	**99.3%**	**100%**												
S23_Kwale	**99.32%**	**99.97%**	**100%**											
S25_Kwale	**99.37%**	**99.42%**	**99.44%**	**100%**										
S18_Machakos	**99.51%**	**99.51%**	**99.53%**	**99.62%**	**100%**									
S36_Taveta	**99.51%**	**99.55%**	**99.53%**	**99.58%**	**99.72%**	**100%**								
S26_Taveta	**99.62%**	**99.62%**	**99.65%**	**99.69%**	**99.83%**	**99.83%**	**100%**							
S31_Kajiado	**99.58%**	**99.58%**	**99.6%**	**99.65%**	**99.79%**	**99.83%**	**99.9%**	**100%**						
S30_Kajiado	**99.53%**	**99.53%**	**99.55%**	**99.6%**	**99.74%**	**99.76%**	**99.86%**	**99.86%**	**100%**					
S13_Makueni	**99.49%**	**99.49%**	**99.51%**	**99.55%**	**99.69%**	**99.69%**	**99.81%**	**99.76%**	**99.72%**	**100%**				
S1_Mt.Elgon	**99.44%**	**99.44%**	**99.46%**	**99.51%**	**99.65%**	**99.69%**	**99.76%**	**99.74%**	**99.67%**	**99.62%**	**100%**			
S6_Trans Nzoia	**99.42%**	**99.42%**	**99.44%**	**99.49%**	**99.62%**	**99.62%**	**99.74%**	**99.69%**	**99.65%**	**99.6%**	**99.6%**	**100%**		
S7_Marakwet	**99.42%**	**99.42%**	**99.44%**	**99.49%**	**99.62%**	**99.62%**	**99.74%**	**99.69%**	**99.65%**	**99.6%**	**99.55%**	**99.53%**	**100%**	
S9_Bomet	**99.28%**	**99.32%**	**99.35%**	**99.39%**	**99.49%**	**99.49%**	**99.6%**	**99.55%**	**99.51%**	**99.46%**	**99.42%**	**99.39%**	**99.39%**	**100%**
	S20_ Kilifi	**S12_ Kajiado**	**S23_ Kwale**	**S25_ Kwale**	**S18_ Machakos**	**S36_ Taveta**	**S26_ Taveta**	**S31_ Kajiado**	**S30_ Kajiado**	**S13_ Makueni**	**S1_ Bungoma**	**S6_**	**S7_ E. Marakwet**	**S9_ Bomet**

The pairwise sequence analysis for MCMV from MCMV isolates from some counties showing high levels of similarities of between 99.28 – 99.97%. Samples from the same geographical region were highly similar as shown in the Table 4.6 above with isolates from neighbouring counties of Trans Nzoia (Elgon), Marakwet and West Pokot

The Kenyan MCMV isolates identified in this study showed 99.75% similarity (Table 4.7) to previously reported isolates from Kenya, Ethiopia (Mahuku et al., 2015) and Rwanda (Adams et al., 2014). Table 4.7 below shows the differences in the seven ORFs, namely P32, P50, P111, P31, P7a, P7b, and CP. below.

Table 4.7: Nucleotide and Amino acid sequence identities (%) between our sample and isolates of MCMV from Kenya, Rwanda, China and USA.

Isolate	Accession	Country	Full genome (nt)	5'UTR (nt)	P32 (aa)	P50 (aa)	P11 (aa)	P7a (aa)	P7b (aa)	P31 (aa)	CP (aa)	3'UTR (nt)
MCMV_T1F6S1	MF510250	Kenya	99.75	100	100	99.5	99.7	100	100	99.6	99.5	100
MCMV_B3_S3	MF510251	Rwanda	99.75	100	99.65	99.5	99.4	100	100	99.2	99.5	100
MCMV_Yunnan2	JQ982468	China	99.02	99.09	98.26	98.8	99.4	100	100	98.2	99.5	99.6
MCMV_Nebraska	EU358605	Nebrask	96.64	97.27	97.23	96.3	97.8	98.5	96.8	93.1	99.1	97.8

Key: ORFs P32, P50, P111, P31, P7a, P7b, and CP functions explained above

The most diverse region was the ORF P31 showing 93.18% identity with an isolate from Nebraska. However, MCMV isolates from these regions were similar in the P32, P11, and P7a ORFs. The CP region was the most similar with an aa identity range of 99.15 – 99.57 % while full genome sequences showed an overall similarity of 96.6 – 99.75%.

Figure 4.6 shows the phylogeny based on the full genomes of MCMV derived from the samples in comparison to isolates from other regions/countries. The genomes assembled in this study were however more divergent from those in Asia, Ecuador and the USA (Fig. 4.6). The phylogeny of MCMV in Fig. 4.6 depicts 3 clades and shows that the samples in this study fall under clade A grouping with other Eastern African MCMV isolates from Kenya, Ethiopia and Rwanda. Clade B had isolates from China while clade C had isolates from the USA and Ecuador.

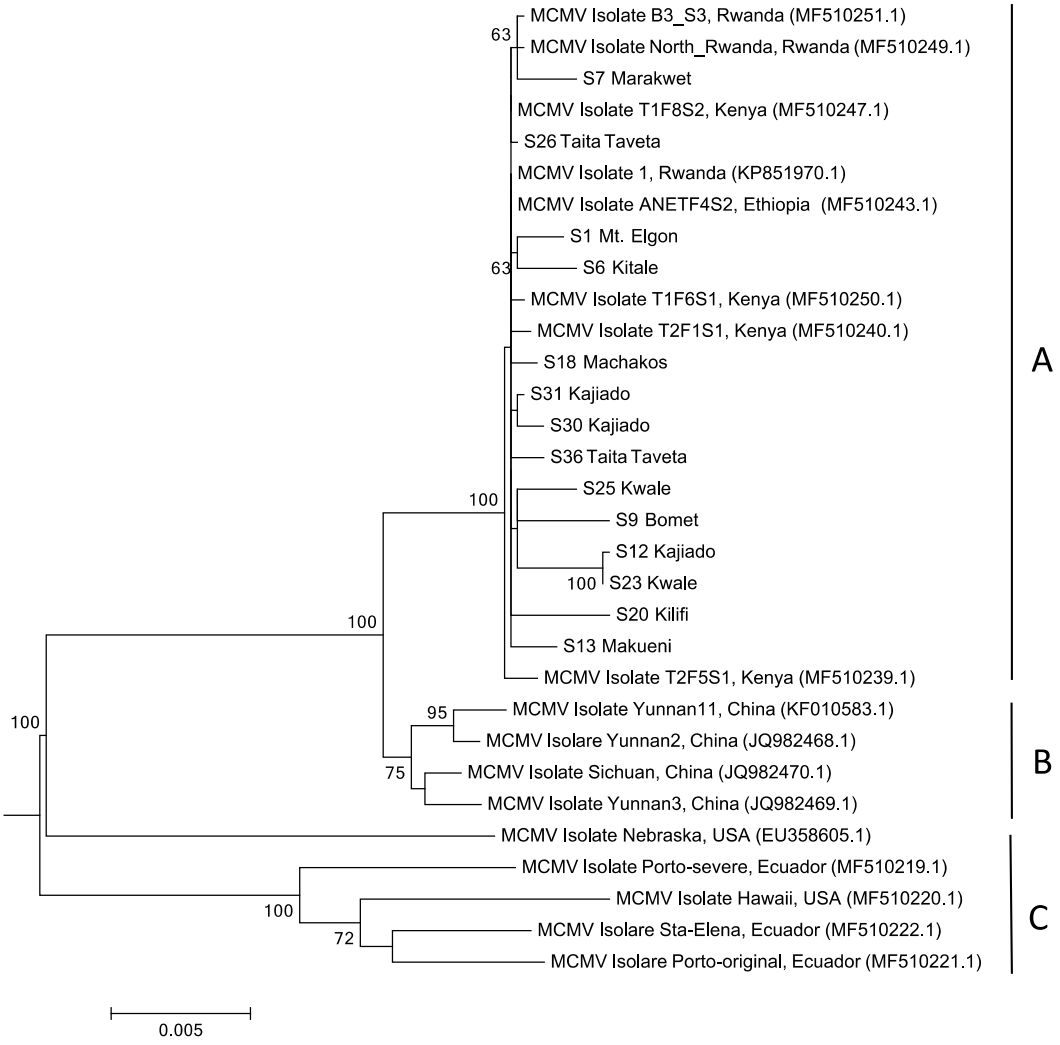


Figure 4.6: Phylogenetic analysis of the Full genomes of 14 MCMV isolates from different counties of Kenya, with other full MCMV genome sequence accessions. The evolutionary history for MCMV viruses was inferred by using the Maximum Likelihood method based on the Tamura 2-parameter model (Tamura et al., 2013).

The phylogeny depicts 3 clades and shows that the samples in this study fall under clade A grouping with other Eastern African isolates of MCMV. The phylogeny was based on aligned 4284 nucleotide bases. Clade B had isolates from China while clade C had isolates from the Americas.

The evolutionary history for MCMV viruses was inferred by using the Maximum Likelihood method based on the Tamura 2-parameter model (Tamura et al., 2013). The phylogeny depicts 3 clades and shows that the samples in this study fall under clade A

grouping with other Eastern African isolates of MCMV. The phylogeny was based on aligned 4284 nucleotide bases. Clade B had isolates from China while clade C had isolates from the Americas.

Eighteen full genomes of SCMV with lengths ranging from 9440-9647 bp (all including the polyprotein and variable lengths in the 5'UTR and 3'UTR) were recovered together with 21 partial SCMV genome sequences (1500bp-6900 bp). In some of the samples, only fragments of the SCMV genome (1500-6900 bp) were assembled (Appendix 3). The genome sequences of SCMV were quite diverse, the sequence identity of the polyprotein gene in our samples ranged from 89.8% to 100%. Amino acids similarity analysis for the SCMV polyprotein revealed that SCMV isolates from neighbouring counties of West Pokot and Marakwet were 99% -100% identical in composition

Eighteen full genomes of SCMV were also assembled from the NGS reads with lengths ranging from 9440-9604 bases (including the polyprotein and variable lengths in the 5'UTR and 3'UTR). The genome sequences of SCMV were quite diverse, the sequence identity (%) of the polyprotein in our samples ranged from 89.81 to 100% as seen in Table 4.8 in pairwise alignment. SCMV isolates from West Pokot, and Marakwet were identical in amino acid composition of their polyproteins.

SCMV sequences are conserved by geographical representation (counties) and this is further supported by the polygenetic tree of SCMV in Figure 4.7. The SCMV phylogenetic tree shows the presence of 2 clades: 11 SCMV genomes detected in this study clustered with SCMV isolates from Rwanda and Ohio, USA, Ethiopia and Iran (Clade A), while 7 SCMV genomes clustered with SCMV strains from China and Mexico (Clade B). There was a distinct difference between sample ancestry in Clade A and B. This is also illustrated clearly in the multiple sequence analysis of SCMV genomes with some selected from the National Center for Biotechnology Information (NCBI) (Fig. 4.8). There were 11 SCMV isolates in group A compared to the seven isolates in group B.

Table 4.8: Identity matrix of the polyprotein (AA) in SCMV isolates recovered from our samples

S3_W.Pokot	100%																	
S6_Trans Nzoia	100%	100%																
S4_West_Pokot	100%	100%	100%															
S7_Marakwet	100%	100%	100%	100%														
S1_Mt.Elgon	98.79%	98.79%	98.79%	98.79%	100%													
S2_Mt.Elgon	98.79%	98.79%	98.79%	98.79%	100%	100%												
S5_Bungoma	98.79%	98.79%	98.79%	98.79%	100%	100%	100%											
S35_Nyeri	98.34%	98.34%	98.34%	98.34%	98.01%	98.01%	98.01%	100%										
S34_Kirinyaga	98.31%	98.31%	98.31%	98.31%	97.98%	97.98%	97.98%	99.96%	100%									
S28_Taveta	97.88%	97.88%	97.88%	97.88%	98.24%	98.24%	98.24%	98.4%	98.37%	100%								
S27_Taveta	97.88%	97.88%	97.88%	97.88%	98.24%	98.24%	98.24%	98.4%	98.37%	100%	100%							
S29_Taveta	90%	90%	90%	90%	90.07%	90.07%	90.07%	90.17%	90.14%	90.3%	90.3%	100%						
S19_Kilifi	89.91%	89.91%	89.91%	89.91%	89.97%	89.97%	89.97%	90.04%	90%	90.17%	90.17%	99.6%	100%					
S11_Kajiado	90%	90%	90%	90%	90.07%	90.07%	90.07%	90.14%	90.1%	90.27%	90.27%	99.77%	99.51%	100%				
S14_Makueni	90%	90%	90%	90%	90.07%	90.07%	90.07%	90.14%	90.1%	90.27%	90.27%	99.77%	99.51%	100%	100%			
S32_Kajiado	90%	90%	90%	90%	90.07%	90.07%	90.07%	90.14%	90.1%	90.27%	90.27%	99.83%	99.57%	99.8%	99.8%	100%		
S25_Kwale	89.94%	89.94%	89.94%	89.94%	90%	90%	90%	90%	89.97%	90.2%	90.2%	99.73%	99.47%	99.7%	99.7%	99.77%	100%	
S21_Tana_River	89.81%	89.81%	89.81%	89.81%	89.87%	89.87%	89.87%	89.97%	89.94%	90.17%	90.17%	99.64%	99.44%	99.6%	99.6%	99.67%	99.57%	100%
	S3_ W.Pokot	S6_ Trans Nzoia	S4_ West_Pokot	S7_ Marakwet	S1_ Mt.Elgon	S2_ Mt.Elgon	S5_ Bungoma	S35_ Nyeri	S34_ Kirinyaga	S28_ Taveta	S27_ Taveta	S29_ Taveta	S19_ Kilifi	S11_ Kajiado	S14_ Makueni	S32_ Kajiado	S25_ Kwale	S21_ Tana_River

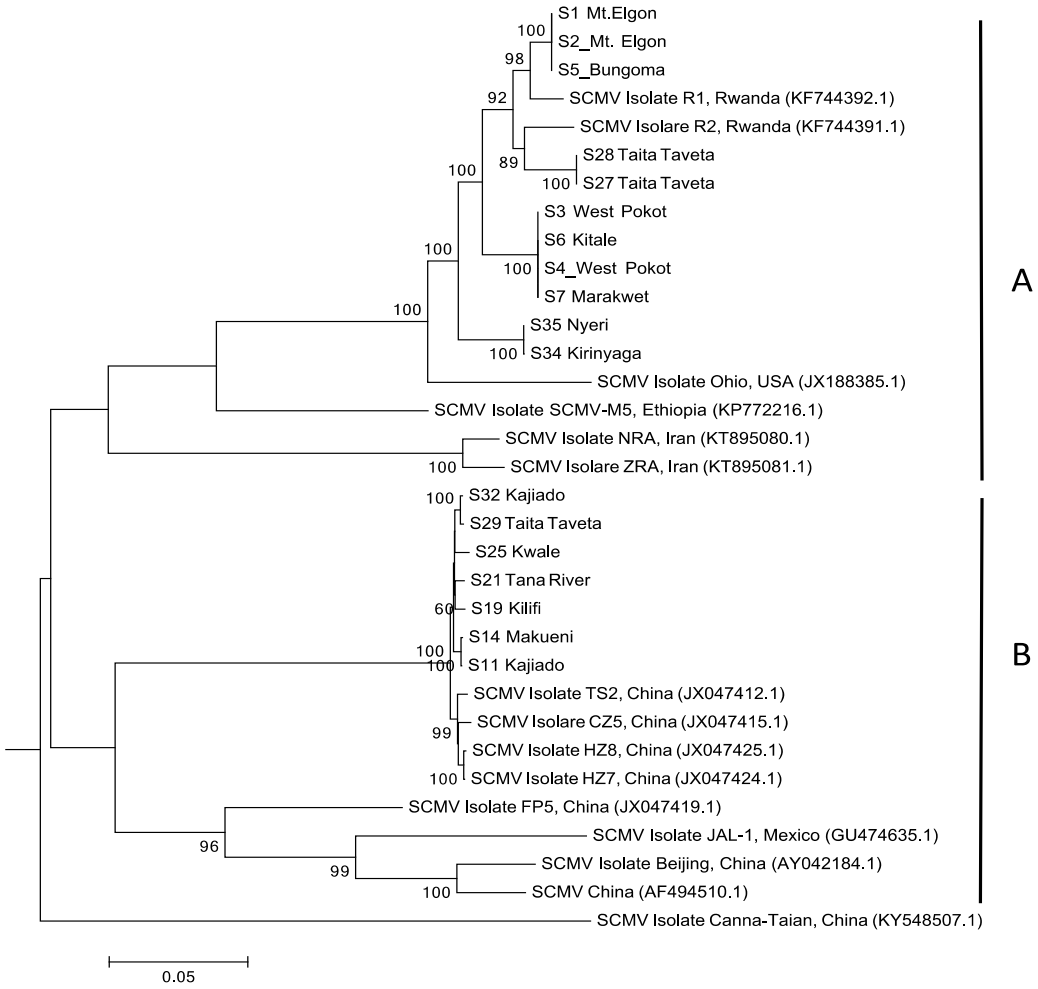
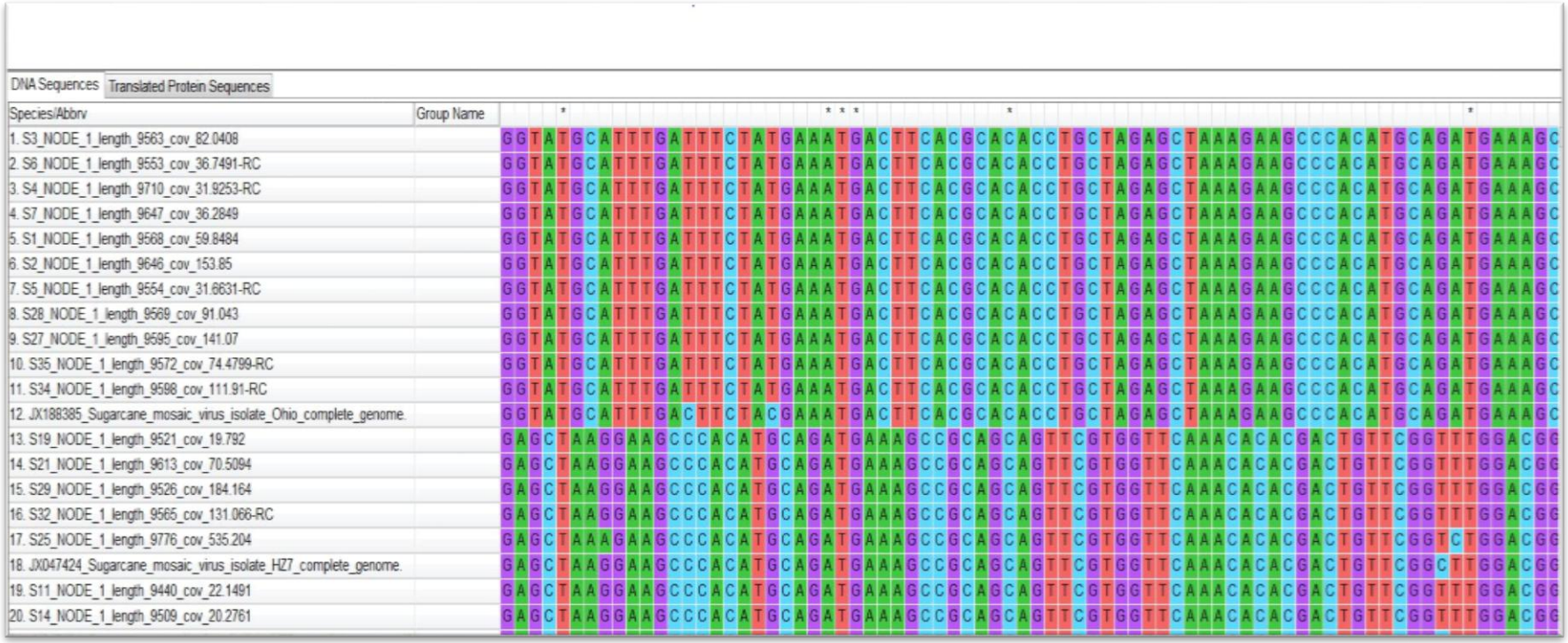


Figure 4.7: Phylogenetic analysis of the polyprotein of 18 SCMV isolates from different counties of Kenya, with other polyprotein SCMV accessions from NCBI. The phylogeny was based on aligned 9099 nucleotide bases. The evolutionary history was inferred by using MEGA version 6, Maximum likelihood method based on the General Time Reversible model at 1000 bootstraps (Tamura et al., 2013).

There were 12 sequences, S1, S2, S3, S4, S6, S7, S8, S25, S27, S28, S34 and S35, which were grouped in a distinct clade hereby denoted A. The other seven samples, S11, S14, S19, S21, S25, S29 and S32 were clearly grouped the second clade B with clear indications of a different strain of this virus. The phylogeny depicts 2 clades and shows that there are seemingly 2 strains of SCMV, and our samples are distributed between the 2 strains.

Table 4.9: Multiple sequence analysis for SCMV samples with some selected from NCBI.



The multiple sequence alignment shows distinct difference between the two strains deduced from this study. There are 12 sequences, S1, S2, S3, S4, S6, S7, S8, S25, S27, S28, S34 and S35which are clearly grouped and they depict some level of similarities compared to the others in this multiple sequence analysis. The other eight samples, S11, S14, S19, S21, S25, S29 and S32 are clearly grouped differently with clear indications of belonging to another different strain.

Seven full genome sequences for MCMV derived from this study were deposited in the NCBI nucleotide database as shown in Table 4.10 below.

Table 4.10: MCMV sequences deposited in the NCBI nucleotide database

MCMV Sequence assembled in the study	NCBI Accession number
BankIt2098945 Seq1	MH238449
BankIt2098945 Seq2	MH238450
BankIt2098945 Seq3	MH238451
BankIt2098945 Seq4	MH238452
BankIt2098945 Seq5	MH238453
BankIt2098945 Seq6	MH238454
BankIt2098945 Seq7	MH238455

4.3.4. MCMV single infection

There were 4 samples where only MCMV as an MLN causing virus was recovered. These were S9-Bomet, S12-Kajiado, S16-Machakos and S-18_Machakos (Table 4.11). These samples also posted medium to high MLN symptoms severity. No SCMV sequences were generated from these samples and the Blast results showed only the presence of MCMV and other short sequences of *Maize Yellow dwarf virus* (Table 4.5). The Krona Blast display also shows only MCMV (Figure 4.5A) while all other samples with MLN showed presence of both MCMV and SCMV (Figure 4.5B).

Table 4.11: Samples that showed only MCMV infection both through NGS and laboratory testing for MCMV and SCMV and their corresponding MLN symptom severity.

Sample ID	Contig Length (bp)	Symptom severity (1-5)
S9_Maize chlorotic mottle virus_Bomet	4405	3.5
S12_Maize chlorotic mottle virus_Kajiado	4403	3.2
S16_Maize chlorotic mottle virus_Machakos	4404	2.1
S18_ Maize chlorotic mottle virus_Machakos	4403	1.9

4.3.5 Viral recombination Analysis for MCMV and SCMV

Potential recombination events were detected in 11 out of 18 SCMV genome sequences but MCMV sequences recovered in this study did not generate any recombinants. However, only 2 SCMV genome sequences were considered to be recombinants with different possible major and minor parents by at least four different RDP4-implemented methods with acceptable P values of $<1.0\times10^{-06}$ (Table 4.12). The recombinant SCMV isolates detected were S2 Bungoma and S35 Nyeri (Table 4.12).

Table 4.12. Detected recombination events of several SCMV isolates by at least 5 recombination evaluation methods. The two from the study samples were S2 Bungoma and S35 Nyeri. The corresponding P values and the recombination sites are illustrated. The methods key; R-RDP, G-GENECOV, B-BootScan, M-MaxiChi, C-Chimaera and S-Siscan were the recombination analysis methods used.

