DETECTION OF *SUGARCANE MOSAIC VIRUS* AND SYMPTOM EXPRESSION OF MAIZE GENOTYPES TO INFECTION BY THE VIRUS IN KENYA

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

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DEDICATION

I dedicate this work to my wonderful parents. My academic journey began with a foundation laid by my parents, who enrolled me for my primary and high school education. My Father Festus Kiambi Mathiu, My Mother Damaris Muthoni Kiambi, whose sacrifices, inspiration and steadfastness, helped me accomplish this dream inspite of all odds.

Their Love, Support, Guidance and Prayers Made All This Possible.

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LIST OF ABBREVIATIONS AND ACRONYMS

ASARECA	Association for Strengthening Agricultural Research in Eastern and Central Africa
AUDPC	Area under Disease Progression Curve
BLAST	Basic Local Alignment Search Tool
BSA	Bovine serum albumin
CIMMYT	International Maize and Wheat Improvement Center
Cp	Coat protein
CTAB	Cetyl trimethylammonium bromide
DAC-ELISA	Direct antibody coating - Enzyme Linked Immunosorbent Assay
DAS-ELISA	Double antibody sandwich - Enzyme Linked Immunosorbent Assay
DNA	Deoxyribonucleic acid
dH_2O	Distilled water
dntp	Deoxyribonucleotide triphosphate
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme Linked Immunosorbent Assay
FAO	Food and Agriculture Organization
FAOSTAT	Food and Agriculture Organization Corporate Statistical Database
HCL	Hydrochloric acid
IITA	International Institute of Tropical Agriculture
IgG	Immunoglobulin G
JGMV	*Johnson Grass Mosaic Virus*
KALRO	Kenya Agricultural and Livestock Research Organisation
KAPAP	Kenya Agricultural Productivity and Agribusiness Project
M	Molar
MCMV	*Maize Chlorotic Mottle Virus*
MDMV	*Maize Dwarf Mosaic Virus*
Mg^{+2}	magnesium cation
mM	millimolar
M-MuLv	Moloney murine Leukemia virus
MLN	Maize lethal Necrosis
MLND	Maize Lethal Necrosis Disease
MOALF&I	Ministry of Agriculture, Livestock, Fisheries and Irrigation

MT/ha	Metric ton per hectare
NaCl	Sodium Chloride
NDM	Non-fat Dry Milk
Na_2SO_3	Sodium Sulphite
PCR	Polymerase Chain Reaction
PBST	Phosphate Buffered Saline + Tween
pH	potential hydrogen
PVP	Polyvinylpyrrolidone
RNA	Ribonucleic acid
RT-PCR	Reverse Transcriptase - Polymerase Chain Reaction
SCMV	*Sugarcane Mosaic Virus*
siRNA	Small interfering RNA
SrMV	*Sorghum mosaic virus*
TRNA	Total Ribonucleic Acid
V4	maize vegetative 4^{th} leaf stage
V8	maize vegetative 8^{th} leaf stage
V12	maize vegetative 12^{th} leaf stage
µM	microMolar

ABSTRACT

Emergence of maize lethal necrotic (MLN) disease, in eastern Africa has threatened maize production in the region. The disease is caused by mixed infection of maize by *Maize chlorotic mottle virus* (MCMV) and a potyvirus, most frequently the *Sugarcane mosaic virus* (SCMV). As a major component in the MLN disease complex, the importance of SCMV in maize production in Kenya has been increasing. Since it was first reported in Kenya in 2012, MLN disease has been persistent due to a favorable environment for survival and spread by insect vectors. Similarly, continuous maize cropping in certain regions and widespread cultivation of susceptible maize germplasm that has not been screened for reaction to SCMV has maintained its persistence. The purpose of this study was to contribute to the management of MLN disease in Kenya through better understanding of SCMV diagnostics and response of two farmer-preferred maize varieties (H614D and DUMA 43) to infection by the viruses causing the disease. Identification of SCMV has been limited due to the apparent vast dissimilarity among the virus as well as its synergy with MCMV. Detection studies of *Sugarcane mosaic virus* in synergism with *Maize chlorotic mottle virus* were carried out by developing and validating PCR assays in MLN disease complex. A field survey was carried out in major maize growing regions of Kenya and leaf samples showing MLN disease symptoms collected. The virus was detected from the leaf samples using enzyme-linked immunosorbent assay (ELISA) and seropositive samples selected to ascertain the identity of SCMV. Polymerase Chain Reaction (PCR) was carried out to further detect and affirm the presence of SCMV in the collected leaf samples. Primers from literature were tested for their reliability in the detection of SCMV. New assays were developed to broaden the scope of SCMV detection. Primer assays from literature were relatively reliable while the developed assay produced the expected band sizes but had different sensitivity. This study also reports that there are two groups of SCMV based on ELISA and PCR detection. The outcomes of this study could be used in the enforcement of quarantine measures that could prevent further spread of the disease across the maize growing regions which do not have the disease. In order to determine the vulnerability period of maize to SCMV, two farmer-preferred maize cultivars H614D and DUMA 43 were inoculated with SCMV at different growth stages (V4, V8 and V12) to evaluate their reaction to infection by SCMV. Symptoms were observed over a period of 35 days at intervals of seven days. The maize plants had varied reactions depending on the genotype and growth stage of infection. The highest area under disease progress curve (AUDPC) was 129.5 and 128 in DUMA 43 and H614D inoculated at V4

growth stage and lowest when inoculated at V12 growth stage with AUDPC mean of 89.2 and 83.1 in the two cultivars, respectively. The V12 growth stage exhibited a rather late reaction to infection in regard to the severity of visual symptoms compared to the other growth stages of which they exhibited symptoms within the first week post inoculation, indicating resistance mechanisms through delayed symptom development. To investigate the effect of consecutive infection on symptom development, two susceptible maize hybrids were each infected with either MCMV or SCMV first and later inoculated with the second virus in the greenhouse. Maize plants pre-inoculated with SCMV followed by MCMV to achieve synergism were observed to have late MLN disease symptoms expressions which were noteworthy. The findings of this study strengthen the possibility that older plants are less vulnerable to SCMV, indicating that management practices aimed at protecting young maize plants at the susceptible growth stages can minimize infection and yield loss due to SCMV. Infection in nature occurs independently rather than concurrently. The results indicated that severity of symptoms in synergy might be as a result of the sequence of infection in maize plants by MLN disease causal agents. SCMV resistance could be incorporated in breeding programs as a necessity in the development of new hybrids. The findings of this study will add to the knowledge gaps in the advancement of an operative management approaches that are ecologically sustainable. There is need for frequent surveys to monitor movement and emergence of new viruses to tailor diagnostic tools to capture putative new viruses and synergisms that could be affecting maize production.

CHAPTER ONE: INTRODUCTION

1.1Background to the study

Maize (*Zea mays*) is a multipurpose crop whose grains are widely consumed as food while stovers are fed to livestock (FAOSTAT, 2018). Critical to smallholder farmers is maize flour and maize meal which are processed from grains and are essential for their livelihoods and the food security of both in the countryside and built-up households in Sub-Saharan Africa (SSA) (Tefera *et al*., 2011). In Africa, maize production is mainly rain fed and with inadequate rainfall it results in famines during occasional droughts. The annual national maize consumption stands at 38 million bags with the deficit being met through imports from Malawi, Zambia, Uganda and Tanzania (FAOSTAT, 2018). For years, maize production in Kenya averages at 1.8tons per hectare, equivalent to 20 bags of 90kg each (Nyoro, 2002). As Uasin Gishu County being the county's food basket with 50% total maize production. Additional significant producing regions include Western, Eastern and Nyanza parts of Kenya which produce an average of 14% each while Central Kenya produces about 6% (Muiru, 2008).

Maize is crucial in food security as it meets most nutritional preferences and thus widely cultivated by various communities in Kenya (FAOSTAT, 2018). In 2011 cropping season, area under maize was 2.13 million Ha; yields realized were 3,375,000 metric tons of dry maize and 414,000 metric tons of green maize (FAOSTAT, 2018). Maize crop total value is worth Kshs.87.8 billion. Hence any threats to maize production from any factors impacts negatively on national economy and food security. In September 2011, Maize Lethal Necrosis (MLN) first cases were reported in Bomet County infecting maize field crops (Wangai *et al.,* 2012). The disease was detected, reported and confirmed in Eastern, Central, Western, Rift Valley, Nyanza and Nairobi provinces within a span of one year. The MLN disease is a result of two viruses in combination; the *Maize chlorotic mottle virus* (MCMV) and *Sugarcane mosaic virus* (SCMV). Aphids are associated with the transmission of SCMV, whereas thrips including a number of other insects are associated in transmission of MCMV. Maize plants infected by the disease portray symptoms that result in the cobs having no grains or rotten grains, thus drastically affecting yields.

1.2 Statement of the problem

Maize is an important dietary crop both as a commercial enterprise and a staple food crop in Kenya. The country's average annual production between 2013 and 2017 was at 40 million tons of maize against a national demand of 52 million bags (MOALF&I, 2019). The emergence of MLN disease however has exacerbated maize yield losses to between 50% - 100% (CIMMYT, 2012). Maize lethal necrosis disease is a maize plant disease caused by a coinfection of *Maize chlorotic mottle virus* (MCMV) and a member of *Potyviridae*, for instance *Sugarcane mosaic virus* (SCMV) which is the most consistently present virus. In mixed infection, SCMV is a key cereal virus that typically produces less severe symptoms than MCMV when it infects maize individually (Mahuku *et al.,* 2015). *Sugarcane mosaic virus* in combination with MCMV in an individual maize plant, a rapid reaction develops due to the synergism which results to enormous damages that may lead to necrosis of the infected plant (Niblett and Caflin, 1978; Uyemoto *et al*., 1981; Wangai *et al*., 2012). Additionally, numerous SCMV genome variations as well as new strains have continuously been documented from various countries (Ha *et al*., 2008; Perera *et al*., 2009; Viswanathan *et al*., 2009; Gao *et al*., 2011; Padhi and Ramu, 2011). There are indications that maize production in Kenya seems to be threatened by the constant presence SCMV in the region for a long period. Therefore, there is need for the identification of the potyvirus SCMV isolate and or strain diversity within the region in interaction with MCMV. The destructive characteristics observed in MLN and the fast spread of the disease is a threat to the livelihoods of many small holder farmers' and their families in the maize farming regions and beyond if it is not curbed in good time. The causative agent SCMV has not been comprehensively studied in Kenya which further compounds the problem. Such information is critical in order to understand, assess, and predict the spatial and temporal spread of the MLN disease. It is also useful in implementing and enforcing phytosanitary measures introduced to avoid disseminating potentially dangerous strains within and between regions and continents. This will eventually provide knowledge to farmers in adopting integrated mitigation measures in controlling these diseases.

1.3 Justification of the study

The potyvirus group is the largest and possibly most economically significant group of the plant viruses (Gibbs *et al.,* 2008) and SCMV is a well-established member in the group (Yang and Mirkov, 1997). The ability of SCMV and/or its strains to combine and result in synergistic interaction with MCMV poses a big challenge to maize production in the SSA

region. Although the genetics and pathogenicity of SCMV have been well studied in crop hosts, its diversity and ecology has received little attention in Kenya. A comprehensive knowledge of the virus' genetic diversity is necessary to elucidate plant–pathogen interactions. *Sugarcane mosaic virus* has been present in Kenya since early 1980s (Louie, 1980). Little attention has been paid to its economic importance towards maize due to development of tolerant hybrids but its importance is commonly exhibited in synergism with other viruses (Paula *et al.,* 2018) This study aimed at evaluating the effectiveness of using Reverse Transcriptase - Polymerase Chain Reaction (RT-PCR) technique in diagnosis of SCMV, evaluating plant response to virus infection as well as its virulence and infectivity using symptom expression and determining infection rate on maize. The findings of the study will contribute to sustainable management of SCMV and ultimately in the management of MLN based on the virus plant interaction on host age and sequence of infection of MLN disease causal agents as well as development and validation for diagnosis. In turn, this could guide in development of specific strain tests for diagnosis. Also findings on synergistic mechanisms of MCMV and SCMV may provide leads to novel control strategies for the disease.

1.4 Objectives

1.4.1 Broad objective

To develop and determine a diagnostic model of *Sugarcane mosaic virus* (SCMV) and evaluate the reaction of maize to infection by the viruses that cause MLN disease in Kenya.

1.4.2 Specific objectives

The specific objectives of the study were:

i. To develop and optimize polymerase chain reaction assays for detection of *Sugarcane mosaic virus.*
ii. To determine the effect of time of infection of maize physiological growth stages by *Sugarcane mosaic virus* on disease development.
iii. To determine the effect of sequence of infection of the causal agents of maize lethal necrosis on disease development.

1.5 Hypothesis

H_0: It is not possible to develop a diagnostic model of *Sugarcane mosaic virus* (SCMV) and evaluate the reaction of maize to infection by the viruses that cause MLN disease in Kenya.

CHAPTER TWO: LITERATURE REVIEW

2.1 Maize production in the world

Maize (*Zea mays* L) is placed as the third most important cereal crop in the world after wheat and rice (FAO, 2015). It is grown all over the world and is a key staple food as well as a cash crop in Sub-Saharan Africa for more than 1.2 billion people (FAOSTAT, 2015) and is therefore a significant contributor to economic and social development. According to International Grains council (2020), in 2018 worldwide production estimate of maize was at 1,147 million tones, more than rice at 500 million tones or wheat at 733 million tones. About 40% of the world harvest was produced by the United States followed by China, Brazil, Argentina, Ukraine, India and Mexico with over 20 million tons (FAOSTAT, 2018). Out of the 6.5% of the maize farmed in Africa, Nigeria is ranked as the major cultivator at nearly 8 million tons ahead of South Africa (FAO, 2016). Maize importation to Africa from countries outside the continent stands at 28% which is considered to be necessary (Shiferaw *et al.*, 2011).

2.2 Economic importance of maize

Maize crop is considered to be important in both farming and in the food systems within developing countries and is the primary source of daily caloric intake for many around the world. Thus, an important staple food which is depended on directly or indirectly in Kenya by a population of approximately 90% for food, labor and income. Being the most popular staple food, maize not only forms a base for food security but also serves as a source of income in most parts of the country through commercial activities (FAOSTAT, 2010). It is projected that by 2050 developing countries will increase their maize demands by two fold, hence becoming the most produced crop globally and in developing countries by 2025 (CIMMYT and IITA, 2010).

