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Determination of Viral Titer and Symptom Severity Variations in Maize Chlortic Mottle and Sugarcane Mosaic Virus Infected Maize Genotypes in Ethiopia

By

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I, the undersigned declare that this thesis and the information presented in are my own and has been generated by me as the result of my own original experimental research.

Kalkidan Tesfu Gebremedhin

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Date ----------

The work has been done under my supervision

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This is to certify that the thesis prepared by **Kalkidan Tesfu** entitled **Determination of Viral Titer and Symptom Severity Variations in Maize Chlortic Mottle and Sugarcane Mosaic Virus Infected Maize Genotypes in Ethiopia** and submitted in partial fulfillment of the requirements for the degree of Master of science in Biotechnology complies with the regulations of the university and meets the accepted standards with respect to originality and quality.

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ABSTRACT

The co-infection of *Maize chlorotic mottle virus* (MCMV) and *Sugarcane mosaic virus* (SCMV) can cause maize lethal necrosis. Though some studies were conducted to determine the incidence of MCMV and SCMV in Ethiopia, the role of their synergistic interaction in enhancing the severity of MLND in Ethiopian maize genotypes received little attention. This study was, therefore, designed to determine the role of MCMV and SCMV interaction in enhancing the severity of MLND in eight Ethiopian maize genotypes. Symptomatic leaves suspected with MCMV and SCMV infection were collected from different parts of Ethiopia. Eight maize genotypes (Melkassa 1, Melkassa 2, Melkassa 4, MH 140, MH 130,MHQ 138, Jibat and CML 445) were also obtained from Melkassa Agricultural Research Center (MARC). The collected leaves were checked for MCMV and/or SCMV infectionby DAS ELISA method. RNA was extracted from each leaf sample using HiPurA plant and fungal RNA Miniprep purification kit. The extracted RNA was then confirmed by RT PCR. Symptom severity was also determined by standard scores. The mean MCMV titer was significantly higher in five of the maize genotypes (Melkassa4, MH140, MH130, CML445 and Jibat) infected by both MCMV and SCMV than those infected by MCMV alone ($P<0.05$ for all). In contrast, there were no significant differences in mean MCMV viral titer between MCMV and MCMV-SCMV infected genotypes in Melkassa 1 ($P=0.860$), Melkassa 2 ($P=0.572$) and MHQ138 ($P=0.734$). Although higher mean SCMV titer was found in all genotypes infected by SCMV than those co-infected by SCMV and MCMV, the difference was not statistically significant ($P>0.05$ for all).As compared to the other genotypes lower score symptom severity was observed in Melkassa 1 and Melkassa 2. This study, therefore, revealed that on the basis of viral titer and symptom severity used to determine disease response, Melkassa 1 and Melkassa 2 have high resistance to MLND. Moreover, MH 138 was also found to have higher resistance to increment in viral titer than the other genotypes. These resistant genotypes might serve as markers for further investigation of resistant maize genotypes in Ethiopia.

Key words: DAS ELISA, Maize genotypes, Maize lethal necrosis disease, RT-PCR, Symptom severity

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LIST OF ABBREVIATION

6K1	First 6K Protein
6K2	Second 6K Protein
BSC	Bundle Sheath Cell
CI	Cylindrical Inclusion Protein
CSA	Central Statistics Agency
DAS-ELISA	Double Antibody Sandwich Enzyme Linked Immunosorbent Assay
Fdv	Ferridoxine-5
FNR	Ferridoxine NADP Oxidoreductase
HC	PRO Helper Component Protein
KALRO	Kenya Agricultural and Livestock Research Organization
MARC	Melkassa Agricultural Research Center
NIa	Nuclear Inclusion Protein a
NIb	Nuclear Inclusion Protein b
OPR	Open Reading Frame
P1	First Protein
P3	Component Protein
PBS	Phosphate Buffer Saline
PTGS	Post Transcriptional Gene Silencing
RdRp	RNA Dependent RNA polymerase
RISC	RNA Inducing Silencing Complex
VPg	Viral Genome Linked Protein
Vpg	Viral Genome-Linked Protein
VSRs	Viral Suppressors of RNA Silencing
ZmEIc	Maize Elongin C Protein

LIST OF VIRUS ACRONYMS

MCDV	*Maize Chlorotic Dwarf Virus*
MCMV	*Maize Chlorotic Mottle Virus*
MDMV	*Maize Dwarf Mosaic Virus*
MMV	*Maize Mosaic Virus*
MRCV	*Maize Rough Chlorotic Virus*
MRFV	*Maize Rayado Fino Virus*
MSV	*Maize Streak Virus*
SCMV	*Sugarcane Mosaic Virus*
TEV	*Tobacco Etch Virus*
PVX	*Potato Virus X*
PVY	*Potato Virus Y*
WSMV	*Wheat Streak Mosaic Virus*

1. INTRODUCTION

Maize or corn belongs to the family *Poaceae*. It is the most important cereal crop in the world (Romay *et al.*, 2013). It grows over a range of agro climatic zones from 58^{o}N to 40 ^{o}S below sea level to an altitude higher than 3000m above sea level. The crop grows in areas that receive from 250 mm to 5000mm of rainfall per year (Piperno *et al.*, 2001; Smith *et al.*, 2001).

Maize is staple food in many parts of the world with total production surpassing that of wheat and rice (Ranum *et al.*,2014;Wallington *et al*., 2012).In 2014, more than 1,022 million tons of maize was produced in more than 170 countries on about 181 million hectares of land (Ranum *et al.*, 2014). The top producers were the United States of America, China, Brazil, Argentina and Ukraine (Ranum *et al.*, 2014; Okweche *et al.*, 2013).

In Africa, 51 countries produced approximately 75 million tons of maize (7.4% of the total world production) on 37 million hectares (20.44% of the total area planted worldwide) (ECEX, 2009). Maize occupies approximately 24% of farm land in Africa, which is more than any other staple crop, and is a food crop accounting for 73% and 64% of the total demand in Eastern and Southern Africa and Western and Central Africa, respectively. South Africa is currently the main maize producer of the African continent, and almost half of its production consists of white maize meant for human consumption (Ministry of Agriculture, 2013).

More than 9 million households, more than for any other crop, grow maize in Ethiopia (CSA, 2011–13 data). The annual rate of growth for the number of households cultivating maize grew at 3.5 % each year between 2004 and 2013, compared to 3.0 % for sorghum, 3.1 % for teff, 2.1 % for wheat, and 1.8 % for barley. At present, as a sub-Saharan country, Ethiopia has the fifth largest area devoted to maize but is second, only to South Africa, in yield and third, after South Africa and Nigeria, in production (Abate *et al*., 2015; Ranum *et al.*, 2014).

Maize currently occupies about 2 million ha with an average yield of upwards of 3 MT/ha. National maize yields have doubled from about 1.50 MT/ha during the early 1990s to 3.23 MT/ha in 2015 (Abate *et al*., 2015). Analysis of FAO data revealed that a highly significant ($p<0.0001$) annual yield gain of 68 kg/ha was recorded for maize in Ethiopia for the period 1990 to 2015. Only South Africa exceeded this figure (119 kg/ ha/yr) in SSA whereas some countries such as Tanzania and Kenya registered negative growth. Ethiopia's figure is superior to Mexico (55 kg/ha/yr), China (55 kg/ha), and India (47 kg/ha/yr). Yield gains grew even faster (120 kg/ha/yr) between 2000 and 2015 (Hake, 2015).

Despite its importance, dramatic reductions of maize yield is reported in many countries of the world including Ethiopia (Abate *et al*., 2015; Ranum *et al.,* 2014). Although several factors such as declining soil fertility, toxicities and low rainfall were stated, diseases such as maize lethal necrosis disease (MLND) and insect pests were reported (Morais and Pinheiro, 2012; Wangai *et al*., 2012).

A major maize pest in Sub-Saharan Africa is *Striga*, a parasitic flowering weed that attacks several crops, resulting in retarded plant growth, stunted and withered crops, and reduced grain yield. Stem borer damage is one of the main causes of low maize yields in Africa. These insects seriously limit the potential yield by infesting the crop during its growth, from seedling stage to maturity (Gressel *et al*., 2004)., Important diseases in maize caused by bacteria are bacterial leaf streak and bacterial whorl and stalk rot. Bacterial leaf streak is caused by *Xanthomonas campestris* pv. *zeae* and was reported for the first time in South Africa in 1949. This bacterium is mainly found in warm and dry maize production areas and is mainly spread by irrigation water, wind and rain, aphids and by plant-to-plant contact. (Kankolongo,2009).