Recombinant	Programs supporting recombination	Major Parent	Minor Parent	P-Value	Recombination sites
S2 Bungoma	RGBMCS	KF744392	S7 Marakwet	2.804×10^{-50}	4619 – 9567
MG 932079.1	RGBMCS	KP860935	S7 Marakwet	6.423×10^{-37}	5532 – 7977
MF467403.1	RGBMCS	MF467404.1	S2 Bungoma	1.359×10^{-16}	4063 – 4137
MF467403.1	RGBMC	S32 Kajiado	S2 Bungoma	$7.728.\times 10^{-16}$	4598 – 9443
JX047419	RBGMCS	S35 Nyeri	MG 932079.1	1.678×10^{-26}	7281 – 8948
MG932076.1	RGMCS	S27 Taveta	KF 744392.1	4.380×10^{-10}	8078 – 9562
S35 Nyeri	RGBMCS	S27 Taveta	S7 Marakwet	6.138×10^{-15}	4653 – 9562
MG930076.1	RGBMC	S27 Taveta	S32 Kajiado	9.607×10^{-141}	8115 – 9571

In the S2 Bungoma recombinant sequence, the major parent was KF744392, an SCMV isolate from Rwanda while the minor parent was S7 Marakwet isolate. S35 Nyeri had S27 Taita Taveta as major parent and S7 Marakwet as the minor parent.

The Neighbor joining trees illustrated in Figure 4.9 shows sample S2 Bungoma and S35 Nyeri as recombinants sequences. Isolate S7 Marakwet was used to infer unknown minor parent and S27 Taita Taveta was identified as the major parent for the S35 Nyeri recombinant sequence.

It was also observed that some SCMV accessions in the GenBank had some of our isolates as minor parents e.g. MG 932079.1, a Kenyan isolate from Kirinyaga county having KP880903, an Ethiopian isolate, as the major parent and S7 Marakwet, as a minor parent. The same was observed for MF467403.1 (Tanzania) which had MF467404.1 (Tanzania) as a major parent and S2 Bungoma as a minor parent. As such, several other SCMV accessions had our sequences either as minor or major parents. There were also weak recombinant signals showing Nigeria and USA isolates as minor parents of our isolates.

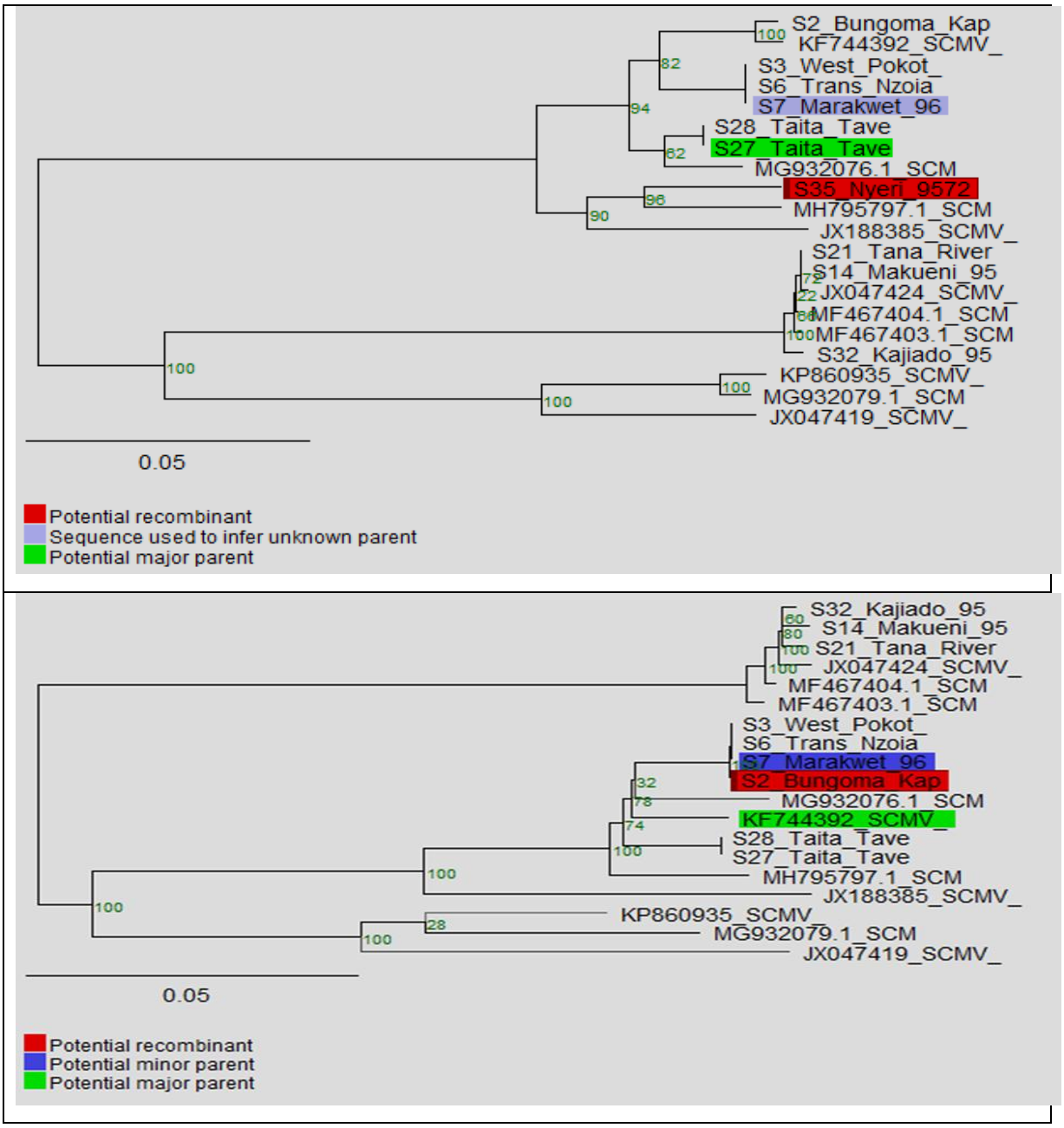


Figure 4.8: Neighbor joining trees generated after accepting S2 Bungoma and S35 Nyeri as recombinants with several recombination events detected. Sample S7 Marakwet and KF744392 are the minor and major parents for the recombinant S2 Bungoma, respectively. The Neighbor joining trees were based on the transition transversion method model at 100 bootstraps (Kimura 1980)

Recombination analysis through RDP4 also allows elucidating the exact points along the viral genome where genetic recombination has occurred, as illustrated in Figure 4.10. The recombination event occurred along position 4619 – 9567on the SCMV genome for sample S2 Bungoma having KF744392 and S7 Marakwet as major and minor parents, respectively. The

size of the fragments from both the major and minor parents can be determined in each recombinant sequence.

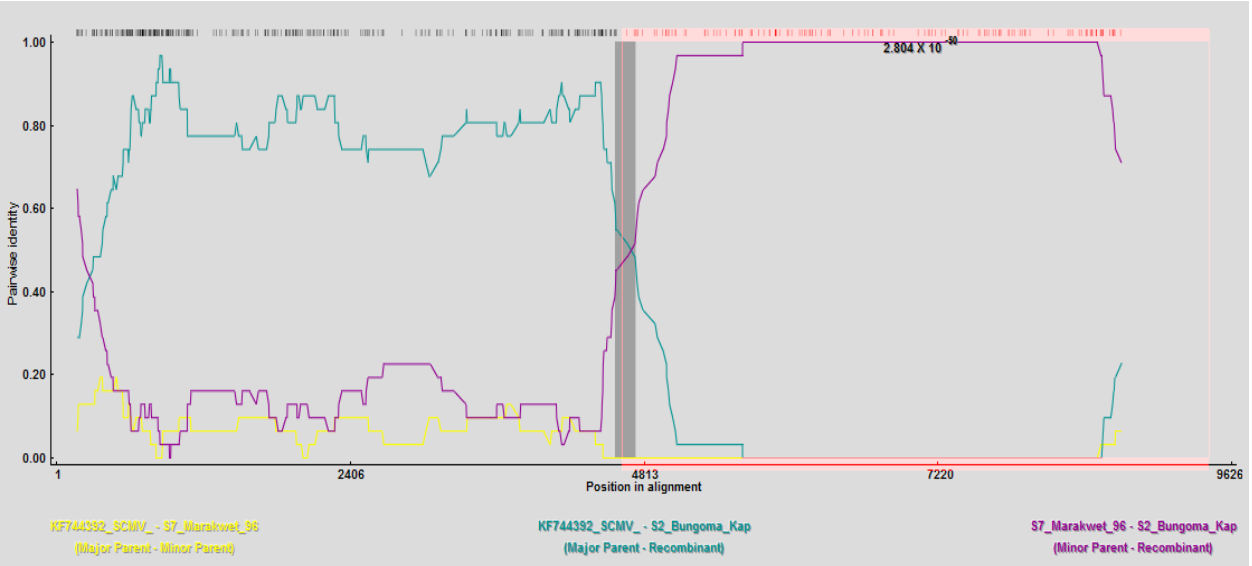


Figure 4.9: Pairwise identity of the sample sequences showing the recombinant S2 Bungoma SCMV sequence and the possible parents of this recombinant sequence: KF744392 identified as the major recombinant parent and S7 Marakwet as a minor parent. Recombination event was detected in the 4619 – 9567 bp region of the genome.

4.4 Discussion

The MLN causing viruses were assessed comprehensively in the country with a pioneer report on the viral recombination profiles for SCMV. Recombination of these viruses which plays an integral role in the evolutionary process of viruses was evaluated in detail. In this study, MCMV was more prevalent than SCMV in all the areas under the survey in farmers' fields. In all the 118 maize composite leaf samples collected, 95 tested positive for MCMV by RT PCR while 85 tested positive for SCMV through DAS-ELISA. Co-infection of the two viruses was witnessed in 73 samples evenly distributed across the country. Previous studies on co-infection of MCMV and SCMV demonstrated increased accumulation of MCMV (Zihao Xia et al 2016). In the same study, synergistic infection of MCMV and SCMV increased remarkably the accumulation of vsiRNAs from MCMV, which were mainly 22 and 21 nucleotides in length. In the United States, it was found out that when maize plants are co-infected with MCMV and one of several potyviruses including *maize dwarf mosaic virus* (MDMV), *wheat streak mosaic virus* (WSMV) or *sugarcane mosaic virus* (SCMV), leaves and stems of infected plants develop a severe systemic necrosis known as corn lethal necrosis (CLN) disease which is MLN (Scheets K. 1989). However, it was observed that only MCMV was present in some

farmers' fields with high incidences and severity of the MLN disease. This indicates that MCMV alone can lead to severe infections like those for plants with coinfection on the aforementioned viruses. This was also demonstrated in the sequencing work where maize leaf samples from farms that had clear symptoms of MLN finally showed 100% infection by only MCMV.

Analysis of sequences through NGS showed that SCMV was the only potyvirus identified in this study that coinfects maize together with MCMV thereby causing MLN. However, MaYMV, a *polerovirus* was also detected in some farms. Coinfection of MCMV and SCMV viruses in farmers' fields was recorded in 73 samples evenly distributed across the country. Moderate to high MLN severity scores were also recorded in these 73 samples during the survey. In the United States, it was found that when maize plants are co-infected with MCMV and one of several potyviruses including MDMV, SCMV or the *tritimovirus* WSMV, leaves and stems of infected plants developed severe systemic necrosis known as corn lethal necrosis (CLN) disease which is an MLN synonym (Scheets K. 1998). The same scenario was observed in this study where samples with both MCMV and SCMV exhibited severe MLN symptoms. Studies on co-infection of MCMV and SCMV demonstrated increased accumulation of MCMV and virus-derived small interfering RNAs (vsiRNAs) from MCMV (Xia et al. 2016). This indicates increased RNA silencing activity by the plant's defense mechanism against the virus infection. It has been demonstrated that the helper component protease (HC-Pro) encoded by potyviruses mediates suppression of post transcriptional gene silencing (PTGS) enhancing the pathogenicity and accumulation of other heterologous viruses (Pruss et al., 1997; Gonzalez et al., 2005; Syller et al., 2012). Though they belong to the same family (*potyviridae*), WSMV HC-Pro has been shown not to influence disease synergism with MCMV (Stenger et al., 2007). Instead, WSMV mediates synergistic interactions with other viruses by utilizing a gene other than HC-Pro for PTGS suppression.

Investigation on ultrastructural damage on chloroplasts in bundle sheath cells of maize leaves infected by both MCMV and SCMV had much smaller starch grains in the chloroplasts (Wang et al., 2017) which indicates that co-infection leads to severity of the disease. It has also been demonstrated that there is an increase in the MCMV virus titer in mixed infections with *Maize dwarf mosaic viru s*(MDMV), strain-B (Goldberge and Brakke, 1987) or with *Johnsongrass mosaic virus* (JGMV), a potyvirus that has been recently reported to co-infect maize with

MCMV causing MLN (Stewart et al., 2017); however, these viruses were not detected in this study.

MaYMV was detected in 5 samples in this study. MaYMV has been detected in maize samples from recent MLN survey studies in eastern Africa and in other MLN endemic countries worldwide (Asiimwe et al., 2019). In eastern Africa, MaYMV has been found present in all recent MLN related studies though some publications have described it as Maize yellow dwarf mosaic virus (MYDMV) (Adams et al., 2017: Massawe et al., 2018: Wamaitha et al., 2018; Read et al., 2019; Asiimwe et al., 2019; Kiruwa et al., 2019: Stewart et al., 2020). Some recent study of maize infecting viruses in South Korea (Lim et al., 2018) identified MaYMV prevalence but no link to MLN related symptoms. Though MaYMV is frequently found in samples with MLN causing viruses, there has been no direct link to it contributing to the MLN disease complex. Polerovirus, just like potyviruses, are known to suppress post transcriptional gene silencing but it is not yet clear if this factor exacerbates MLN proliferation (Baumberger et al., 2007).

However, there were some farmers' fields that were SCMV negative by DAS-ELISA and MCMV positive by RT-PCR but showed severe MLN symptoms. These isolates were S9-Bomet, S12-Kajiado, S6-Machakos and S18-Machakos. These farms posted moderate to high MLN symptom severity scores (1.9-3.5 on a 1-5 MLN severity scale) showing full development of MLN just like those with co-infected with SCMV (Table 4.10). Farmers field samples S9 and S12 had symptom severity scores of 3.5 and 3.2 respectively. This indicates that MCMV alone can lead to severe infections like those arising from coinfections. This observation was also supported by the NGS data where samples that had clear MLN symptoms showed infection by only MCMV through Blastn results the recovered MCMV sequences (Tables 4.5 and 4.11).

MCMV genome sequence comparison with accessions from China, Africa and the USA reveals that MCMV is a highly conserved virus with identities ranging from 96% to 99%. This is typical of members of *Tombusviridae* where the diversity of nucleotides documented are between 0-0.02 with MCMV in the genus *Machlomovirus* recording lowest nucleotide diversities of 0.01 (Varanda et al., 2014 and Braidwood et al., 2018). Comparison of the amino acid sequences of the viral proteins also exhibited high similarities, especially in the P7a, P111 and the CP regions as indicated in Table 4.7. The 5' and 3'UTRs were highly conserved in all the isolates under

investigation confirming reports from previous studies (Mahuku et al., 2015a; Braidwood et al., 2018; Wamaitha et al.,2018).

Phylogenetic analysis suggested a potentially common origin for Eastern Africa and Asian MCMV isolates. The isolates clustered in clade A (Figure 4.6) together with MCMV isolates from Kenya, Ethiopia and Rwanda indicating a very close relationship of MCMV strains circulating in Eastern Africa. The closest neighbor (clade B) contains MCMV isolates from China suggesting that the MCMV strains endemic in Eastern Africa may have had its origin from China. The MCMV isolates characterized seemed to be more divergent from the MCMV isolates in the USA and South America. Recent studies on global phylogeny of MCMV by Braidwood and colleagues (Braidwood et al., 2018) also showed close similarity between the China and Eastern Africa isolates. However, a more distinct strain in East Africa which has proved to be more virulent compared to the strains of the Americas and Asia (CIMMYT MLN Epidemiology research Project report 2019). This is evident from the reported yield losses of 50-100% in Kenya (Mahuku et al., 2015a; De Groote et al., 2016) and up to 90% in Ethiopia (Girma et al, 2018). Initial reports alluded to MCMV not varying in its infection and pathogenicity (Wang et al., 2017) but the infection patterns in eastern Africa show a different scenario. Materials tolerant to MCMV in the US have been found to be susceptible to MLN in eastern Africa indicating differences in the pathogenicity of the strains in the US and those circulating in eastern Africa (CIMMYT, 2017).

Phylogenetic analysis of SCMV recovered sequences revealed 2 distinct clusters of SCMV (Figure 4.7). This analysis also showed some geographical clustering as seen for the isolates from Trans Nzoia, West Pokot and Marakwet counties which border each other in the north rift region of the country. The same trend was observed with SCMV isolates from counties of Kajiado, Taita Taveta, Kwale, and Tana River in South rift and coast region. There were no other potyviruses recovered through NGS in this study, contrary to other studies where a potyvirus, *Johnsongrass mosaic virus* (JGMV) (Stewart et al., 2017; Wamaitha et al 2018) was found to be present in MLN infected plants.

Viral recombination of the identified viruses was evaluated using the recombination detection program RDP4 v4.84 (Martin et al., 2015). Farms in Bungoma and Nyeri indicated strong recombination signals and were recombinants as explained earlier. The recombination events were also identified amongst SCMV isolates found in the eastern Africa region with the exception of one isolate from China (JX047419), which had our isolate S35 Nyeri and another

Kenyan isolate MG932071 (Wamaitha et al., 2018) as a major and minor parent, respectively. Generally, recombination signals were strong amongst eastern Africa isolates mostly from Kenya, Rwanda, Tanzania and Ethiopia. A typical case is a Tanzanian isolate, MF 467403.1 which had our sample S32 Kajiado as a major parent and S2 Bungoma as a minor parent. This is expected for Kajiado borders Tanzania and this isolate MF 467403.1 (Kiruwa et al.,2019) originates in Arusha, a district that borders Kenya.

Recombination signals were not detected in the MCMV genomes analyzed. This is partly due to the fact that the virus is largely conserved with little genetic variation across the globe (Braidwood et al., 2018). As illustrated in the MCMV phylogenetic analysis, MCMV isolates are highly similar hence genetically conserved with minimal evidence of rapid evolution. There is a clear separation of MCMV isolates from different world regions, indicating that there has been no recombination between MCMV genomes in geographically isolated regions (Braidwood et al., 2018).

Viral genetic recombination is a natural phenomenon and has been demonstrated to play an important role in the evolution of viruses (King et al., 1982). Recombination in viruses has also been observed to be a pervasive process that generates diversity in most viruses (Valli et al., 2007 and Martin et al., 2015). It occurs when at least two viral genomes coinfect the same host cell and exchange genetic segments possibly creating new variants for viruses to adapt to new hosts and environments by selective pressures (Perez-Losada et al., 2015). Similar studies on SCMV diversity in Shanxi, China revealed that SCMV not only evolves by divergence from common ancestors but also by inter-viral recombination (Xie et al., 2016). A considerable number of *potyviruses* can, in fact, be regarded as successful products of several recombination events (Goncalves et al., 2011). Intra-species recombination is important in *potyviridae* evolution (Li YQ et al., 2013) as demonstrated for the eastern Africa SCMV isolates.

The evolutionary pattern of SCMV needs to be continuously assessed in the country to determine if there are any virulent or more severe strains for which commensurate management strategies must be designed. Molecular diagnostic protocols need to be updated by incorporating new primer sequences designed by analyzing the new SCMV sequences generated and publicly available. It has been shown previously by KEPHIS (KEPHIS annual report 2016) that primer sequences for SCMV from other sources do not work for the Kenya

SCMV isolates. This was the case where the SCMV primers used did not amplify the target Kenyan SCMV isolates hence the reason why DAS-ELISA was used in detecting SCMV. This confirms the high level of diversity in the SCMV sequences across the globe hence the need for specific primers for the local isolates. Real time qPCR primers were designed for these Kenyan SCMV isolates. The primers and probe targets the region 7375 – 7521 with an expected amplicon of 186 bp.

4.5 Conclusion

The MLN disease complex in Kenya is caused by MCMV in synergy with SCMV, the only potyvirus detected from the countrywide survey of maize fields. However, MaYMV, a poleovirus was also detected in a few farms signalling the potential of these virus to playing a role in MLN development. Only SCMV exhibited recombination contributing to the rapid evolution of this virus. There is need for further studies to ascertain the sum effect of other viruses, or abiotic factors that complicate the aetiology of MLN in Kenya and by extension in eastern Africa.

CHAPTER FIVE

DEVELOPMENT OF A REVERSE TRANSCRIPTASE-LOOP MEDIATED ISOTHERMAL AMPLIFICATION (RT-LAMP) ASSAY FOR THE DETECTION OF MAIZE CHLOROTIC MOTTLE VIRUS (MCMV)

5.0 Abstract

Monitoring of MCMV is important in farmers' fields, seed fields, seedlots, in grain and seed for trade in the region. There is therefore need for a sensitive and affordable diagnostic method of testing for MCMV both in the laboratory and in the field. A reverse transcription loop-mediated isothermal amplification (RT-LAMP) assay with a two end point analyses was developed to detect MCMV in maize during active vegetative stages of maize crop. Six sets of specific primers were designed from a consensus sequence after multiple sequence alignment of various MCMV NCBI accessions with majority from the eastern Africa region. The selected primer set targeted the region 3760-3955nt of the MCMV genome sequences. The primers' efficiency was evaluated for specificity and sensitivity. Amplification in the GenieII model GEN2-02 LAMP for this RT-LAMP assay was very efficient with sharp visible curves displayed between the 10^{th} and the 20^{th} minute of the reaction hence quite rapid. The reactions were fully completed in 30 minutes compared to 1 hour for the colour changing, dye-based (Sybr Green) end point analysis LAMP assays. The assay discriminated against the common viruses infecting maize in the eastern Africa region namely SCMV, MDMV, WSMV, PMV, MSV. The real-time amplification analysis showed excellent specificity of this assay to MCMV detection with absolutely no chances of cross contamination. The assay cost for detection of MCMV was $7.5 compared to $35 for RT-PCR. The simplicity, rapidity, and inexpensiveness of this technique makes it a suitable choice for large-scale sample processing, especially by laboratories with limited resources and for the field analysis by regulatory agencies in the country.

5.1 Introduction

Maize chlorotic mottle virus (MCMV) belongs to the family *Tombusviridae* and is the only species in the genus *Machlomovirus* (Nutter et al., 1989). The virions of this single-stranded RNA virus are isometric, and the single-component particles have a smooth spherical or hexagonal shape (Scheets, 2000). Previously, two genetically and geographically distinct strains of MCMV were reported, MCMV-P (Peru) and MCMV-K (Kansas) (Nyvall, 1999). However, recent studies on global phylogeny of MCMV reveal distinct MCMV strains for East

Africa, North America, China and South America (Braidwood et al. (2018). The virus was first reported to infect *Zea mays* in Peru (Castillo and Hebert, 1974). MCMV is not widespread in the United States, having been reported only in Nebraska, Kansas (Uyemoto et al., 1980), and Hawaii (Niblet and Claflin, 1978). Globally, the virus occurs in Argentina, Mexico, and Peru and lately Eastern Africa, China, Spain and Ecuador (Quito-Avila et al., 2016). Maize Lethal Necrosis has spread fast in the eastern and central Africa regions after it was first reported in Kenya (Wangai et al., 2012). For instance, in Tanzania, the disease was first reported in 2012 in regions around Lake Victoria and Arusha (CIMMYT, 2012). The government of Tanzania and The Maize and Wheat Improvement Center (CIMMYT) carried out surveys and tests which revealed the presence of MCMV and SCMV in maize leaf samples (CIMMYT, 2012). MLN was also reported in Uganda in 2012 in the Kenyan border districts of Busia and Tororo (CIMMYT, 2012) and has been detected in eastern Uganda districts of Iganga and Mbale (Kagoda et al., 2016). Currently, the disease is present in the western, south western and recently in the northern parts of Uganda (NaCCRI, 2017, 2019). In Rwanda, it was first reported in 2013 and was found endemic in all maize growing districts (Adams et al., 2014). The disease was officially reported in the Democratic Republic of Congo (DRC) predominantly in western provinces of North and South Kivu in 2014 (Lukanda et al., 2014). In Ethiopia, maize plants with MLN symptoms were first observed in 2014 prompting surveillance which led to the first report in 2014 (Mahuku et al., 2015a). Nowadays, the disease is endemic in Gambela, Oromia, Amhara, SNNPR and Gumuz administrative regions of Ethiopia (EIAR, 2019). There are reports of MLN in Southern Sudan (Mahuku et al., 2015b) and Burundi Institute of Agronomic Sciences (ISABU), 2017). MLN has had a serious impact on maize production and grain yield in eastern Africa. In Kenya, maize yield losses of 23-100% were estimate in maize growing counties in 2012-2013. For instance, 26,000 ha of maize were affected by MLN in Kenya in 2012 estimated losses of up to US$ 52 million (Ministry of Agriculture, Kenya, 2012), and by 2013 (De Groote et al., 2016).