2.3 Maize production challenges

Maize is cultivated all over the world, but has varying yield and that is also inconsistent to the different region and the season during which it is grown. Over the past 10 years its production is viewed to have increased, but various challenges have rendered this increase insufficient in comparison to the human population growth (FAOSTAT, 2018). The average maize yield per hectare is low in many African countries. Cultivation of maize is continuously curbed by biotic and environmental factors that result in partial or total crop

failure (Nyoro, 2002; Cairns *et al*., 2012). An array of environmental factors that affect maize production include; poor seed quality, little use of farm inputs especially fertilizers, abrupt changes in climate, degraded soil fertility; slow adoption rates of novel technologies and proper agronomic practices (Shiferaw *et al.,* 2011). Biotic stresses arise consequently due to damage done by other living organisms to the plants. These threatening organisms are pests, nematodes (Gressel *et al.,* 2004), bacteria, viruses (Shepherd e*t al.,* 2014., fungi and weeds while abiotic stresses are impacted by non-living factors on plants in a specific environment (Mahuku *et al*., 2015). Other crop losses are as a result of wildlife invasions on farm that border forested areas, bird's damage and post- harvest losses (Nyoro, 2002).

Climate change influences are also experienced in maize cultivation by farmers within the developing countries since it is rain dependent (Shiferaw *et al.,* 2011). Rainy seasons have become unpredictable and sporadic greatly affecting farmers' capability to plan for timely land cultivation activities. This makes the crop particularly vulnerable to intensive heat and water stressed thus drought leading to potential crop failure. Furthermore, fast exhaustion of soil nutrients and quality resulting in poor yields is attributed to the short fallow periods and nonstop cultivation. Maize production in Africa is still below 1 MT/ha due to unavailability of key inputs (especially quality improved maize seed and fertilizers), poor levels of mechanization or none, and compromised post-harvest management (Shiferaw *et al.,* 2011). Insect pests also have their share in maize production challenge with an example of the stem borer which is a major menace to maize yields with a crop loss of 20-40% (De Groote *et al*., 2011). Furthermore, fungal diseases such as downy mildew, rust, gray leaf spot and bacterial diseases such as bacterial leaf streak and bacterial whorl and stalk rot are recognized as maize yield-limiting diseases (Olga and Tibor, 2015). The major viral diseases affecting maize include: *Sugarcane mosaic virus, Maize streak virus, Maize dwarf mosaic virus* and *Maize chlorotic mottle virus;* and more recently the devastating maize lethal necrosis (MLN) disease that has resulted to the total loss of maize crops in some parts of Kenya.

2.4 Maize lethal necrosis disease

Maize lethal necrosis (MLN) disease was initially reported in the USA in 1976 (Niblett and Claflin, 1978) and 2011 in Kenya (Wangai *et al.,* 2012). In open cultivations, different maize varieties are known to be affected by the disease causing severe stunting on the plants, chlorotic mottling on the leaves and necrosis most often resulting to plant death (Mahuku *et al.,* 2015). Maize lethal necrosis disease is as a result of mixed infection between *Maize*

chlorotic mottle virus (MCMV, genus *Machlomovirus*) and *potyviruses* infecting maize. In Kenya, *Sugarcane mosaic virus* (SCMV) was found to synergize with MCMV (Adam *et al*., 2012) resulting in MLN disease. In MCMV or SCMV single infection, symptoms expressed are slight mild mosaic or mottling and a possible stunted growth and low risk crop failure.

2.5 Viruses

In plant viral infection, viruses have to survive the responses of host defense under which they must move from cell to cell within its crop host and replicate to induce a disease (Boevink and Oparka, 2005). The biological understanding of virus mechanisms in overcoming the host growth patterns has disentangled significant genetic and biochemical occurrences similar to all living organisms (Hull, 2009). Viruses show their ecological importance in environment since they are attached to all major groups of organisms (Breitbart and Rohwer, 2005). Historically, viruses effects are expressed in their cause of odd development and damaging effects by causing losses in directly or indirectly infected plants and or animals (Agrios, 1997; Hull, 2009). Different reactions of the host to virus activity may result to physiology changes, metabolic movements variations or otherwise the plants anatomy when it's infected by viruses (Hull, 2009). Each year, enormous crop losses estimated to 500 billion dollars losses are as a result of viral infection (Fermin *et al*., 2000). Majority of viruses in single infections cause mild or no symptoms to plants (Valverde *et al*., 2007), whereas in mixed infection often one of the viruses lead/or aid the second one and the dual virus infection results to synergism more or less depicted by increased titres and severe symptoms (Hull, 2009).

A virus major adaptive strategy is to efficiently use the limited genome nucleic acids it possesses, as they may survive in their host more beneficially given their host size. Viruses have adapted multifunctional roles by use of evolving protein gene products (HC-Pro and CP) especially in the genus *potyviruses* (Hull, 2009). In nature, plants harbor a wide range of viruses and due to their interaction and frequent distinctions within the infected organisms; they lead to rise of new viruses that are diverse in sequence (Domingo, 2010). The adaptability of viruses within host is immensely affected by their vast variability regardless of the environmental dynamics perhaps giving them the independence of movement from one host to a new host (Kikkert *et al*., 1999). Integrated within the viral genome is an important gene (*RNaseIII*) and is best likely involved in strengthening their resilience in the defense

system of some viruses in the *Closteroviridae* family as they could have co-evolved with its host (Kreuze *et al*., 2005).

2.5.1 Viruses affecting maize

Viruses around the world are recognised in causing plant diseases and a significant amount of financial loss (Kang *et al*., 2005; Gomez *et al.*, 2009). The occurrence of diseases caused by viruses is present all over the world particularly in maize-growing regions and can or has caused significant losses for producers. Maize as a crop has been identified to be infected by over 50 viruses (Lapierre and Signoret, 2004). Several of these viruses are of narrow importance despite being extensively spread, while some arise sporadically as natural infections or probably systemic infections. Normally, virus infection is identified by observable, mostly foliar symptoms more or less frequently as mosaics, streaks and chlorosis (Roossinck, 2013). Reddish or purple pigments may be observed on leaves from older plants while those infected early in development portray dwarfing or stunting symptoms. Symptoms on young plants are easier to distinguish than on plants after silking (Anderson *et al.,* 2004; Cleaveland *et al.,* 2007). Insect vectors are the primary mode of transmission for the maize infecting viruses, thus in open fields infected plants will either be scattered throughout or concentrated at the edges of the farms based on the vector invasion (Anderson *et al.,* 2004).

In light of symptom variability, diagnosis based on symptoms expression in maize in comparison to other crops is quite tasking due to the plant genetic range, diverse environmental conditions and likelihood of mixed infections (Kannan *et al.,* 2018). Thus, serological tests and molecular tests support and are used to verify the visual diagnosis. Some of the commonly known and tested maize viruses are *Maize streak virus* (MSV) genus G*eminivirus*, *Maize mottle/chlorotic stunt virus* (MMCSV), *Maize stripe virus* (MStpV) genus *Tenuivirus*, *Maize chlorotic mottle virus* (MCMV) genus *Machlomovirus*, *Maize dwarf mosaic virus* (MDMV) genus *Potyvirus*, *Maize eyespot virus*, and *Guinea grass mosaic virus* (GGMV) genus *Potyvirus*, *Sugarcane mosaic virus* (SCMV) genus *potyvirus* (Margaret and José, 2014). Among these, the MSV is largely studied due to its high yield loss potential. Emerging viruses continue to pose problems in the seed system, thus limiting commerce for seed and grain producers. This is likely to become worse as acreages increase with the demand for biofuels and year-round planting of maize in the tropics (Thottappilly *et al.,*

1993). As a consideration in maize resistance breeding program, extensive studies are required on viral diseases and their strains particularly in major maize growing regions.

2.5.2 Potyviruses

The family *Potyviridae* has six genera; *Macluravirus*, *Bymovirus*, *Rymovirus*, *Tritimovirus, Ipomovirus* and *Potyvirus* which constitutes as the largest genus. These viruses genomic constitution is monopartite with the exception of *Bymovirus* which is bipartite (Regenmortel *et al*., 2000). Aphids are the main mode of spread of the members in *Potyvirus* genus in a non-persistent manner. The members of genus Potyvirus are transmitted in a non-persistent manner by aphids. During feeding the virus is taken up via the aphids stylet together with the plant sap and is infectious for a very limited period of time of which it may infect numerous species in the *Gramineae* (Maia *et al*., 1996; Ng and Falk, 2006). In genus *Rymoviruses* and *Tritimoviruses*, spread is mainly done by mites of genus *Abacarus* and *Aceria*, correspondingly (Regenmortel *et al*., 2000). *Ipomoviruses* are mostly transmitted by whiteflies in a non-persistent manner. *Potyviruses* comprise of a major and significant group of plant viruses economically, as they account of possibly more than 200 members constituting nearly 30% of the identified plant viruses (Khan and Dijkstra, 2002). They have virion particles that are flexous, nonenveloped and filamentous 680-900 nm long by 11-15 nm wide (Harrison *et al*., 1971; Adams *et al*., 2005). They are distinguished by their characteristic in establishment of virus encoded cytoplasmic cylindrical inclusion (CI) bodies within the cytoplasm (Regermortel *et al*., 2000). There are four distinct potyviruses infecting maize, namely *Sugarcane mosaic virus* (SCMV), *Maize dwarf mosaic virus* (MDMV), *Johnson grass mosaic virus* (JGMV), and *Sorghum mosaic virus* (SrMV). They are serologically closely related but very distinct in relation to the host reaction, cytopathology, amino acid sequencing, peptide profiling of the coat proteins and nucleotide sequence analysis.

2.5.2.1 Sugarcane mosaic virus

Sugarcane mosaic virus (SCMV) is commonly associated to sugarcane mosaic disease which is an important disease in many maize and sugarcane growing countries in the world (Kulkarni, 1973). Its infection is reported on three major crops: sorghum, sugarcane (10–35% yield loss), and maize (20–50% yield loss), and is economically and significantly associated in damaging plant (Rybicki, 2015). The genome contains a single positive sense strand of linear RNA monopartite, of about 9.5kb (Berger *et al.,* 1988; Jilka, 1990). The polyprotein of

potyviruses, including SCMV, contains an open reading frame. The genome organization (Figure 1) which is cleaved by margin lines to indicate the putative cleavage sites of the polyprotein. The 5'- and 3'- untranslated regions are also indicated (single thick lines at both ends) and encode's a large virion that is processed into 10 mature proteins products by virus-encoded proteases including: P1, Hc-Pro and NIa-Pro (Reichmann *et al*., 1992). The functional proteins are P1 proteinase (P1-Pro), helper component proteinase (Hc-Pro), third protein (P3), 6kDa protein 1 (6K1), cylindrical inclusion protein that is an RNA helicase (CI), 6kDa protein 2 (6K2), nuclear inclusion protein a (NIa), which can be further be processed into the viral overlapping protein genome-linked (VPg) and NIa proteinase (Pro). The last two proteins are the nuclear inclusion protein b (NIb) which acts as the RNA-dependent RNA polymerase (replicase) and the (CP) (Shukla *et al*., 1988; Padhi and Ramu, 2011).

Figure 1: *Sugarcane mosaic virus* (potyvirus) genome organization.

Abbreviations: PI, first protein; HC-Pro, helper component-protease; P3, third protein; $6K_1$, putative 6k peptide; CI, protein with RNA helicase activity; $6K_2$, second putative 6k peptide; NIa, Nuclear Inclusion 'a' protein (VPg and protease); Nib, Nuclear Inclusion 'b' protein (presumed RNA polymerase); and CP, coat protein. The genome has a 5'-terminal genome linked protein (VPg) and a 3' untranslated region between the coat coding region and the poly (A) tail (Yongwang *et al.*, 2004; Viral zone, 2016).

2.5.2.2 Diversity of Sugarcane mosaic virus

One way through which diversity arises is by recombination and pseudo recombination (Padidam *et al*., 1999) resulting in the emergence of strains causing severe SCMV symptoms or synergism. *Sugarcane mosaic virus* in maize has been reported in over 70 countries (Jeffrey *et al*., 1998), with its occurrence in Kenya detected from 1977 (Louie, 1980) and in Madagascar, Réunion (Autrey, 1983) and South Africa (Handley *et al*., 1998). In maize, it was initially known as *Maize dwarf mosaic virus* strain B MDMV-B (Shukla and Ward, 1994). Based on their difference in host range, SCMV consists of at least 14 strains designated 'A' through 'N' that are worldwide (Australian strains SC, BC and Sabi, and USA *Maize dwarf mosaic* strain B; USA strains A, B, D and E). Broad successful diagnostic tests for potyviruses in general (Pappu *et al.,* 1993) have been developed based on Polymerase chain reaction (PCR) primer pairs for specific strain/isolates of SCMV (Smith and van de

Velde, 1994). Recombination is the key dynamic factor in the evolution and emergence of several new variants of SCMV with recombination break points, present mainly within 6K1-VPg-NIaPro-NIb region, thus indicating a region for recombination hotspot (Padhi and Ramu, 2011). Molecular characterization of the complete CP gene or the whole genome of SCMV strains in Kenya is necessary. It has been noted that within the host selection pressure and various environmental isolation conditions, SCMV has genetically evolved for a long period of time leading to different strains (Xu *et al*., 2008).

2.5.3 Symptom manifestation of plant viruses

Diagnosis based on a characteristic symptom expression of a specific disease is normally considered as simple of plant viruses. Presence of viruses in most cases is detected by the symptoms they exhibit on plant host given their small sizes and the inability to visualize them by naked eyes or through a light microscope. Symptoms induced also vary depending on; environmental factors, host plant nutrition, host genetic make-up, time of infection, strain and number of viruses infecting the host. Some viruses cause symptomless infections (Vicente and Juan, 2011). Although symptoms provide baseline evidence on virus diseases, vast field of knowledge is necessary when coming to a conclusion on symptomatology alone (Naidu and Hughes, 2001). Therefore, it is important to combine visual symptom observation in the field with other verification tests to ensure accurate diagnosis of viral attack (Block, 1982). Infection of maize crops with SCMV is systemic thus presence of detectable virus in the entire plant including roots. Brandes (1919) thus described the virus from sugarcane in relation to the symptoms it caused on the plant. The mosaic diseases has many variants with the most prevalent type consisting of dark-green patches through to light-green, or yellow areas on the leaves often accompanied by raised blister-like spots. However, the symptoms (mosaic and/or necrosis) are mostly noticed on the leaves and sometimes the stems. In other cases the whole plant is stunted. Sugarcane mosaic disease symptoms are characterized by a mottled pattern on the leaves produced by contrasting light green to yellow and dark green patches observed on the leaves and sometimes the stems. The patches are unbalanced in form appearances and have diffuse margins. Infected plants appear paler, more yellow and stunted than healthy plants.