Maize gray leaf spot is caused by the fungus *Cercospora zeae-maydis* and was first observed in the US. Nowadays, gray leaf spot is recognized as one of the most destructive and yield-limiting diseases of maize worldwide, and has also become pandemic in Africa (Wegary *et al.*,2004).

More than a dozen of viral diseases have been reported to be prevalent and cause a great economic loss in several maize producing countries including Ethiopia (Redinbaugh and Pratt, 2008; Lapierre and Signoret, 2004). Maize lethal necrosis (MLN) which is caused by co-infection of maize chlorotic mottel virus (MCMV) and sugar cane mosaic virus (SCMV) is one of the most devastating viral diseases of maize (Redinbaugh and Pratt, 2008). MCMV causes a variety of symptoms including excessive tillering, severe stunting resulting in premature plant death, shortened male inflorescences, severe leaf mottling, dry leaves and a rotting cob which develops very few grains as it matures (Nelson and Brewbaker, 2011).

MCMV is the only species in the genus *Machlomovirus*, in the family *Tombusvirideae*. It is single stranded RNA virus and first reported to infect maize in Peru (1973) and then in Argentina and Mexico (Navarro *et al.,* 2012). It can spread from plant to plant by vectors like maize thrips, rootworms, leaf beetles, seeds, maize residues (leaves), farming implements and animals (Nyvall, 1999).

SCMV is one of the major viruses in the genius *Potyvirus*, family *Potyvirideae*. It is single stranded positive sense RNA genome with single large open reading frame (ORF) which is translated into a single polyprotien and then digested in to ten functional proteins (Kreuze,2002).

The mechanism by which MCMV increases during the interaction of MCMV and SCMV as compared to SCMV is not fully understood. However, Mbega *et al*., 2016 have reported that there is a region in SCMV genome which mediates synergism. This region encodes polyprotiens such as: P1 and HC-Pro both of which have multifunctional ability .The HC-Pro component of the polyproteins is mainly involved in viral vascular movement suppression of an antiviral defense mechanism in plants (Savenkov and Valkonen, 2001; Xie *et al*., 2016).

In Ethiopia, the MLND was first reported in 2013/14 cropping seasons where it caused damage ranging from low infection rate to total crop failure (CSA, 2013). Maize producing areas like East Shewa zone of Oromia Region and Duguna Fango area of

Wolayta zone in SNNPR, the disease has been reported to cause total crop failure (CSA, 2013). It has also reported that, moderate MLND mediated crop damage in Jinka area of SNNPR,North Amhara region and Benishangul Gumuz (Mengistu *et al.*, 2017).

Despite the role of maize as a source of food in Ethiopia and the effect of MLND in damaging this valuable crop of the country, there is no study that determines the effect of MCMV and SCMV interaction in enhancing the severity of MLN disease in the available maize genotypes of the country. The aim of this study was, therefore, to determine the effect of MCMV and SCMV synergetic interaction in enhancing the severity of MLND in eight maize genotypes from Ethiopia.

2. SIGNIFICANCE OF THE STUDY

Though few studies were conducted MLND incidence is rising generally in the world and also in Ethiopia. Moreover, the hypothesis that MCMV and SCMV interaction will increase MCMV titer and enhance the severity of MLND received less consideration. Determining the effect of MCMV and SCMV synergetic interaction in enhancing the severity of MLND in this study will, therefore, have several significances. First, it helps to design appropriate MLND prevention strategies which have the potential to reduce further MLND mediated maize yield reduction and the spread of MLND in Ethiopia. Second, it increases maize productivity through identification of MLND resistant maize genotypes. Moreover, the resistant genotypes found in this study could be utilized to improve genotypes with MLND resistance and other relevant traits.

3. OBJECTIVES

General Objective

To determine the role of Maize Chlortic Mottle and Sugarcane Mosaic Virus interaction in enhancing the severity of Maize lethal necrosis disease in Ethiopia.

Specific Objectives

- To detect Maize Chlortic Mottle and Sugarcane Mosaic Virus.
- To determine variation in symptom severity between SCMV and MCMV co-infected maize genotypes.
- To determine variation in MCMV and SCMV viral titer between maize genotypes co-infected by MCMV and SCMV and those infected only by MCMV and SCMV.

4. LITERATURE REVIEW

4.1 Maize Production

Maize is cultivated throughout the year in almost every part of the world. About 875,226,630 tons of maize was produces in 2016 alone and production has increased by 600 million metric tons since 1990 (Incarbon, 2013; Sheets, 1998). World maize production has grown at roughly the same rate as consumption. One mechanism that can be used to increase maize production is increasing the amount of land dedicated to producing it and the area of harvested maize has increased at a rate of 1.32% annually since 1990. Similarly, world maize yield increased at the rate of 1.3% per year from 1990-2016 (FAO, 2017).

In addition to producing maize locally, many African countries import additional maize for food and feed consumption (Trevor *et al.,* 2015). In contrast, some African countries such as South Africa, Uganda, Tanzania, Rwanda and Namibia are important exporters of maize. In 2013, 20% of the worldwide export of maize flour came from Africa, while the USA and France accounted for 14.9% and 10.5%, respectively (Daly *et al*., 2016).

More than 75 % of maize production in Africa is done by small-scale farmers, while some large-scale farmers mainly work for global export (Nuss and Tanumihardijo, 2010).In Ethiopia, smallholder farms account for more than 95 % and use draft animals for land preparation and cultivation. Approximately 88% of maize produced in Ethiopia is consumed as food, both as green and dry grain (Demeke, 2012).

4.2 Maize Production Constraints

Socio-economic, technological, policy, abiotic and biotic factors are some of the constraints of maize production (Oscar, 2009). While factors like decline in soil fertility, low soil pH with associated nutrient deficiencies and toxicities make the abiotic factors; insect, viral and fungal infections are the biotic constraints, which affect maize production(Ali *et al*., 2011).

Viruses are among the most abundant forms of biotic factors in maize production, and exceed the number of host cells by at least one order of magnitude (Cheng *et al*., 2008; FAO, 2006). Worldwide, more than 50 viruses have been reported in maize causing an array of symptoms in single or mixed infections (Lapierre and Signoret,2004; Redinbaugh and Zambrano,2014). Of these, at least dozen viruses have been considered important in terms of prevalence and economic losses in several maize producing areas (Redinbaugh and Pratt, 2008). MLN is one of the most devastating viral diseases reported (Mahuku*et al*., 2015a).

In Ethiopia, maize production is characterized by variability of yield due to different factors including size of area cultivated, amount and quality of improved seeds, chemical fertilizers and policy environment. There are also different risk factors, which adversely affect maize yield. Weather risk and market risks are the major challenges for farmers. Various biotic, abiotic and socioeconomic constraints such as weeds, pest and diseases, erratic rainfall, erosion, low soil fertility, poor infrastructure, and post-harvest crop losses also adversely affect production (Cheng *et al*., 2008; FAO, 2006). MLN is currently considered as an emerging diseases and a top priority research question. The disease was caused by double infection of maize plants by Maize chlorotic mottle virus (MCMV) in combination with any of the cereal viruses in the family *Potyvirideae*, such as Sugarcane mosaic virus (SCMV), Maize dwarf mosaic virus (MDMV) or Wheat streak mosaic virus (WSMV) (Cabanas,2013). Among maize production constraints MLND is the main concern of this study.

4.2.1 Maize Lethal Necrosis Disease (MLND)

MLN, also termed corn lethal necrosis (CLN), was first reported in Peru in 1973 (Hebert and Castillo, 1974) with losses of 10% and 15% in floury and sweet corn varieties, respectively. MLN was then reported in Nebraska (Doupnik, 1979), Hawaii (Jensen *et al*., 1990; Jiang *et al*., 1992), China (Xie *et al*., 2011), Kenya and Tanzania (Wangai *et al*., 2012), Uganda, Rwanda and Democratic Republic of Congo (Adams *et al.,* 2014; Lukanda *et al.,* 2014) and Ethiopia (Mahuku *et al*., 2015a). Since its first record in East Africa, MLN has spread and emerged as a threat to maize based food security in Sub-Saharan Africa (Mahuku *et al*., 2015b; Kiruwa *et al*., 2016).

MLN is a serious disease of maize which is now found in many countries of East Africa where maize is grown. The disease naturally affects varieties of maize resulting in chlorotic mottling of the leaves, severe stunting and necrosis, often leading to plant death.

Most frequently it is SCMV in synergism with MCMV which cause MLND. Single infections of MCMV or SCMV cause only mild mosaic or mottling symptoms and a moderate reduction of growth. In mixed infections, early infected plants appear stunted and show a general chlorosis, leaf bleaching and necrosis (Cabanas *et al*., 2013).