Continuous maize production in the field greatly increases the incidence of MCMV, because the virus can be spread by insect vectors, mechanically, and by seed at very low rates of 0.003% (Jensen et al., 1991). Additionally, MCMV is possibly transmitted through infested soil, as the virus can survive in maize plant residues (Nyvall, 1999). Therefore, it is essential to test for the presence of this virus in seed lots and in the field aiming its management and to limit its spread.

Disease diagnosis and virus detection in maize is important to monitor maize diseases through surveillance programs and to facilitate implementation of appropriate management measures (Riley et al., 2002). MCMV has been detected in leaves, pollen, female and male inflorescences, ear husks, cotyledons, and seeds (pericarps, endosperm, cotyledons, and embryo) (Scheets, 2004). The most reliable methods currently used for detecting MCMV in host tissues include ELISA (enzyme-linked immunosorbent assay), Northern blots, and polymerase chain reaction (PCR). *Maize chlorotic mottle virus* infection may be difficult to diagnose based on symptoms alone, because some of its symptoms (stunting, chlorosis) resemble those caused by nutrient deficiencies, moisture stress or other maize infecting viruses like *Maize mosaic virus, Maize streak virus* and *Maize stripe virus* (Marchand et al., 1995).

PCR has been cited for lower levels of sensitivity and specificity compared to quantitative real-time PCR (qPCR) (Mackay et al, 2002). However, qPCR involves acquisition of expensive equipment that are not affordable by most laboratories in developing countries. It is against this background that a simple, versatile and cheap molecular based method of diagnostics for MCMV need to be developed. The Reverse Transcriptase Loop Mediated Isothermal Amplification (RT-LAMP) was therefore a preferred choice. LAMP is a powerful innovative gene amplification technique used as a simple and rapid diagnostic tool for detection and identification of microbial diseases. This method first described by Notomi et al., (2000) employs a DNA polymerase and a set of four specially designed primers that recognize a total of six distinct sequences on the target DNA. An inner primer containing sequences of the sense and antisense strands of the target DNA initiates LAMP. Amplification can be achieved in 1 hour under isothermal conditions with a set of six primers in a single tube. Amplification can be detected by a turbidimeter, colourimetric detection (Wong et al., 2015), agarose gel electrophoresis or by a real time fluorimeter platform. Since its discovery, LAMP has been widely used for diagnosis of various plant and animal disease worldwide (Parida et al., 2004, 2006).

The current documented LAMP assays for detection of MCMV (Chen et al., 2016; Zhanmin et al., 2016) exploit the colour change end point analysis which have limitations of uncertainty in correct colour change contrast observations and the high risk of false positives due to contamination of the testing equipment and environment. The other documented LAMP assay for MCMV detection (Xu et al., 2017) employs the colorimetric and turbidity visual assessment also with limitations as earlier indicated.

MCMV is a highly stable virus (Wang et al., 2015) hence the opening of reaction tubes for incorporating the DNA intercalating dyes leads to massive contamination and false positives (Hsieh et al., 2014) as used in the aforementioned MCMV LAMP assays.

As such, an extra real time end point analysis and a colourimetric end point analysis assay which does not involve handling of dyes and reaction tubes opening is vital. The objective was to develop a LAMP assay with a two-end point analysis for the detection of MCMV. The two-end point analysis include the observation of colour change of the SYBR green dye from orange to green without opening the tubes and real-time graphical plots of the amplification based on the detection of fluorescence in the samples using the Genie II platform. The Genie II allows real-time isothermal amplification to be performed on a low power portable platform hence the assay can be performed in the field. The closed tube system used in Genie II avoids any post-amplification handling, eliminating laboratory contamination from the amplified product. The isothermal amplification master mix that allows fluorescence detection of the product on the Genie II platform can also be used on generic qPCR instrumentation.

5.2 Materials and methods

5.2.1 Plant preparation in the greenhouse

Maize plants were planted and maintained in the quarantine screen house in Ohio Agricultural Research Development Center (OARDC), Ohio State University, USA. The maize hybrid grown in the screen house was DEKALB DKC55-84RIB. The plants were raised in pots with soils sterilized by heat treatment. Normal agronomic practices were observed during the growth period. Pure cultures of the viruses under study were inoculated 3 weeks after transplanting by gently rubbing the infected leaf sap extracts mixed with carborundum on the leaves. The viruses inoculated and maintained were *Maize dwarf mosaic Virus* (MDMV), *Wheat streak mosaic virus* (WSMV), *Panicun mosaic virus* (PMV), and the MLN causing viruses *Maize chlorotic mottle virus* (MCMV) and *Sugarcane mosaic virus* (SCMV). Plants were observed for symptoms development for the viruses inoculated. Plants inoculated for each virus were maintained in separate chambers to avoid cross contamination hence maintenance of pure cultures.

5.2.2 Ribonucleic acid (RNA) extraction and detection of MCMV by conventional RT-PCR.

Total RNA was extracted from fresh leaf samples infected with MCMV at active vegetative stage. The ZR RNA MiniPrep™ kit (Zymo Research, USA) was used for RNA extraction from the samples. The quality and concentration of RNA was tested using a NanoDrop ND-1000 Spectrophotometer (Thermo Fischer Scientific, Wilmington USA). An RNA Agarose gel electrophoresis was used for quality check of the extracted RNA. A 2-step RT-PCR was performed with the samples to verify the presence of MCMV using primers (MCMV F 5' – CCG GTC TAC CCG AGG TAG AAA – 3' and MCMV R 5' – TGG CTC GAA TAG CTC TGG ATT T – 3') - Research unit at OARDC, Ohio State University. The first step involved cDNA synthesis using Maxima First strand cDNA synthesis kit for RT-qPCR, with ds DNase (Thermo Fisher Scientific, USA). The 20 µl reaction mixture contained 4 µl 5X reaction mix, 2 µl Maxima Enzyme mix, 1 µl of the RNA template and 13 µl of nuclease free water. The mixture was incubated at 25 ^{o}C for 10 min, 50 ^{o}C for 15 min and the reaction was terminated at 85 ^{o}C for 5 min. The cDNAs synthesized were used in conventional PCR with a reaction mixture of 50 µl. The mixture had 25 µl of the DreamTaq PCR Master Mix, 0.1ul of both reverse and forward primers, 2.0 µl of cDNA and 22.8µl of nuclease free water.

The following cycle parameters were used: denaturing 95 ^{o}C for 30 sec, annealing at 49 ^{o}C for 30 sec and Extension 72 ^{o}C for 1 min. Final extension was 72 ^{o}C for 15 min. PCR was done using the GeneAmp 9700 PCR system thermocycler (Applied Biosystems, Foster City, CA, USA) programmed as shown in Table 5.1 below.

Table 5.1: Conditions for PCR reactions for MCMV detection.

Step	No. of cycles	Temperature (^{o}C)	Time
Initial denaturation	1	95	5 min.
Denaturation		95	30 sec.
Annealing	25	49	30 sec.
Extension		72	1 min.
Final extension	1	72	15 min.

5.2.2 RT-LAMP Primer design

Complete sequence genomes for MCMV were extracted from NCBI Nucleotide database for use in this work. The accession numbers for the used sequences were KJ782300 MCMV isolate from Taiwan (Deng et al., 2014), EU358605 MCMV isolate from Nebraska USA (Stenger and French 2008), KF744396 MCMV Rwanda isolate (Adams et al., 2014), KF010583 MCMV China isolate (Wang et al., 2011) and the Kenyan MCMV isolate (Mahuku et al., 2015). The sequences were subjected to multiple sequence alignment using the ClustalW Omega software (http://www.ebi.ac.uk/ Tools/msa/clustalo/2015) to obtain the consensus sequences. The target primer design was accomplished by using the Primer Explorer V4 software [LAMP primer designing support software program, Netlaboratory, Japan (http://venus.netlaboratory.com), 2015].

The four key factors in the LAMP primer design that were considered as per the instructions in the Primer Explorer V4 manual for the software were;

i. Melting Temperature (Tm).
ii. Stability at the end of each primer,
ii. GC content, and
iii. Secondary structure.

The stability of the 3' end at the region F2, 5' end at the region F1c, 3' end at the region B2, and the 5' end at the region B1c is important for LAMP primer amplification efficiency. These are the starting positions of DNA synthesis initiated by primers hence their end stability is important. The primers were specifically checked to see whether the change of free energy (ΔG) (stability) is -4.0 kcal/mol or lower. Only those that met these criteria were selected as possible candidates. The change in free energy (ΔG) is the difference between the product free energy and the reactant free energy.

Primers were designed so that their GC content is between about 40% to 65%. Primers with GC content between 50% and 60% tend to give relatively good primers. As such, the range that was chosen for this parameter was for GC content of 50 - 60%. The parameter was also set automatically selected GC rich sections along the entire target sequence.

The melting temperature (Tm) for each region was designed to be about 65°C (64 – 66°C) for F1c and B1c, about 60°C (59 – 61°C) for F2, B2, F3, and B3, and about 65°C (64 – 66°C) for

the loop primers. The differences in the Tm were set to about 5°C for the primers (regions F2 and F1c, regions B2 and B1c). To facilitate forming this loop, F1c (B1c) was set at a Tm value of 5°C higher than those of the other primers. To prevent the formation of primer dimers, only primers with non-complementary 3' ends were selected.

All these parameters were considered for more than 30 possible LAMP primers generated by the software and six sets were selected for meeting all the requirements i.e., the primer melting temperature (Tm), stability at the end of the primers, the GC content and the inability to form secondary structures as described above.

The sequences of the six designed primer sets were selected based on the described parameters described are shown in Tables 5.2 below. These primers were synthesized by Integrated DNA Technologies (San Diego, California 92121, USA). All the six sets for each primer candidate were HPLC-purified.

Table 5.2: The six MCMV primer sequence options derived from LAMP primer designing support software program, Netlaboratory, Japan. Six primer set options were obtained with the inner primers (F3 and B3), outer (FIP and BIP) and the Loop primers (LF and LB)

MCMV LAMP PRIMER SETS	PRIMER TYPE		PRIMER SEQUENCE
MCMV K2- (2527-2704) ID:5 176bp	1F3	F3	ACAGGTTGAGTTTTGCCAGA
	1B3	B3	TCCTGCTTTACGGGGAGG
	1FIP	FIP	CCTTGGACATGGTGGTGCGTG-TGCGACCAGTCTTTGACG
	1BIP	BIP	CCTTTTAACACGCCAACCGCAG-GTAAGGCTGAGTCCGCATTC
	1LF	LF	GTCTCTCATCATGGTGTATTGTGT
	1LB	LB	AGGAGGTGGATGAGAGCAGTT
MCMV K2-(2020-2233)ID:1 209bp	2F3	F3	GGCTATCCAGAGCTTCTCAC
	2B3	B3	TCAGGACGTTGTCATCTCCA
	2FIP	FIP	CTGGTAGTTGAACGCGCCGTC-GCGGTGGCAGATTCACAA
	2BIP	BIP	ATTGGGCAATTGCATCCTGGC-GGATCCCCAGCTTGGTCA
	2LF	LF	GAAGCGTATGCTGTTCCACGA
	2LB	LB	CACAGCAATCACACATGACTTCG
MCMV NEW K1 (463-636) ID:19 173bp	3F3	F3	CAAGTGCCAGGCATGTGAT
	3B3	B3	TGTGGGTTGGATAGGTCTCT
	3FIP	FIP	TCGTCTCCGGATTCCGGCAA-CCAGACTTTCAAGCACGGTC
	3BIP	BIP	TGCGGAGTGGATGGAGCAAG-TGGTTGGAATGGGGTCATCA
	3LF	LF	AAGCAGAGCCCGCGAGA
	3LB	LB	ACACTCCAGATGCAGAATTGTTCGT
MCMV NEW K1 (46-249) ID:5 203bp	4F3	F3	TCTTGCATCCTGTGAGAGCT
	4B3	B3	GGATCTGTTGCGCAATTCGT
	4FIP	FIP	GGTCGCCATATGTGCACGGAG-CGTGGGAATTTGCCCCTG
	4BIP	BIP	GGAAGCTGTGGTGGCCGATG-CGCGTCAAACCATTCCTCAA
	4LF	LF	GGCATGAAACCTGATTGCCA
	4LB	LB	AACGGAAGATGACGGTGGT
MCMV NEW K3-3760-3955 ID:21 195bp	5F3	F3	CGCGGCTGACAAGCAAAT
	5B3	B3	ACTGGTTGTTCCGGTCTTG
	5FIP	FIP	CACGGTAGGACACGGAGTACGA-ATTGTGGCTATCCCCAAAGC
	5BIP	BIP	CCTACAACCTGCCCTGGTTCC-TCATGCCGGCTCACCTTA
	5LF	LF	CCTACCGCTTGTGTTGCAC
	5LB	LB	CAGGGCTGGCAAATCATTGA
MCMV NEW K3-3707-3874 ID:24 165bp	6F3	F3	CGGACGATTCATTGTGGCTA
	6B3	B3	TGGTTGTTCCGGTCTTGC
	6FIP	FIP	CACGGTAGGACACGGAGTACGA-CCCCAAAGCTAGTGCAACAC
	6BIP	BIP	CCTACAACCTGCCCTGGTTCC-TCATGCCGGCTCACCTTA
	6LF	LF	TTTTGATTTGGCCTACCGCTT
	6LB	LB	GCCCAGGGCTGGCAAAT

5.2.3 RT – PCR Using the LAMP inner primers

RT-PCR was performed on various MCMV samples using the inner LAMP primers. All the 6 sets of inner primers amplified MCMV positive samples. This gave an indication that the

primers can amplify the target MCMV genome sequence. Viruses were detected using a one-step reverse transcription PCR (RT-PCR) assay performed in a GeneAmp 9700 PCR System thermocycler (Applied Biosystems, Foster City, CA, USA). LAMP primer set 5 was subsequently used on all the LAMP primer evaluation experiments.

5.2.4 LAMP Primer sets evaluation through initial RT LAMP Sybr Green colour change Reaction

The modified NEB LAMP protocol was used for LAMP primer evaluation. Thermoscript Reverse transcriptase enzyme was used due to its ability to withstand high temperatures. All the components of the reaction mixture that gave amplification are as shown in Table 5.3. The 25µl reaction tubes with LAMP reaction contents were incubated at 65 $^{\circ}$C for one hour and the reaction terminated by inserting the tubes in a water bath at 80 $^{\circ}$C. The colour change was visualized by adding 1µl of Sybr Green dye in the cap to avoid aerosol contamination by the amplified product.

Analysis of the RT-LAMP products was analysed by observing colour change of the reaction mixture from orange to green for positive samples (Soliman and El-Matbouli, 2006). This is due to intercalation of the dye with the amplified RT- LAMP products produced. The best primer combination was replicated 4 times to determine its reproducibility.

Table 5.3: Modified NEB LAMP protocol for MCMV

Components	Stock Concentration	Final Concentration	Volume in 25 µl rxn
dNTPs	10 mM	1.4 mM	3.5 µl
Isothermal amplification Buffer	10X	10X	2.5 µl
Primer Mix (F3/B3,FIB/BIL and FLP/BLP)	100 µM	-	2.5 µl
MgSO4	100 mM	6 mM	1.5 µl
Bst DNA Polymerase	8000 U/mL	8U	1.0 µl
Thermoscript RT	200 U/mL	8U	0.33 µl
RNA Template	-	-	1.0 µl
RNase free Water	-	-	12.67 µl
RT-LAMP Reaction volume	-	-	**25.0 µl**

5.2.5 Sensitivity of RT-LAMP, conventional PCR and qRT-PCR assays

The sensitivity of detection between RT-LAMP, conventional PCR, and conventional qRT-PCR were compared using nine10-fold serial dilutions of the virus-positive total RNA extracts. In all these assays, the original concentration used was 25ng/µl. These dilutions gave the following concentrations: 25 ng, 2.5 ng, 0.25 ng, 0.025 ng, 0.0025 ng, 0.00025 ng, 0.000025

ng, 0.0000025 ng, 0.00000025 ng. In all the runs, the detection limits were determined by the lowest RNA concentration that gave a positive result.

Sensitivity tests were not done for the Sybr green visual assay for it is difficult to apportion colour change with minor changes in the RNA quantity used as opposed to the real-time analysis where you can compare the amplification curves. The sensitivity (limit of detection) analysis was also done for the same concentrations on RT- qPCR and conventional RT-PCR for comparison with the LAMP assay.

5.2.6 Real time endpoint analysis using GenieII LAMP

Genie II (Figure 5.1) is a compact, lightweight and robust instrument suitable to use in the field or in the laboratory. It is designed to run any isothermal amplification method that employs target detection by fluorescence measurements. The device has two heating blocks each of which takes a single 8-microtube strip that is specifically designed for the instrument. The tubes feature locking caps that do not open after a run, so preventing any contamination. The blocks can be controlled independently or run together for processing up to 16-samples. It includes a rechargeable Lithium-Polymer battery that can keep it running for a full working day.

Figure 5.1: GenieII model GEN2-02. This was used for real time RT LAMP protocol

The Real-time RT-LAMP protocol was used. The first steps involved wiping the working benches with 10% Sodium Hypochlorite then drying with 70% ethanol and cleaning the pipettes, pipette tips and racks with 70% ethanol.

The primer mix preparation was constituted for MCMV GenieII LAMP assay (Table 5.4).

Table 5.4: Primer mix preparation for the MCMV LAMP assay.

Reagent stock	Final concentration (μM)	MCMV Primer volumes (μl)
FIP (50μM)	20	10
BIP (50μM)	20	10
F3 (20μM)	5	40
B3(20μM)	5	40
FL (100 μM)	10	20
BL (100μM)	10	20
SDW		60
TOTAL		200

For 8 reactions, the following master mix in Table 5.5 below was prepared.

Table 5.5: Master mix preparation for the MCMV LAMP for the GenieII LAMP assay

Reagent	Volume (μl)
Enzyme mix	115
Primer mix	23
Thermoscript RT	3
SDW	43

For each reaction, 23 µl of the master mix was dispensed in the reaction tube and 2 µl of RNA was added. Prior to adding the sample to the aliquoted master mix, the equipment was set to run the assay. The wells were labelled, and the amplification step was set at 65˚C for 60 minutes. All the settings were saved to avoid changing the reaction parameters by mistake. The RNA sample was added in a separate area from where the master mix was prepared (to avoid contamination). The tubes were then gently vortexed before loading them in the GenieII machine. The assay was then run, and the results were observed in real time. Amplification commenced at the 7-10th minutes in many assays. The data was saved for further analysis.

5.2.7 Specificity analysis

For determination of the specificity of the primer set, LAMP assay was carried out on samples with other viruses commonly infecting maize namely *Maize dwarf mosaic Virus* (MDMV), *Wheat streak mosaic virus* (WSMV), *Sugarcane mosaic virus* (SCMV) and *Panicun mosaic virus* (PMV). A combination of MCMV and SCMV was also included in the specificity evaluation. The MCMV LAMP protocol for the visual colour change and the GenieII were used to assess the specificity of the assay.

5.3 Results

5.3.1 LAMP outer primers testing

The RT–PCR test results using the LAMP inner primers showed amplification of all the positive MCMV samples was achieved as illustrated Figure 5.2. The FIB (F) and BIP (R) primers from the LAMP primer 5 alternative were used. All the RT-PCR evaluation with the inner primers for the LAMP assay amplified MCMV in the samples tested.

The gel picture below (Figure 5.2) shows the amplification of positive MCMV samples by the LAMP outer primers.

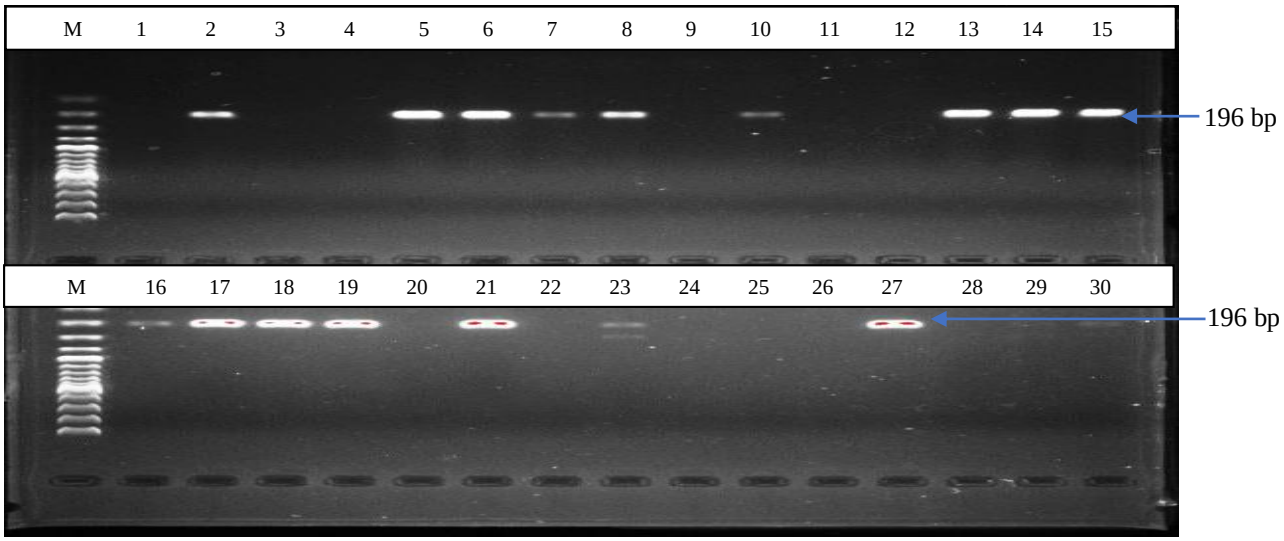
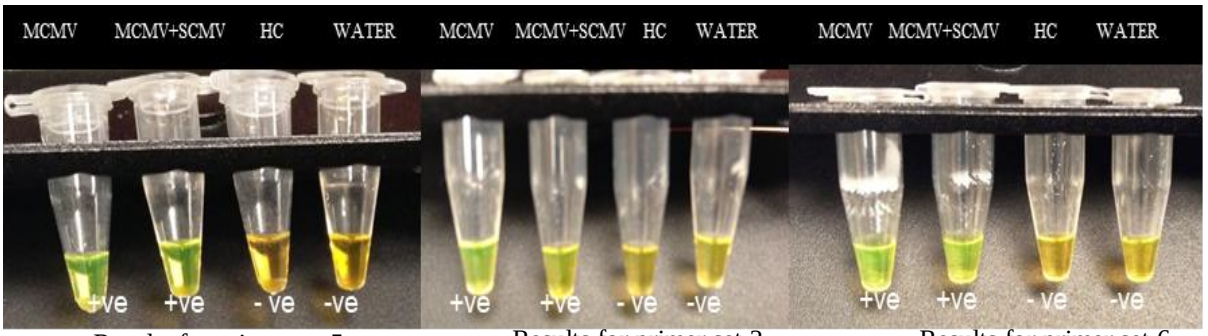


Figure 5.2: Gel electrophoresis image of 30 RNA samples extracted from maize leaves showing the samples with MCMV amplified by the LAMP inner primers, 5FIP and 5BIP through an RT-PCR assay. Amplicons size was 196bp as compared to the 1kb marker(M)

5.3.2 Primer efficiency evaluation

Amplification for MCMV and a combination of MCMV and SCMV by colour change (Sybr Green) assay and in Genie II was evaluated and recorded. Primer efficiency was analysed by colour change intensity. All primer sets gave amplification for MCMV but at different efficiency levels. The colour changes were analysed visually for the ones having clear green colour change from the orange colour of the cyber green dye (Figure 5.3).



Results for primer set 5 Results for primer set 2 Results for primer set 6

Figure 5.3: Primer efficiency evaluation for primer sets 5, 2 and 6 through the RT-LAMP assay using the visual colour change (SYBR Green). Tubes with green colour depicts positive amplification for MCMV while the orange ones show negative amplification for MCMV.