2.5.4 Biological detection of potyviruses

Accurate detection and diagnosis of plant viruses is highly dependent on biological assay methods. However, in relation to time they are more demanding in comparison to other test

methods (Matthews, 1991). In order of sensitivity; biological indexing and serology are highly utilized methods in symptom diagnosis despite their limited reliability and sensitivity. To detect and verify the viral strains existent in a diseased plant, various tests will have to be carried out. Plant viruses' detection has utilized various techniques not limited to; liquid chromatography, molecular beacons, Enzyme-linked Immunosorbent Assay (ELISA), Nucleic Acid Sequence Based Amplification (NASBA), Immune-capillary Zone Electrophoresis (I-CZE), (Wong, 2002), Loop-mediated isothermal Amplification (LAMP) (Notomi *et al.,* 2000), deep-sequencing of siRNA (Kreuze *et al*., 2009) and PCR. In routine procedures of viral diagnosis, serological tests are recognized to be more convenient and ease in standardization thus incorporated as a more practical method. Despite being a rapid test, ELISA may be limited detection technique when virus titre is low thus reporting false negatives (Stynen *et al.,* 1995).

In multiple viral infections and or the presence of genotypic differences of the viral strains during synergism, specificity of antibodies could be hindered thus affecting the accuracy of serology tests (Tairo *et al.,* 2006). Thus need for result validation with successive confirmatory tests to affirm and support previous serology tests. *Potyviruses* are complex and unstable thus crucial to have confirmatory test since they may have cross reactivity due to their serological relationship (Shukla *et al*., 1994). Since the early 1990s virus diagnostics in plants through viral nucleic acid detection has heavily relied on PCR as molecular based techniques (Wong, 2002). The information derived from readily available genetic alignment sequences has enabled viruses as well as virus strains detection with gene specific primers designed with more or less accurately (Abad and Moyer, 1992; Colinet *et al*., 1998). Complementary DNA (cDNA) is used as a template for PCR which has to be reverse transcribed on the virion RNA viruses. In detection of several viruses simultaneously, multiplex PCR is used (Mumford *et al.,* 2000); while for determining viral concentration real-time PCR enables quantification as other PCR techniques.

Sugarcane mosaic virus is highly considered important and prevalent viral pathogens of *poaceae* family universally, causing the disease simply known as mosaic (Koike and Gillaspie, 1989). It is amongst the known plant viruses and accounts for almost 25% as it causes disease in almost all cultivated crops. In the SCMV genome group of strains, the most sequenced and characterized region is the 3' terminal genes for the nuclear inclusion protein encoding polymerase (NIb) and capsid coat protein (Cp) (Gough *et al.,* 1987; Frenkel *et al.,*

1991). One of the established SCMV strain dominant in Australia has been used successfully to develop PCR gene specific primer pairs for diagnostic tests based on its published homology sequences (Smith and van de Velde, 1994) widely for potyviruses (Pappu *et al.,* 1993). *Potyviruses* are considered as the major and economically most important family of plant viruses. Currently as of this study, no PCR detection techniques for SCMV strains' occurring in Kenya has been developed especially in relation to synergisms for diagnosis test.

2.5.5 Synergism of plant viruses

Viral synergism is as a result of two unrelated viruses co-infecting same host plant commonly in nature, leading occasionally to more severe symptoms or increased titres of one or both viruses (Untiveros *et al.,* 2007; Xia *et al.,* 2016). It is still not clear on the mechanisms within synergistic interactions among the single viruses in mixed infections despite them being well documented. When co-infections occur, viruses may or may not synergistically interact. Majority of the documented cases of plant virus synergism one of the viruses involved is mostly in the genus potyvirus (Mbega *et al.,* 2016). A number of devastating crop diseases are the outcomes of viral synergism especially in maize (Wangai *et al.,* 2012; Adam *et al.,* 2012). Studies have indicated that the order of infection of crop plants with the causal agents of the resultant disease likely influences the severity of symptoms and titres of either virus in synergistic infection in the host plants (Mcgregor *et al.,* 2008). Maize Lethal Necrosis disease outbreak has become a menace in maize production thus food security in Sub-Saharan Africa (Mahuku *et al*., 2015b; Kiruwa *et al*., 2016).

CHAPTER THREE: MATERIALS AND METHODS

3.1 Sampling of maize leaves

Maize leaf samples from both symptomatic and asymptomatic plants were collected during the Maize Lethal Necrosis (MLN) disease survey conducted by multi-institutional teams in two phases in the months of June and September, 2014. The field survey focused on maize producing regions of Kenya as well as on yield losses caused by maize lethal necrosis disease (30 - 100%). The field survey and sampling was conducted in: North Rift (Nakuru, Baringo, West Pokot, Elgeyo Marakwet, Laikipia, Nandi and Uasin Gishu Counties), South Rift (Narok, Kajiado and Bomet Counties), Central Kenya (Kiambu, Murang'a, Kirinyaga, Nyandarua and Nyeri Counties), Nyanza (Migori, Nyamira, Kisii, Kisumu, HomaBay and Siaya Counties), Upper Eastern (Embu, Meru and Tharaka Nithi Counties), Western Region (Kakamega, Bungoma, Trans Nzoia and Busia Counties), Coast region (Kilifi, Tana River, Lamu Taita Taveta and Kwale Counties) and Lower Eastern region (Machakos, Makueni and Kitui Counties). In each County, 10 farms were randomly surveyed and in each, five symptomatic and five asymptomatic maize leaf samples were collected. The maize leaf samples were collected from farmers' fields mostly from maize crops at the vegetative growth stage avoiding any plant cross contamination. The symptomatic maize plants varied from yellow spotting (early-stage), streaking (mid-stage) or necrosis of the leaf margin (late stage). The leaf sample were packaged in zip lock bags and put in Styrofoam foam boxes containing icepacks to maintain the integrity of the samples. They were thereafter transported to the KALRO plant molecular laboratory and then stored in -80^{o}C freezers ready for nucleic acid extraction.

3.2 Developing and optimizing PCR assays for detection of *Sugarcane mosaic virus*

3.2.1 Direct Antigen Coating Enzyme-Linked Immunosorbent Assay

Direct Antigen Coating Enzyme-Linked Immunosorbent Assay (DAC – ELISA, Agdia) was used to confirm the presence of SCMV. Sap from the leaf samples was used at 1:1 dilution. The sample, homogenized in carbonate coating buffer was loaded in duplicates in an ELISA plate and incubated at 37ºC in a Thermoshake THO500/1 (Gerhardt analytical systems, Germany) for 1 hour. The sap from the healthy and known SCMV positive sample leaves served as in house controls to validate the assay and calculate sample results. The SCMV 1mg/ml polyclonal antiserum in 5% NDM of PBST-PVP-BSA was used at 1:1000 dilutions (University of Minnesota, USA). In the assay, anti rabbit IgG conjugated with enzyme

alkaline phosphatase in 5% NDM of PBST-PVP-BSA (University of Minnesota) was used at a concentration of 1:10000. The plates were rinsed three times with PBST at 2 minutes interval between the steps. Colour development with the enzyme substrate p-nitrophenyl phosphate in diethanolamine buffer, pH 9.5 (1 mg / ml) was recorded at A405nm in the ELISA reader. A405nm values were determined after 1 hour substrate incubation as negative wells remained virtually clear. The ELISA cut off point was calculated from the mean values of the negative control. The value of infected samples was considered as positive if mean absorbance value was two times that of the cutoff point.

3.2.2 Analysis of seropositive samples for PCR assay

A total of 39 seropositive samples from the MLN disease survey were selected from an initial screening using Direct Antigen Coating- Enzyme Linked Immunoabsorbent Assay (DAC-ELISA) technique. The samples tested positive with polyclonal potyvirus antibodies carried out to detect SCMV. They were then amplified using diverse sets of primers whose sequences were obtained from literature (Table 1).

3.2.3 Total RNA extraction

Total Ribonucleic acid (T-RNA) was isolated using a modified protocol based on the Cetyl trimethylammonium bromide- Lithium Chloride (CTAB-LiCl) extraction method as described by Song *et al*. (2011) for the 39 field samples. A frozen maize leaf sample of 100-200 mg was homogenized to a uniform suspension in a sterile pestle and mortar with 800 µl of extraction buffer (2% CTAB, 100mM Tris HCL pH 8´0, 25mM EDTA, 2M NaCl with [1% Na_2SO_3 and 2% PVP-40 added fresh]). The suspension (800µl) was transferred to a 2ml centrifuge tube and 800µl of chloroform-isoamyl alcohol (24:1, v/v) was added and mixed by inverting the tube. The phases were separated by centrifuging at maximum speed for 10 minutes at room temperature (23 ± 2^{o}C). The upper aqueous phase was collected and 1/3 volume of 10M LiCl was added, mixed well and incubated at 4^{o}C overnight. Centrifugation was done at 12,000 *g* for 10 minutes at room temperature, the RNA pellet was re-suspended in 300µl of Diethyl pyrocarbonate (DEPC) water then added 30µl of 3M sodium acetate and 700µl of chilled absolute ethanol, mixed well and incubated at -20^{o}C for 10 minutes. The RNA pellet was collected by centrifuging at 14,000g, washed with 70% ethanol, air dried and re-suspended in 50µL of DEPC water and stored at -70°C. The integrity of total RNA was confirmed using a NanoDrop™ 2000c Spectrophotometer (Thermo Scientific) by making

assessment of the purity of RNA at A260/A280 wavelength (most values for RNA samples ranged from 1.8 to 2.0).

3.2.4 cDNA synthesis

Reverse transcription was performed on T-RNA catalyzed by the enzyme M-MuLV Reverse Transcriptase (New England BioLabs inc.) primed with oligo(dT_{18}). The cDNA synthesis was performed in a total volume of 20µl. The mixture contained T-RNA 1µl, Primer 1µl, 10mM dNTP mix 2µl and nuclease free water 10.5µl. The mixture was then incubated by heating at 65ºC for 5 minutes and immediately chilled on ice for at least 1 minute. This was followed with a master mix of 10X RT buffer 2µl, M-MuLV Rt enzyme 1µl and Nuclease free water 2.5µl whereby the master mix was vortexed gently to mix and 5.5µl aliquot was dispensed into each 14.5µl reaction tube containing Master Mix I making up a total volume of 20µl. Reverse transcription was performed using the following steps; An initial incubation at 42^{o}C for 60 minutes. The reaction was then terminated at 85^{o}C for 5 minutes. The resulting cDNA samples were stored at -20ºC awaiting further polymerase chain reaction (PCR) analysis.

3.2.5 Detection of SCMV using Polymerase Chain Reaction

Primer specificity was determined by establishing the detection of different gene specific primer sets obtained from literature in the identification of SCMV in Kenya (Table 1). Different parts of the virus cDNA was amplified using primer pairs spanning the most conserved regions of SCMV polyprotein (Table 1). Endpoint RT-PCR assays were carried out in a reaction volume of 20µl containing 2µl of 10X PCR Buffer +Mg (Dream taq), 0.4µl (10µM) each of primers, 0.4µl, 10µM each dNTP mix 2µl of cDNA as a template, 0.2µl 5U/µl of taq DNA polymerase and 15.6µl of nuclease-free water (Qiagen). Reactions were performed in a BioRad TM 100 thermocycler (BioRad). The cycling parameters consisted of an initial denaturation at 94°C for 2 minutes, followed by 35 cycles of denaturation at 94°C for 45 seconds, annealing at 55°C for 45 seconds, and extension at 72°C for 1 minute. A final extension was performed for 5 minute at 72°C. Positive and negative (non template control; water) controls were included in each round of PCR amplification. The PCR products were electrophoresed for analysis.

Table 1: Primer sequences obtained from literature and tested for detection of *Sugarcane mosaic virus* isolates collected from maize fields in Kenya

Lab No	Primer Name	Sequence (5'- 3')	Virus Region amplified	Fragment length	Reference
1	BHF	AGCTCCATATATTGCAGAAACAGC	Cp	884bp	Smith and Van de Velde, 1994
	Gsr	AGTCAAAGGCATACCGCGCTA			
2	Gsf	ACACAAGAGCAACCAGAGAGG	Cp	510bp	Smith and Van de Velde, 1994
	Gsr	AGTCAAAGGCATACCGCGCTA			
3	SCMV-cp-2 F	TCAACCACCAGCAAGTGGAG	Cp	735bp	Prof Ben Lockhart (*per comm.*)
	SCMV-cp-2 R	GTGTTTGAACCACGGACT			
4	SCMV F4	GTTTYCACCAAGCTGGAACAGTC	Cp	900bp	Haider *et al*., 2009
	SCMV R3	AGCTGTGTGTCTCTCTGTATTCTCT			
5	SCMV-F1	TCT GGA CGG AAA TGT CGG C	Cp	253bp	Geering *et. al*., 2004
	SCMV-R1	CCT GTR TCC TGC AGA CTG G			
6	SCMV-2-F1	CAATCTTGAGGAATGCGGAAAAC	Cp	719bp	Guohui *et. al*. 2009
	SCMV-2-R1	ATCGATAGGCCCACAAATGAGTCT			
7	8679F	GCAATGTCGAAGAAAATGCG	Cp	950bp	Wangai *et.al.*, 2012
	9595R	GTCTCTCACCAAGAGACTCGCAGC			
8	SCMV CP-F	CCA GGC CAA CTT GTA ACA AAG C	Cp	500bp	Adams *et al*., 2012
	SCMV CP-R	CATCATGTGTGGATAAATACAGTTGAA			
9	SCMV HC-pc-F	TCGTGCGTGGAAGGATGC	Helper Component protein cistron	1582bp	Chaves-Bedoya *et al.*, 2011
	SCMV HC-pc-R	GAGATAAGCACGGTAGGG			
10	SCMV-Cp-c-F	TCCGGAACTGTTGATGCGGGTGTACAAG	Cp Cistron	950bp	Chaves-Bedoya *et al.*, 2011
	SCMV-Cp-c-R	CTAGTGGTGATGCTGCACTCCCAACAGA			

3.2.6 PCR product analysis using agarose gel electrophoresis

Agarose (1.5%) was dissolved fully in 0.5X TBE buffer (Tris-base, Boric acid, 0.5M Na_2EDTA, pH adjusted to 8.0 with NaOH or HCl) by heating and stained with ethidium bromide (5%). The matrix was allowed to cool and harden. Three microlitre of the PCR product and 5µl of 1x loading dye (Xylene Cyanol and Orange G) were mixed by pipetting up and down on a parafilm then loaded into the wells of the hardened gel in the gel tank. The gel was electrophoresed at 5V/cm for approximately one hour. At the end of the electrophoresis, the gel was carefully removed and the bands visualised under UV Labnet Enduro™ Gel Documentation system and captured. Four microliters of 1 kb plus ladders (Invitrogen) was also used parallel with the samples to confirm the product size.