4.2.2 Maize Chlorotic Mottle Virus (MCMV)

MCMV is a single-stranded RNA virus with isometric virions, single-component particles and have a smooth spherical or hexagonal shape (King *et al*., 2011). At least two genetically and geographically distinct strains of MCMV have been reported, MCMV-P (Peru) and MCMV-K (Kansas) (Nyvall 1999).

MCMV transmission occurs through insect vectors, mechanically, and by seed at very low rates (Jensen *et al*., 1991). It is also possibly transmitted through infested soil, as the virus can survive in maize residue (Nyvall, 1999). In addition, leaf beetles such as *Chaetocnema pulicaria* and *Diabrotica* can transmit this virus over a short period of time. Reports also indicated that it is transmitted at very low rates via infected seed and continuous maize production in a field greatly increases the incidence of maize chlorotic mottle virus (Makone*et al*., 2014).

MCMV infected young leaf shows fine chlotoric spots that coalesce and develop into broad chlorotic stripes along the veins. These chlorotic stripes contrast with dark green tissue when observed against the light. Leaves showing chlorosis finally die. Plants are stunted because of shortened internodes. Infected plants produce fewer and smaller ears. In most cases, the male inflorescence is malformed.

4.3 MLND Symptom and Transmission

Age at the time of infection, environment, and maize variety or genotype is some of the factors affecting MLND symptoms (Scheets, 2004). Symptoms of MLND include leaf mosaic with fine chlorotic, longitudinal yellow streaks parallel to leaf veins developing

about 10 days after inoculation. Streaks may coalesce to create chlorotic mottling. Chlorotic mottling may be followed by leaf necrosis, stunting, shortening of Ears, often with prematurely aged husks and plant death (Nelson *et al*., 2011).

Main symptoms incited by MLN-interacting viruses in a susceptible host include: yellow streaks parallel to leaf veins, chlorotic mottling, leaf necrosis which, may lead to "dead heart" symptom and plant death; premature aging of the plants (Incarbon 2013, Sheets 1998; Uyemoto1981), sterility in male plants and failure to tassel; malformed or no ears, rotting of cobs and failure of cobs to put on grains (Nelson *et al*., 2011; Makone *et al*., 2014). MLN has been identified as the most devastating foliar disease responsible for highest yield loss in maize (Ochieng *et al*., 2012). The two catch terms; ''lethal" and ''necrosis" describe two conditions. The first portrays a disease that kills infected plant and the second term means a disease which seriously kills infected cells. If the viral pathogens succeed to colonize the host, MLN disease symptoms can develop. Most of the developed symptoms listed above have direct effect on plants growth and development (Mbega *et al*., 2016).

4.4 Host Range for MCMV

Maize is the only natural host reported for MCMV. Hosts that can be infected experimentally are limited to the grasses in the family *Poaceae.*Among these grasses, 73 plant species in 35 genera have been tested for susceptibility to virus strains MCMV-Kansas and MCMV-Peru (Scheets, 2004).

4.5 Mechanism of Synergism

Viruses, vectors and susceptible maize cultivars in a suitable environment are the three main components for the MLN disease to occur (Redinbaugh and Zambrono, 2014). For the virus to invade the host it must enter a plant cell, replicate in primarily infected cell and move within cells i.e. cell to cell through plasmodesmata and long-distance (leaf to leaf) movement through the vascular system (phloem). Movement of viruses from cell to cell in plants involves one or more viral proteins with special functions.

The MLN-causing *Potyviruses* i.e. SCMV, WSMV or MDMV are single stranded, positive-sense RNA genome. They are characterized by induction of pinwheel or scroll-shaped inclusion bodies in the cytoplasm of the infected cells (Edwardson, 1974). These viruses contain a single large open reading frame (ORF) in their genome that is translated into a single polyprotein, which is then automatically digested into about 10 functional proteins: the first protein (P1), helper component proteinase (HC-pro), the third protein (P3), the first 6K protein (6K1), cylindrical inclusion protein (CI), the second 6K protein (6K2), viral genome-linked protein (VPg), nuclear inclusion protein a (NIa), nuclear inclusion protein b (NIb) and coat protein (CP) (Gough *et al*., 1987; Kreuze, 2002).

It is only known that, the region of the potyviral genome that mediates synergism encodes a polyprotein comprising of two potyviral gene products; P1 and HC-Pro, which are both multifunctional (Verchot*et al*., 1991; Verchot and Carrington, 1995; Brantley and Hunt, 1993). The HC-Pro of *Potyviruses* is involved in viral vascular movement and suppression of an antiviral defense mechanism in plants (Savenkov and Valkonen, 2001). It also has a central domain (200 aa) which affects long-distance movement and replication-maintenance functions of the virus, and a C-proximal (150 aa) domains which is a cystein-type proteinase that plays a role in virus cell-to-cell movement (Kasschau and Carrington, 2001; Syller, 2012). It doubtlessly seems that the presence of a *Potyvirus* in the synergistic interaction is very important for development of the disease that seems to be primarily resulting from MCMV.

The virus removes the protein coat and nucleic acid enters the nuclear membrane and alters the host DNA replication process by changing its RNA to complementary DNA

(cDNA) to mimic its host maize DNA so as to produce many of its copies. When more copies of viral particles have been created, they can move between cells through plasmadermata and the whole maize plant through phloem then colonize a susceptible host (Mbega*et al*, 2016). In synergism, the presence of one virus leads to the increased replication of another. In MLN, concentration of the *Potyvirus* in the synergism is similar to that in a single infection whereas the concentration of MCMV is increased clearly (Xie *et al*., 2016).

Two *Potyvirus* genes, the helper component gene and the gene for nuclear inclusion proteins are potentially avirulent in that they reduce the capacity of maize plants to inhibit the replication of MCMV (Rajamaki and Valkonen, 2009). HC-pro of *Potyviruses*is known to enhance pathogenicity and accumulation of other viruses (Prusse*t al.,* 1997). However, it is also clear that MLN induction is independent of the HC-Pro for a *Potyvirus* wheat streak mosaic virus (WSMV), suggesting that this virus utilizes a gene other than HC-Pro to suppress posttranscriptional gene silencing (PTGS) and mediate synergistic interactions with MCMV (Stenger*et al*., 2007).

In the MLN history, SCMV offers two proteins that aggravate MCMV replication and severity of symptoms: HC-Pro and nuclear inclusion protein-a and viral genome-linked protein (NIa/VPg) (Kreuze, 2002). SCMV VPg is known to interact with maize elongin C protein (ZmElc) leading to its reduced production as detected in all maize organs, but most highly in leaves and pistil extracts (Zhao*et al*., 2014). The reduction in the expression of ZmELc gene that produces ZmElc protein causes increased replication of MCMV. SCMV VPg is also believed to enhance cell to cell and long distance (systemic) movement of its own virus particles as well as those of MCMV (Barker, 1989; Cronin *et al*., 1995).

The most important role of the *Potyvirus* HC-pro though is to function in a counter defensive capacity as a suppressor of PTGS (Kasschau and Carrington, 2001). Furthermore, and in similarity with NIa/VPg, HC-Pro of the *Potyvirus* SCMV interacts with ferredoxin-5 (FdV) of maize (Cheng *et al*., 2008) resulting into disturbance in its posttranslational import into maize bundle-sheath cell (BSC) chloroplasts. Ferrodoxins play a key role in the distribution of electrons transferred from photosystem I of

photosynthesis to a range of electron acceptors. In leaves under optimal conditions the majority of electron flux through ferrodoxins is used to reduce NADP+ via a ferrodoxin NADP oxidoreductase (FNR). Of the three maize photosynthetic ferredoxinisoproteins (FdI, FdII and FdV), HC-Pro interacts specifically with FdV. The disruption of chloroplast function in maize due to concurrent infection by MCMV and SCMV leads to two things: (1) Production of less ATP required driving the Calvin Cycle through electron flow around photosystem I, which directly leads to low yield and (2) Inadequate production of chlorophyll and symptom expression (Mbega *et al*., 2016).

4.6 Infection Cycle

The classical infection cycle of plant viruses includes entry into the cell, disassembly of the virus capsids, genome replication and transcription, and the translation of the viral RNA (Kasschau and Carrington, 2001). Resistance of maize plants to virus infection primarily owes to PTGS. PTGS is a conserved sequence-specific RNA degradation mechanism in most eukaryotic organisms (Incarbone and Donoyer, 2013). It is often associated with methylation of the transcribed region of the silenced gene and with accumulation of small RNAs (21 to 25 nucleotides) homologous to the silenced gene (Molnar *et al.,* 2005).