The intensities in colour change considerably differed when the primer set 5 was used, providing the best colour intensities and sharp contrast.

5.3.4 Specificity analysis

For the determination of specificity of the MCMV primer set, LAMP assay was carried out on samples with other viruses commonly infecting maize namely *Maize Dwarf Mosaic Virus* (MDMV), *Wheat Streak Mosaic virus* (WSMV), *Sugarcane Mosaic virus* (SCMV) and *Panicum Mosaic Virus* (PMV). A combination of MCMV and SCMV was also included in the specificity evaluation. The sample with only MCMV or with a combination with SCMV gave colour change to green showing the amplification for MCMV (Figure 5.4). This clearly shows that the primers were only specific to MCMV.

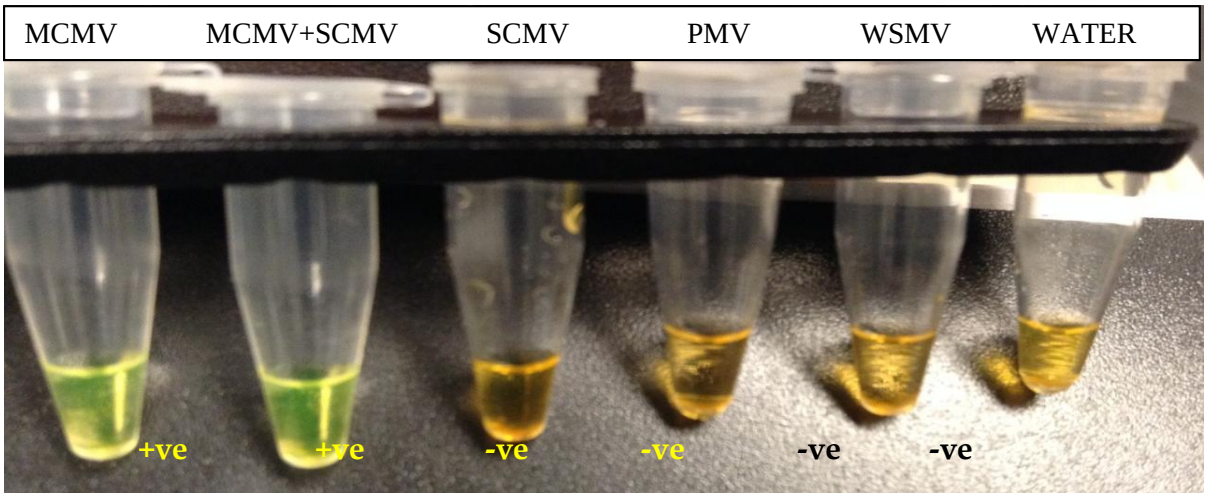


Figure 5.4: Specificity assessment test using SYBR green dye in the RT-LAMP assay for MCMV.

All samples with MCMV alone or in combination with another maize infecting virus changed colour from orange to green showing the distinct level of specificity of the LAMP assay. The samples containing other maize infecting viruses SCMV, PMV, WSMV and control (water) had no colour change.

5.3.3 Real time endpoint analysis using GenieII LAMP

Specificity analysis was repeated using the Gennie II LAMP assay with real-time evaluation. The amplification was only evident with MCMV samples or samples with MCMV mixed with other maize viruses. Other common maize infecting viruses were not picked by the assay hence demonstrating the high level of specificity of the assay as illustrated in Figure 5.5 where only sample MCMV and MCMV (2) were amplified showing the presence of the virus in the samples.

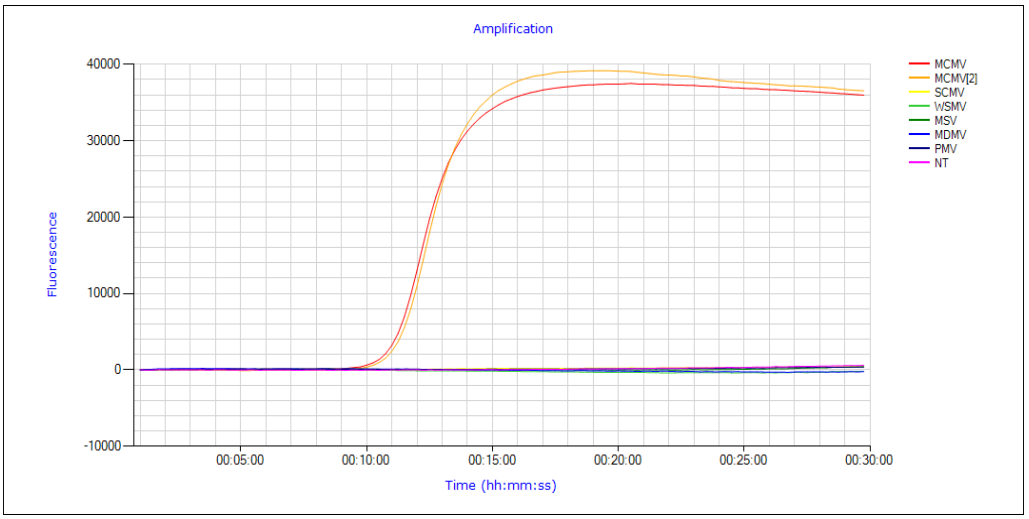


Figure 5.5: Specificity of the Tests using primer set 5.

Amplification for MCMV and MCMV2 samples were distinct at the 10th minute of the assay. The other maize viruses SCMV, MDMV, WSMV, and PMV were all negative with no amplification up to the 30th minute.

Genie1 II works under an isothermal amplification temperature and can be programmed for different temperatures. For the specific primer set the optimum temperature was determined as 65˚C with detection duration of approximately twenty minutes. The subsequent melting process (in GenieII called annealing process) starts with 98˚C and ends with a temperature of 70˚C with a ramp rate of 0.05˚C/sec.

There was strong visible annealing activity for the primer set 5 used in the specificity evaluation. Annealing was best at 89 ºC. Only samples with MCMV demonstrated annealing activity of the amplified products. All the other samples did note hence negative.

Only MCMV samples showed the annealing activity which further describes the specificity of the LAMP primer sets shown in Figure 5.6. There was no primer annealing activity with samples without MCMV further demonstrating the specificity of the LAMP primer sets designed and synthesised.

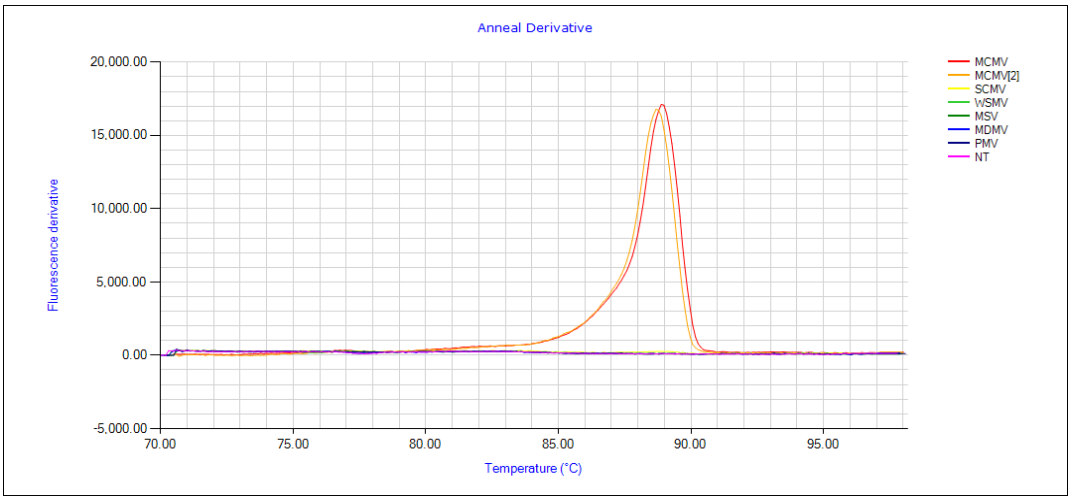


Figure 5.6: Annealing temperature analysis for primer set 5.

5.3.5 MCMV RT-LAMP limit of detection (Sensitivity) results

The MCMV LAMP assay hereby developed demonstrated a high level of sensitivity with samples diluted 1 million-fold being detected (Figure 5.7). This was equivalent to a concentration of 0.0000025 ng/µl. Samples with concentrations from 25 ng/µl to 0.0000025 ng/µl of total RNA were detected by the assay (Figure 5.8). The annealing activity was active only with MCMV samples with total RNA concentrations between 25 ng/µl and 0.0000025 ng/µl (Figure.5.9). The same MCMV concentrations evaluated under the MCMV qRT-PCR also detected MCMV up to 1 million-fold dilutions as in the LAMP assay (Figure 5.10). However, the conventional RT-PCR was able to detect MCMV up to 100,000-fold dilution at 000025 ng/µl. This is shown in Figure 5.11 with amplification on the gel electrophoresis picture showing bands only in Lane 2 – 6.

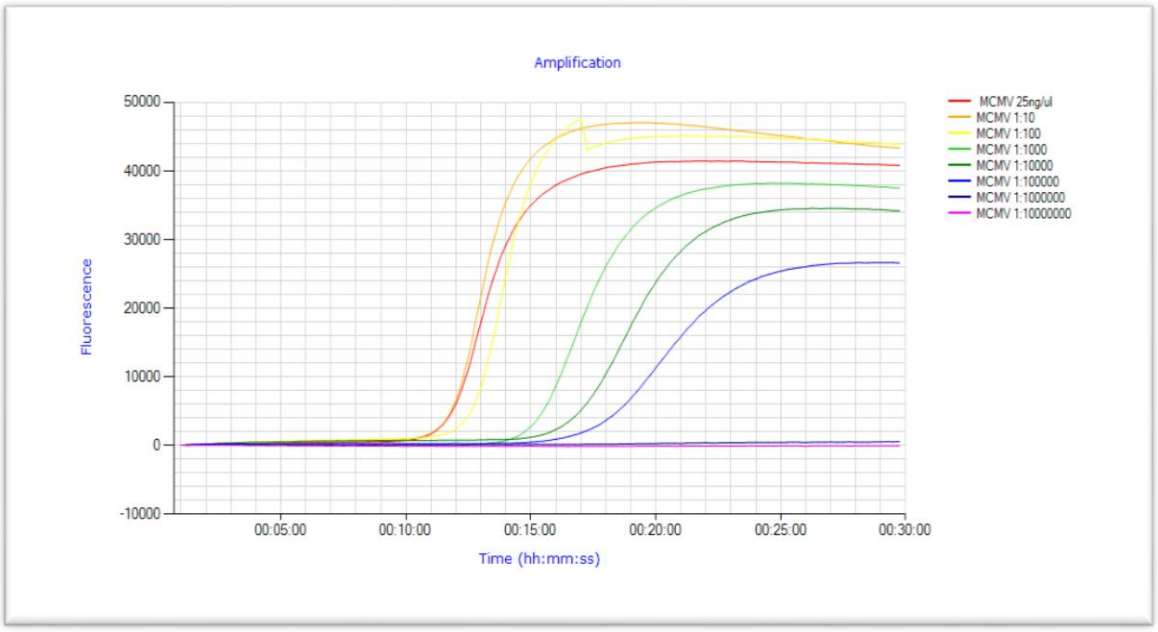


Figure 5.7: Limit of detection detection evaluation comparison with MCMV dilutions using RT- LAMP assay and the Genie II platform. Amplification was distinct for samples infected with MCMV concentrations of between 25ng/µl – 0.25pg/µl

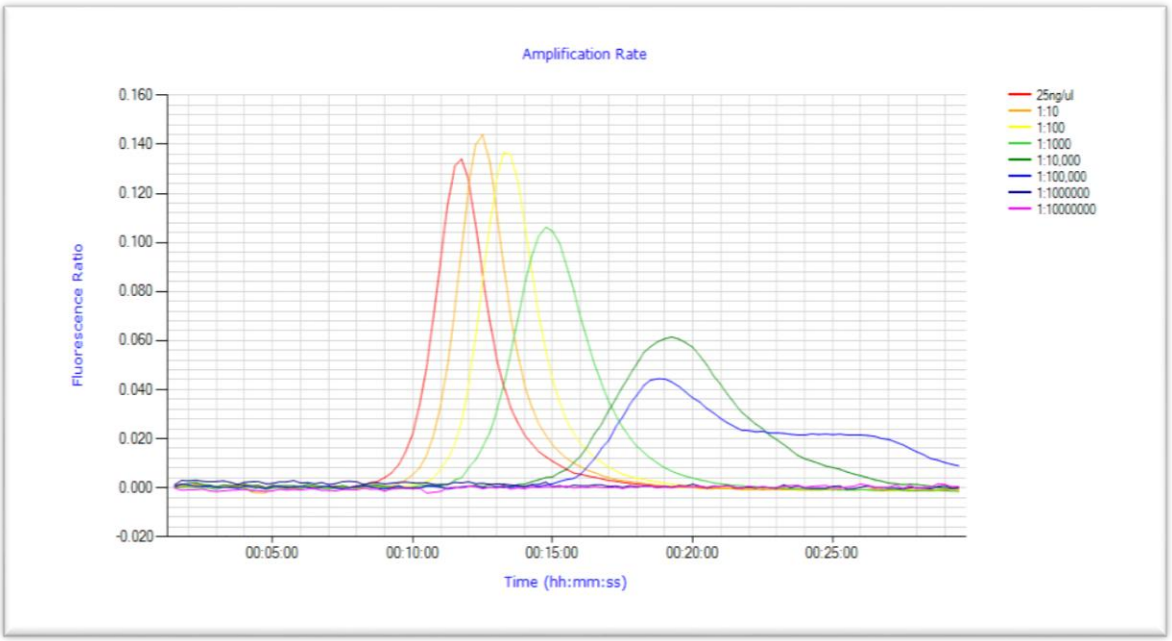


Figure 5.8: Amplification rates of the different MCMV viral dilutions. Amplification is evident for 0.25ng/ul – 25ng/ul at 10^{th} – 13^{th} minute. More dilute samples had amplifications at 15^{th} – 20^{th} minute. All detectable samples showed at the 25^{th} minute.

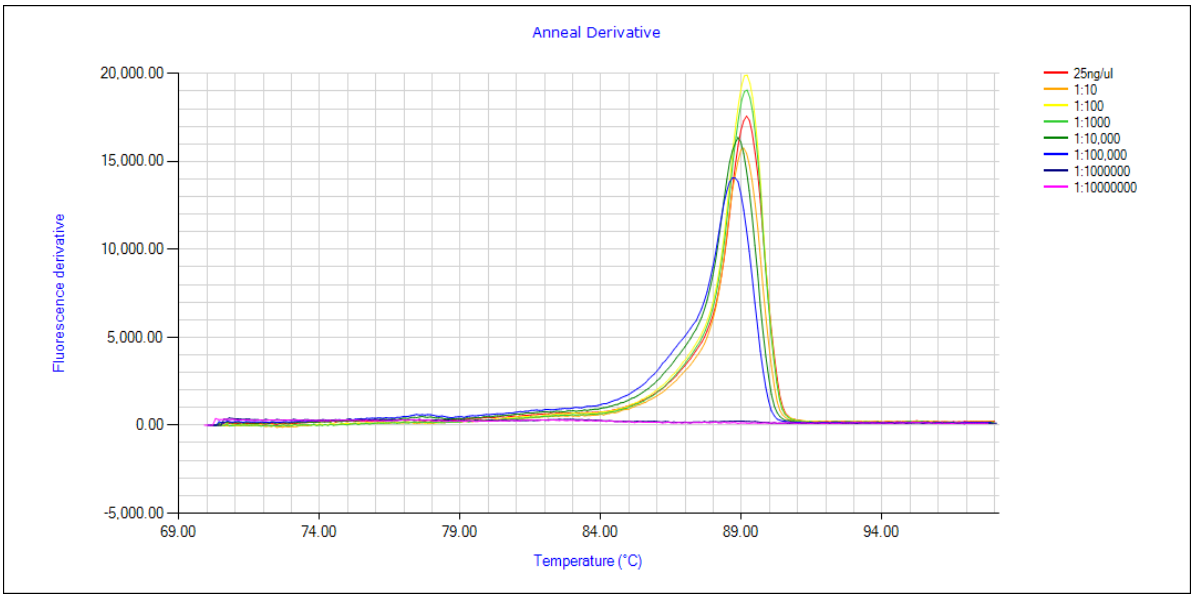


Figure 5.9: Annealing Temperatures analysis for primer set 5 with the different MCMV dilutions. There was strong visible annealing activity only for the MCMV viruses containing samples up to 1:1000,000 which depicts the high sensitivity of this MCMV LAMP assay.

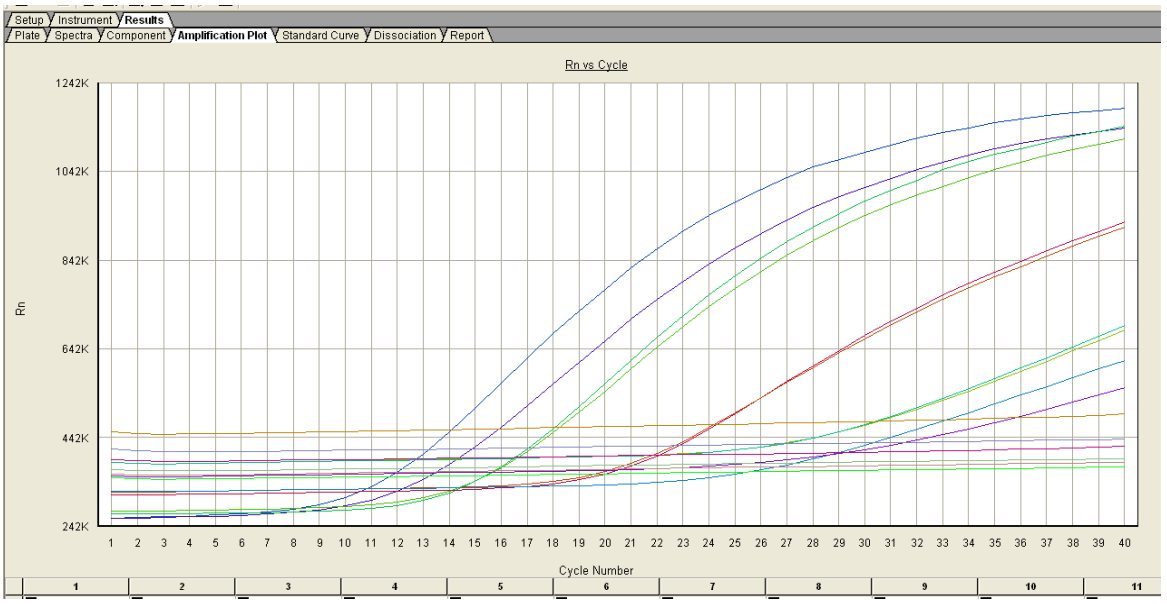


Figure 5.10: Limit of detection for MCMV virus by qRT-PCR. Sensitivity analysis using qRT-PCR for the same MCMV dilutions as for LAMP assay. Key: Neat-blue curve, 25 ng/µl, 1st Purple curve 2.5 ng/µl, 1st Green curve 0.25 ng/µl, Red curve 0.025 ng/µl, 2nd Green 2.5 pg/µl, 2nd Blue curve 0.25 pg/µl, 2nd Purple 25 fg/µl

Figure 5.11: Gel electrophoresis picture showing the limit of detection for MCMV virus by Conventional RT-PCR. The inner primers FIB and BIP were used for this assay. M-Ladder, 1-2.5 fg/ µl, 2-25.0 fg/ µl, 3-0.25 pg/ µl, 4-25ng/ µl, 5-2.5 ng/ µl, 6-0.25 ng / µl, 7-0.025 ng/ µl, 8-2.5 pg/ µl

5.4 Discussion

Several remedial actions have been taken to reduce the devastating effects of MLN which has threatened food security and people's livelihoods in the Eastern Africa region (Mahuku et al., 2015a). This includes breeding and deployment of resistance varieties and observing several cultural and agronomic practices. Seed transmission has been attributed to the rapid spread of the disease in the eastern Africa region (Mahuku et al., 2015a: Prasanna et al., 2020). Therefore, testing for the disease during active vegetative growth and in seed is vital for both seed certification agencies and seed producers to limit seed contamination and designing feasible management practices (Uyemoto, 1983). There is a thin line between seed contamination and seed transmission but seed contamination by MCMV leads to seed transmission of this virus (CIMMYT, 2019).

Currently, seed certification and plant health agencies in eastern Africa use RT-PCR for testing the virus in seed lots as a certification procedure and for confirmation in plant health laboratories. This involves expensive equipment for the tests in the laboratory and long turnaround time for the test results. The RT-PCR test is also expensive with some agencies charging up to USD 35 per sample tested. A detailed two end point analysis RT-LAMP was developed under this study to provide a reliable, effective efficient, sensitive, cost effective and field deployable molecular based assay for the detection of MCMV. The existing MCMV LAMP assay developed by Chen et al., 2016 and Zhanmin et al., 2016 only reports one end

point analysis of the use of SYBR green dye and hydroxynapthol blue dye respectively. In these assays, the dye was added in the tubes after incubation which can lead to cross contamination (Hsieh et al., 2014) hence false positives when not handled carefully. LAMP assays result in millions of copies of the target nucleic acid and therefore chances of cross contamination in the lab are quite high. The equipment is also far much cheaper than PCR machines with costs ranging from $10,000 to $16,000. The cost of analysing a sample for MCMV in Kenya by KEPHIS is $35 using qRT-PCR while the cost for the same sample using RT-LAMP is $7.5.

All the LAMP primer sets developed during this study amplified the target regions of the MCMV genome but with varying efficiencies in MCMV detection. The six sets yielded amplicons of between 165 – 209bp hence quite suitable for the maximum activity of the DNA displacement Taq Polymerase (Ignatov et al., 2014). However, primer set 5 and 6 gave the best amplifications with the clearest contrast on colour change from orange to green and sharp curves on the Genie II platform. Primer set five was selected for all subsequent tests in this study and is the one already adopted by KEPHIS and KALRO for their MCMV testing on the LAMP Genie II platform. As indicated earlier, the assay was replicated four times and hence proved to be reproducible. The primer sets also discriminated against the common viruses infecting maize in the region namely SCMV, MDMV, WSMV, PMV and MSV. The real-time amplification was carried out in the Genie II LAMP instrument as earlier mentioned. This instrument features low power requirements and includes a rechargeable lithium-polymer battery that can keep it running for a full working day. The lithium battery can be charged on the power outlets or using the car charger system hence suitable for field deployment. The Genie II assay analysis also showed the excellent specificity to MCMV (Figures 5.4, 5.5 and Figure 5.6) of this assay with absolutely no chances of cross contamination if all precautions are considered. This is because all the reaction tubes are not opened during the assay and are discarded safely after every reaction procedure. In the Genie II platform, an annealing curve generated in the fluorimeter confirms the presence of the amplification product. This eliminates the need for gel electrophoresis or turbidity detection and allows for a closed-tube system hence eliminating cross contamination that can lead to false positives.

The test also showed the same level of sensitivity with Real time RT–PCR detecting up to 10^6 dilutions of the initial RNA concentration of 25 ng/ µl, (Figures 5.7, 5.8, 5.9, and 5.10). The test was however more sensitive than the conventional RT–PCR which detected up to 4 (10^4) fold dilutions Figure 5.11. In a study comparing RT-LAMP and RT-PCR for MCMV, the

detection limit of RT-LAMP assay was 280 fg of MCMV RNA, which was 100 times more sensitive than that of RT-PCR assay (Shan et al., 2017). In the same study, high species-specificity of RT-LAMP method was confirmed by the assay of 5 pathogens such as MCMV, SCMV, MDMV, WSMV and *Maize white line mosaic virus* (MWLMV). Seed infected with MCMV can be identified through a grow out test using this method with the same sensitivity as qRT-PCR. Further sensitivity optimization may yield a protocol that can be utilized in detecting these viruses directly in seed maize without a grow out test which requires both time and resources to grow the seedlings in germination chambers.

It is worth noting that the Real-time PCR machines are expensive with prices over $60,000 hence not within purchasing capability of many laboratories in the developing world. These PCR based assays cannot be deployed in field situation for detection pathogen detection. They are set up in relatively advanced laboratories in few institutions.