3.2.7 Designing of new primers

Three Next-Generation Sequencing (NGS) generated sequences of SCMV isolate from Rwanda infected maize samples were initially compared to known viral sequences using the BLAST program available at the National Centre for Biotechnology Information (NCBI) (http:www.ncbi.nlm.nih.gov=BLAST=). The sequences were aligned using the CLC Genomics Workbench V9.5 program to obtain the consensus sequences of the capsid genes. The SCMV primer pairs were designed from different positions of the coat protein because the genomic sequences from that region were determined to be highly conserved according to the alignment of multiple strains/isolates. The primer pairs were designed to generate PCR products of different sizes for virus that would be discernible by agarose gel electrophoresis. Additionally, Primer PREMIER 5.0 and Integrated DNA technologies online software was used to ensure that these primers had relatively similar annealing melting temperatures (Tm), did not form secondary structures and did not form primer–dimers during PCR assays. Potential interactions among primers were also analyzed. The sequences were sent to Biosciences Eastern and Central Africa - International Livestock Research Institute (BecA-ILRI) Hub (Nairobi, Kenya) for primer synthesis.

3.2.8 Initial Polymerase chain reaction using designed primers

Previously synthesized cDNA products from the 39 seropositive maize leaves described in Section 3.1.0 were used to test the amplification efficiency of the newly designed primer sets presented in Chapter Four, Table 3. A modified version of the PCR protocol was used to test each primer set for detection of SCMV. Unless otherwise specified, conditions for cDNA amplification included incubating samples in a Gene Amp PCR System 9700 thermal cycler

were as described in Section 3.1.2. The PCR products were either stored at -20ºC or processed immediately by gel electrophoresis (Sambrook *et al*., 1989).

3.2.9 Validation of newly designed primers

Maize leaf samples (symptomatic and asymptomatic) were collected from four maize producing representative Counties in Kenya (Table 5). The selection criterion was based on the incidence of MLN/SCMV Kenya productivity and Agribusiness Project (KAPAP) survey conducted in 2014 and on their differences in agro-ecological zones in order to single out effects of agro-ecological zones in SCMV diversity. T-RNA was extracted from the maize leaf samples using a modified CTAB-LiCl method as described in Section 3.1.1. Reverse transcription of T-RNA was carried out as described in Section 3.1.2 and each resulting cDNA was used as template for PCR amplification with five SCMV of the designed primers.

3.3 Effect of time of infection by *Sugarcane mosaic virus* on development of maize lethal necrosis disease

The experiment was carried out in two seasons during the months of May – June and August – November 2017 and the trends observed in both experiments were similar. To test the effect of maize plant age on SCMV infection and disease development, plants were inoculated at V4, V8 and V12 growth stages and observation made during the assessment period.

3.3.1 Viral isolate

The *Sugarcane mosaic virus* used in this study was originally from infected maize collected in Bomet County, in the South Rift-Valley Region of Kenya. The isolate was maintained in maize cultivar H614D by serial transmission from characteristically symptomatic plants by mechanical inoculation. This is at a secluded greenhouse at the Kenya Agricultural and Livestock Research Organisation - National Agricultural Research Laboratory (KALRO-NARL). Strict quarantine measures are observed in the greenhouse to avoid cross contamination with any other viruses.

3.3.2 Growth of maize test plants

Maize seeds were planted in 45cm diameter pots, filled with sterilized soil to approximately ¾ full. Diammonium Phosphate (DAP 18:46:0) fertilizer was mixed with the soil before planting at a rate of approximately five grams per plant. The pots were pre-watered to moisten the soil for ease in planting. Two maize cultivars, H614D and Duma 43, which are extensively grown in most parts of Kenya, were used. Certified maize seeds were sown every

4 days over a period of 21 days to produce four different growth stages. At each planting time, five seeds were placed in each pot, spreading them across the soil and pushed for about 1 inch into the soil. The plants were later thinned to four per pot at two-leaf stage. The experimental design was a complete random design (CRD) consisting of four replications and three different ages of plant with four plants in each replication (pot). The pots were watered every other day. The plants were top dressed with calcium ammonium nitrate (CAN, 26%N) fertilizer at a rate of approximately 2.5 grams per plant at V8 stage (Neild and Newman, 1990).

3.3.3 Inoculation of maize plants with *Sugarcane mosaic virus*

The SCMV used for inoculating the plants was prepared by extracting sap from leaves of 21 days maize crop previously confirmed to be infected and symptomatic. Briefly, the inoculum was prepared by grinding the SCMV infected maize leaves with mortar and pestle in buffer in the ratio of 1:10 (w/v) of 0.1M phosphate buffer pH 7.0 (KH_2PO_4-4.8g, K_2HPO_4-10.8g, Na_2SO_3-1.26g in 1 liter of dH_2O, pH7.4) from primary inoculum of maize infected with *Sugarcane mosaic virus* obtained from the KALRO - NARL. Debris was removed by filtering the extracts through muslin cloth. A small amount (Approximately 0.1g) of a mild abrasive (carborundum) was added to the inoculum. Inoculation on the plants was done by rubbing lightly with two fingers distributing the virus inoculum over the entire surface of young fully expanded leaves. The leaves were rinsed with distilled water after 30 minutes to get rid of the excess inoculum and carborundum. Plants at different growth stages (V4, V8 and V12, corresponding to the different planting times) were inoculated at the same time. Inoculation was repeated after seven days to ensure there were no escapes. Plants were observed for virus symptom development and recorded at weekly intervals.

3.3.4 Disease assessment

The manifestation of mosaic and yellowing symptoms covering the total expanded foliage of individual plants served as the basis for determining the symptom severity. Disease incidence and severity were recorded at weekly intervals starting seven days post inoculation (DPI) for seven weeks. Severity was evaluated based on subjective visual scale of 1-5 as described by Rosenkranz and Scott (1978) where: 1 = No symptoms; 2 = <10% of plant leaf surface showing mosaic; 3 = 11-30% plant leaf surface showing mosaic, yellowing; 4 = severe in 31-50% of plant leaf showing mosaic and yellowing; and 5 = extensive leaf necrosis in >51% of plant leaf showing mosaic and yellowing. Disease incidence was determined as the ratio of

symptomatic plants/total number of plants and presented as a percentage of the number of plants showing disease symptoms. The presence of SCMV on the inoculated plants was confirmed by DAC-ELISA. The inoculated plants showing positive reactions were regarded as SCMV infected.

3.4 Effect of sequence of infection of the causal agents of Maize Lethal Necrosis on disease development in maize

The experiment was carried out in two seasons during the months of May – June and August – November 2016 and the trends observed in both experiments were similar. The maize plants in the different set of treatments were inoculated with either SCMV and or MCMV at V4 and the subsequent second virus was introduced after seven days to achieve MLN.

3.4.1 Virus isolates

The SCMV and MCMV virus isolates used in the study were previously collected from infected maize crops from farmers' fields; purified and propagated in H614D maize variety which also served as the source material for the inoculum. The isolates were maintained in separate secluded greenhouses to avoid any form of contamination with other viruses.

3.4.2 Growth of maize plants in sequence of infection

Potting and planting of maize plants was as described in Section 3.3.2 with H614D and Duma 43. Viral inoculum was prepared as described in Section 3.3.3. Each variety of maize was inoculated with either of the virus; SCMV or MCMV inoculum at V4 growth stage. Plants were allowed to grow for seven days before the second virus was introduced to have a combination sequence of SCMV followed with MCMV (SCMV + MCMV) or MCMV followed with SCMV (MCMV + SCMV) to produce MLN (Table 2). Dual infection (MLN) and the single virus inoculated plants were also established as controls in the experiment. Symptom severity was observed and recorded based on the severity scale of 1-5 adopted from Gowda *et al.* (2015), where 1 = no visible MLN symptoms, 2 = fine chlorotic streaks mostly on older leaves, 3 = chlorotic mottling throughout the plant, 4 = excessive chlorotic mottling on lower leaves and necrosis of newly emerging leaves (dead heart), and 5 = complete plant necrosis.

Table 2: Treatment sequences of infection inoculations with the either of the virus

Treatments	V4 growth stage	Seven days after 1st inoculation	Final Treatment Sequence Scenario
1	MCMV+SCMV	MCMV+SCMV	MCMV+SCMV
2	MCMV	SCMV	MCMV/SCMV
3	SCMV	MCMV	SCMV/MCMV
4	SCMV	SCMV	SCMV/SCMV
5	SCMV	none	SCMV only
6	MCMV	none	MCMV only
7	MCMV	MCMV	MCMV/MCMV

3.5 Data analysis

The PCR data results were analyzed on the basis of PCR products by validating the presence or absence of a specific segment of DNA with different primer assays. This was used to determine the sensitivity of the assays vis-a-vis variability of SCMV. Individual plant severity score data recorded was used to calculate the relationship between plant age at the time of inoculation and the symptom expression based on the severity visual score scale. Disease severity data was analyzed using Genstat statistical program, 12th Edition. Analysis of Variance (ANOVA) was used to test for significant differences and means separated using Fischer's Protected least significant difference at P = 0.05. Disease severity data was used to compute AUDPC (Area under the disease progress curve) score and was calculated for each individual plant in order to compare different growth stages of plant against time of infection through the season. The AUDPC is ----calculated using the midpoint rule method (Campbell and Madden, 1990) using the formula:

$AUDPC = \Sigma_{i=1}^{n-1} [(t_{i+1} - t_i)(y_i + y_{i+1})/2]$

Where "t" is time in days of each reading, "y" is the percentage of affected foliage at each reading and "n" is the number of readings.

CHAPTER FOUR: RESULTS

4.1 Development and optimization of PCR assays for detection of *Sugarcane mosaic virus*

4.1.1 Amplification of SCMV using different primers from literature

There was variation in primer pair (Table 1) amplifications between SCMV F/R1, SCMV cp-2F/R and BHf/GSr which amplified at approximately 235bp, 735bp and 880bp fragment, respectively from the maize leaf samples, thus indicating possible genetic variation among SCMV isolates collected from Kenya. The 39 seropositive maize leaf samples were analyzed using Polymerase Chain Reaction and their amplification products documented as shown in Figure 2. The samples had previously tested positive with the Direct Antibody Coating Enzyme Linked Immunoassay (DAC-ELISA). Out of the 10 SCMV pairs of primers, amplification was detected on only four primer pairs after optimization. This was observed in primer SCMV F/R1 (Table 4); out of the 39 samples, 26 samples tested positive for SCMV with a band size of 253bp (Figure 2A). In primer F3/R3 (Table 4) there were five positive samples out of the 39 samples tested with a band size of 735bp (Figure 2B). Amplification with primer 1 resulted in nine PCR products out of 39 samples.

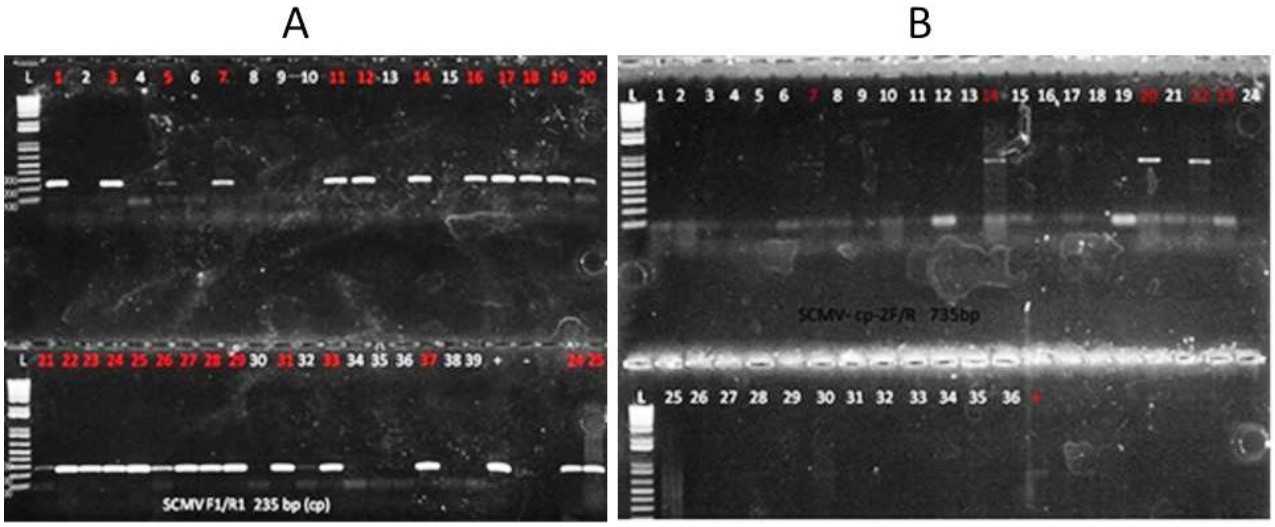


Figure 2: Polymerase Chain Reaction amplifications of *Sugarcane mosaic virus* (SCMV) using two primer sets.

A: Amplification of 235 bp using primer SCMV F1/R1, B: 735 bp bands amplification using primer SCMV – cp – F/R. All samples had tested positive to SCMV using Direct Antibody Coating-Enzyme Linked Immunoassay.

4.1.2 PCR assays based on newly designed primers

Direct software comparison between primer and target sequenced RNAs showed that three out of the 10 primer sets described in Table 1 and after electrophoresis as described in Section 3.2.6 are useful for detection of SCMV. The specificity and efficiency of the SCMV RT-PCR with designed primers were assessed in the tests that were previously described in Section 3.2.3 for cDNA synthesis and Section 3.2.4.