In order for viruses to infect and cause disease in plants they have to suppress this gene silencing strategy. One strategy used by plant viruses to affect this silencing machinery is by expressing viral suppressors of RNA silencing (VSRs) at a multiple stage (Pumplin and Voinnet, 2013). Those VSRs are among a major requirement for successful colonization of the host plant by the virus. For viral infection to occur, there must be cell-to-cell movement as well as long distance transport of the virus through vascular tissues, which requires one or more viral proteins that supply the dedicated movement functions (Syller, 2012).

In the interaction between a *Potyvirus* and MCMV, the main causative components of MLN, this dedicated movement function seems to be carried out by the *Potyvirus*. In the recent study by Xia *et al.* (2016), the accumulations of both MCMV and MCMV-derived siRNAs in maize seemed to be higher during the synergistic infection (with SCMV and

MCMV) compared to single infection. This implies that the presence of *Potyvirus* was not only in favor of its own multiplication within the host but also catalyzing the multiplication of the partner co-infecting virus (Mbega*et al*., 2016).

4.7 Plant Resistance for Viral Disease

Virus infection in a host plant activates a defense mechanism related to PTGS that causes degradation of viral RNA and slows down or limits virus accumulation and systemic infection. This process is triggered by double-stranded RNA (dsRNA), produced by replicative intermediates of single- stranded RNA (ssRNA) viruses, or by genomic or defective viral ssRNAs with extensive secondary structure, which is cleaved by Dicer-like enzymes to produce 21–24 nucleotide (nt) fragments called small interfering RNAs (siRNAs) (Cheng *et al*., 2008).

The siRNAs are incorporated into a large ribonucleoprotein complex (the RNA inducing silencing complex, RISC) that guides sequence-specific cleavage of the target RNA. siRNAs may also play a role in amplification of dsRNA by a plant encoded RNA-dependent RNA polymerase (RdRp), that in turn is degraded by Dicer-like enzymes to produce secondary siRNAs (Cheng *et al*., 2008).

As a counter defence, plant viruses encode proteins that suppress RNA silencing to overcome this defence mechanism (Dıaz- Pendon and Ding, 2008). Temperature affects plant-pathogen interactions, and a higher growth temperature may either increase or decrease disease resistance, thus reflecting a differential influence of the same temperature variation on different plant-pathogen systems (Incarbon 2013, Sheet 1998; wangai *et al*., 2012). Symptoms induced by many plant viruses are attenuated when plants are grown at high temperature, a phenomenon that might be related to different efficiency of the host defense system (Vela *et al.,* 2010).

4.8 Maize Resistance for Viruses

The most effective means of managing MLN would be the use of tolerant or resistant varieties. A trial performed by Nelson *et al*. (2011) in Hawaii found many tropical inbred lines and varieties to be highly resistant to MCMV. According to the report, 30 out of 40

(75%) of University of Hawaii bred field maize inbred lines tested positive for resistance; however, no complete immunity was observed. Almost all temperate climate inbred lines and hybrids are highly susceptible to the virus (Nelson *et al*., 2011). The level of MCMV resistance varies widely among pure lines that have been tested in Hawaii, so it is considered a quantitative trait (Nelson *et al*., 2011). Preliminary inheritance studies on the inheritance of traits suggest a polygenic control of the disease, with resistance being partially dominant. This encourages the commercial production of hybrids only if both parents are resistant to the pathogen.

In Kenya, varieties are being screened for resistance by KARI and CIMMYT in two sites;Naivasha and Bomet. Preliminary data from 43 pre-commercial maize hybrids and seven commercial hybrids at Bomet, Chepkitwal and Naivasha, and 200 elite inbred lines at Naivasha, during one season of screening under natural disease pressure, suggest that MLN-resistant maize germplasm can be identified and developed quickly. KARI, CIMMYT and other partners will be expected to reconfirm the potential resistance of pre-commercial hybrids and inbred lines that show the lowest susceptibility to MLN and work urgently to develop resistant varieties (Makumbi and Wangai, 2013).

As MLN is due to the co-infection of two viruses, resistance against any one of the viruses would substantially reduce the damage due to the disease. Results of a trial of elite CIMMYT inbred lines under artificial SCMV inoculation showed several highly-resistant lines (Makumbi and Wangai*et al*, 2013). In the long run, deployment of varieties that are resistant to both MCMV and SCMV will be the best means of managing MLND (Incarbon, 2013; Sheets, 1998; Uyemoto, 1981).

4.9 Effect of MLND

MLN is expected to threaten maize production in developing countries. Maize is ranked the third most important cereal crop after wheat and rice (Khalili*et al*., 2013) and that more than 1.2 billion small scale farmers in Latin America and Sub-Saharan Africa depend on it as their main staple food and livestock feed (Iken and Amusa, 2004). It has been estimated that highly MLN-affected areas can experience a massive yield loss

(Incarbon 2013,Sheet 1998;Uyemoto1981). Due to dependence of farmers on maize as their main food crop, shortage in its supply can be synonymous with food insecurity.

The MCMV alone has a big potential to establish in warm arid, semi-arid and sub-humid tropics (Isabirye and Rwomushana, 2016). Of the identified *Potyviruses*, MDMV and SCMV are wide spread and cause diseases in maize worldwide (Mahuku*et al*., 2015b). Their widely presence can be indicative of their adaptation and interaction with host plants in areas where MCMV is considered new. Since they are adapted, they have a full machinery to attack the host, and the host has ways of resisting attack from the virus (Redinbaugh and Zambrano ,2014).

Since MCMV is new to the crop system, plants have little or lack resistance to the pathogen, and thus the additional weakened effect by the *Potyviruses* and/or abiotic stress favor their full colonization to maize host (Nelson *et al.,* 2011). As no single germplasm has been identified as resistant to the synergistic-interacting viruses as whole, serious maize loses are expected in Africa. Estimates made with an ecological niche models using a genetic algorithm (GARP) by Isabirye and Rwomushana (2016) showed that, suitable habitats for MCMV is as high as 662,974 km^2 in Ethiopia, 625,690 km^2 in Tanzania, 615,940 km^2 in D. R. Congo, 361,556 km^2 in Angola, 298,402 km^2 in South Africa and 265,564 km^2 in Madagascar. Swaziland, Burundi, and Rwanda lose 100% each and Uganda 88.1% in terms of national maize production area.

In a synergistic interaction of MCMV with a *Potyvirus*, higher damage to maize crop is expected as it is clear that effects are higher when in combination compared to when MCMV or a *Potyvirus* infects the host individually (Xia *et al.,* 2016).

In Kenya, in areas where MLND was very serious, farmers experienced extensive or complete crop loss (Incarbon 2013; Wangai *et al*., 2012). The infected plants are frequently barren; the ears formed are small, deformed and set little or no seeds, drastically reducing the yield. The areas affected constitute major maize production acreage and given the recorded loss of up to 100%, it has become an important food security issue in Kenya. Infection rates and damage can be very high, seriously affecting yields and sometimes causing complete loss of the crop (Adams *et al*., 2012). Infected

plants are frequently barren; ears formed may be small or deformed and set little or no seed.

The impact of the disease can be felt in the whole maize value chain. To control MLND, the maize seeds have to be dressed with an insecticide in addition to a fungicide seed dressing. Seed producers have incurred an extra cost in the production of seed maize.

4.10 Virus Diagnosis

Identification of MLND and the viruses involved in the disease complex is generally by observation of symptoms in the field. However, because single infections of the viruses and early stages of the disease are often inconspicuous and resemble physiological disorders, specific diagnostic tests are to be applied to confirm virus presence and to adequately detect/identify the viruses in the mixed infection (DSMZ, 2014)ELISA tests are the most reliable assays for detection of these viruses in maize as both viruses reach concentrations in maize that can easily be detected by ELISA. Specific polyclonal antibodies developed at the DSMZ Plant Virus Department allow a sensitive and reliable detection and identification of MCMV and SCMV in a standard double antibody sandwich enzyme linked immuno-sorbent assay (DAS-ELISA)(Cabanas *et al*., 2013).

PCR method is also used in many applications (Dougharie*t al*., 2009) including diagnostics of plant virus diseases (Henson and French, 1993; Hadidi*et al*., 1995; Lopez *et al*., 2003) because of its speed, specificity, sensitivity, and versatility (Naidu *et al*., 2003). Apart from detection of viruses, PCR products can be sequenced to provide further data on strain types (Webster *et al*., 2004). Sequencing is a very reliable technique for virus identification and has led to development of strain specific probes and primers from extensive sequence data available from many viral isolates. Next-generation sequencing (NGS) is one of the modern techniques that have been used in the diagnosis of new unidentified viral plant diseases (Punja*et al*., 2007).