LAMP assay on the on the other hand is simple because it involves only one instrument, The Genie II LAMP machine. The simplicity, rapidity, and inexpensiveness of this technique makes it a suitable choice for large-scale sample processing, especially by laboratories with limited resources. Currently, phytosanitary regulatory institutions are using conventional and real time PCR for routine screening of seed material for MCMV which is quite expensive. However, trial runs for Genie II RT-LAMP for MCMV optimization done in KEPHIS prompted the Kenyan NPPO to adopt the use of this developed assay in their Plant pathology laboratories both in the KEPHIS headquarters and in The Plant Quarantine and Biosecurity station. The assay has also been adopted by the Kenya Agriculture, Livestock Research Organization (KALRO).

5.5 Conclusion

Strict regulation coupled with internal controls for self-regulation by seed companies utilizing clean seed maize in conjunction with growing resistant varieties and monitoring new virus strain emergence are necessary measures to prevent the spread of MCMV throughout the maize industry and in the region. MLN free seed Protocols (https://mln.cimmyt.org/) developed for clean seed production by CIMMYT and partners in eastern Africa (Prasanna et al., 2020) will also go a long way in ensuring low or nil incidences of MLN viruses in both farmers' fields and commercial seed fields. This will further contribute immensely in the efforts towards limiting the spread of this deadly disease to the southern African region where maize is a vibrant industry.

CHAPTER SIX

REVERSE TRANSCRIPTASE–RECOMBINASE POLYMERASE AMPLIFICATION (RT-RPA) FOR RAPID DETECTION OF MAIZE CHLOROTIC MOTTLE VIRUS (MCMV)

6.0 Abstract

Diagnostics of the MLN-causing viruses is key in the management of MLN through country and regional surveillance programs and in testing seed for zero tolerance of MCMV in final seedlots. This requires a customised MCMV detection assay that is specific, sensitive, affordable, and portable. RT-RPA was a perfect choice for it meets those conditions and it is a molecular based assay that targets the viral genome for detecting the virus. RPA is a rapid isothermal nucleic acid amplification and detection platform that is based on patented Recombinase Polymerase Amplification (RPA) technology. An RPA diagnostic method for the detection of MCMV was developed. Primer sets targeting MCMV genome section of 2765bp-2948bp were designed with a complementary probe since this assay is probe based as qPCR in the Primer Quest tool with guidelines from the Agdia AmplifyRP Handbook. The primer sets targeted the MCMV genome at position 2765bp -2948bp (MCMV_gp2 replicase gene). All the essential parameters were evaluated including sensitivity, specificity and reproducibility for the assay. The assay discriminated against other maize infecting viruses hence specific to MCMV. The assay took only 20 mins and its detection limit of 10^{-4} was well comparable to RT-PCR and other molecular based detection assays. MCMV was also detected directly from leaf saps without the nucleic acid extraction step hence suitable for on-farm testing. This assay is suitable for detection of MCMV in field surveys, seed certification processes in the field on leaf tissues and for seed.

6.1 Introduction

A variety of isothermal amplification methods have been recently developed which can be deployed in the field for easier and faster diagnostics solutions (Piepenburg et al., 2006). One example is the ESEQuant Tube Scanner device (Qiagen Lake Constance GmbH, Stockach, Germany). This device is portable, battery operated fluorimeter which enable on-site, real-time detection. It has an advanced fluorescence sensor which slides back and forth under a set of eight tubes, collecting fluorescence signals over time and allowing for real-time documentation of increasing fluorescence signals. The Tube Scanner can be used to show nucleotide amplification by the recombinase polymerase amplification (RPA) technique. RPA is a novel DNA amplification technique at a low isothermal condition.

RPA exploits isothermal recombinase-driven primer targeting of template material with strand-displacement DNA synthesis (Piepenburg et al., 2006). It achieves exponential amplification with no need for pre-treatment of sample DNA. Reactions are sensitive, specific, and rapid and probe based.

There are five main components of RPA: template DNA; a primer–recombinase complex to bind to template and initiate the copying process; nucleotides; a polymerase to synthesize nascent strand; and single-stranded DNA-binding proteins (SSBs) to prevent dsDNA to anneal, resulting in an exponential increase in the DNA sample (Mary Hoff 2008). RPA combines with a sequence specific fluorescent probe for real-time detection. Probes are made up of an oligonucleotide backbone that contains an abasic nucleotide analogue, a tetrahydrofuran residue (THF), flanked by a dT-fluorophore and a corresponding dT-quencher group (Euler et al., 2012a; 2012b). The probes are also blocked at the 3' end by a modification group (such as a C3-spacer). Any fluorescent signal generated by the fluorophore will normally be absorbed by the quencher located 2-6 bases 3' to the fluorophore. Once the probe has paired with its target sequence the THF residue presents a target for the DNA repair enzyme, exonuclease III. The exonuclease cleaves the probe at the THF position, separating the fluorophore from the quencher to generate a detectable fluorescent signal (Haberstroh K., 2017). This occurs only when the probe has annealed to its target sequence within the amplification product and is an indication that amplification has occurred. The increase in signal allows real-time monitoring of the reaction.

Apart from the ability of RPA to operate under a low constant temperature, its reagents, and enzymes are lyophilized into individual reaction pellets. They are only activated in the field for reactions by mixing with the relevant rehydration buffer solution. The current documented RT-RPA for MCMV detection (Jiao et al., 2019) employs the end-point analysis of gel electrophoresis which limits it only to laboratory conditions since it is not practical to run gels in the field.

RPA extends the application of DNA amplification in fieldwork and in laboratories where thermocycling instruments are not available. As such, this study aimed at developing a real time recombinase polymerase amplification assay for the detection of MCMV both in the laboratory and in field conditions. This assay to complements the loop mediated isothermal amplification assay developed in Chapter five.

6.2 Materials and Methods

6.2.1 Ribonucleic acid (RNA) extraction and detection of MCMV

RNA was extracted from several maize plants' leaf samples infected by inoculation with MCMV grown and maintained in the quarantine screen house in Ohio Agricultural Research Development Center (OARDC), Ohio State University, USA. RNA from maize leaf samples showing symptoms of MLN was also extracted for subsequent specificity analysis of the assay developed in this study. The ZR RNA MiniPrep™ from Zymo Research was used for RNA extraction from the samples. RNA is isolated from homogenized leaf samples using Fast-Spin column technology. RNA is eluted into volumes of 25 µl or more suitable for use in RT-PCR and other RNA-based procedures. RNA samples were also obtained from maize plants infected with *Maize Dwarf Mosaic Virus* (MDMV), *Sugarcane Mosaic Virus* (SCMV), healthy maize plants, and grass samples infected with *Panicum Mosaic virus* (PMV). The routine testing in the OARDC laboratories for MDMV, SCMV and PMV ensured only positive samples for these viruses were used in evaluating this assay.

The quality and concentration of RNA were tested using a NanoDrop ND-1000 Spectrophotometer (Thermo Fischer Scientific, Wilmington USA). A formaldehyde RNA denaturing agarose gel electrophoresis was also performed to determine the integrity of the extracted RNA. RT-PCR was performed on the samples to ascertain the presence of MCMV presence using the primers designed by the USDA – Research unit at OARDC, Ohio (MCMV F 5' – CCG GTC TAC CCG AGG TAG AAA – 3' and MCMV R 5' – TGG CTC GAA TAG CTC TGG ATT T – 3'). The detection MCMV was done in a two-step RT-PCR. The first step involved cDNA synthesis using the Maxima First Strand cDNA synthesis kit (ThermoFisher, Waltham, MA) for RT-qPCR with dsDNase.

6.2.2 RPA Primers and Probe design

The AmplifyRP Handbook (AmplifyRP Discovery Kit Assay Design Help Book, Agdia 2014) (https://www.agdia-emea.com/en/product_category/amplifyrp_discovery_kit) was used for guidance in designing RPA primers and probes for this assay. The primers were designed using the Primer Quest tool, Integrated DNA Technologies Inc. (https://www.idtdna.com/Primerquest/Home/Index)

The primers and probes were designed targeting position 2765 to 2948 and 1869 to 2067 on the MCMV consensus genome sequence. The MCMV sequences used in the alignment were

MCMV Taiwan – KJ782300, MCMV Nebraska USA – EU358605, MCMV Rwanda – KF744396, MCMV China – KF010583 and the Kenyan MCMV sequence, KP798454.1. Alignment was done using the ClustalW alignment algorithm in the MEGA X (MX) Alignment Explorer (Kumar et al., 2018) with the sequences to get consensus regions with conserved sequences for primer and probe design (Figure 6.1).

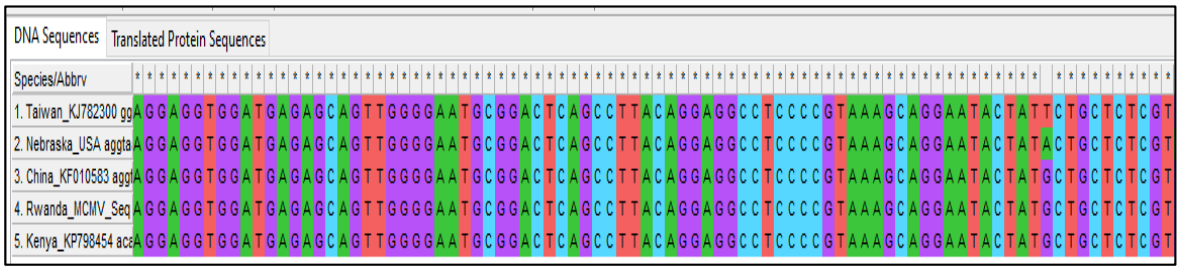


Figure 6.1: MCMV sequence alignment using ClustalW algorithm in MEGA X

The XRT probes were designed manually by getting a reverse complementary sequence in the targeted region and modified with a Fluorophore (dT-Fam), tetrahydrofuran residue (THF, H), and a quencher dye (dT- Q) as per the AmplifyRP guidelines. The probe was designed from the sequences in the amplification region targeted by the forward and reverse primers without overlapping the priming regions denoted by the red colour of the bases (Figure 6.2). Complementary bases to these chosen regions were generated manually yielding the probe denoted in blue in Figure 6.2. The dT-Fam, -H and the-dT-Q- were plugged in the probe sequence by replacing the area with TTT (highlighted yellow in Figure 6. 2). The final designed raw and modified probe had the following sequence:

AGTAACGAGCGCTTTCTTGGACCTCCTGCCATTTGCGATTGGATAGTTTGC
AGTAACGAGCGCTTTCTTGGACCTCCTGCCA--dT-Fam-H-dT-Q-
GCGATTGGATAGTTTGC

The probe position in the sequence to be amplified was 2813 – 2863 as shown in Figure 6.2 below.

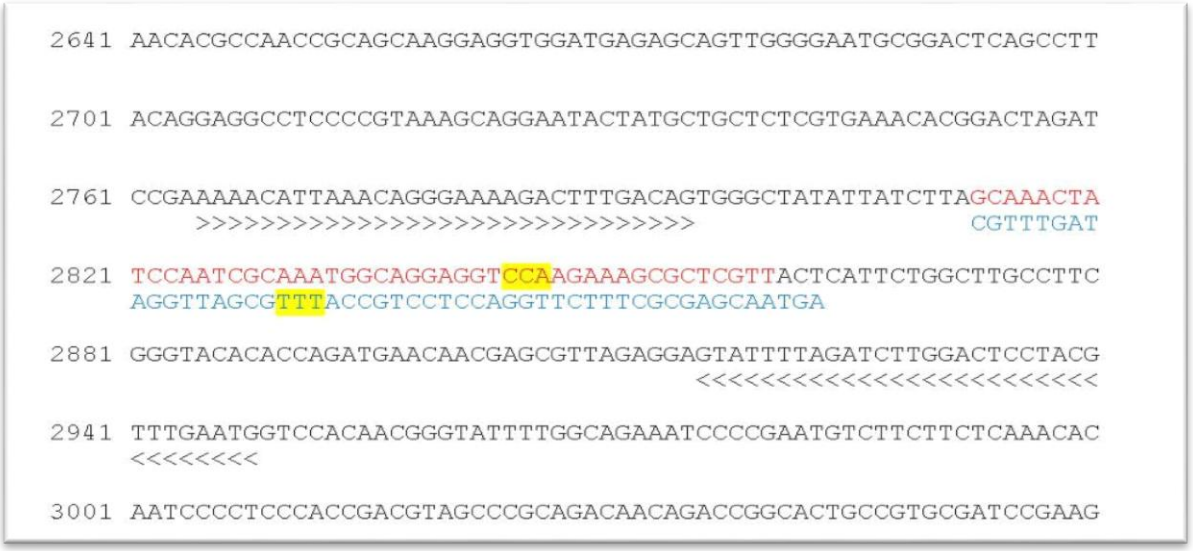


Figure 6.2: Primer design output from the IDT primer design program. The forward primers start at 2765–2775 denoted by the greater than sign (>>) while the reverse primer at position 2920 – 2948denoted by the less than sign (<<). The probe is denoted by the blue colour nucleotide sequence.

The primers and probes were designed by considering several parameters that are important in designing RPA primers and probes. The size of the amplicons produced should be 200bp or less with the primers typically be 30 to 35 nucleotides long. Shorter oligonucleotides are bound less efficiently by the recombinase. A "G/C clamp" at the 3' end was adopted. The GC content between 30% and 70% was employed since Tm is not a factor in RPA.

Primer sets with long strings of repeated bases as well as excessive numbers of short repeats were avoided. As with PCR, sequences that could contribute to primer-primer interactions, hairpins, and secondary structures were avoided. This can affect the sensitivity of the assay by competing for resources in the reaction mix.

Below are the RPA primers and probes designed (Table 6.1). For each probe, three sets of F and R primes were designed.

Table 6.1: The primer and probe options designed for the RT- RPA assay

1st Primer and Probe Option - TARGET 2765-2948			
MCMV-K1A	**F**	AAAACATTAAACAGGGAAAAGACTTTGACAG	**Product size**
MCMV-K1A	**R**	CATTCAAACGTAGGAGTCCAAGATCTAAAATAC	**184bp**
MCMV-K1B	**F**	TGAAACACGGACTAGATCCGAAAAACATTAAACAG	**Product size**
MCMV-K1B	**R**	GAGTCCAAGATCTAAAATACTCCTCTAACGCTCGT	**185bp**
MCMV-K1C	**F**	CACGGACTAGATCCGAAAAACATTAAACAGGGA	**Product size**
MCMV-K1C	**R**	CAAACGTAGGAGTCCAAGATCTAAAATACTCCT	**192bp**
Probe		AGTAACGAGCGCTTTCTTGGACCTCCTGCCA-dT-Fam-H-dT-Q-GCGATTGGATAGTTTGC- C3-Spacer3’	
2nd Primer and Probe Option - TARGET 1869-2067			
MCMV-K2A	**F**	GAAAACATTTGTCAAAGCAGAAAAGATCAATC	**Product size**
MCMV-K2A	**R**	AATCTGTTCAACCGAGTAACCTTTCATGATA	**199bp**
MCMV-K2B	**F**	CTAAACTGAAAACATTTGTCAAAGCAGAAAAGATC	**Product size**
MCMV-K2B	**R**	TTCATGATAGTCGGACCACCCCAAATCTTGTCTAT	**184bp**
MCMV-K2C	**F**	GATGCTAAACTGAAAACATTTGTCAAAGCAGAAAA	**Product size**
MCMV-K2C	**R**	TAACCTTTCATGATAGTCGGACCACCCCAAATCTT	**194bp**
Probe		CGTGTGATCCAACCCCGTGCTCCCCGGTACAA-dT-Fam-H-dT-Q-GTGGAACTGGGTAGGTAT-C3-Spacer3’	
Key		dT-Fam- Flourophore H- Tetrahydrofuran (THF) residue dT-Q- Quencher	

The first set of primers and the probe (Table 6.2) were ordered from Integrated DNA Technologies (IDT, 1710 Commercial Park Coralville, Iowa 52241 in October 2014. The primers and probes were reconstituted upon receipt and stock of 100 µM were prepared for each primer.

Table 6.2: RPA primers under evaluation identification and their corresponding sequences

Primer	Primer Synthesis ID	Primer Sequence
1AF	MCMV-K1A F	AAAACATTAAACAGGGAAAAGACTTTGACAG
1AR	MCMV-K1A R	CATTCAAACGTAGGAGTCCAAGATCTAAAATAC
1BF	MCMV-K1B F	TGAAACACGGACTAGATCCGAAAAACATTAAACAG
1BR	MCMV-K1B R	GAGTCCAAGATCTAAAATACTCCTCTAACGCTCGT
1CF	MCMV-K1C F	CACGGACTAGATCCGAAAAACATTAAACAGGGA
1CR	MCMV-K1C R	CAAACGTAGGAGTCCAAGATCTAAAATACTCCT

6.2.3 RPA primers and probe evaluation for testing MCMV in Maize leaves

The following experiments were designed to evaluate the primers and the probe.

The primers and the probe for the first option were the ones that were ordered and used in screening the primers. A primer combination approach was used. Each forward primer was screened against the three reverse primers. The primer combinations were arranged and carried out as shown below;

i. 1AF - 1AR, 1BR, and 1CR
ii. 1BF - 1AR, 1BR, and 1CR
iii. 1CF-1AR, 1BR, and 1CR

The Agdia XRT-RPA protocol was used to make the reaction master mix for the RT-RPA reactions (AmplifyRP Discovery Help Book (2), 2015). Each primer combination was screened with a positive MCMV and a water control. The rehydration solution for the RPA experiments was prepared as follows; 14.75 µl rehydration solution, 1.05 µl of the forward and reverse primers, 0.3µl XRT probe, 0.25 µl of reverse transcriptase (Protoscript II, New England Biolabs (NEB), Ipswich, Massachusetts, United States), 4.37 µl of water and 1.0µl of the RNA template. The total working volume of the rehydration mixture was 22.75 µl.

For each reaction, 22.75 µl of rehydration mixture was added to the reaction pellet for each sample. This was mixed briefly by pipetting up and down. The reaction tubes were caped immediately, spun briefly, and 1.25 µl of 280 mM magnesium acetate solution was added to start the reaction. The tubes were then vortexed and spun briefly before they were loaded into the fluorimeter (ESEQuant TS95, ID: ESTS10-MB-3020 Serial No.: 0014 from Qiagen). Fluorescence measurements (excitation, 470nm; detection, 520 nm [FAM channel]) were performed at 39 °C for 20 min. This reaction temperature was found to yield the best performance in terms of sensitivity in a range tested from 39 °C to 42 °C. The tube scanner software offers threshold validation, i.e. evaluation of fluorescence by increase of fluorescence above three standard deviations over the background determined in minute 1 (adaptable) of the reaction.

The reactions were then monitored from the display on the connected computer screen.

For the first set of experiments, the first forward primer (1AF) was combined with all the reverse primers under the first set (1AR, 1BR and 1CR).

These runs were repeated three times to assess the reproducibility of the assay. The same process was done for the evaluation of primer set 2; 1BF-1AR, 1BR, -1CR and set 3; 1CF-1AR, and 1BR-1CR.

6.2.4 Assessment of RPA performance using fresh maize leaf crude extracts

Extracts from fresh maize hybrid DEKALB DKC55-84RIB leaf samples from the OARDC quarantine Screen house infected with MCMV, MCMV+SCMV, SCMV and a healthy control (HC) were obtained using the General Extraction Buffer 3 (GEB3) from Agdia. The GEB3 extraction buffer was reconstituted from the components as per the user guide from Agdia. To make 1000 ml of GEB3 sample extract buffer, a smooth slurry was made by adding a small amount of water to 48 g of powder. Then while mixing, Tween-20 was added to the slurry. Water was added to bring the final volume to 1 litre. The mixture was stirred for 30 minutes.

The maize leaf extracts from maize plants infected singly by MCMV, MCMV+SCMV, SCMV and a healthy control (HC) were evaluated for the RPA assay in this experiment. The experiments were set up using the protocol outlined earlier in section 6.2.3. RPA primer 1BF and 1AR in combination with probe1were used in subsequent tests.

6.2.5 Determination of specificities for MCMV detection by the RPA primers/ probe

The viruses tested were the common ones that infect maize namely *Maize streak virus* (MSV), potyviruses that co infect maize with MCMV namely *Sugarcane mosaic virus* (SCMV), *Maize dwarf mosaic virus* (MDMV) and *Wheat streak mosaic virus* (WSMV). The closest relative of MCMV, *Panicum mosaic virus* (PMV) was also used in evaluating the primers and probes for specificity. RNA was extracted from the maize plants infected by these viruses in the glass house chambers in OARDC (OSU). The primer sets IBF and 1AR were used together with probe 1. These runs were replicated three times for consistency and reproducibility assessment.

6.2.6 Determination of RPA sensitivity for MCMV detection

One positive sample from the primer and probe testing experiment was selected and quantified with the Nanodrop to be used in the sensitivity analysis. The total plant RNA concentration was found to be 701 ng/ul. This was then standardized to 100ng/ul. This was followed by six 10-fold serial dilutions, yielding samples of 100 ng/ul, 10 ng/ul, 1 ng/ul, 0.1 ng/ul, 0.01 ng/ul, and 0.001 ng/ul. These MCMV viral dilutions were analysed in the RPA assay for MCMV to determine the limit of detection.

6.3 Results

6.3.1 Testing Primer combinations

Amplification of positive samples occurred between the 6th and the 10th minute for samples which had high titers of the viral pathogen under investigation (MCMV) and the 10th and the 20th minute for lower titer samples. By the 20th minute, all the sample status had been determined as either positive or negative for MCMV. All the primer and probe optimization and combinations were evaluated to get the most efficient combinations. The combination of 1CF-1BR gave the best amplification with a sharper curve at the 5th minute showing it was the most efficient in terms of amplifications (Figure 6.3). This was followed by 1AF-BR, 1AF-AR and 1CF-AR. All the primer evaluations are shown in Table 6.3 below with each primer combination reaction in a specific tube.

Table 6.3: RPA Primer combinations for Efficiency analysis

Tube No.	**Tube 1**	**Tube 2**	**Tube 3**	**Tube 4**	**Tube 5**	**Tube 6**	**Tube 7**	**Tube 8**
Primers	1BF-1BR	1AF-1AR	1AF-1BR	1CF-1BR	1AF-1CR	1CF-1AR	1BF-1AR	1BF-1CR

This combination of primers 1CF-1BR was used for subsequent experiments for evaluation on other maize viruses, sensitivity test and on crude samples from maize tissues. However, other primer combinations worked well but not as efficient and precise as the combinations of 1BF-1AR and 1CF-1BR. These primer combinations were 1AF-1BR and 1A-1AR. The remaining primer combinations did not yield any amplifications whatsoever.

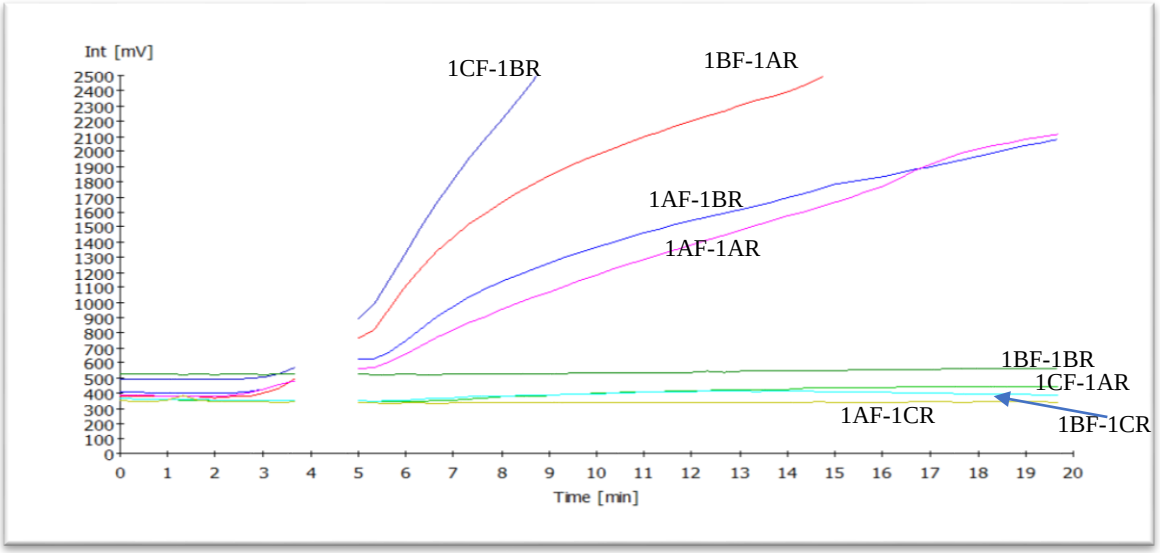


Figure 6.3: Primer efficiency analysis of the various primer combinations. Primer combinations 1CF-1BR, 1BF-1AR, 1AF-1BR and 1AF-1AR amplified the targeted viral RNA. The others 1BF-1BR, 1CF-1AR, 1BF-1R and 1AF-1CR did not amplify the targeted viral RNA.