Table 3: List of primers designed using sequences of *Sugarcane mosaic virus* (SCMV) isolates from Rwanda and used to detect SCMV isolates collected in Kenya using polymerase chain reaction

Primer No.	Primer Name	Sequence (5' – 3')	Region of Amplification	Expected band size
1	F1(19 mer)	GGAACAACACCACCAGCAA	Cp	924bp
	R1(18 mer)	TCCCAACAGAGAGTGCAT		
2	F2(21 mer)	CACAGCAGCAAGACATATCAA	Cp	566bp
	R2(21 mer)	TCCCAACAGAGAGTGCATATT		
3	F3(18 mer)	CAAGAGCAACCAAGGAAG	Cp	505bp
	R3(18 mer)	GCTGTGTGTCTCTCTGTA		
4	F4(18 mer)	CGGAAACCAAGGAACAAC	Cp	933bp
	R4(18 mer)	CCCAACAGAGAGTGCATA		
5	F5(17 mer)	GATCTGGTAGCGGAACA	Cp	819bp
	R5(19 mer)	CTGTATTCTCCTGGGTCTC		

4.1.3 Specificity of designed primers on detection of SCMV

Five sets of primers were designed and evaluated for detection of SCMV. Specificity of the newly designed primers on SCMV was compared with those published previously (Table 4). The PCR protocol included primer pair SCMV F1 and SCMV R1 (Table 1) with the new primers at a relatively wide range of annealing temperature, and 55◦C as the lower limit. The usefulness of the primer designed for detection of SCMV was tested using the 39 samples described in Section 3.1.0. All five primer sets displayed their specific expected band products after RT – PCR for the maize leaf samples. The band sizes of the RT-PCR products were confirmed by gel analysis in Primer set F3/R3 (Table 3) detected the highest number of tested samples and had expected band sizes that were strong and thus enabled positive identification. Primer F3/R3 detected 14 samples while primer F1/R1 had two, F2/R2 had five, F4/R4 had one and F5/R 5 had six out of a total of 39 seropositive samples. Therefore,

the primer pair 3 seems to be the most suitable for identification of SCMV (Table 4). However, the sensitivity of the primer pairs varied based on their reaction, the positive samples were inconsistent in relation to the different regions sampled as compared to the primer designed by Geering *et al.* (2004) presented in Table 1. Further comparison of the sequences of this primer pair with SCMV sequences showed that primer 3 had more SCMV positive samples in comparison to the other designed primers (Table 4).

Table 4: Summary of RT-PCR assay for the detection of SCMV with designed primers versus literature primers

Counties	Seropositive ELISA	Primers designed in the current study					Reference primers from Literature		
		Primer 1	Primer 2	Primer 3	Primer 4	Primer 5	BHf/Gsr	SCMV cp-2F/R	SCMV F1/R1
		924 bp	566 bp	505 bp	933 bp	819 bp	884 bp	735 bp	253 bp
Baringo	7			2		1			4
Bomet	8		1	5			3	2	6
Embu	2			2					2
Kirinyaga	5	2	3	3	1	1	2		5
Meru	1								
Nakuru	9		1	2		3	2	1	4
Narok	7					2	2	1	3
Total positive samples	39	2	5	14	1	7	9	4	24

Footnote;

RT – PCR = Reverse Transcriptase – Polymerase Chain Reaction.
SCMV = *Sugarcane mosaic virus*
ELISA = Enzyme Linked Immunosorbent Assay

Maize leaf samples collected from the field in four counties afresh were used to evaluate designed primers independently for detection of SCMV. RT-PCR results with candidate newly designed primers were simultaneously carried out successfully, and in most cases their results were consistent (Primer 3 and Primer 2) with the seropositive samples tested earlier (Table 5). However, in terms of detection and comparison of the newly designed primers and the in-house reference primer (Geering *et al.*, 2004) there was some indication that there might be genetic variability in the current SCMV isolate from Kenya. The primer analysis results are shown in Table 5.

Table 5: Summary of RT-PCR assay in validation of SCMV detection with different designed primers

County	Total samples	Primer 1	Primer 2	Primer 3	Primer 4	Primer 5	Ref	ELISA
Narok	12		2	3		1	7	4
Nyeri	18		5	7		1	13	8
Bomet	18		6	5			7	10
Nakuru	22		6	10		1	14	11
Total +ve samples	70	0	19	25	0	3	41	33

Footnote;
Ref = Reference
ELISA = Enzyme Linked Immunosorbent Assay

4.2 Effect of time of infection by *Sugarcane mosaic virus* on development of Maize Lethal Necrosis Disease

4.2.1 Description of the disease symptoms

The susceptibility level in the different growth stages of the two cultivars was revealed in their disease severity and incidence indices respectively. The first symptom of the disease, by the *Sugarcane mosaic virus* appeared 7 dpi as pale patches on the leaves, not of uniform width and confined to the young leaves on plants inoculated at V4 growth stage. The patches then gradually progressed and coalesced to narrow chlorotic streaks extending parallel to the veins which eventually became more abundant even to the older leaves. Stems showed mottling and were small in width with the affected areas later becoming necrotic. This was observed 15 dpi on plants inoculated at V4 growth stage. The SCMV symptoms were confined to the young and emerging leaf at the V8 growth stage and were visible 15 dpi. However, most of the plants developed visible symptoms at 30 dpi. In maize inoculated at the V12 growth stage, symptoms were confined to the emerged leaves and particularly the leaf blade and basal central region. More so, symptom appearance was delayed and did not manifest until about 30 dpi. Infected leaves showed distinct mosaic-bright yellow islands in the distribution of normal green color on the leaf bases and chlorotic streaks extending parallel to the veins.

As the plants approached reproductive stage, for maize plants inoculated at V4 and V8 growth stages, the mosaic and streaks were more visually visible and more covering a greater area of the maize plants (Figure 3). The infected maize plants at V4 growth stage had immature male inflorescence or nubbins, with short branches and fewer spikelets at the base of the central spike (Figure 3C).

Figure 3: *Sugarcane mosiac virus* symptoms observed on the tassle and leaves of maize plants. (Photo courtesy of Roy G. Kiambi, 2016)

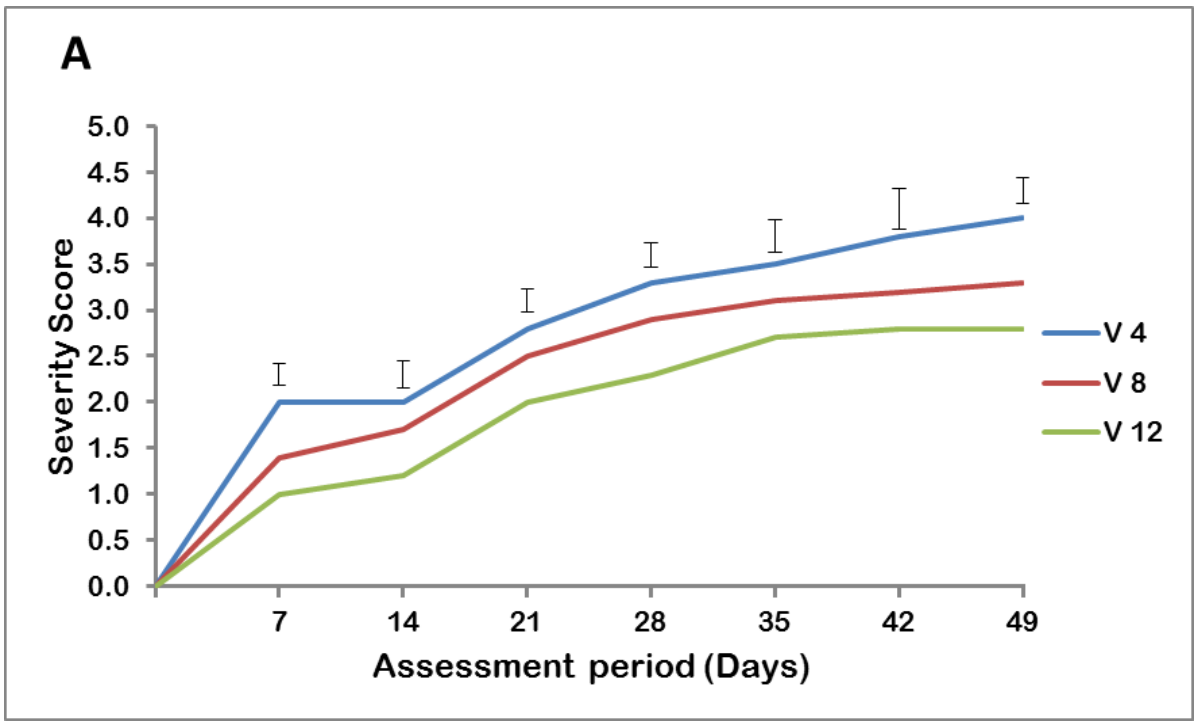
A: Immature tassel observed on SCMV infected maize plants, B: Maize plant showing green islands on emerging second and third leaves, which will develop into broken streaks along leaf veins, thus presence of SCMV virus. C: Young maize emerged leaves showing symptoms of SCMV.

4.2.2 Disease severity and infection rates

A continuum of reactions between the genotypes and the isolates was observed in the period of symptom assessment. After seven weeks, disease symptoms were observed in all plants inoculated from V4 – V12 growth stages. Symptom manifestation varied with different growth stages on the test maize plants. Maize plants in V4 growth stage had immediate reaction to the virus and had a severity score of 2 in all of the replicates within the seven days post inoculation (dpi) while plants inoculated at growth stages V8 and V12 had no visible reactions to the virus in the first seven dpi.

However, in all the growth stages the maize plants were susceptible but had varied reaction to SCMV over the time of observation. The lowest and highest severity scores of 2.8 and 4 were observed 49 dpi on DUMA maize plants variety inoculated at V12 and V4 growth stages respectively. In greenhouse trials, maize plants inoculated at V4 growth stage or maize plants inoculated while younger than V10 growth stage were highly susceptible whereas plants

inoculated after V12 growth stage developed symptoms late characterized by low disease severity compared to plants inoculated at a younger age. After 30 dpi, all the plants produced visible symptoms. There was a significant (P = 0.05) difference in the severity of SCMV infection when it was inoculated at different growth stages of maize for both cultivars (Figure 4) at 4.0_a, 3.3_b, 2.8_c and 3.9_a, 3.3_b, 2.8_c; V4, V8, V12 respectively for maize cultivar DUMA 43 and H614D respectively. Difference in disease severity was influenced by growth stage of maize plant at the time of infection and slightly by maize variety from 14 dai (Figure 4, A & B).



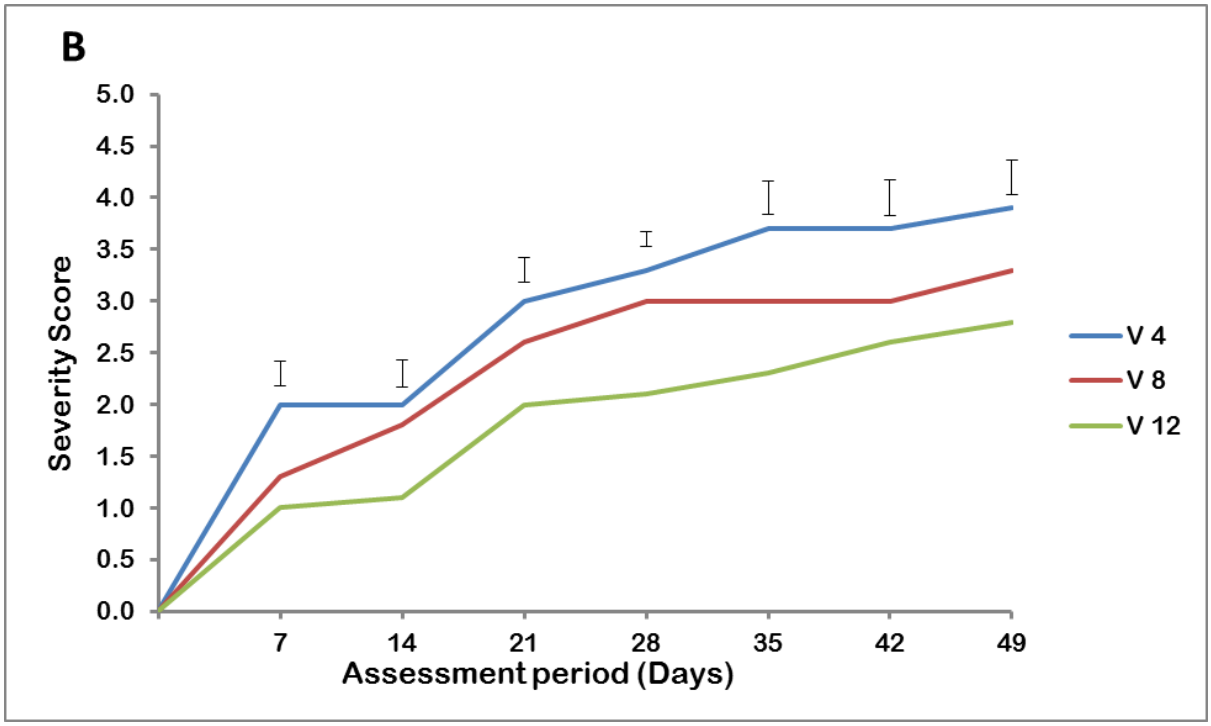


Figure 4: Severity scores of *Sugarcane mosaic virus* on Duma 43 (A) and H614D (B) varieties inoculated at three different growth stages over the weekly assessment period. Bars indicate standard error of the means for each of the assessment periods.

Key: V4, V8, and V12 = maize plant physiological growth stages

Incidence of SCMV on the maize plants at varied with plant age at the time of inoculation. The maize plants inoculated at V4 growth stage had 100% infection seven dpi for both varieties. Disease incidence of the plants inoculated at V12 growth stage had no infection seven dpi while the incidence was at 100% seven dpi on maize plants inoculated at V4 growth stage. At 21 dpi, disease incidence was 100% for inoculation at all the three growth stages on both maize varieties (Figure 5).