4.11 Prevention and Control Mechanism

Some management principles such as plant quarantine, pathogen eradication, avoidance, plant protection and use of plant resistance has been reviewed by Kiruwa *et al.* (2016). In Africa where MCMV is considered new, scarce information is available on management of MLN. In other countries such as Hawii, integration of cultural practice, host tolerance and suitable insecticides has been used (Nelson *et al.*, 2011).

Work in developing suitable management options such as screening for MLN-tolerant/resistant germplasm and vector control is going on in countries heavily attacked by MLN in Africa (Mahuku *et al*., 2015b).

Few studies have determined the incidence of MLND and diversities in SCMV and MCMV in some parts of Ethiopia, the synergetic interaction of SCMV and MCMV, as well as the possible role of such interaction in increasing the severity of MLND received little attention.

5. MATERIALS AND METHODS

5.1 Sample Collection

Symptomatic leaves infected with MCMV and/or SCMV were collected from upper Awash, Ziway, Hawassa and Ambo areas using 50 ml anhydrous calcium chloride filled falcon tube. Seeds of eight maize genotypes (Melkassa 1, Melkassa 2, Melkassa 4, MH 140, MH 130, MHQ 138, Jibat and CML 445) were also obtained from Melkassa Agricultural Research Center (MARC).

5.2 Detection of Maize Chlortic Mottle and Sugarcane Mosaic Virus

5.2.1 Double Antibody Sandwich Enzyme-Linked Immunosorbent Assay (DAS - ELISA)

DAS ELISA was used to determine the presence of MCMV and/or SCMV according to Clark and Adams (1977). Briefly, specific antibody was diluted in coating buffer i.e. 20µl in 20 ml buffer at a recommended dilution of 1: 1000. After 200µl coating buffer was added to each well of the microtiter plate; the plates were covered and incubated at 37 °C for 2hrs. Then the plate was washed with PBS-Tween and soaked for a few minutes two times after that blot plates were tapped upside down on tissue paper and 200 µl aliquots of the test sample was then added to the duplicated wells. After covering and incubating overnight at 4 °C,the plates were washed three times. Then 200 µl enzyme conjugate was added in each well and incubated at 37 °C for 2 hours and it was washed three times.two hundred µl aliquots of freshly prepared substrate (1 mg /ml para- nitrophenyl- phosphate in substrate buffer) was added to each well and the plate was covered and incubated at 37°C for 30-60 min, positive and negative controls were also added in each plat .Finally, results were assessed by Spectrophotometric ELISA reader (HUMAREADER ELISA) measurement of absorbance at 405 nm, when the absorbance value was at least two times greater than that of the negative control, samples were considered to be positive.

5.2.2 RNA Extraction

For confirmation of DAS ELISA, Reverse Transcription PCR (RT-PCR) was performed. RNA was extracted from approximately dried 100 mg tissue from each leaf sample using HiPurA plant and fungal RNA Miniprep purification kit. Briefly, 1 ml of RNA-XPress Reagent was added to the ground plant material, the mixture was transferred to 2.0 ml capped collection tube then samples were incubated for 5 minutes at room temperature (10-25°C) to permit the complete dissociation of nucleoprotein complexes, after that 200 µl of Chloroform per ml of RNA-X Press reagent was added. Sample was covered tightly then shaked vigorously for 15 seconds and allowed to stand for 5-10 minutes at room temperature (10-25°C) after that the mixture was centrifuged at 12,000 x g (»13,000 rpm) for 15 minutes at 4°C. The aqueous phase containing RNA was transferred to a fresh tube and 1 ml of Binding Solution was added. The entire solution was transferred to a 1.5 ml tube then 0.5 volumes (usually 775 µl) of ethanol (96-100%) were added to the above solution. All samples were applied on the HiElute Miniprep Spin Column placed in a 2 ml uncapped collection tube then the flow-through was discarded and 700 µl of Prewash Solution was added. After centrifugation the flow through was then discarded and 500 µl of diluted wash Solution was added (Farrell *et al*., 1998).

5.2.3 RNA Quantification

The concentration of RNA was determined at A260 with an UV-spectrophotometer (ND-8000, 8-sample Spectrophotometer). UV absorbance measurements were acquired by diluted RNA sample (2 ul) under the RNA-40 settings of ND-8000 V2.0.0 software (Sambrook, 1989).

5*.2.4 Reverse Transcription PCR*

Two microliters of total RNA solution was used in each 12.5 µl reverse transcription reaction to synthesize and amplify cDNA for RT-PCR using one step RT PCR Promega protocol. Briefly, the reaction mixture by combining each component was prepared (Nuclease free water, Reaction buffer, dNTPs, forward primer, reverse primer and

$MgCl_2$) in a 2 ml reaction tube on ice. Then Reverse Transcriptase and DNA polymerase was added to the reaction. Primers MCMV F1 and MCMV R1 were used to amplify MCMV and SCMV F1 and SCMV R1 to amplify SCMV. MCMV and SCMV fragments were amplified by RT PCR after the cDNA was synthesized. The first strand of the cDNA was synthesized at 42 °C for 50 minute, followed by one cycle of 95 °C for 12 minute to inactivate the reverse transcriptase. Then the second strand of cDNA and synthesis of DNA was conducted at 95 °C for 30 seconds of denaturation followed by annealing at 55 °C for 30 seconds and then extension at 72 °C for one minute for 35 cycles with a final extension of 10 minute at 72 °C with holding at 10 °C (Sambrook, 1989; Farrell *et al*., 1998).

TABLE 1. List of MCMV and SCMV primers

No	Primer	Sequence (5′→ 3′)	Loci	Size
1	MCMV-F1	GTCCTGGCCTCAGTGGTTAAGG	3226-3247	478 bp
2	MCMV-R1	CGCACAGAGTTGAACACAATTGT	3703-3681	
3	SCMV-F1	AGCTAAG(a)GAAGCCCACATGCAG	9169-9190	319 bp
4	SCMV-R1	AGAAGACACTGGGTCCAACCCTG	9487-9465	

5.2.5 Analyzing RNA and RT-PCR Product

The RNA and PCR products were analyzed by 2% agarose gel electrophoresis. Briefly, agarose powder was dissolved in 100 ml TAE buffer which melts completely after heating. The melted agarose was poured in to the gel cast tray on leveled bench and then allowed the gel to solidify for 20 minutes. A total of 2µl extracted RNA were mixed with 2µl of 6x loading dye and 2µl of RT PCR with 2µl of 6x loading dye product then apply electric field with controlled power supply at 135V for 30 minute. Visualization and image capturing were done by using Bio-Rad gel doc system (Farrell *et al*., 1998).

5.3 Preparation of Inocula

The inoculum were prepared from MCMV and SCMV positive leaf samples following the methods of Jones (1977). Briefly, MCMV/SCMV positive leaves were grounded in a 0.1 M potassium phosphate in a 1:10 dilution ratio (1 gram of tissue to 10 milliliters of the 0.1M, 7.0 pH potassium phosphate buffers) using mortar and pestle followed by 2 min centrifugation at 12,000 rpm. For mixed inoculations, equal amount of (10 ul) MCMV sap was added to SCMV sap. The concentration of MCMV and SCMV was the same in single and mixed inoculations.

5.4 Plant Growth Condition

Eight maize genotype (three samples per genotype) were planted at National Agricultural Biotechnology Research Center (NABRC) green house (28 °C day and 22 °C night, 12 h light and 12 dark) in perforated plastic pots filled with sterilized soil. Before inoculation, DAS ELISA was conducted from random samples of young leaves to confirm the seeds purity. Then, MCMV, SCMV and mixed inocula treatments were applied at three leaf stage (~at the age of 15 days) Treated genotypes were observed for 60 days post inoculation (dpi) (Kareem and Taiwon, 2007).

5.5 Artificial Inoculation

Eight maize genotypes were inoculated with MCMV, SCMV and with both MCMV and SCMV separately. The same maize genotypes were inoculated with only buffer as a negative control (sodium phosphate). Carborundum-dusted leaves were inoculated by adding 20 ml of solution and stroking three times with a gloved finger. Following mechanical inoculation, plants were rinsed with water and plants were kept in a dark and humidified area for 24 hours and were moved to the previous growth chamber the next day according to Kareem and Taiwon (2007).