6.3.2 Specificity analysis of the RT-RPA Assay.

The evaluation of the specificity of the developed assay was paramount. The samples with MCMV, SCMV, PMV, MDMV and WSMV were found to be well discriminated by the assay as illustrated in Figure 6.4. The Healthy control (HC) and Water were used as controls. The primer set, 1CF-1BRwas used in this trial as indicated earlier. The assay was found to be highly specific picking up only MCMV from plants infected with other maize viruses.

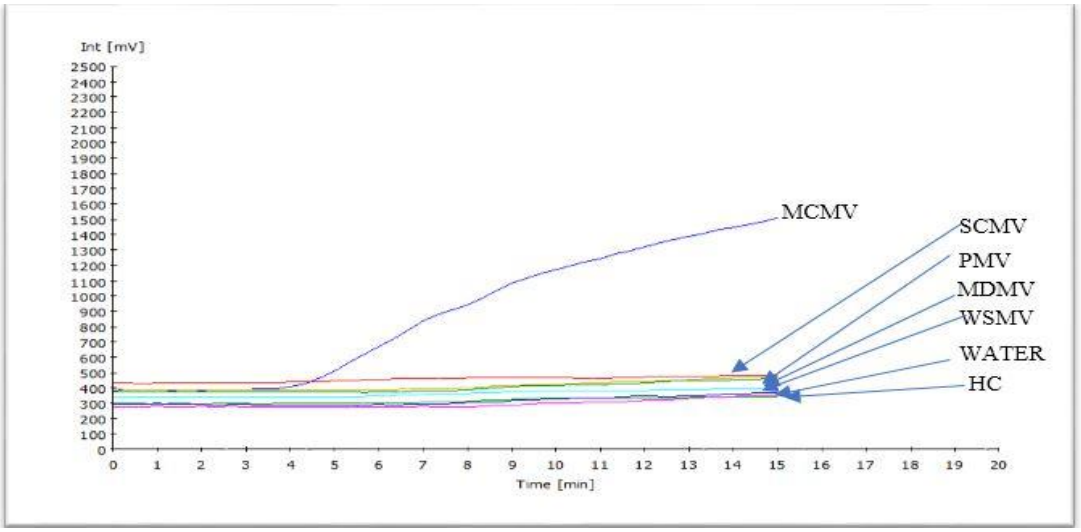


Figure 6.4: RPA Specificity analysis for MCMV against other viruses infecting maize, SCMV, PMV, WSMV and MDMV. Amplification for sample with MCMV was evident

from the 4th-5th minute. All samples with other maize infecting viruses did not show any amplifications.

6.3.3 RT-RPA MCMV Sensitivity analysis results.

The RT-RPA assay for MCMV demonstrated the ability to detect MCMV to the 10^{-3}dilution with the 10^{0} being 100ng/ul. All the four dilutions amplified between the 4th and the 8th minute of the assay (Figure 6.5). This is consistent with the initial amplifications recorded in the primer evaluation stage. The healthy control (HC) and water registered no amplification as expected which showed that the assay was successfully executed.

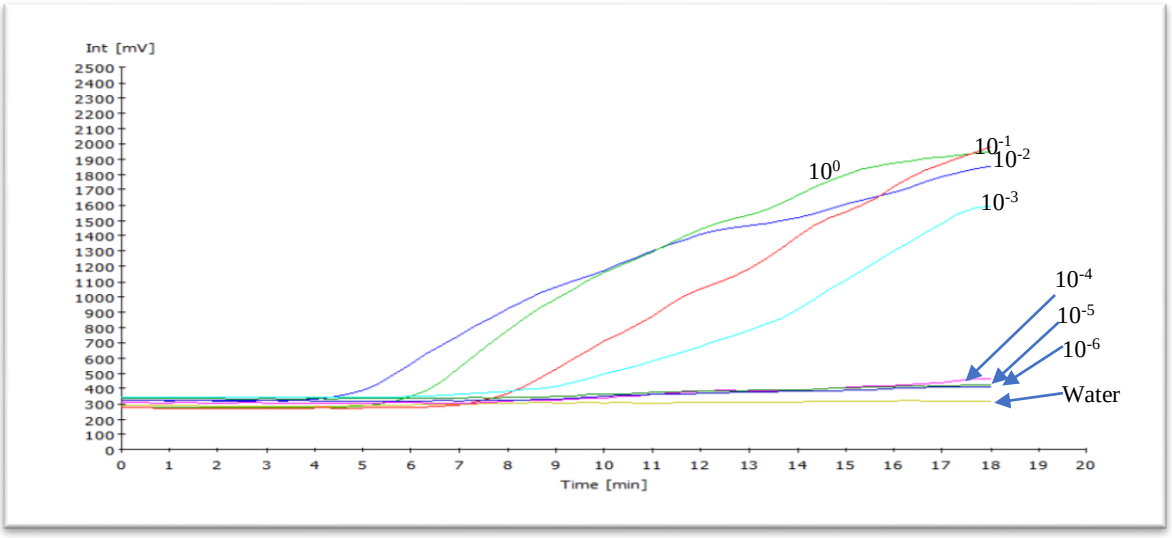


Figure 6.5: RT-RPA MCMV Sensitivity analysis. The dilutions used were from 1 ng/µl - 0.00001 ng/µl (10^{0}– 10^{-6})

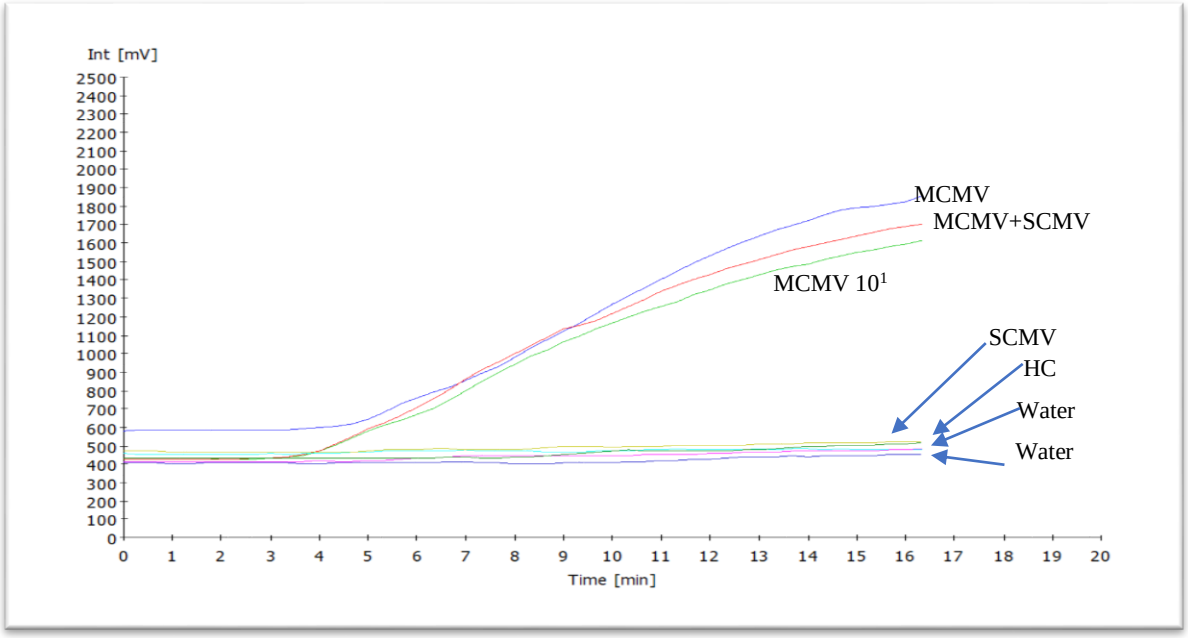


Figure 6.6: Further RPA MCMV virus segregation tests. Samples with either MCMV only or mixed with SCMV were amplified.

The RPA assay was able to segregate samples without MCMV hence displaying a high level of specificity (Figure 6.6). Only the three samples with different levels of MCMV were detected with the assay while SCMV was not detected by the assay as demonstrated with other maize infecting viruses.

6.3.4 RT-RPA Assay for MCMV using direct fresh leaf extracts

The MCMV RT-RPA assay was able to amplify samples with MCMV from maize hybrid DEKALB DKC55-84RIB leaf samples sap solution after grinding the leaf samples in the GEB extraction buffer. There was no RNA that was extracted from these leaf samples, yet amplification was realised as seen in Figure 6.7 below. The assay also illustrated the specificity power by posting amplifications only with samples with MCMV. Amplification was not realised in samples with SCMV and PMV or HC. Amplifications were delayed by a few minutes in some samples, but early amplifications started at minute 5 which is quite comparable with MCMV RNA samples.

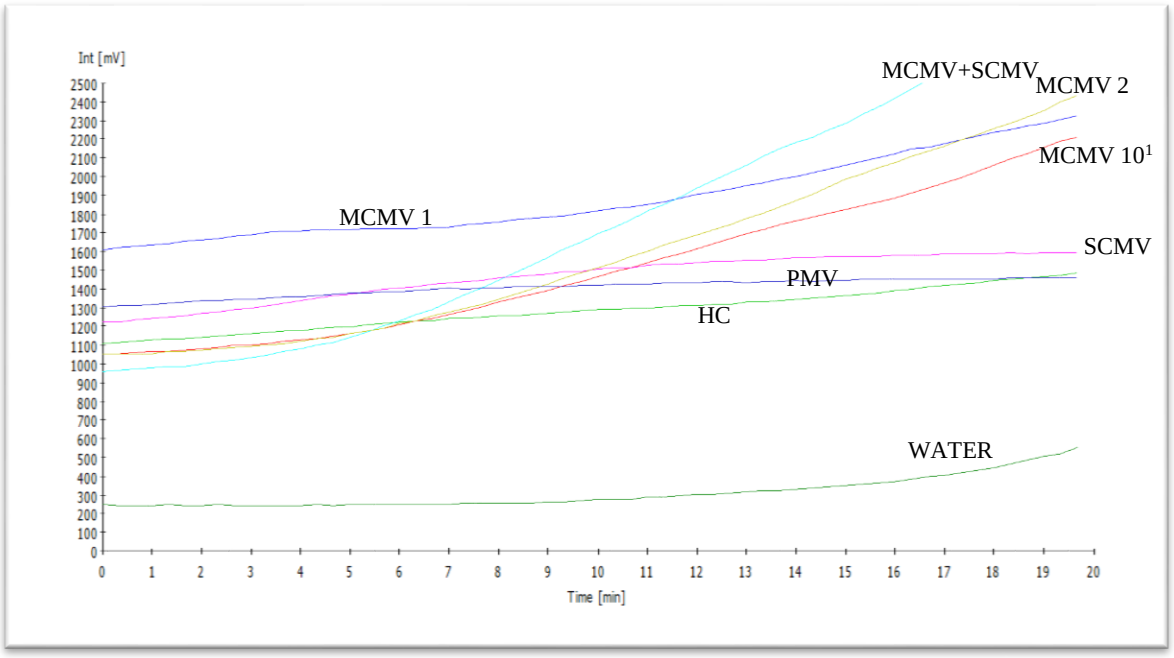


Figure 6.7: RPA for MCMV from fresh leaf samples. Samples with MCMV showed amplification but not with samples of other maize viruses (PMV, SCMV), healthy control and water.

All the fresh leaf samples with MCMV or a combination of MCMV and SCMV amplified and gave slightly higher fluorescent signals than the controls. This is an indication that indeed the RPA method for the detection of MCMV may work with fresh maize leaf samples without isolation of RNA, which is a strenuous and expensive process. The test was run with leaves infected with other maize viruses for segregation. Only samples infected with MCMV tested positive. The samples of MCMV were in different concentrations and one sample had both MCMV and SCMV but the assay was able to amplify only samples with MCMV.

6.4 Discussion

Maize chlorotic mottle virus has been declared a regulated non-quarantine pest in Kenya and in eastern Africa where it has been endemic for the last seven years and a quarantine pest in the non-endemic countries in the region. This is due to its serious effect on maize yield in the MLN disease complex and it's spread through infected and contaminated seed (Min of Agriculture and Livestock development, 2014; De Groote et al., 2015, Marenya et al., 2018). Since its first report in Bomet County, Kenya in 2012, the disease spread fast to other countries in the eastern and central Africa region (Wangai et al., 2012). Several prediction models show that the disease may spread to the central and western Africa region in the next five years (Isabirye and

Rwomushana, 2016). As such, strengthening of phytosanitary regulatory systems in these countries bordering the MLN endemic countries to prevent the introduction of the disease in both new areas in endemic and non-endemic countries is crucial.

One of the important phytosanitary tools towards this effort is a dependable, efficient, effective, and affordable diagnostic tool for MCMV. The most reliable methods for detecting MCMV in host tissues include the enzyme-linked immunosorbent assay (ELISA), Northern blots, and polymerase chain reactions (PCR) for the detection of virus RNA (Mary Hoff 2008). Immunostrips are also handy for testing MCMV in the field (CIMMYT, 2017). Currently, there are Immunostrips for detection of MCMV namely MCMV Agristrips from Bioreba, Switzerland, and MCMV Immunostrips manufactured by Agdia, USA. It is difficult to diagnose MCMV based on symptoms alone for some of its symptoms (stunting and chlorosis) resemble those caused by nutrient deficiency (Marchland et al., 1995). It has also been demonstrated that some infected plants may not exhibit MCMV or MLN symptoms early enough hence are potential in spreading the disease in a localized manner. Molecular based diagnostic assays have been adopted by several National Plant Protection Organizations (NPPOs) and seed certification agencies for testing MCMV in fresh leaf tissues and seed. This is mostly from surveillance programs, for seed certification and phytosanitary requirements for trade. Of these molecular methods, qRT-PCR has been used due to higher sensitivity levels in comparison with conventional RT-PCR (Mackay et al, 2002; Liu et al., 2016). This has led to high testing charges up to USD 35 per sample due to the expensive equipment, maintenance and reagents for the qRT-PCR MCMV assay. On the other hand, conventional RT-PCR is also used but the laborious process of gel-electrophoresis is a setback. The tests also require highly trained/skilled personnel and take up to 3 hours excluding the RNA extraction process or more to generate enough copies of the amplicons for visualization under real-time endpoint analysis. It is also worth noting that the RT-PCR assays are only limited in laboratories hence are not field deployable like the RT-RPA assay developed.

An RT-RPA diagnostics method for the detection of MCMV was developed. RPA is a rapid isothermal nucleic acid amplification and detection platform that is based on patented RPA technology (Piepenburg et al., 2006). The method achieved amplification within the 4^{th} to the 6^{th} minute from the start of the reactions (Figure 3 and 4) compared to the 20^{th} minute to 1hr for the MCMV Loop-mediated isothermal amplification (LAMP) assays (Zhanmin et al., 2016; Chen et al., 2016). This demonstrates how fast the assay is in detecting the virus. The primer

combination 1CF-1BR and Probe1 demonstrated its high level of specificity by only targeting the MCMV viral genome. The test discriminated against the viral RNA from PMV, SCMV, MDMV, WSMV, and in samples that had both MCMV and SCMV. This is an important aspect for testing of MCMV since other isothermal assays like LAMP that use colour change due to DNA intercalating dyes are prone to cross-contamination leading to false positives (Liu et al, 2015 and Chen et al., 2016). The LAMP MCMV assays require the opening of the reaction tubes to add the dyes for visualization of the amplification through colour change. False positive results can have devastating economic effects on seed companies, especially when testing seed lots for MCMV in seed certification procedures which are rejected and only sold as grain. In RPA reactions, the reaction tubes are sealed after all the reagents are added and analysed in real-time due to fluorescence from the probe activity (AmplifyRP Discovery Help Book,2014; Piepenburg et al., 2006).

The assay was also evaluated for sensitivity which showed detection limits of up to 0.1 ng/µl. The advantage of RPA over qPCR is that it misses the range of equivocal or false positives of the tested real-time PCRs for all the amplifications that are over in 20 minutes (Euler 2012b). In this study, AmplifyRP reagents were used to successfully amplify target RNA in a procedure that does not require nucleic acid extraction and purification steps. Amplification was recorded from leaf sap samples extracted using the GB33 extraction buffer (Agdia) (Figure. 6), but the differentiation between positive samples and controls was not as sharp as with RNA samples. Many common sample extraction buffers, such as those used in Agdia's Immunostrips and ELISA test kits are suitable for use with AmplifyXRT format which was used to develop this MCMV RT-RPA assay. Physical extraction of plant tissue or insect vectors can be performed using any common laboratory technique such as macerating with a mortar and pestle and/or mesh extraction bags (RP Amplify Help book, 2014).

The rapid real-time RT-RPA MCMV test hereby developed facilitates faster testing periods and is easily deployable in the field. The small fluorimeter which runs on a lithium phosphate battery is well adopted for field conditions. The lyophilized kits contain pre-mixed enzymes and reagents in a pellet form necessary for the amplification. The reagents can be carried around in the field without storage/transport problems and with low risk of degradation. This makes the reaction amenable to field use or any other point of use applications. The cost per sample was evaluated by CIMMYT in the MLN Epidemiology project and found to be USD 4.0 which

was quite affordable compared to USD 35 for qRT-PCR and USD 7.5 for LAMP (CIMMYT, 2018).

6.5 Conclusion

The rapid and highly sensitive isothermal real-time RT- for the detection of MCMV and by extension MLN developed in this study is well adapted for both laboratory and field usage hence quite mobile. This methodology could be utilized by both regulatory agencies and seed maize production entities for surveillance, quality control, regulation, and internal self-regulation.

The MCMV RT-RPA method has been recommended to the Kenya Plant Health Inspectorate Service (KEPHIS) management, other regional Plant Protection, seed certification agencies, and seed companies for adoption due to the short turnaround time, short time in sample preparation and flexibility in point of use (KEPHIS, 2018). in this study.

CHAPTER SEVEN

EFFICIENCY OF TRANSMISSION OF MAIZE CHLOROTIC MOTTLE VIRUS (MCMV) THROUGH SEED

7.0 Abstract

Maize seed has been found to harbour plant diseases including viral diseases like *Maize chlorotic mottle virus* (MCMV). Investigating the efficiency of transmission of viruses through infected seed is vital for putting up remedial actions to prevent its spread.

The rate of transmission of MCMV in Kenya was investigated under natural and artificial infection of maize plants with MLN causing viruses (MCMV and SCMV). Seventeen hybrids were used in this study. They included hybrids popular with Kenyan maize farming communities, hybrid candidates in CIMMYT breeding programs, and hybrids selected from the Water Efficiency Maize for Africa (WEMA) breeding program. Split plot design was adopted for this experiment where plots of these hybrids were randomised in two blocks and exposed to MLN viruses under natural MLN infection by insect vectors in the field and under artificial inoculation with MCMV infected maize plant extracts. Seeds from these plots in the two blocks were grown out and evaluated for the presence of MCMV at the four-leaf stage using the DAS ELISA MCMV test kit.

It was established that low levels of transmission of MCMV occurred through seed but higher than those previously reported in studies conducted in temperate countries. Out of the total 42,010 seeds evaluated through planting and rigorous testing using ELISA, only 522 tested positive for MCMV. This translates to a transmission rate of 1.2%. Seed transmission rate of MCMV in MLN inoculated fields were found to be slightly higher (1.45%) compared to the rates recorded for the seeds from naturally MLN infected fields that posted a transmission rate of 1.04%.

7.1 Introduction

Transmission of plant viruses through seed is a property intrinsic to members of at least 21 known groups of plant viruses (Tolin S.A. 1999). Approximately 18% of the described plant viruses are seed-transmitted in one or more hosts, and it is estimated that one third of the plant viruses will eventually prove to be seed-transmitted in at least one host (Johansen et al., 1994) The importance of seed transmission and the availability of new techniques to study host-virus interactions have stimulated interest in the virus-host interactions that result in seed

transmission (Stace-Smith and Hamilton 1988). Several reviews on seed transmission has provided current views in the quest of understanding this subject (Johansen et al., 1994).
Approximately 20% of plant viruses are transmitted from generation to generation in the seed (Matthews, 1991; Mink, 1993), and yet very little is known about the mechanism(s) involved. Seed transmission can be a fundamental problem with some plant viruses. In the case of *Barley stripe mosaic virus* (BMSV), seed transmission is the major mechanism of spread of the virus (Timian R.G., 1974). With other viruses, seed transmission may be rare but significant in the epidemiology only as a means for the introduction of viruses into new areas where they may further spread and become established if suitable vectors and hosts are available. This mode of virus spread is of great concern in modern agriculture, where it is common for substantial amounts of seed stock to be moved between regions or continents to take advantage of different growing seasons.

The process of virus seed transmission is environmentally influenced and is a consequence of a specific interaction between the virus and the combined physiology of two generations of the host plant (Carroll, 1981). The host genetic basis for this interaction has been studied in various plant viruses including the classical case of BSMV in barley; in this host, a single recessive gene was implicated in the regulation of seed transmission (Carroll et al., 1979). In no case has it been determined whether it is the genetic complement of the maternal or progeny tissues that determines the efficiency of seed transmission.
Very low levels of seed transmission of viruses in maize have been documented in several reports. *Maize dwarf mosaic virus* (MDMV) was transmitted at a low level, with only one seed transmission in 22,189 seedlings in one study (Mikel et al., 1984), one seed transmission in 11,448 seedlings in another study (Hill et al., 1974), and two transmissions in 29,735 seedlings in a third study (Williams et al.,1968). Similarly, Zhu et al., 1982 reported 14 seed transmissions of MDMV-B, now known as *Sugar cane mosaic virus-MDB,* in 22,925 seedlings. Shepard and Holdeman., 1965 found somewhat higher transmission of MDMV, with 17 transmissions from 9,485 seeds, but 14 of these came from one lot of 3,163 seeds.

Many species of insect vectors are known to move MCMV under long distances but not much is known under tropical conditions. Some seed transmission of MCMV has been documented (Jensen et al., 1991). It is becoming clear that seed transmission of MCMV is playing a role in the rapid emergence of MLN across eastern Africa. Circumstantial evidence also suggests recent introduction of MCMV to Africa, probably through contaminated maize seed (KEPHIS,

2018) though this is still a subject of discussion. Although MCMV was previously shown to be transmitted through seed at very low frequencies of <0.003 percent (Jensen et al. 1991), preliminary results from recent studies suggest much higher rates of seed infection by MCMV in Kenya (Mahuku et al. 2015). Significantly high rates of MCMV infection (>70%) were found in an irrigated hybrid seed production field, increasing the potential for dissemination of MCMV through seed. A very high level of sequence identity among MCMV isolates in Africa (as well as with isolate from SE Asia) suggests a single virus source contributing to the on-going epidemic in eastern Africa.

Jensen et al., 1991 found 21 seed transmissions of MCMV in over 42,000 seeds tested. Again, there was one source of seed that had most of the seed transmissions, so there may be a genotype or a point of origin influence on seed transmission.

There is scanty unpublished information on the rate of transmission of MCMV in Eastern Africa region especially in fields which are naturally infected with MLN and under artificial inoculation with the MLN causing viruses. This objective was to evaluate the rates of transmission of MCMV under natural and under artificial infections.

7.2 Materials and Methods

7.2.1 Growing selected hybrids under natural and artificial infection of MLN viruses

Seventeen different hybrids were used for this study as described in appendix 4. These included the CIMMYT derived hybrids under the MLN breeding program (CMKMLN) series, the hybrids released under the Water Efficiency Maize for Africa (WEMA) series, and the popular commercial hybrids currently on the market in Kenya as shown in Table 7.1 below.

Table 7.1: Hybrids used in the MCMV seed transmission experiment from various sources.