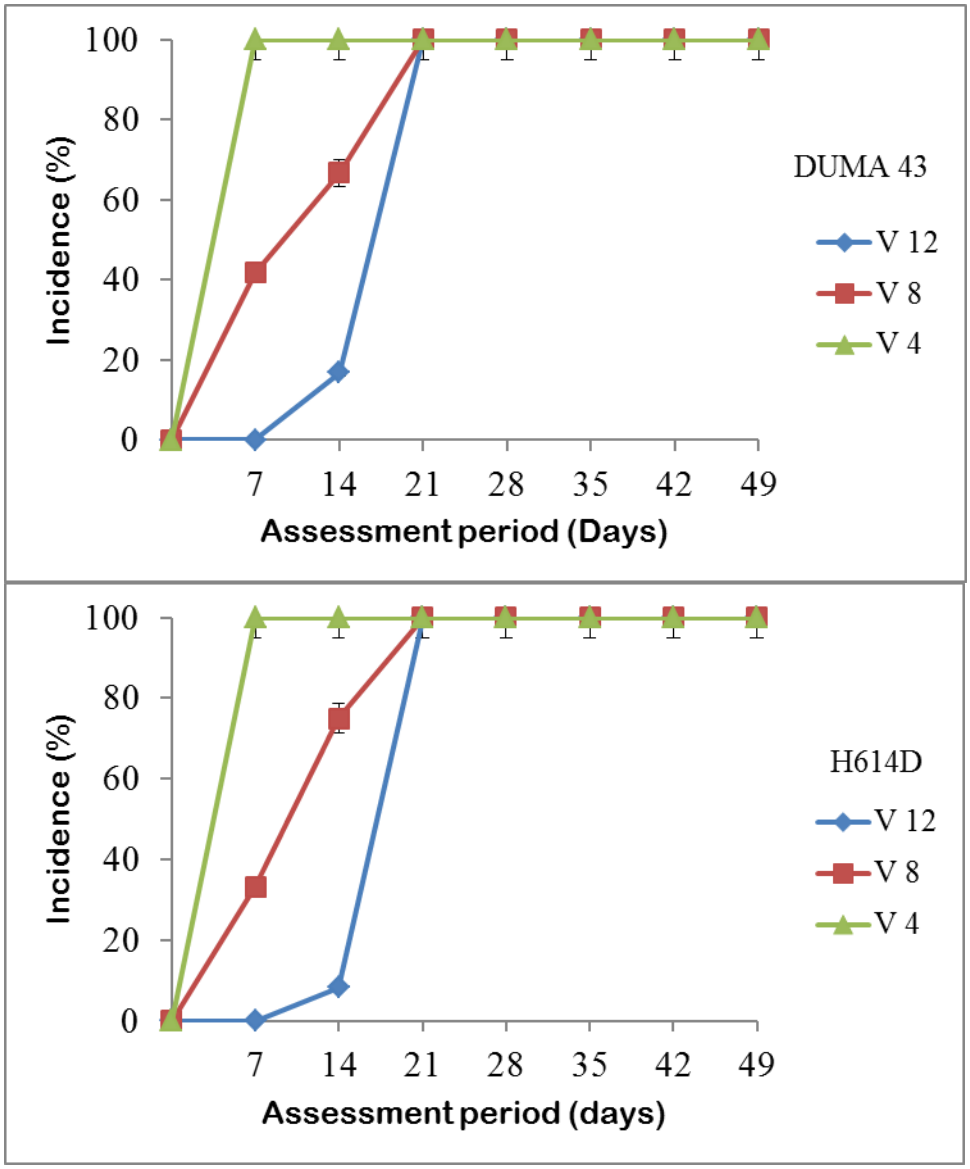


Figure 5: Incidence (%) of *Sugarcane mosaic virus* on DUMA 43 and H614D maize varieties inoculated at three differrent growth stages.

Bars indicate standard error of the mean for each of the assessment periods.

4.2.4 Disease progress on maize plants infected with SCMV at different growth stages

Maize plants inoculated with SCMV at the V4 growth stage had the highest mean AUDPC value during the entire observation period at 128_a and 129.5_a for H614D and DUMA 43, respectively (Figure 6). The lowest mean AUDPC scores recorded were 83.1_c for H614D and 89.2_c for DUMA 43 at V12 growth stage. AUDPC scores for plants inoculated at V12 growth stage were significantly different from those inoculated at V8 and V4 growth stages, indicating a delay in symptom development with inoculation at the V12 growth stage. Disease severity, as measured by the AUDPC scores, indicated that symptoms on maize

plants inoculated at the V12 growth stage were delayed and limited compared to inoculation at the other two growth stages.

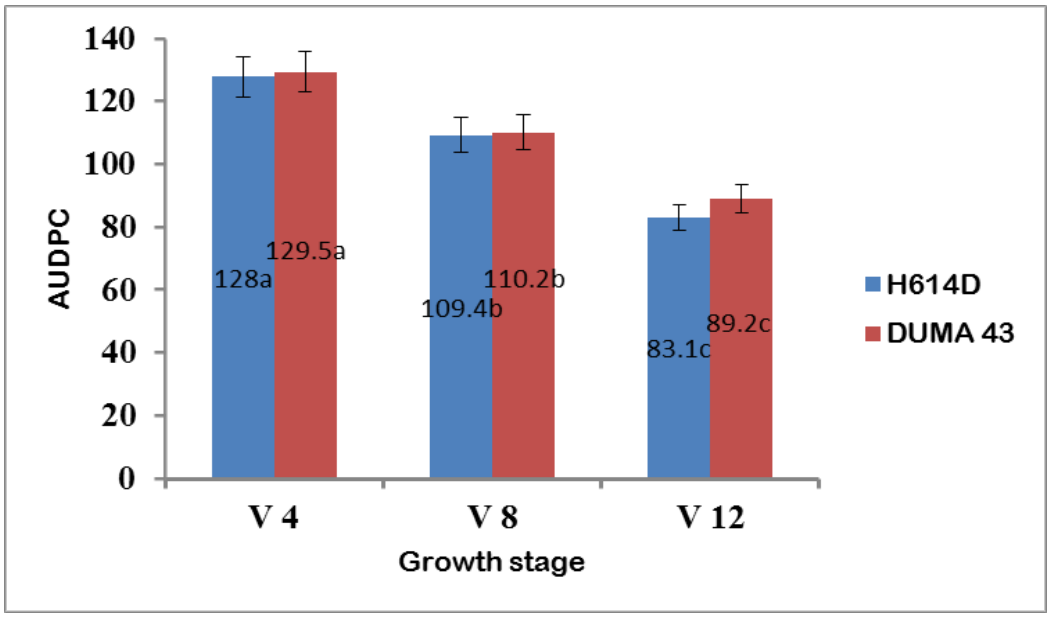


Figure 6: Mean area under disease progress curve calculated from disease severity ratings 49 days post-inoculation for two maize varieties inoculated at three growth stages.

Bars indicate standard error of the means.

Varietal differences were also observed with regard to rate of infection of SCMV. Both cultivars showed mosaic and chlorosis symptoms but DUMA 43 had relatively higher mean AUDPC scores compared to H614D variety (Figure 6). In DUMA 43 (Figure 7), visual disease severity was comparatively higher in H614D plants inoculated at V4 growth stage, with the plants producing visible symptoms 15 dpi. After 30 days, all the maize plants inoculated at the V4 growth stage (128.0_a and 129.5_a H614D and DUMA 43 respectively) exhibited severe mosaic symptoms unlike the mild symptoms observed on plants inoculated at V8 growth stage (109.4_b and 110.2_b H614D and DUMA 43 respectively) as they appeared less severely infected in their mature stages.

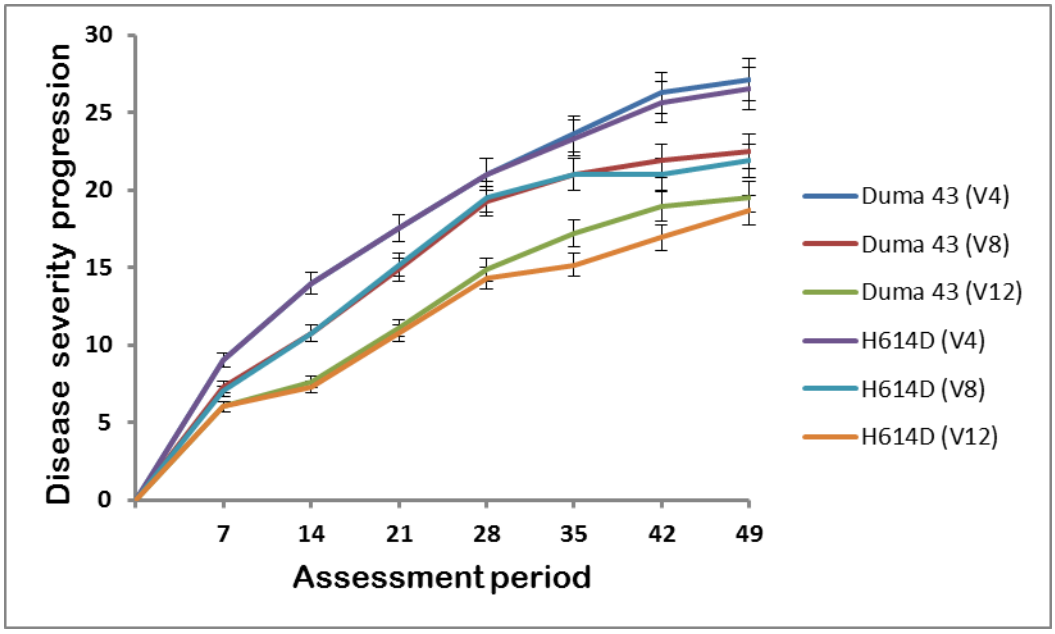


Figure 7: The disease severity progress curve on maize varieties H614D and DUMA 43 inoculated with SCMV at different growth stages.

Bars indicate standard error of the means.

4.2.5 Serological confirmatory test

Maize plants inoculated with SCMV at the V12 growth stage had delayed and/or no infection thus only 33% of the plants exhibited visible symptoms 15 days after inoculation. However, more than 90% of the plants were later infected at 30 days after inoculation and exhibited distinctive symptoms of SCMV. Within the first thirty days after inoculation, all the plants showed visible symptoms and were positive for SCMV with DAC-ELISA detection method (Section 3.2.1).

4.3 Sequence of infection of the causal agents of maize lethal necrosis on disease development in maize

4.3.1 Disease development

Reactions amongst the varieties (DUMA 43 and H614D) and the treatments were observed in the period of symptom scoring based on Section 3.4.2. Maize plants inoculated with SCMV had immediate reactions and had an average severity score of 3 in some of the replicates within the first seven days post inoculation. The reaction on the plants however, did not progress rapidly as compared with those which had been inoculated with MCMV. Maize plants inoculated with the dual viruses had the visual symptoms appear continuously and the infection progressed gradually as the severity of the symptoms increased in each successive week. The other treatments (Table 2) such as co-infection of MCMV/SCMV and MCMV independently had no symptoms manifestation in the first seven dpi. The symptoms rapidly developed from mosaic to tissue necrosis with severe plant stunting and death in some replicates 21 dpi for MCMV+SCMV and MCMV/SCMV treatments (Table 2) with a severity score of 4.5 (Figure 8) while the single infections with MCMV and SCMV showed mild symptoms.

The first systemically infected leaves of co-infection became symptomatic expressing chlorosis at 9 dpi and developed necrotic areas at 10 dpi while the leaves of SCMV or MCMV single infection showed consistent mosaic or chlorotic symptom by 10 dpi, respectively. The systemic symptoms caused by multiple viruses were initially similar to the single virus infection. At 14 dpi, a symptom rating score of 4 was recorded with MLN; characterized by mild streaking, mosaic and mottling patterns usually starting from the base of the young leaves in the whorl and extending upwards toward the leaf tips. At 18 dpi, symptoms observed in the MCMV/SCMV inoculated plants were significantly more severe than all other treatments (Table 2) except MCMV+SCMV. At 40 dpi, maize plants initially inoculated with MCMV followed by SCMV were more diseased than maize plants inoculated initially with SCMV followed by MCMV but less than MCMV+SCMV (Figure 8). As of 30 dpi, the symptoms observed on maize plants initially infected with SCMV/MCMV were more severe than those for singly infected plants (Figure 9B).This was followed by shortened internodes, dwarfing and necrosis of young leaves in the whorl before expansion leading to a ‘dead heart’ symptom.

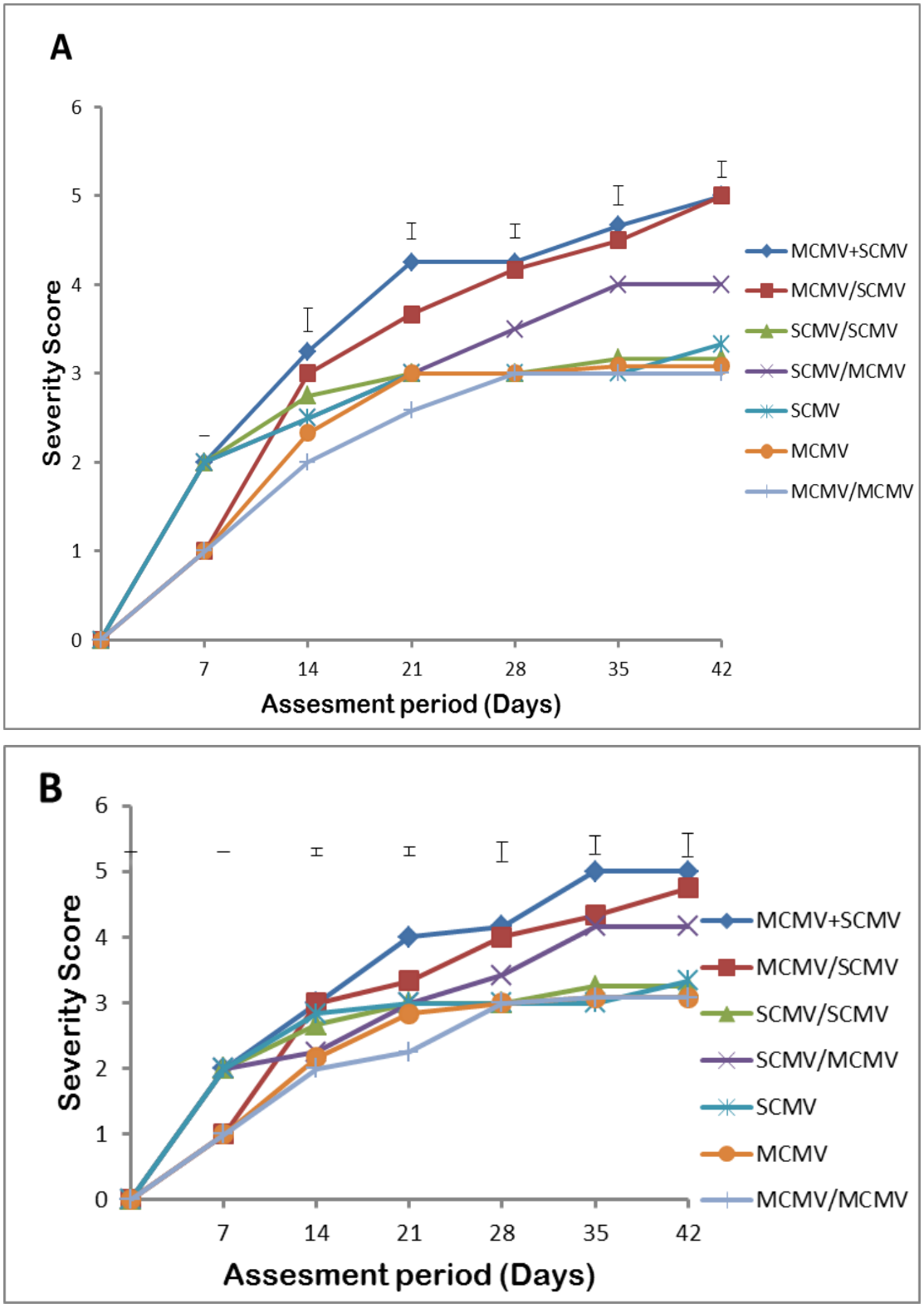


Figure 8: Severity scores of Maize Lethal Necrosis disease symptoms in plants initially infected with *Sugarcane mosaic virus* (SCMV) followed by *Maize chlorotic mottle virus* (MCMV) and plants initially infected with MCMV followed by infection with SCMV on Duma 43 (A) and H614D (B) varieties.