5.6 Phenotypic Data Collection

The symptom of viral and buffer inoculated genotypes wererecorded beginning 7-15 days post inoculation and rating continued every 7 days until 60 days post inoculation. Disease severity score was determined on a scale of 1 to 5 (1 = no visible MLN,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), 5 = complete plant necrosis) according to Gowda *et al.*(2015)

5.7 Sample Collection after Inoculation

Leaf samples were first collected 15 days post inoculation. Samples were collected in each 7 days interval and it lasted for 8 weeks for DAS ELISA to quantify the viral titer (7-mm-diameter discs weighing 4–5 mg).

5.8 Viral Titer Quantification

Commercially available DAS ELISA kits were used to determine relative titer of MCMV and SCMV (Clark and Adams, 1977). Briefly, specific coating antibody (IgG) of the respective viruses (MCMV and SCMV) was diluted with coating buffer; i.e. 1 µl of IgG in 1 ml of coating buffer with a recommended dilution of 1:1000. Then 100 µl of diluted IgG was added to each duplicated wells of the ELISA plate by leaving both upper and lower borders to prevent border effects. Each plate of the respective viruses were sealed with ELISA plate cover and incubated for 2 hrs at 37°C. After the incubation plates were washed 3 times in 3-minute interval after adding the washing buffer (PBS). leaf sample (0.05 g) were put into mortal for crashing using liquid nitrogen and then 2500 µl of extraction buffer were added. After drying the plates with tissue paper 100 µl of sample antigen (plant sap) were added to each well in replicate. Positive and negative controls were also added in each plate with a similar fashion as the samples. All the plates were sealed with ELISA plate cover and incubated overnight at 4°C.

The ELISA reactions were read about one hour after substrate addition at 405 nm in a microtitre plate reader HUMAREADER ELISA. Samples having an absorbance value of at least two times greater than that of the negative controls were considered to be positive. Viral titer was taken in each week for each genotype until week eight.

5.9. Statistical Analysis

The data was analysed by SPSS software (Version 24). The viral titer data obtained for each week after inoculation (week 1-8) is graphically presented using Microsoft Excel. MeanMCMV or SCMV for each genotype was determined by ANOVA.Values were considered to be statistically significant when P-values are less than or equal to 0.05.

6. RESULTS

6.1. Virus identification by ELISA and Reverse Transcription PCR

Values that are two times greater than the mean of the negative control considered as a positive. Strongly reacted samples to the virus specific antibody showed strong yellow color as shown in Figure 1 below.

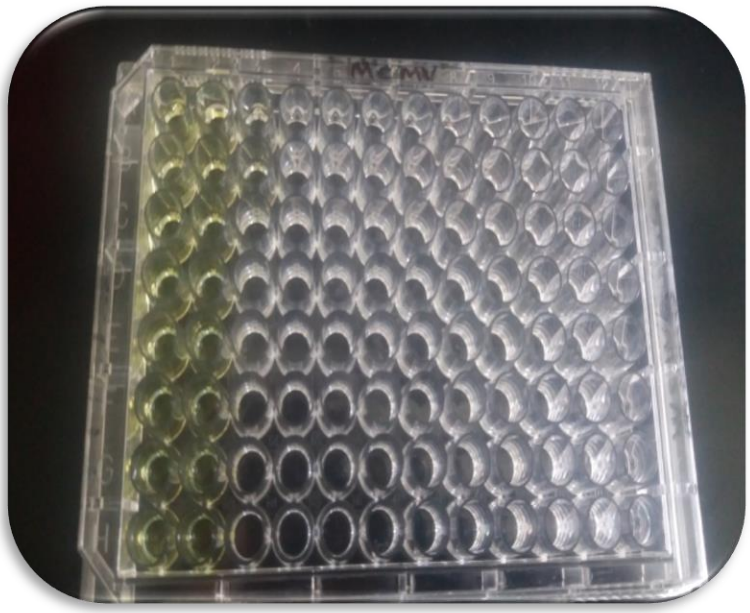


Figure 1: DAS-ELISA results and reaction to virusspecific antibodies

The agarose gel electrophoresis band below shows RT PCR positive samples which were also positive for MCMV and SCMV by DAS ELISA. The bands were analyzed by comparing with the 1Kbp DNA ladder. Specific bands which were at a position corresponding to the expected size of primers used for both viruses (478 bp for MCMV and 319 bp for SCMV) are considered as positive. Therefore, it has been considered the result of both DAS ELISA and RT-PCR before using the samples for further investigation.

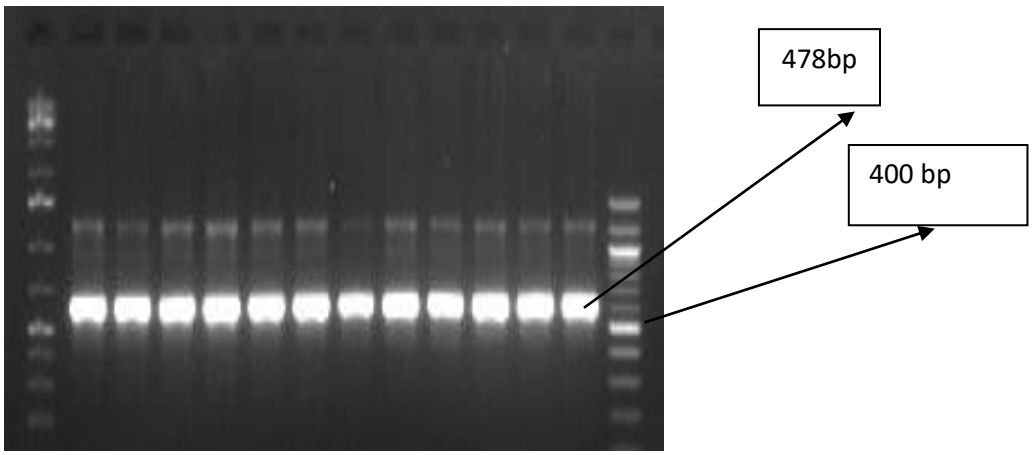


Figure 2. One step RT PCR positive samples for MCMV

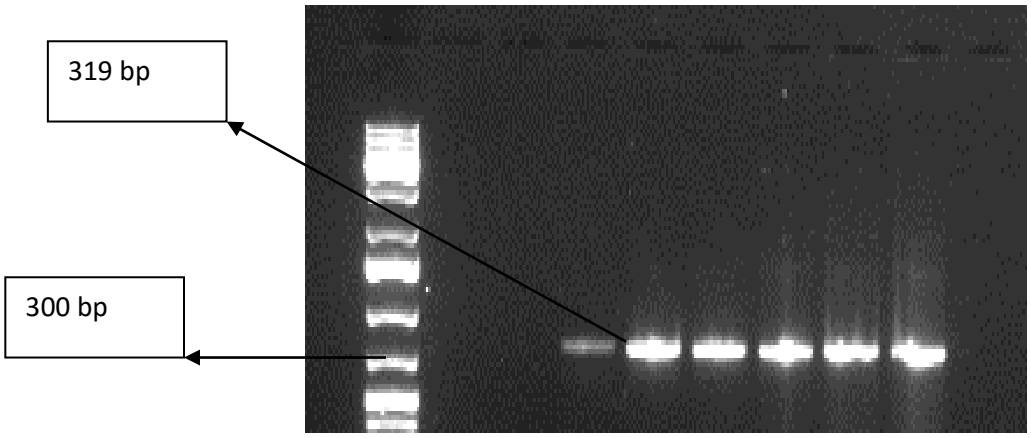
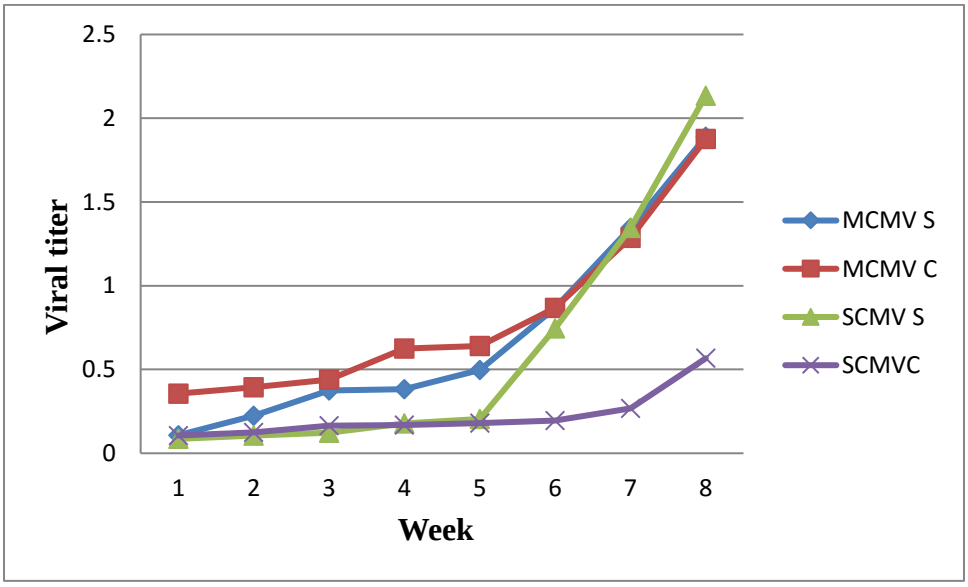