Source/Program	Maize Hybrid
CIMMYT derived hybrids (CMKMLN)	CKMLN150067, CKMLN150072, CKMLN150073, CKMLN150074, CKMLN150075, CKMLN150076, CKMLN150077,
Water Efficiency Maize for Africa (WE) series	WE5135, WE5136, WE5137, WE5138, WE5139
Popular commercial hybrids currently on the market in Kenya	Duma-43, PH3253, PIONEER30G19, KH500-33A, DK8031

The entire experiment was laid out in the CIMMYT MLN Screening facility located at Naivasha (latitude 0°43′S, longitude 36°26′ E, 1896 m ASL) in Kenya from December 2016 to July 2017.

The experimental design adopted was Split plot design. The 17 hybrids were planted each in sub plots of 4m long and 3m wide at a spacing of 75cm x 25cm on 12th Dec 2016. This gave a plant population of 60 plants per plot. Each block had 17 variety subplots for the 2 plots under natural MLN infection and those under artificial MLN inoculation. Natural infection of MLN occurs in the field where insect vectors for MCMV and SCMV transmit the viruses to healthy plants. In artificial infection, plants are physically inoculated with the two viruses at a ratio of 1:4 by weight for SCMV and MCMV respectively by high pressure spraying of the extract sap mixture. The maize plants were sprayed adequately ensuring complete cover. There were two biological replications, but each subplot had 3 technical replications with 20 plants per replication.

The subplots in blocks under artificial inoculation were inoculated with MLN causing viruses i.e. MCMV and SCMV twice at a ratio of 1:4 by weight. The first inoculation was done on 21st

Jan 2017 and the second one done on 27th Jan 2017. The blocks under natural infection were left for MLN infection to occur naturally from the existing vectors in the field. No spraying was done to control the vectors for MLN viruses in the blocks under natural MLN viruses' infection. This was to make sure that we have the natural infections progress going on well in the plots where no inoculation was done.

Data was collected from all the plots which included the symptoms severity, incidences, no. of plants infected and stand counts per plot. The symptom severity scale rating was based on MLN severity scoring scale (1 to 10) (CIMMYT, 2016). This data was collected at 2 weeks' intervals to monitor the development of the MLN symptoms with time. The plots were allowed to progress to maturity and harvesting of the cobs from each plot was done.

7.2.2 Estimating the rates of seed transmission of MCMV.

Maize cobs from MLN infected maize plants in each plot were harvested and shelled. Most plants were affected by the disease and indeed some died especially for the commercial hybrids. Seed was harvested from the plants that survived and produced some cobs. The harvested seeds were sun dried until they attained a moisture content of between 13.5% – 15%. Seed from each hybrid from the two experiments were bulked and subsequently used in the grow out test for MCMV rate of transmission evaluation. The seeds were planted in 5 l plastic pots filled with soil sterilized by heat treatment. Twenty-five seeds for each variety (hybrid) were planted in each pot. A total of 25 pots for each hybrid from the natural and artificial MLN infection were planted giving a total of 625 seeds for each hybrid under investigation. This was replicated twice giving a total of 1250 seeds per hybrid for the natural infected plants and the same (1250) for the artificial infected plants. The pots were placed in the screen houses at the Naivasha screening facility to ensure the correct isolation, insect control, strict confinement to limit any chances of reinfection or contamination by the virus (MCMV).

The pots were maintained with moisture at levels optimum for germination of seeds and growth of the seedlings. For the clarity of the results and to reduce any chances of mixing the seed pots planted from the two experiments, the pots for two experiments were placed in separate screen houses. The moderately elevated temperatures in the screen houses prompted faster growth of the seedlings with majority having four to five leaves at the 14th day after germination.

The rate of transmission of MLN was assessed by testing all the grow-out plants for MCMV to ascertain the percentage of plants with MCMV infection through seed. MCMV Double Antibody Sandwich-enzyme Linked Immunosorbent Assay (DAS-ELISA) kit was used.

Two young apical and actively growing leaves were picked from each plant from each pot forming a composite sample for ELISA testing for MCMV for that particular hybrid. The composite sample leaves from 25 plants weighing 3g were ground in sample extraction buffer at a 1:20 ratio (tissue weight in g: buffer volume in ml) in mesh bags. ELISA test was repeated for individual plants for pots that tested positive to ascertain the number of plants infected in that particular pot.

Commercially available antisera kits of DAS-ELISA (DSMZ DAS ELISA) were used for the detection of MCMV. The specific antibody for the MCMV antigen was diluted in a coating buffer i.e. 20µl in 20 ml buffer at a recommended dilution of 1:1000 or 40µl in 20 ml buffer at a recommended dilution of 1:500. The microtiter plate was prepared and 200µl of the antibody was added to each well. The plates were covered and incubated at 37 °C for 2- 4 h. The plates were washed with PBS-Tween using wash bottle, soaked for a few minutes and washing was repeated two more times. The plates were dried by tapping upside down on tissue paper. Aliquots of 200 µl of the test sample extracts were added to duplicate wells. The plates were covered and incubated overnight at 4 °C. The plate was washed three times as described earlier. The aliquots of 200 µl of the enzyme conjugate with a recommended dilution of 1:1000 was added in the conjugate buffer. The plates were covered and incubated at 37 °C for 2- 4 hours. The plate was washed three times again. Aliquots of 200 µl of freshly prepared substrate (1 mg/ml para- nitrophenyl- phosphate in substrate buffer) was added to each well. The plates were covered and incubated at 37°C for 45 min which was adequate for clear reactions. The data was analysed by visual observation and by spectrophotometric measurement of absorbance at 405 nm in an ELISA reader.

7.3 Results

The seeds in the field blocks germinated from the fourth day after planting and the germination percentage was evaluated and found to range between 95%-99%.

Symptom expression was detected one week after inoculation in the plots under artificial inoculation whereas natural MLN infection plots showed symptoms later and in a slow but progressive manner. The artificial inoculated plots exhibited rather uniform MLN symptoms in

the four weeks after inoculation. Table7.2 shows the symptoms severity of the maize plants planted under artificial infection at three weeks' interval while Table 7.3 shows for the maize plants under natural infection.

Table 7.2: Means of severity symptoms under artificial infection at three weeks' interval after the 1st assessment on 10/02/2017.

Entry	Name	Pedigree	No. of rows	Stand Count	Symptom Severity	Symptom Severity	Symptom Severity	Mean Symptom Severity
1	CKMLN150067	(CKDHL0159/CKDHL0500)//CKDHL120312	4	47	2	3	4	3.0
2	CKMLN150072	(CKLMARSI0037/CKLTI0139)//CKDHL120312	4	48	2	2	3	2.3
3	CKMLN150073	(CML543/CML4940)//CKDHL120312	4	49	2	2	3	2.3
4	CKMLN150074	(CKLMARSI0037/CML543)//CKDHL120312	4	50	2	3	4	3.0
5	CKMLN150075	(CKLTI0137/CKLMARSI0022)//CKDHL120313	4	49	2	2	3	2.3
6	CKMLN150076	(CKLMARSI0022/CKLTI0136)//CKDHL120312	4	44	2	3	4	3.0
7	CKMLN150077	(CKLMARSI0037/CKLTI0138)//CKDHL120312	4	52	2	3	4	3.0
8	WE5135	WE5135	4	48	3	4	4	3.6
9	WE5136	WE5136	4	49	3	5	6	4.7
10	WE5137	WE5137	4	44	4	5	6	5.0
11	WE5138	WE5138	4	47	4	5	6	5.0
12	WE5139	WE5139	4	49	3	5	6	4.7
13	DUMA-43	DUMA-43	4	52	5	7	8	6.7
14	PH3253	PH3253	4	51	5	7	8	6.7
15	PIONEER 30G19	PIONEER 30G19	4	46	5	6	7	6.0
16	KH500-33A	KH500-33A	4	41	5	6	7	6.0
17	DK8031	DK8031	4	47	5	6	7	6.0
1	CKMLN150067	(CKDHL0159/CKDHL0500)//CKDHL120312	4	44	3	4	4	3.7
2	CKMLN150072	(CKLMARSI0037/CKLTI0139)//CKDHL120312	4	49	2	2	3	2.3
3	CKMLN150073	(CML543/CML4940)//CKDHL120312	4	51	2	3	3	2.7
4	CKMLN150074	(CKLMARSI0037/CML543)//CKDHL120312	4	49	2	3	4	3.0
5	CKMLN150075	(CKLTI0137/CKLMARSI0022)//CKDHL120313	4	44	3	3	3	3.0
6	CKMLN150076	(CKLMARSI0022/CKLTI0136)//CKDHL120312	4	43	2	3	4	3.7
7	CKMLN150077	(CKLMARSI0037/CKLTI0138)//CKDHL120312	4	51	3	4	4	3.7
8	WE5135	WE5135	4	51	3	4	4	3.7
9	WE5136	WE5136	4	50	4	5	6	5.0
10	WE5137	WE5137	4	49	4	5	6	5.0
11	WE5138	WE5138	4	51	4	5	6	4.7
12	WE5139	WE5139	4	49	3	5	6	4.7
13	DUMA-43	DUMA-43	4	52	5	7	8	6.7
14	PH3253	PH3253	4	44	5	7	8	6.7
15	PIONEER 30G19	PIONEER 30G19	4	48	5	6	7	6.0
16	KH500-33A	KH500-33A	4	50	5	6	7	6.0
17	DK8031	DK8031	4	50	5	6	7	6.0

Table 7.3: Means of severity symptoms under natural infection at three weeks' interval after the 1st assessment on 10/02/2017.

Entry	Name	Pedigree	No. of rows	Stand Count	Symptom Severity	Symptom Severity	Symptom Severity	Mean Symptom Severity
1	CKMLN150067	(CKDHL0159/CKDHL0500)//CKDHL120312	4	52	1	2	2	1.7
2	CKMLN150072	(CKLMARSI0037/CKLTI0139)//CKDHL120312	4	51	1	2	2	1.7
3	CKMLN150073	(CML543/CML4940)//CKDHL120312	4	45	1	3	2	2.0
4	CKMLN150074	(CKLMARSI0037/CML543)//CKDHL120312	4	50	1	3	3	2.3
5	CKMLN150075	(CKLTI0137/CKLMARSI0022)//CKDHL120313	4	44	1	3	3	2.3
6	CKMLN150076	(CKLMARSI0022/CKLTI0136)//CKDHL120312	4	42	2	3	3	2.7
7	CKMLN150077	(CKLMARSI0037/CKLTI0138)//CKDHL120312	4	50	2	3	3	2.7
8	WE5135	WE5135	4	44	2	4	4	3.3
9	WE5136	WE5136	4	50	3	5	5	4.3
10	WE5137	WE5137	4	51	4	5	5	4.7
11	WE5138	WE5138	4	50	4	5	5	4.7
12	WE5139	WE5139	4	47	4	5	5	4.7
13	DUMA-43	DUMA-43	4	48	5	7	7	6.3
14	PH3253	PH3253	4	50	5	7	7	6.3
15	PIONEER 30G19	PIONEER 30G19	4	47	4	5	7	5.3
16	KH500-33A	KH500-33A	4	48	4	5	6	5.0
17	DK8031	DK8031	4	47	4	5	5	4.7
1	CKMLN150067	(CKDHL0159/CKDHL0500)//CKDHL120312	4	43	2	3	2	2.3
2	CKMLN150072	(CKLMARSI0037/CKLTI0139)//CKDHL120312	4	43	1	3	2	2.0
3	CKMLN150073	(CML543/CML4940)//CKDHL120312	4	45	1	2	2	1.7
4	CKMLN150074	(CKLMARSI0037/CML543)//CKDHL120312	4	42	2	3	3	2.7
5	CKMLN150075	(CKLTI0137/CKLMARSI0022)//CKDHL120313	4	40	2	3	3	2.7
6	CKMLN150076	(CKLMARSI0022/CKLTI0136)//CKDHL120312	4	37	2	3	3	2.7
7	CKMLN150077	(CKLMARSI0037/CKLTI0138)//CKDHL120312	4	50	2	2	3	2.3
8	WE5135	WE5135	4	52	3	4	4	3.7
9	WE5136	WE5136	4	48	3	5	5	4.3
10	WE5137	WE5137	4	45	4	4	5	4.3
11	WE5138	WE5138	4	49	4	4	5	4.3
12	WE5139	WE5139	4	44	3	5	5	4.3
13	DUMA-43	DUMA-43	4	40	5	7	7	6.3
14	PH3253	PH3253	4	47	5	7	7	6.3
15	PIONEER 30G19	PIONEER 30G19	4	48	5	7	7	6.3
16	KH500-33A	KH500-33A	4	37	4	6	6	5.3
17	DK8031	DK8031	4	48	4	6	5	5.0

Table 7.4 and 7.5 shows the response of difference maize hybrids to Maize Lethal Necrosis disease under artificial infection and natural infection respectively. The figures recorded for stand count, plants infected with MLN, Symptom severity and MLN incidence are means from the two replications. The rate of infection was calculated as the percentage of the plants that tested positive for MLN from the total number of maize plant seedlings that germinated from the two replications for each hybrid.

Table 7.4: Response of difference maize hybrids to Maize Lethal Necrosis disease under artificial infection.

Variety	Stand count	Natural Infection			
		No. infected	Incidence	Severity	Rate of transmission
CKMLN150067	45.5±0.6a	43.9±1.15def	92.6±0.58d	3.7±0.58bc	0.5±0.053efg
CKMLN150072	48.5±1.2ab	39.9±0.57g	81.3±1.15e	2.3±0.58c	1.0±0.09de
WE5137	46.5±0.0ab	44.3±0.57def	100.0±0.00a	5.0±0.00ab	2.1±0.09b
CKMLN150074	45.9±0.6abc	50.3±0.57abc	100.0±0.00a	3.0±0.00bc	0.2±0.10fg
PH3253	47.5±0.6abc	50.9±1.15ab	100.0±0.00a	6.7±0.58a	2.2±0.09b
WE5136	49.5±0.6abc	48.6±1.15abc	100.0±0.00a	5.0±0.58ab	0.3±0.09fg
CKMLN150077	51.5±0.6abc	50.6±0.58abc	96.3±0.58bc	3.7±0.00bc	0.2±0.09g
WE5138	49±1.6abc	47.3±0.58bcde	100.0±0.00a	4.7±0.58bc	1.4±0.09dc
DUMA-43	52±0.0bcd	52.0±0.58a	100.0±0.00a	6.7±0.58a	0.6±0.09fe
KH500-33A	45.5±0.0bcd	41.3±1.15fg	100.0±0.00a	6.0±0.00a	1.1±0.09dc
WE5139	49±1.6cde	49.9±1.15abc	100.0±0.00a	4.7±0.58ab	1.4±0.09dc
PIONEER 30G19	47±0.6cde	46.3±0.58cde	100.0±0.00a	6.0±0.58a	3.1±0.09a
DK8031	48.5±0.6cde	47.0±0.58bcde	100.0±0.00a	6.0±0.58a	2.6±0.09b
CKMLN150073	50±0.0def	47.3±0.00bcde	96.6±0.00bc	2.7±0.00c	1.5±0.1c
CKMLN150075	46.5±1.2ef	46.6±0.58cde	94.3±0.58dc	2.9±0.00dc	1.3±0.09dc
WE5135	49.5±1.2ef	48.3±0.00abcd	100.0±0.00a	3.7±0.58bc	0.5±0.09efg
CKMLN150076	43.5±0.6f	43.±0.00efg	98.3±0.00dc	3.7±0.88c	1.1±0.09dc
Grand mean	48	46.63	97.38	5.27	1.24
l.s.d (0.05)	3.9	4.01	2	2.66	0.48
F(16,50) Value	15.25	23.06	169.94	12.35	94.01
CV	2.674	2.837	0.679	16.657	12.825

Severity – MLN symptoms severity scored using the revised CIMMYT severity scale of 1-10 Plants infected – These are the number of plants recorded with MLN symptoms in the plots against the stand count per plot Incidence – This was calculated at by the percentage of plants with MLN symptoms against the total number of plants in the plot.

Table 7.5: Response of difference maize hybrids to Maize Lethal Necrosis disease under natural infection.

Variety	Stand count	Artificial infection			
		No. infected	Incidence	Severity	Rate of infection
CKMLN150067	47.5±0.6a	41.3±1.2d	87.1±1.2d	1.7±0.0d	0.6±0.14ih
CKMLN150072	47±1.2ab	29.1±1.2e	60.3±0.6e	1.7±0.0d	0.4±0.05ih
WE5137	48±0.0ab	51.7±0.6a	100±0.00a	4.7±0.6abc	3.5±0.09a
CKMLN150074	46±0.6abc	46.7±0.0abc	95.3±0.00c	2.3±cd	0.6±0.05ih
PH3253	48.5±0.6abc	50.3±1.2a	100±0.00a	7±0.6a	2.6±0.05c
WE5136	49±0.6abc	50±1.2a	100±0.00a	4.3±0.6abc	2.0±0.05d
CKMLN150077	50±0.6abc	50±1.2a	100±0.00a	2.7±0.6cd	0.7±0.09gh
WE5138	49.5±1.6abc	47±1.2abc	100±0.00a	4.7±0.0abc	0.6±0.05ih
DUMA-43	44±0.0bcd	48.3±0.0ab	100±0.00a	6.3±0.6a	1.1±0.05f
KH500-33A	42.5±0.0bcd	48.3±1.2ab	100±0.00a	5.0±.6ab	1.0±0.09fg
WE5139	45.5±1.6cde	47.6±0.6abc	100±0.00a	4.7±0.6abc	2.1±0.09d
PIONEER 30G19	47.5±0.6cde	47±0.6abc	100±0.00a	5.3±0.6a	1.6±0.05e
DK8031	47.5±0.6cde	47±0.6abc	100±0.00a	4.7±0.6abc	3.0±0.05b
CKMLN150073	45±0.0def	43.3±1.2cd	98.3±0.6b	2.0±0.0d	0.7±0.07gh
CKMLN150075	42±1.2ef	41.3±1.2d	97.6±0.6b	2.3±0.6cd	0.3±0.05i
WE5135	49.5±1.2ef	44±0.6bcd	100±0.00a	3.3±0.6bcd	1.1±0.05f
CKMLN150076	39.5±0.6f	42±0.6d	100±0.00a	2.7±0.6cd	2.7±0.12c
Grand mean	48	45.4	96.35	4.4	1.46
l.s.d(0.05)	3.9	4.79	1.95	2.66	0.35
F(16,50) Value	15.25	34.01	717.59	12.23	217.36
CV	2.674	3.461	0.666	20.089	8.055

Severity – MLN symptoms severity scored using the revised CIMMYT severity scale of 1-10 Plants infected – These are the number of plants recorded with MLN symptoms in the plots against the stand count per plot Incidence – This was calculated at by the percentage of plants with MLN symptoms against the total number of plants in the plot.

In each experiment, MLN symptoms severity was well comparable for the plants under artificial inoculation and those naturally infected by MLN viruses. This scenario replicated itself for the disease incidence where incidence figures in the two experiments were in the same range for the same hybrids. The MLN incidence was evaluated in three stages during the entire growing period of the plants hence the last incidence recorded was the one used in this assessment. There were high MLN incidences in the artificial inoculated plants compared to the plots with natural MLN infection.

There was a general trend of MLN disease severity progressing along the hybrids with CIMMYT derived hybrids posting symptom severity levels that are lower and significantly lower than those exhibited by the common and popular commercial hybrids in Kenya. The same trend was evident with the disease incidences though some CMKMLN hybrids that posted high incidences under artificial and natural infections. However, there is significant difference in severity and incidence between popular commercial hybrids found on the market, WEMA hybrids and the CIMMYT derived lines tolerant to MLN (Table 7.4 and 7.5).

Parameters investigated are stand count, plants infected with MLN, disease symptoms severity and the disease incidence.

There was a total of 524 seedlings which tested positive for MCMV out of the total 42,010 seedlings tested. Out of these, 305 seeds tested positive for MCMV out of the 21,014 from the fields planted and artificially inoculated with MLN viruses. As for the fields under natural infection of MLN, there were 219 seeds that tested positive for MCMV out of the total 20,996 seeds planted and germinated in this study.

The positive samples were well distributed amongst the seed lots of the hybrids. However, there were samples from some seed lots which showed no significant difference in MCMV transmission in the seed lots between the artificially inoculated fields and the natural infected fields. These were CKMLN150077 and CKMLN150075 from the inoculated fields and the natural infection fields respectively. There were higher disease severity and incidence levels from the hybrids already on the market, i.e. the commercial hybrids in this study compared to the CIMMYT derived hybrids under development for MLN tolerance (the CKMLN series). Most of the commercial hybrids posted incidences of 100% as early as the 2nd month of planting for the inoculated plants and later for the natural infection. MLN infection was uniform in those

fields artificially inoculated compared to the natural infection fields but eventually all the fields developed the full symptoms of MLN infection with time.

7.4 Discussion

This study confirmed the previous reports of notably low transmission rates of MCMV through seed (Jensen et al., 1991). Out of the total 42,010 seeds from MCMV infected plants evaluated through planting and rigorous testing using ELISA, only 524 plants tested positive for MCMV. This translates to a transmission rate of 1.2% that is slightly higher than the reported 0.003% representing MCMV transmission in 17 seeds out of the 42,000 (Jensen et al., 1991). However, the transmission rate of MCMV in inoculated fields were slightly higher (1.45%) compared to the rates recorded for the seeds from naturally infected fields at 1.04%. Seed transmission of MDMV, another potyvirus known to synergise with MCMV to cause MLN showed very low levels of seed transmission, i.e. one of 22,189 seeds (Mikel et al., 1984). In another study, only one seed transmission out of 11,448 seedlings transmitted the disease in the seedlings (Hill et al., 1974). However, in both cases, these rates are quite lower than the MCMV transmission rate found in this study. In one of the studies done in eastern Africa where seed harvested from an MCMV-infected maize plant was tested for the presence of MCMV by RT-PCR, 18 out of 25 seeds (72%) were positive for MCMV (Mahuku et al., 2015). Under the same study, MCMV was detected in 12 of 26, 10-seed samples pooled from 26 lots of locally purchased seed. This indicated quite high levels of seed contamination with MCMV though transmission through seed was not evaluated.

Most of the previous studies for the seed transmission for MCMV and MDMV were carried out in temperate countries as opposed to the tropical conditions of Eastern Africa. These slightly higher levels of transmission can be attributed to both the prevailing climatic conditions and genotype related factors like elevated levels of susceptibility to MCMV. Recent studies by Braidwood et al., 2018 on the global phylogeny of MCMV reveals a distinct strain in Eastern Africa which has proved to be more virulent compared to the strains of the Americas and Asia. However, materials tolerant to MCMV in the US were brought for screening in the CIMMYT Naivasha MLN screening facility where they were inoculated with the local strains of MLN viruses. All the materials were severely infected by MLN, signalling the possibility of differences in the pathogenicity of the strains in the US and in Eastern Africa. Another possible explanation is the increased virulence of MCMV due to the synergistic interaction with SCMV (Xia et al., 2016). This disease commonly referred to as corn

lethal necrosis (CLN) is associated with MCMV in synergy with WSMV in United States (Niblett and Caflin 1978; Scheets, 1998).

In this study, MCMV transmission through seed cuts across all hybrids with a bearing on genotypes with varying rates of transmission. MCMV seed transmission results in Table 7.4 and 7.5 indicates significant differences between the popular commercial varieties on the market and the CIMMYT derived lines. The materials had a potential of transmitting the virus to the offsprings at different rates and influenced by the type of the hybrid as seen in Table 7.4 and 7.5. The fact that all the seed materials here in the study have a potential of transmitting MCMV at different proportions may perhaps explain why this disease has rapidly spread in the region from the time it was first reported in the year 2012 (Wangai et al., 2012, Mahuku et al., 2017 and Kiruwa et al., 2015). MLN has spread rapidly in countries neighbouring Kenya in a span of just 12 months from the time it was first officially reported. Subsequently, the disease was detected in Tanzania (CIMMYT, 2012; Mexico, C. I. M. M. Y. T. (2012); Miano et al., 2013), Uganda (IPPC, 2014), Rwanda (Adams et al., 2014) and in DRC (Lukanda et al 2014). There are unconfirmed records from South Sudan about the presence of this disease there. There is now an increasing realization that commercial seed flows have been the initial source of the dissemination of the MLN-causing viruses over large distances in Kenya and Eastern Africa. MCMV was detected in commercial seed lots in Rwanda and Ethiopia. Once MCMV is established in a country, it spreads mechanically or easily via insect-vectors during the growing season, making eradication very difficult or impossible. Transmission by maize thrips (*Franklinellia williamsi*) and other insect vectors is likely the major source of plant-to-plant spread within fields, and between neighbouring fields (Nyasani et al 2015). Experience in Hawaii has shown that insect-vectors like thrips can be blown considerable distances by storms (Jiang et al., 2009), but this mode of vector movement is likely less important in east Africa where weather patterns are different. MCMV transmission is also possible via field operations (mechanical means), and possibly through irrigation water, but insects are the major factor in virus spread within fields and to nearby fields (Bragard et al., 2013).