Bars indicate standard error of the means.

Some of the treatments such as co-infection of SCMV/MCMV, MCMV+SCMV and SCMV in single infections had an immediate symptom expression with a severity score of 2 in some of the replicates within the first seven days post inoculation (dpi). The first leaves in these treatments developed bright mosaic, which developed to tissues chlorosis (Figure 9).

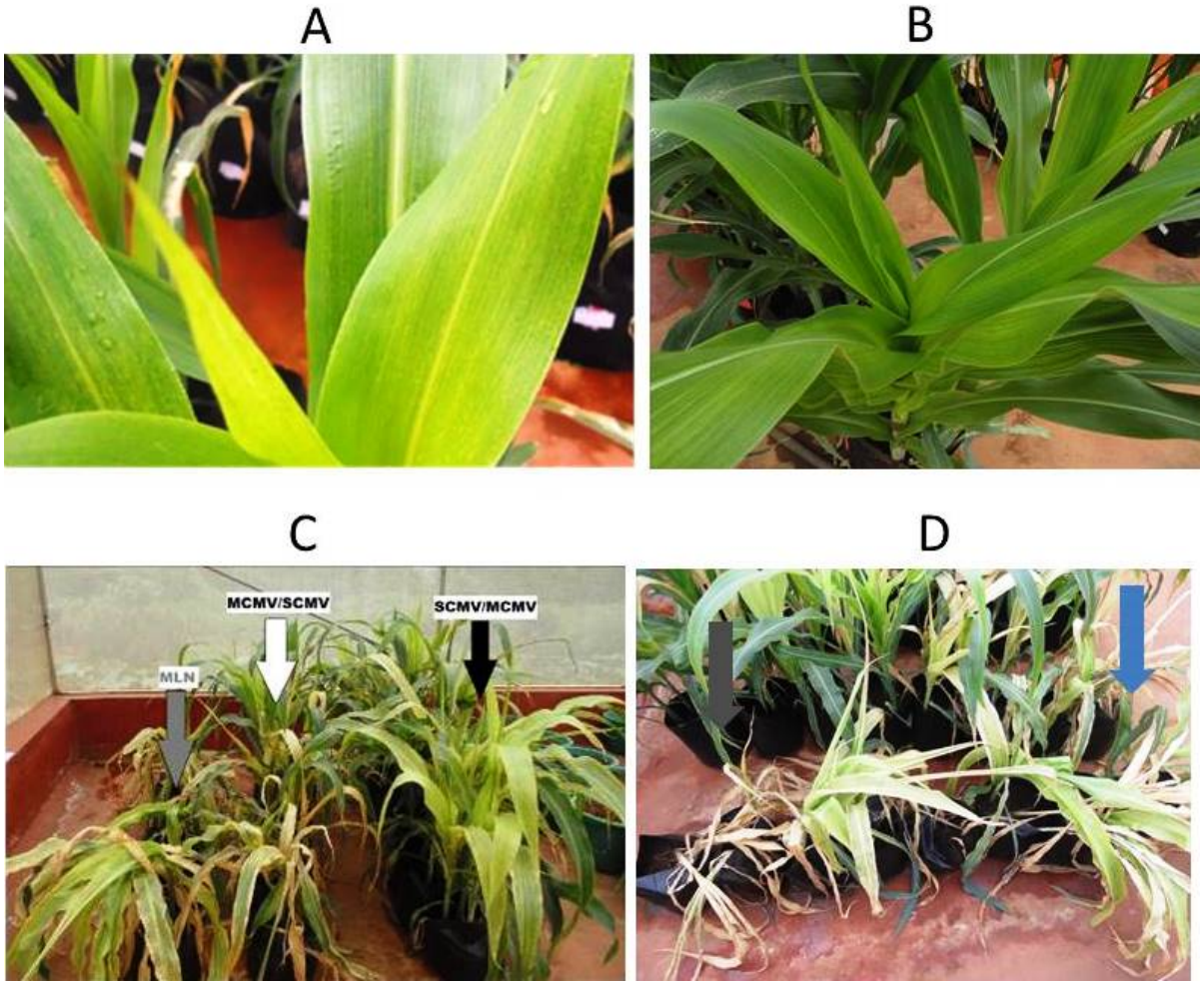


Figure 9: Expression of symptoms on maize plants inoculated with Maize Lethal Necrosis disease viruses independently and in mixture. (Photo courtesy of Roy G. Kiambi, 2016).

A: Mild mosaic and chlorotic symptoms in maize plants inoculated with SCMV alone. **B**: Mild mottling symptoms on maize plants inoculated with MCMV alone. **C**: Maize plants showing MLN symptoms based on sequence of infection with MCMV followed by SCMV. **D**: Maize Lethal Necrosis symptoms from maize plants inoculated with both viruses (MCMV+SCMV) at the same time (Blue arrow showing maize plants showing MLN symptoms with excessive chlorotic mottling with leaf necrosis, Grey arrow showing complete plant necrosis).

4.3.2 Area under disease progress curve in sequence of infection

Maize plants inoculated with both SCMV and MCMV at the same time at the V4 growth stage had the highest mean AUDPC value for the observation period at 137.7 and 139.4 for H614D and DUMA 43, respectively. The lowest mean AUDPC scores recorded were 86.61 H614D and 88.1 DUMA 43 with MCMV treatment. Area under disease progress curve scores for MCMV+SCMV and MLN were significantly higher than scores of SCMV+MCMV (Figure 10).

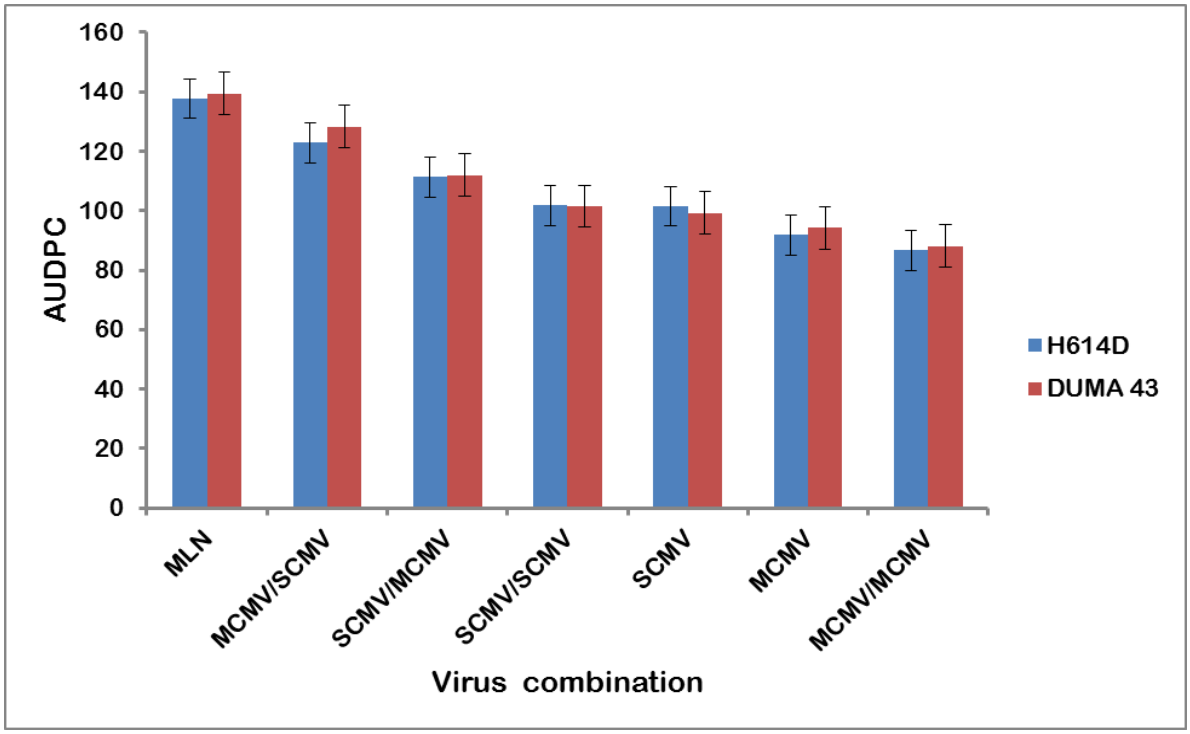


Figure 10: Mean AUDPC scores of different virus combination treatments calculated from disease severity rating on H614D and DUMA 43 maize varieties inoculated as described on Section 3.4.2 Table 2.

Bars indicate standard error of the means among each virus combination treatment.

CHAPTER FIVE: DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 Discussion

Maize is considered an important cereal crop in Eastern Africa with a 30% caloric consumption as Kenya is leading in the region at more than 50% of the caloric consumption (Derek and Carl 1997; FAOSTAT, 2015). *Sugarcane mosaic virus* in co-infection with *Maize chlorotic mottle virus* is a major threat to maize production in Kenya. Advances in research are critical to avoid significant crop yield loss. Though breeding and cultivating disease-resistant varieties remain an important control method, accurate and early disease identification in the laboratory and the field is desirable.

Diagnosis by symptom expression is important but often has imprecise results, because of the synergisms of different viruses infecting the host and high variable visual symptoms due to interactions between host and virus or by abiotic stresses (Strange, 2005). The basis of diagnosis in crop protection is to manage plant diseases and to predict the crop loss by infection of plant pathogens (van der Want and Dijkstra, 2006). Thus, for any crop management system, reliable diagnostic platforms which can be accepted and utilised universally are required (Aboul-Ata *et al*., 2011). Generally SCMV+MCMV (MLN) and MCMV infections result to visual symptoms which are relatively similar in especially older crop, thus difficult to include or rule out SCMV in the synergisms for its epidemiology and distribution studies. It is however important to note that SCMV still has the potential to affect the maize industry due to the synergism with MCMV resulting in MLN disease and measures should be put in place to mitigate its impacts.

The ELISA detection method was first used in virus disease diagnostics of the maize samples as a rapid method and due to its relative sensitivity, adaptability and economy in use of reagent. This study focused on detection of *Sugarcane mosaic virus* carried out using various diagnostic PCR assays on sampled farmers' maize crops infected with MLN disease and possibly in synergism. In East Africa, including Kenya, epidemics of the MLN disease have been observed over the past 8 years and SCMV was the most consistent potyvirus reported in the synergism with MCMV to cause MLN (Wangai *et al.*, 2012; Adam *et al.*, 2012; Mahuku *et al.*, 2015). A total of 39 samples turned out positive for SCMV by ELISA method. Results by ELISA method may be inconsistent due to false positives which are mainly as a result of non-specific reactions or cross-reactivity with certain factors in samples (Kfir and Genthe,

1993). Use of ELISA is helpful in detection of closely related viruses especially in seed certification programs. However, due to lack of specificity, antibody used in ELISA can respond to many strains leading to misdiagnosis and lack of clarity on the specific particular disease causative agent. Hence, strains of virus closely related cannot be distinguished precisely by ELISA (Boonham *et al*., 2014). In contrast to MCMV, potyviruses infecting maize are incredibly diverse within and between species which is a limitation with serology detection based technique due to them being closely related. Potyviridae evolution features extensive intra-specific recombination (Sztuba-Solińska *et al.,* 2011).

Molecular diagnostics of SCMV in maize plants is essential to abate the spread of MLN disease as well as an important link in forecast of plant virus disease, quarantine of imported and exported seeds and control of plant virus disease epidemics (Makkouk and Kumari, 2006). Polymerase chain reaction is currently considered as the foundation of all diagnostic methods and has been incorporated with other detection methods (Lopez *et al.,* 2008). In the effective diagnostics of viruses, PCR is frequently used as a classic molecular technique due to the specificity of primers. In this study, new PCR assays were developed from most conserved region of SCMV consensus alignment of homologous sequences, derived from closely-related strains. The reference assay (Geering *et al.,* 2004) had a higher number of positive samples compared to the newly developed assays. Thus, the reference primer turned out to be more suitable for a broader detection of SCMV isolates during PCR assay. The RT-PCR assay also had wide range of reaction and sensitivity with inclusion of both the reference assay and newly developed assay. In addition, there could be a possibility of genomic diversity in the SCMV considering its history in the region. One way through which diversity arises is by recombination which can promote nucleotide diversity by mixing lineages and pseudo recombination (Padidam *et al.,* 1999) resulting in the emergence of strains causing severe SCMV symptoms.

According to the phylogenetic analysis of the complete genomic sequences (Gao *et al.,* 2011), there is a large number of related SCMV isolates and currently with an expanded distribution worldwide (Grisham *et al*., 2013). This may have had an implication on the wide range of reactions among the designed and also the reference primer. Worth noting is that the reference assay had a short PCR product coverage of 253bp while the designed assays had a PCR product coverage of >500bp. This could also have been an added advantage to the reference primer to the designed primers. Probably with the high variability of SCMV (Luke

et al., 2019), with large PCR products this may have led to mismatches leading to low numbers being detected. The PCR process heavily relies on a target nucleic acid sequence in addition to species specific primer assays and any evolution radiation that creates diversity alters the results. Recombination is established to be one of the important evolutionary drivers in the evolution and divergence of several positive sense RNA viruses, including several members of Potyviridae (Chare and Holmes, 2006). In genetic variability studies, it is essential to select an appropriate target and including the required hierarchical level for species and strain-specific primer during primer design (Bromham and Penny, 2003). From this study, SCMV was grouped into two isolates (samples positive for SCMV as detected by ELISA and RT-PCR and samples positive for ELISA and negative for RT-PCR). There is need to do further tests with other primers targeting different regions, or other specific techniques.