Figure 3. One step RT PCR positive samples for SCMV

6.2. MCMV and SCMV Viral Titer and Weeks of Inoculation in Different Maize Genotypes

6.2.1 MCMV and SCMV Viral Titer and Weeks of Inoculation in MHQ138

MHQ 138 maize genotype inoculated with both MCMV and SCMV have relatively higher MCMV viral titer until week six than those inoculated with MCMV alone. And the highest increase in MCMV titer was observed in week one, two, three and four. There were 3.6, 1.7 ,1.0 and 1.6 fold increase in MCMV titer in week one, week two and week four , respectively, in genotype infected with both SCMV and MCMV than those infected with only MCMV. In the first five weeks of post inoculation, the SCMV titer in MCMV and SCMV co- infected genotype and only SCMV infected genotype was almost similar. In contrast, SCMV titer in SCMV infected genotype increased dramatically than MCMV and SCMV co-infected genotypes after week five (Figure 4).

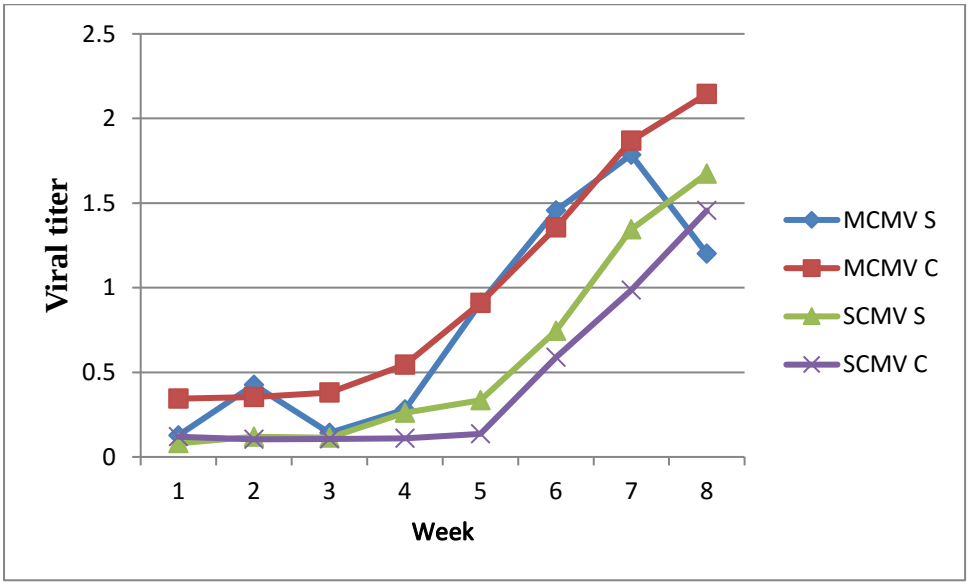


s=single c=co infection

Figure 4: SCMV and MCMV viral titer in MHQ 138

6.2.2 MCMV and SCMV viral titer and weeks of inoculation in Melkassa 2

MCMV viral titer rose steadily in MCMV and SCMV co-infected genotypes from week one to week eight. There was also an increase in viral titer from week four to week six in MCMV single infected, then a higher increase in early week seven until it reaches 1.2 OD in week eight. SCMV viral titer in MCMV and SCMV co-infected and in those infected with SCMV alone leveled off from week one to week three, and then rose steadily from week four to week eight. However, from week four to week eight there was higher SCMV viral titer in single SCMV infected than those infected with both MCMV and SCMV (Figure 5).



s=single c=co infection

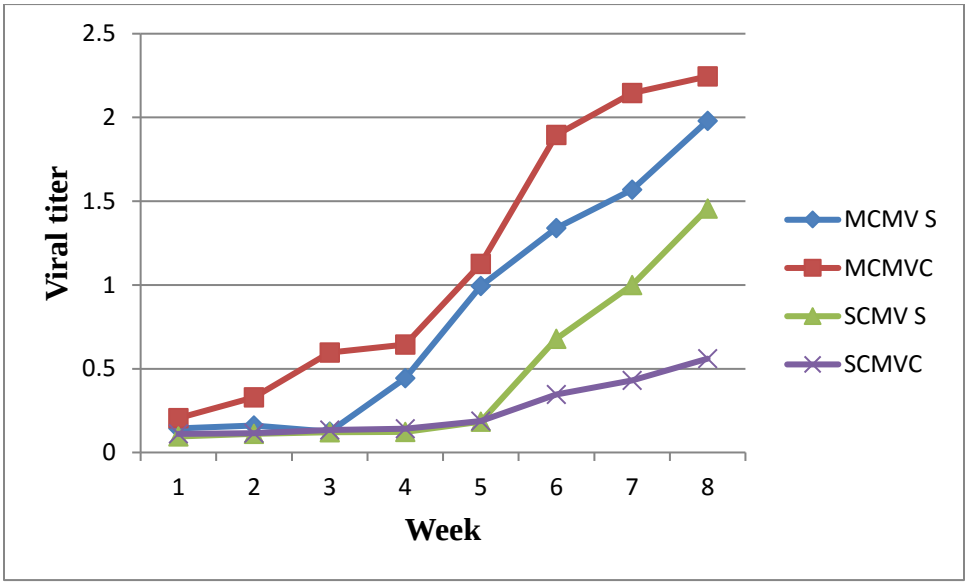
Figure 5: SCMV and MCMV viral titer in Melkassa 2

6.2.3 MCMV and SCMV viral titer and weeks of inoculation in Melkassa4

From week one to week three MCMV titer in MCMV and SCMV co- infected genotype rose steadily, and then leveled off until week four. Then there was a gradual increase in MCMV titer from week four to week eight with a peak of 2.24 OD at week eight. In

contrast, from week one to week three MCMV titer leveled off, and then gradually increased from week three to week eight in genotypes infected with MCMV alone.

SCMV titer leveled off from week one to week four in both SCMV and MCMV co-infected and SCMV single infected genotypes. From week five to week eight, however, there was higher increase in SCMV titer in genotypes infected with SCMV than those infected with both SCMV and MCMV. The highest SCMV titer was recorded in week eight for both SCMV single infected (1.46 OD), and SCMV and MCMV co-infected genotypes (0.56 OD) (Figure 6).



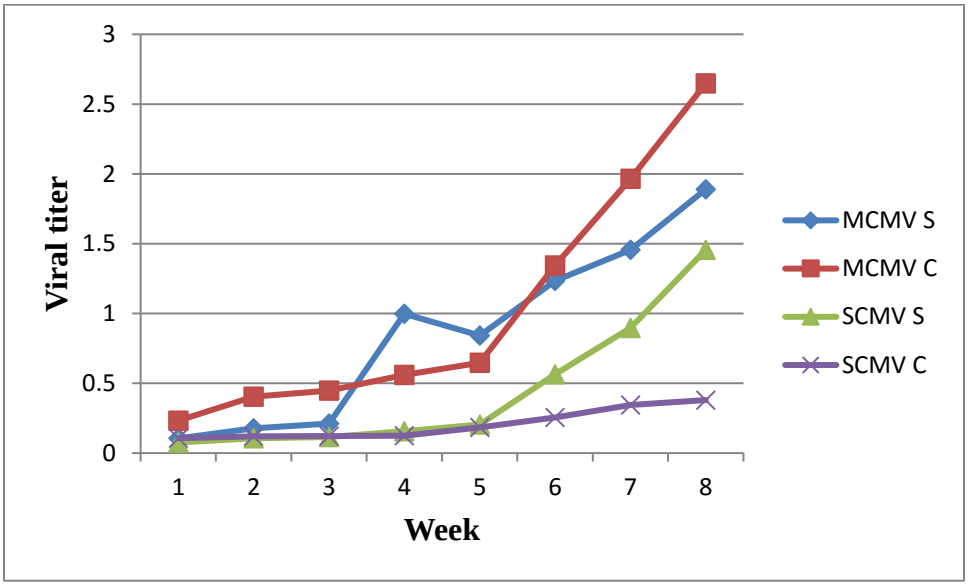
s=single c=co infection

Figure 6: SCMV and MCMV viral titer in Melkassa 4

6.2.4 MCMV and SCMV viral titer and weeks of inoculation in MH130

There was higher MCMV titer from week one to week three in MCMV and SCMV co-infected genotypes than those infected with SCMV alone. From week three until week six, however, MCMV titer was higher in MCMV infected than those infected with both SCMV and SCMV. MCMV titer raised steadily from 1.34 OD in week six to 2.65 OD in

week eight. Although lower than MCMV and SCMV co-infected plants, there was a gradual increase in MCMV titer in genotypes infected with MCMV alone. From week one to week five, SCMV titer leveled off in genotypes infected with SCMV alone and those co-infected with SCMV and MCMV, and then steadily increased from week five to eight. The increase in SCMV titer was, however, higher in genotypes infected with SCMV than co-infected with both SCMV and MCMV (Figure 7).