The rates of transmission of MCMV through seed in artificially MLN infected plants was 1.42% compared to the seeds from naturally infected plants at the rates of 1.02%. One probable reason is that infection in artificially inoculated fields starts with a higher inoculum dose hence more accumulation of the virus compared to naturally infected plants. This difference needs to be investigated further to determine the factors that dictate slightly higher rates in artificially infected crop.

Given poor phytosanitary systems, porous borders, and weak regulatory regimes in the East and Central Africa countries, the disease has spread broadly to all countries bordering Kenya (FSNWG, 2012). It is difficult to explain this rapid spread to be due to insect pests. Insect pests spread has been found to be a little more rapid within shorter distances. Despite this rapid spread, role of potential vectors in the disease epidemiology and sustainable vector management strategies are poorly understood in eastern Africa (Nyasani et al 2015). Maize thrips is the only widely distributed vector of MCMV in East Africa and is likely the primary vector. It transmits MCMV in a non-persistent manner. Although maize is the preferred host, maize thrips can survive on many plants including cassava, beans, maize, sorghum, onions, various grasses, rice, peppers coriander, peas, and the weedy species *Bidens pilosa* and *Tithonia diversifolia* (Capinera 2008; ICIPE Thrips 2011; Frison and Feliu 1989; King and Saunders 1984). This however falls short of explaining the spread of MCMV through vast distances in Eastern Africa within a short period of time. At the same time, MCMV has shown up in areas within the same country surmounting natural barriers like forested land and vast game reserves. This is a typical situation with Kenya where the disease was detected in the coastal region the same year it was detected in the Rift valley. During surveillance programs by KEPHIS, startling findings have pointed to maize fields grown inside forests under the shamba system showing MLN symptoms while the crops around in the proximal areas of the forests were healthy and MLN free. The only probable method of transmission of MCMV in these cases described points to transmission through seed by few plants but rapidly in the fields through the insect vectors which are abundant in the maize growing regions of the country. Currently, environmental conditions favouring MLN emergence and rapid spread remain poorly understood, making design of temporal and spatially explicit mitigation and response strategies difficult (Isabirye and Rwomushana 2016)

7.5 Conclusion

MCMV transmission rate through seed is low but the rates in artificially inoculated plants is slightly higher compared to those under natural infection. It is clear that the rapid spread of MLN in Kenya and neighbouring countries was through MCMV infected seed. Location spread however was mainly attributed to insect vectors, the maize thrips which are abundant in maize growing regions in Kenya. There is need for harmonized phytosanitary regulations for movement of seed maize within the country and in the region to minimize the spread of MCMV through seed. The lucrative seed market in the country and the region may fuel MLN spread if not well addressed and regulated.

CHAPTER EIGHT

8.0 GENERAL DISCUSSIONS, CONCLUSION AND RECOMMENDATION

8.1 General discussion

There is no doubt that MLN is one of the most devastating diseases of maize in recent times in Kenya and the entire eastern African region. The disease has been around in other parts of the world, but the yield losses have not been as severe as those reported in the tropical countries of eastern Africa.

The current situation of the disease has been analysed and the causative agents were further evaluated. Viral diversity is important since it informs both researchers and the policy makers of the breeding programs to address the specific pathogens identified. This is of paramount importance since materials which are otherwise resistant to MCMV, the major virus in the MLN disease complex in the Americas cannot be deployed in the eastern African region and confer the same attributes as exhibited in their native regions. The need for locally bred materials customized to the prevailing biotic and abiotic constraints are of vital importance in Kenya. This is only possible when the genetic diversity of the strains of both MCMV and SCMV are well understood and their phylogeny in relation to other strains around the globe are well studied and understood. The diversity within MCMV in Kenya is quite small as determined in this study and other previous studies. Most of the samples analysed showed similarities to other strains in Asian countries. However, there are some genetic differences from MCMV strains of Americas and of Asian regions.

SCMV was found to infect maize synergistically with MCMV with devastating MLN symptoms. The genetic diversity and molecular evolution of SCMV capsid protein (CP) gene were investigated with the nucleotide sequences available in the GenBank database. Phylogenetic analyses revealed that SCMV isolates clustered in relation to their original hosts, and geographically distinct isolates from maize or sugarcane clustered differently. There were two major clusters of the African isolates and the Asian isolates. High genetic diversity was recorded with ranges between 86% - 90%. Kenyan SCMV sequences were 95% - 97% similar to the Rwanda and other Kenyan isolates

A detailed two end point analysis RT- LAMP was developed under this study to provide a reliable, effective, efficient, sensitive cost effective and field deployable molecular based assay for the detection of MCMV.The existing MCMV LAMP developed (L. Cheng et al 2016) only

reports one end point analysis with the use of Syber green dye that has been reported to lead to cross contamination hence false positives. LAMP assays result in millions of copies of the target nucleic acid and therefore chances of contamination in the lab are quite high.

An RPA diagnostics method for the detection of MCMV was also developed. RPA is a rapid isothermal nucleic acid amplification and detection platform that is based on patented recombinase polymerase amplification (RPA) technology. The method achieved amplification within the 4^{th} to the 6^{th} minutes from the start of the reactions demonstrating the strongpoint of rapidity of this assay in testing MCMV. The fact that the fluorimeter can be operated by a battery pack demonstrates that it is field deployable hence the tests can be done in the fields. The Primer combination 1BF X 1AR and Probe1 demonstrated high level of specificity by only targeting the MCMV viral genome. The test discriminated against the viral RNA from *Panicum mosaic virus* (PMV), *sugarcane mosaic virus* (SCMV), *Maize dwarf mosaic virus* (MDMV) and *Wheat streak mosaic virus* (WSMV). The test is less laborious since it can be executed without the nucleotide's extraction step from leaf samples.

The notably low transmission of MCMV through seed was confirmed as per the previous reports on the same study. It was established that only 524 tested positive for MCMV out of the total 42,010 seeds evaluated through planting and rigorous testing using ELISA. This translates to a transmission rate of 1.2% that is slightly higher than the reported 0.04% representing MCMV transmission in 17 seeds out of the 42,000 evaluated (Jensen et al., 1991). It has been quite difficult to explain the rapid spread of MLN in the region. There is now an increasing realization that commercial seed flows have been the initial source of the dissemination of the MLN-causing viruses over large distances in East Africa. MCMV was detected in commercial seed lots in Rwanda and Ethiopia. Once MCMV is established in a country, it spreads easily via insect-vectors during the growing season, making eradication very difficult or impossible. In this study, MCMV transmission through seed cuts across all hybrids at varying transmission rates. Higher rates of transmission were noted in artificially infected maize plants and in hybrids popular on local markets. There are no significant differences between the popular commercial varieties on the market and the CIMMYT derived lines showing tolerance to MLN

8.2 Conclusions

This study confirmed that MCMV infects maize synergistically with SCMV, a potyvirus in Kenya. Many a times, the disease is described as being caused by MCMV and any other potyviruses in Kenya and in the region (Wangai et al., 2012: Mahuku et al., 2015). A polerovirus, MaYMV was also detected in some farms where MLN was detected but its synergistic interaction with MCMV for MLN development is not yet understood (Adams et al., 2017). In eastern Africa, MaYMV has been found present in all recent MLN related studies though some publications have described it as Maize yellow dwarf mosaic virus (MYDMV) (Adams et al., 2017: Massawe et al., 2018: Wamaitha et al., 2018; Read et al., 2019; Asiimwe et al., 2019; Kiruwa et al., 2019: Stewart et al., 2020). SCMV partial sequences of between 8984bp and 9607bp were generated from this study. It is also quite evident that SCMV is highly genetically diverse in this country with similarities ranging from 86% to 90%.

Phylogenetic analyses revealed that SCMV isolates clustered in relation to their original hosts where isolates in the North rift clustered together. SCMV isolates from the south rift and Coast regions also clustered together. It is worth noting that the MCMV isolates derived in this study were in three major clusters of the African, Asian and American isolates that were well segregated.

The MCMV contigs from this study generated one full genome sequence of 4438bp and several partial sequences between 4416bp – 4427bp.Sequences obtained from this MCMV isolate was 99% identical to one isolate previously reported from Kenya, Ethiopia and Rwanda. The contigs sequence also had 99% identity with MCMV isolates from maize and sugarcane collected in Yunnan and Sichuan, China; 98% identity with another MCMV isolate from Yunnan, China (KF010583.1); and 96 to 97% identical to genome sequences of MCMV isolates from Kansas and Nebraska in the United States. Similarly, partial sequences of the MCMV coat protein for isolates from the country were 99% identical to those from Rwanda, KP851970.1. These results suggest a potentially common origin of MCMV for eastern Africa and some Asian MCMV isolates.

The development of the RT-LAMP assay is timely with two custom end point analysis employing colour change (Sybr Green) and real time amplification plots of the Genie LAMP diagnostics equipment. The simplicity, rapidity, and inexpensiveness of this technique make it a suitable choice for large-scale sample processing, especially by laboratories with limited resources. Currently, phytosanitary regulatory institutions are using conventional and Real time

PCR for routine screening of seed material for MCMV which is quite expensive. Seed infected with MCMV can be identified using this method with the same sensitivity as conventional PCR. Further sensitivity optimization may yield a protocol that can be utilized in detecting these viruses in maize seed before symptoms appear in the seed fields. Currently this assay has been officially adopted by the Kenyan NPPO and the official seed certification agency, KEPHIS for routine testing for MLN viruses both in leaf tissues and in seed. Strict regulation coupled with internal controls for self-regulation by seed companies utilizing clean maize seed in conjunction with growing resistant varieties and monitoring new virus strain emergence are necessary measures to prevent the spread of MCMV throughout the industry and the region.

The need for several versatile molecular based diagnostics assays for MLN viruses demanded research and development of such assays. As such, the development of a very rapid and highly sensitive isothermal real-time RPA for the detection of MCMV and by extension MLN was vital. This assay is well adapted for both lab and field hence quite mobile and the best molecular based assay choice for MLN viruses testing. This methodology will be utilized by both regulatory agencies and seed maize production entities in the regions where MLN is endemic if adopted.

Seed transmission of MCMV is playing a role in the rapid emergence of MLN across eastern Africa. Circumstantial evidence also suggests that recent introduction of MCMV to Africa was probably through contaminated maize seed. Although MCMV was previously shown to be transmitted through seed at very low frequencies, the transmission rates are comparatively higher in this region as revealed by this study. This partly explains the reason why the disease rapidly spread in the Eastern Africa region.

8.3 Recommendations

The several outputs from these studies can be utilized appropriately in the management of this deadly disease infecting maize in Kenya and beyond. The molecular breeders can use the sequences generated for the identification of QTLs and development of molecular markers and for Gene editing to facilitate accelerated breeding for MLN resistance.

The precise mapping of the disease in the country can also inform extension officers and other collaborators to intensify awareness of the disease and the current available management practices. This information also facilitates the seed companies to grow seed maize with minimum MLN viruses' infection to avoid massive losses due to rejection by the KEPHIS through the seed certification process.

Both RT–LAMP and RT-RPA for MCMV diagnostics has been recommended to the Kenya Plant Health Inspectorate Service (KEPHIS) management and other regional Plant Protection and seed certification agencies for adoption due to the short testing time and the short sample preparation time.

Full genome sequences for the local strains of MCMV and SCMV generated from this study can be used by various groups of scienetists in further studies and applications. Molecular Breeders can use the sequences generated from this study to breed for resistance to local strains of MCMV and SCMV. Specific primers for the Kenyan and East African isolates of SCMV can be designed from the SCMV sequences from this study for accurate molecular-based diagnostics.

The fact that the transmission rates for MCMV through seed are higher than previously thought, suggests that stringent phytosanitary measures need to be put in place to limit seed contamination and eventually seed transmission of MCMV. Seed companies also need to step up internal regulation strategies to minimize their seed lots from contamination by MCMV. This will ultimately reduce loses from rejection of seedlots infected by the viruses through the seed certification process.

There is a need to implement mitigation strategies simultaneouslyto effectively combat this devastating maize disease based on proposed MLN management models (Hilker et al., 2017). There is an initiative to strengthen the National Plant Protection Organizations (NPPOs) capacity to test for MLN viruses, especially MCMV, in seed lots for seed certification and on seed and grain movement across borders. Adoption of MLN free seed production protocols developed by partners and seed companies in eastern African countries where MLN is endemic will reduce seedlots infected with MCMV (Prasanna et al., 2020). With the current extremely high levels of MCMV and SCMV infections in seed fields, this initiative is very valuable. There is also an initiative aiming at studying various factors affecting MLN epidemiology in eastern Africa (CIMMYT annual report 2018). Several studies are being pursued to understand MCMV transmission through commercial seed in countries where the virus is endemic to facilitate more effective control (Annual MLN Epidemiology project report 2019). Highly important is generating knowledge about the relationship between seed infestation and seed transmission of MCMV, agronomic mitigation practices, crop rotations (especially with legumes), and prevention measures for the spread of MCMV from endemic to non-endemic areas. More studies on MCMV pathogenicity is required especially for any synergistic role of MaYMV.

There is also a need for further studies to ascertain the sum effect of other viruses and abiotic factors that complicate the etiology of MLN in Kenya and by extension in eastern Africa.

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APPENDICES

Appendix 1. MLN surveillance protocol for farmers and seed fields

Surveyor's Name: _____

Country/Institution: _____

Date of Survey (d/m/y): _____ / _____ / _____

Location: _____

.

Latitude (decimal degrees): N S

.

Longitude (decimal degrees): E W

Elevation: _____ (meters above sea level)

Survey Site: 0 Farmer's field 0 Seed Production Field

Growth Stage: Vegetative (VE-VT) _____ Reproductive: R1. Silk R2. Blister R3. Milk R4. Dough R5. Dent R6. Maturity

Date of Planting (d/m/y): _____/_____/_____

Field area size: _____ ha Variety: _____

Disease Present (Y/N) Plot Incidence (% of plot infected) Plot Severity (Avg % severity on plants)

1. MLN (visible symptoms)
2.
3.
4.
5.

MLN Control Measures: None Insecticide Removal of Infected Plants

Insecticide used: _____ Dose (l/ha):_____ Date of Last Application:_____/_____/_____

MLN-infected Leaf samples collected: Y N Number of Leaf samples collected:

*Indicate specific source from where the leaf sample was collected (e.g., Variety X if several varieties are grown in the same field)

MLN FIELD Survey Form

Farmer's Name: _____

Is maize cultivated continuously? Y N

Previous Crop: _____

Is maize planting synchronized in the locality? Y N

Has the farmer seen MLN symptoms before? Y N

Does the farmer have access to extension agent? Y N

No. of extension visits / season? _____

Additional Comments / Observations:

Notes on filling MLN FIELD survey form:

- Disease Table: Primary focus of survey is MLN, but if other diseases are observed and can be identified record them in the disease column and score plot incidence and severity.
- If unknown viral symptoms are observed, use the following 6 symptom categories for recording – Unknown virus symptoms – Mosaic; Chlorotic stripes; Yellowing; Necrotic leaf margin; Dead heart; Dead plant

Appendix 2. MCMV Full genome characterization. The seven MCMV ORFs; P32, P50, P111, P31, P7a, P7b, and CP with their positions on the genome are shown. The percentage base in each sample sequence are also shown

ID	Length	A%	C%	G%	T%	P32	P50	P111	P31	P7a	P7b	CP
S1	4416	27.2	25.1	25.2	22.5	121-990	140-1456	140-3037	2998-3837	2998-3204	3206-3400	3387-4097
S6	4319	27.2	25.0	25.2	22.6	25-894	44-11360	44-2941	2902-3741	2902-3108	3110-3304	3291-4001
S7	4407	27.1	25.0	25.3	22.6	109-978	128-1444	128-3025	2986-3825	2986-3192	3194-3388	3375-4085
S9	4405	27.2	25.2	25.2	22.5	110-979	129-1445	129-3026	2987-3826	2987-3193	3195-3389	3376-4086
S12	4403	27.2	25.2	25.2	22.4	109-978	128-1444	128-3025	2986-3825	2986-3192	3194-3388	3375-4085
S13	4403	27.1	25.1	25.3	22.5	109-978	128-1444	128-3025	2986-3825	2986-3192	3194-3388	3375-4085
S14	4098	27.1	25.2	24.8	22.9	111-980	130-1446	130-3027	2988-3827	2988-3194	3196-3390	3377-4087
S18	4403	27.1	25.1	25.3	22.6	109-978	128-1444	128-3025	2986-3825	2986-3192	3194-3388	3375-4085
S20	4405	27.0	25.1	25.4	22.5	109-978	128-1444	128-3025	2986-3825	2986-3192	3194-3388	3375-4085
S23	4404	27.2	25.2	25.2	22.4	108-977	127-1443	127-3024	2985-3824	2985-3191	3193-3387	3374-4084
S25	4413	27.1	25.2	25.2	22.5	112-880	131-1447	131-3028	2989-3828	2989-3195	3197-3391	3378-4088
S26	4389	27.1	25.1	25.2	22.6	98-967	117-1433	117-3014	2975-3814	2979-3185	3187-3381	3364-4074
S30	4404	27.1	25.1	25.3	22.5	108-977	127-1443	127-3024	2985-3824	2985-3191	3193-3387	3374-4084
S31	4406	27.1	25.1	25.3	22.5	109-978	128-1444	128-3025	2986-3825	2986-3192	3194-3388	3375-4085
S36	4402	27.1	25.1	25.2	22.6	109-978	128-1444	128-3025	2986-3825	2986-3192	3194-3388	3375-4085

Appendix 3. SCMV Full genome characterization.

Key; P1; protein 1, HC-Pro; helper component proteinase, P3; protein 3, 6K1 (6K protein 1), CI; cylindrical inclusion protein, 6K2 (6K protein 2), VPg; viral protein genome-linked, NIa-Pro; nuclear inclusion protein, 6K2 (6K protein 2), VPg; viral protein genome-linked, NIa-Pro; nuclear inclusion protein a-proteinase, NIb; nuclear inclusion protein b, CP; coat protein,

ID	Length	A%	C%	G%	T%	P1	HC-Pro	P3	6K1	CI	6K2	NIa-VPg	NIa-Pro	NiB	CP
S1	9568	34.4	19.4	21.1	25.1	137-835	836-2215	2216-3256	3257-3457	3458-5371	5372-5530	5531-6097	6098-6823	6824-8533	8534-9370
S2	9596	34.4	19.4	21.1	25.1	133-831	832-2211	2212-3252	3253-3453	3454-5367	5368-5526	5527-6193	6192-6819	6820-8529	8530-9366
S3	9563	24.6	19.3	20.9	25.2	137-835	836-2215	2216-3256	3257-3457	3458-5371	5372-5530	5531-6097	6098-6823	6824-8533	8534-9370
S4	9540	34.7	19.3	20.9	25.2	133-831	832-2211	2212-3252	3253-3453	3454-5367	5368-5526	5527-6193	6192-6819	6820-8529	8530-9366
S5	9554	34.5	19.4	21.0	25.1	137-835	836-2215	2216-3256	3257-3457	3458-5371	5372-5530	5531-6097	6098-6823	6824-8533	8534-9370
S6	9553	34.7	19.3	20.9	25.2	148-846	847-2226	2227-3267	3268-3468	3469-5382	5383-5541	5542-6108	6109-6834	6835-8544	8545-9381
S7	9604	34.5	19.3	21.0	25.1	135-833	834-2213	2214-3254	3255-3455	3456-5369	5370-5528	5529-6095	6096-6821	6822-8531	8532-9368
S11	9440	33.6	19.8	21.8	24.8	135-833	834-2213	2214-3254	3255-3455	3456-5369	5370-5528	5529-6095	6096-6821	6822-8384	8385-9323
S14	9509	33.4	19.8	22.0	24.8	135-833	834-2213	2214-3254	3255-3455	3456-5369	5370-5528	5529-6095	6096-6821	6822-8384	8385-9323
S19	9521	33.4	19.8	22.0	24.9	135-833	834-2213	2214-3254	3255-3455	3456-5369	5370-5528	5529-6095	6096-6821	6822-8384	8385-9323
S21	9576	33.5	19.8	21.9	24.8	137-835	836-2215	2216-3256	3257-3457	3458-5371	5372-5530	5531-6097	6098-6823	6824-8386	8386-9325
S25	9561	33.4	19.7	22.0	24.9	140-838	839-2218	2219-3259	3260-3460	3461-5374	5375-5533	5534-6100	6101-6826	6827-8389	8390-9328
S27	9595	34.5	19.4	21.0	25.1	138-836	837-2216	2217-3257	3258-3458	3459-5372	5373-5531	5532-6098	6099-6824	6825-8534	8535-9371
S28	9569	34.5	19.4	21.0	25.1	138-836	837-2216	2217-3257	3258-3458	3459-5372	5373-5531	5532-6098	6099-6824	6825-8534	8535-9371

S29	9526	33.4	19.8	22.0	24.8	140-838	839-2218	2219-3259	3260-3460	3461-5374	5375-5533	5534-6100	6101-6826	6827-8389	8390-9328
S32	9565	33.3	19.8	22.0	24.9	140-838	839-2218	2219-3259	3260-3460	3461-5374	5375-5533	5534-6100	6101-6826	6827-8389	8390-9328
S34	9598	34.5	19.6	21.0	24.9	138-836	837-2216	2217-3257	3258-3458	3459-5372	5373-5531	5532-6098	6099-6824	6825-8534	8535-9371
S35	9572	34.6	19.6	21.0	24.9	143-841	842-2221	2222-3263	3263-3463	3464-5377	5378-5536	5537-6103	6104-6829	6830-8539	8540-9376

Appendix 4. Description of maize hybrids used in MCMV seed transmission evaluation.

The CIMMYT derived hybrids (CMKMLN) were under research hence their attributes were not publicly described. These are CKMLN150067, CKMLN150072, CKMLN150073, CKMLN150074, CKMLN150075, CKMLN150076 and CKMLN150077.

Hybrid	**Descriptor**
PIONEER 30G19	Average standing power. Lodging is definitely the weakness of this variety despite its yield potential being right up there with 691. 10 days earlier to maturity in most areas, and produces brilliant white grain
DK8031	DK 8031 maize is suitable for dry areas which takes three months to mature and can produce 34 bags of maize. This variety is drought tolerant and takes only 3months to mature
Duma-43	Very Early, drought resilient variety with wide adaptability scope. It is a very early white Maize Streak and Mottle Viruses tolerant hybrid, with a relatively short, flinty ear and excellent yield stability over a range of environments. The maturity period of 90 days has exhibited yields of ranging between 30-32 bags(90kg) per acre.
KH500-33A	A medium altitude variety that is flint and thus sweet to taste. It matures in 120-140 days with average yield is 35-40 bags per acre. It has a good standability and it is resistant to MSV & tolerant to smut Flinty white grains, tightly packed Large cobs & stalk Good taste when roasted
PH3253	Is a drought tolerant variety with a mean plant height of 75.2 cm. It is more adaptable and uses nutrients more efficiently and grows taller than most short season cultivars in dry areas.
WE5135	Moist transitional and moist midaltitude regions of lower and upper eastern, Central (Mukuyuni, Kathiani, Kangundo, Mwea, and Kianjai) Thika and Nyanza (Homabay) Kenya Yields 3.5–7.1
WE5136	transitional and moist mid-altitude regions of lower and upper eastern with average yields of 3.5–5.5

WE5137	transitional and moist mid-altitude regions of lower and upper eastern with average yields of 3.5–6.0
WE5138	transitional and moist mid-altitude regions of lower and upper eastern with average yields of 4.0–6.5
WE5139	transitional and moist mid-altitude regions of lower and upper eastern with average yields of 3.5–6.4