The age at which susceptible plants are infected is often an important factor in determining loss of yield in economically important crops in relation to disease manifestation and symptom expression (Scott *et al*., 1977; Moriones *et al*., 1998). *Sugarcane mosaic virus* was reported as early as 1924 in maize and sugarcane in South Africa (Storey, 1924) and in East Africa in 1935 in sugarcane (Hansford, 1935), where later it was identified as a pathogen of maize in 1973 (Kulkarni, 1972) and few studies on it have been undertaken. However there is limited information on the response of SCMV infection in maize worldwide and very little about the disease has been documented in Kenya. This study showed that the SCMV symptom expression on maize plants was affected by plant growth stage at the time of infection. Hence, there was significantly different and high symptom expression on early infected maize plants (V4) which was evident based on the disease scores recorded compared to maize plants infected at late growth stages (V8 and V12). There was no significant difference in the disease progression despite DUMA 43 having a higher disease score compared to H614D. Thus symptom development was rapid and significantly higher on maize crops inoculated at V4 growth stage on both varieties. Data provided by this study offers valuable information to support decisions in management practices to control SCMV epidemics in maize. The knowledge of loss per plant could be linked, if available, to information on disease progress to predict loss for the whole crop (Madden and Nutter, 1995) as a tool to make strategic decisions on integrated control programs for SCMV and likewise MLN disease. Results from the trials enabled the identification of a period during which inoculation led to stunting and mosaic on the plants; this period was designated the ‘window

of vulnerability' as at V4 and V8 growth stage. Maize plants inoculated within the window of vulnerability expressed early visual symptoms and were stunted with severe mosaic symptoms to the end of the experiment. The results presented here demonstrate the importance of defining the window of vulnerability for effective disease control. By avoiding, the primary spread of MLN during this period, plant mortality and yield losses may be minimized. The best planting period could also be guided in time of minimal vector influx or before migration. Early planting as a management strategy would help the seedlings in the fields escape the main flights of aphid and will be past their vulnerable stages of infection by the time the vectors invade. It is evident that one of the greatest approaches to understanding and solving virus problems is ecological. It will be key continue developing various cultural control measures to minimize the spread of viruses by vectors.

In nature, virus co-infections in plants is common, and results into a potential devastating disease. In plants, viral synergistic interactions between members of other genus and potyviruses resulting in mixed infections have been well documented (Syller, 2012) but the mechanism underlying these interactions remains elusive. For a long time SCMV has been present in Kenya (Kulkrani, 1972) but with the emergence of MCMV, they have overlapped in distribution, and both symptom expression and disease development in both single and co-infections based on sequence infection with both viruses. Maize seedlings at the third leaf stage were inoculated with a single virus of SCMV and/or MCMV; after seven days they were later inoculated with the independent viruses (SCMV and or MCMV) interchangeably to achieve MLN. In this study, the disease expression levels were enhanced and persistent symptoms observed in MCMV + SCMV co-infected maize plants compared with that in SCMV + MCMV infected maize plants. The fact that symptom severity was low with no visual symptoms in SCMV + MCMV after seven dpi, suggests that the plant resistance mechanism was established when the plant was initially infected with only SCMV, and it is not fully overcome when MCMV infection takes place.

When MCMV is in mixed infection with SCMV, the disease progression as well as chlorosis and necrosis visual symptoms were greater in comparison to the effects of individual virus independently (Niblett and Claflin, 1978). Nevertheless, the basis for the synergism of MCMV and members of the Potyvirus genus in molecular level has not been fully elucidated. In other cases of synergistic plant virus mixed infection that include a potyvirus, the potyviral silencing suppressor enables and enhances the second virus (Pruss *et al*., 1997). In synergism,

the existence of one virus leads to the increased replication of another otherwise less economically important virus. In MLN (SCMV + MCMV) instance, concentration of the potyvirus (SCMV) in the synergism is similar to that in a single infection whereas the concentration of MCMV is increased markedly (Xie *et al.*, 2016). The virus replication in singly infected maize crop is not accelerated as there is no virus interaction or the crop is actively suppressing one virus, thus the mild expression of symptoms. In the potyvirus-MCMV synergistic interaction more factors are involved; two of the SCMV proteins [HC-Pro and Nuclear Inclusion protein A and viral genome-linked protein (NIa/VPg) (Kreuze, 2002)] are involved aggravating MCMV replication and thus suggesting the severity of symptoms. It would be of interest to study the profiles of vsiRNAs from SCMV and MCMV simultaneously infected maize plants to understand the role of RNA silencing in the synergistic interaction between SCMV and MCMV in maize plants. RNA silencing is crucial in antiviral defence mechanism in plants, which triggers the generation of vsiRNAs during viral infection. It is widely accepted that vsiRNAs play a vital role in the interactions between plants and viruses as well as symptom expression (Xia *et al.*, 2016).

Viruses are ranked as the second most significant plant pathogens after fungi (Vidaver and Lambrecht, 2004). Plant viral diseases have resulted to significant economic losses and there is no commercialized chemical to manage them (Hull, 2002). Viruses cause epidemics on all major food crops of agronomic importance, representing a serious threat to global food security. As strict intracellular pathogens, they cannot be managed chemically and prophylactic measures involved are; destruction of infected plants and excessive pesticide applications to limit the population of transmission vectors. A powerful alternative frequently employed in agriculture relies on the use of crop genetic resistance, an approach that depends on mechanisms governing plant–virus interactions. Hence, knowledge related to the molecular bases of viral infections and crop resistance is key to face viral attacks in fields. Over the past 80 years, great advances have been made on understanding of plant immunity against viruses.

5.2 Conclusion

Sugarcane mosaic virus is widely distributed in Kenya and exists as numerous strains with distinct genetic diversity. The present study using previously developed RT-PCR assays for detection of SCMV revealed the presence of genetic diversity existing within strains of *Sugarcane mosaic virus* presently found in Kenya. The detection of SCMV virus in the

varying reaction by RT-PCR assays from maize samples surveyed from both hotspots and other regions strongly suggest the presence of well-established virus strains. There are multiple potyviruses present in the region but SCMV was the most detected partner in mixture with MCMV. This has implications on movement of germplasm by stakeholders involved in promotion of maize growing in Kenya. The developed assays may therefore be useful and can be applied in detection and sequencing of SCMV samples obtained from different regions of Kenya. Variation in the detection by different PCR assays despite being designed from homologous SCMV genome suggests wide diversity within the SCMV isolates.

Based on this study, it is postulated that age-related resistance could have implications for disease management strategies. Cultural control strategies will be of practical importance in viral disease management to guard young plants from infection as long as possible especially when growing non-resistant host cultivars, in order to take advantage of improved disease tolerance conferred on plants by age. Accordingly, by the end of each growing season, farmers should invest all efforts possible to remove the infected tissues from the farms and surrounding farms to avoid early infections.

Depending on the order of virus infection, synergism was the same but evidently more expressed by dramatic increase in severity of symptoms based on the sequence of infection by the two individual viruses on the host maize plant and subsequent reduction in yield. In addition; with the synergistic interactions of MLN viruses (Adams *et al.*, 2016), there is need to focus efforts in the direction of natural or transgenic resistance of MCMV in maize cultivars it being the new virus (Wangai *et al.*, 2012).

5.3 Recommendations

The findings of this study could lead to further studies in areas that will improve virus diagnosis and understanding of the *Sugarcane mosaic virus* in maize population in Kenya. The following recommendations are drawn from this study:

i. With the ever increasing disease incidence but mild to moderate severity, there is need to study the factors contributing to synergism of MCMV and any of the potyviruses.
ii. In management practices, seed has been identified as a source and carrier of MCMV, there is need to its association with SCMV as it is widely distributed.

iii. There is also need for frequent surveys to monitor movement and emergence of new viruses to tailor diagnostic tools to capture putative new SCMV strains and synergisms that could be affecting maize production.

iv. In addition to growth stage at time of infection, it is important to evaluate the role of other factors like elevation, temperature and rainfall in development of MLN.

v. With climate changes, possible vector variations and their acquisition and transmission of the virus are greatly influenced by environmental factors. Hence detailed studies should be undertaken on the other possible vectors of SCMV on the host *gramineae* family.

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APPENDICES

Appendix A: RT-PCR assay for the detection of SCMV with different primers

S	County	Subcounty	Primer 1	Primer 2	Primer 3	Primer 4	Primer 5	BHf/Gsr	Gsf/Gsr	SCMV cp-2F/R	SCMV F4/R4	SCMV F1/R1	SCMV-2-F1/R1	8679F/9595R	SCMV cp-F/R	SCMV-HC-pc-F/R	SCMV-Cp-c-F/R
			Designed Primers					Assay from literature									
			924 bp	566 bp	505 bp	933 bp	819 bp	884 bp	360 bp	735 bp	900 bp	253 bp	720 bp	950 bp		1582 bp	950 bp
1	Kiriny aga	Mwea E		+	+	+		+				+					
2	Meru	Igembe S															
3	Baring o	Mariga t										+					
4	Baring o	Mariga t			+												
5	Bomet	Konoin			+							+					
6	Baring o	Baring o C															
7	Bomet	Sotik			+			+				+					
8	Narok	Transmara E															
9	Bomet	Chemamer															
10	Narok	Transmara E															
11	Embu	Manyatta			+							+					
12	Embu	Manyatta			+							+					
13	Narok	Transmara W															
14	Narok	Transmara W					+	+		+		+					
15	Narok	Narok S															
16	Bomet	Konoin										+					

17	Kirinyaga	Mwea W				+			+
18	Kirinyaga	Mwea	+	+	+				+
19	Baringo	Baringo C				+			+
20	Bomet	Sotik		+	+		+	+	+
21	Baringo	Marigat							+
22	Bomet	Bomet C			+		+	+	+
23	Narok	Transmara W					+		+
24	Kirinyaga	Mwea E							+
25	Kirinyaga	Mwea E	+	+	+		+		+
26	Baringo	Marigat			+				+
27	Bomet	Konoin			+				+
28	Nakuru	Rongai			+				+
29	Nakuru	Molo					+		+
30	Nakuru	Kuresoi S							
31	Nakuru	Nakuru N		+	+				+
32	Nakuru	Subukia							
33	Nakuru	Nakuru N				+			+
34	Nakuru	Nakuru W				+			
35	Bomet	Sotik							
36	Baringo	Marigat							
3	Nakuru	Subuki					+	+	+

7		a														
3	Nakuru	Gilgil														
8																
3	Narok	Narok														
9		S				+										
4	Narl	Greenh														
0		ouse	+				+	+		+						
	Total positive samples		2	5	14	1	6	9	0	4	0	24	0	0	0	0	0

Key: + = positive sample, blank = negative sample with the PCR assays

Appendix B: RT-PCR assay for validation for the detection of SCMV with different designed primers against serology

S	County	Sub County	Primer 1	Primer 2	Primer 3	Primer 4	Primer 5	Reference Primer
1	Narok	Narok North						
2	Narok	Narok North		√				√
3	Narok	Narok North						
4	Narok	Narok North			√			
5	Narok	Narok North			√			
6	Narok	Narok North						√
7	Narok	Mulot						√
8	Narok	Mulot						√
9	Narok	Mulot						√
10	Narok	Mulot						
11	Narok	Mulot						
12	Narok	Mulot						√
13	Nyeri	Kieni						√
14	Nyeri	Kieni						
15	Nyeri	Kieni						
16	Nyeri	Kieni						√
17	Nyeri	Kieni						√
18	Nyeri	Kieni						√
19	Bomet	Konoin						√
20	Bomet	Konoin						√
21	Bomet	Konoin						
22	Bomet	Konoin						
23	Bomet	Konoin						√
24	Bomet	Konoin						
25	Nyeri	Mathira West						
26	Nyeri	Mathira West			√			
27	Nyeri	Mathira West						
28	Nyeri	Mathira West						√
29	Nyeri	Mathira West						
30	Nyeri	Mathira West			√			√
31	Nakuru	Bahati						

32	Nakuru	Bahati	√				√
33	Nakuru	Bahati	√	√			√
34	Nakuru	Bahati					
35	Nakuru	Bahati	√	√			
36	Bomet	Bomet Central					
37	Bomet	Bomet Central	√	√			√
38	Bomet	Bomet Central					
39	Bomet	Bomet Central	√				
40	Bomet	Bomet Central	√	√			√
41	Bomet	Bomet Central					
42	Nakuru	Molo(Elbu)					√
43	Nakuru	Molo(Elbu)					
44	Nakuru	Molo(Elbu)					√
45	Nakuru	Molo(Elbu)		√			√
46	Nakuru	Molo(Elbu)	√	√			√
47	Nakuru	Molo(Elbu)					√
48	Nakuru	Molo(Elbu)					√
49	Bomet	Bomet Central	√	√			√
50	Bomet	Bomet Central	√	√			√
51	Bomet	Bomet Central					
52	Bomet	Bomet Central		√			
53	Bomet	Bomet Central	√				
54	Bomet	Bomet Central					
55	Nyeri	Mathira West		√			√
56	Nyeri	Mathira West	√	√			√
57	Nyeri	Mathira West	√	√			√
58	Nyeri	Mathira West	√	√		√	√
59	Nyeri	Mathira West	√	√		√	√
60	Nyeri	Mathira West	√			√	√
61	Nakuru	Njoro(F8)					
62	Nakuru	Njoro(F8)		√			√
63	Nakuru	Njoro(F8)		√			√
64	Nakuru	Njoro(F8)		√			√
65	Nakuru	Njoro (Kalro farm)		√			√

66	Nakuru	Njoro(F6)		√				
67	Nakuru	Njoro(F6)			√			√
68	Nakuru	Njoro(F6)						√
69	Nakuru	Njoro(F6)						
70	Nakuru	Njoro(F6)		√	√			
71	Nakuru	Njoro(F6)		√				
72	Nakuru	Njoro(F6)		√	√			√
	Totals positive samples		0	20	25	0	3	40

Key: √ = positive sample, blank = negative sample with the PCR assays