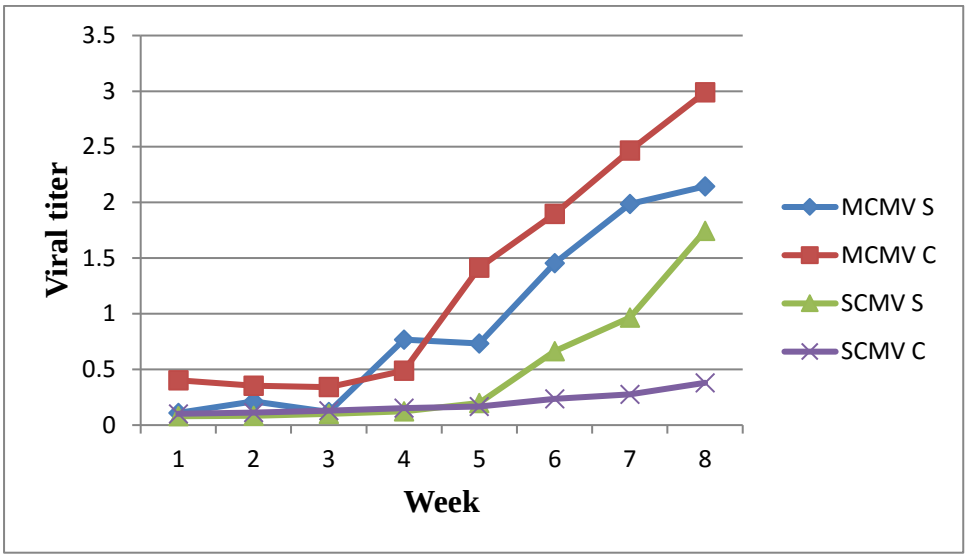
s=single c=co infection

Figure 7: SCMV and MCMV viral titer in MH130

6.2.5 MCMV and SCMV viral titer and weeks of inoculation in MH 140

There was a slight increase in MCMV titer in genotypes infected with MCMV alone from week three to four. Moreover, there was a slight dip in MCMV titer in week five, followed by a gradual rise until it reached to a peak of 2.14 week eight. MCMV titer in genotype co-infected with MCMV and SCMV leveled off from week one to week four, and then steadily increased from 0.49 OD in week four to 2.99 OD in week eight. SCMV titer was almost similar in genotypes infected with both SCMV and MCMV and those infected with SCMV alone from week one to week five. From week five to week eight,

however, there was a steady increase in SCMV titer from 0.2 OD in week five to1.75 in varieties infected with SCMV alone (Figure 8).

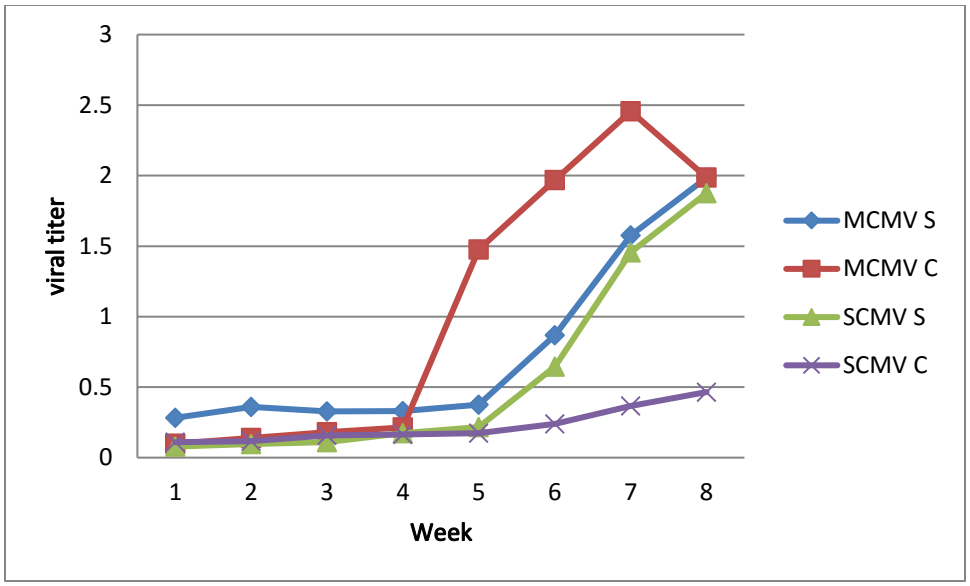


s=single c=co infection

Figure 8: SCMV and MCMV viral titer in MH140

6.2.6 MCMV and SCMV viral titer and weeks of inoculation in Jibat

MCMV titer in MCMV and SCMV co infected genotypes rose steadily from week four to week seven with a peak of 2.45 OD in week seven, and then decreased gradually until week eight. Though lower than the observed MCMV co infected titer increase in only MCMV infected genotype, there was a steady increase in MCMV titer in MCMV infected genotypes. Moreover, there was higher SCMV titer increase from week five to week eight in genotypes infected with SCMV than those co-infected with SCMV and MCMV (Figure 9).

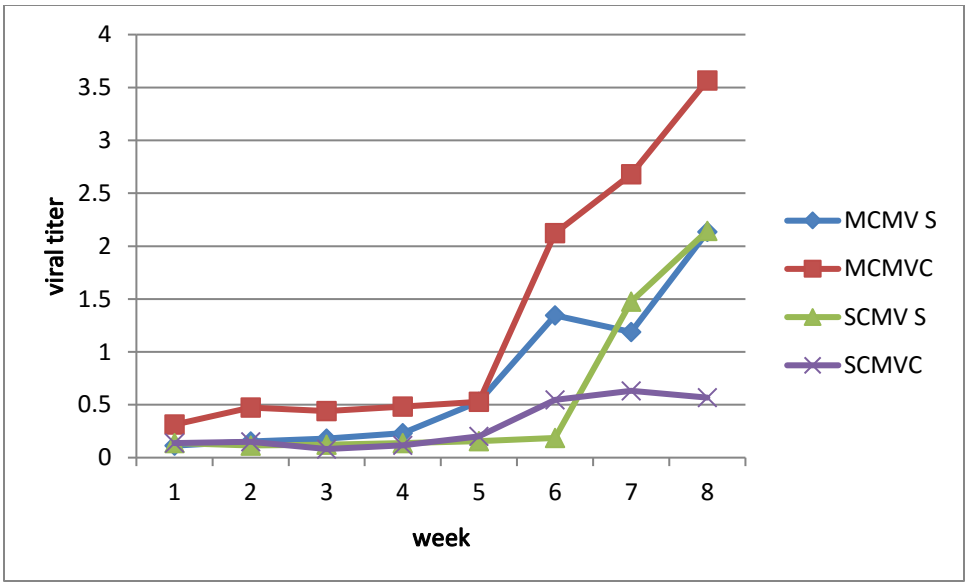


s=single c=co infection

Figure 9: SCMV and MCMV viral titer in Jibat

6.2.7 MCMV and SCMV viral titer and weeks of inoculation in CML445

SCMV titer in genotypes co-infected with SCMV and MCMV, and those infected with SCMV alone was nearly similar from week one to week four. Although there was a slight higher SCMV titer from week five (0.2 OD) to week six (0.546 OD) in SCMV and MCMV co-infected genotypes and there was higher increase in SCMV titer in single (week seven=1.48, week eight=2.15) infected than co-infected (week seven=0.632, week eight=0.57) in the remaining weeks (. In contrast, MCMV titer was higher in genotype infected with both MCMV and SCMV genotypes than those infected with MCMV alone (Figure 10).

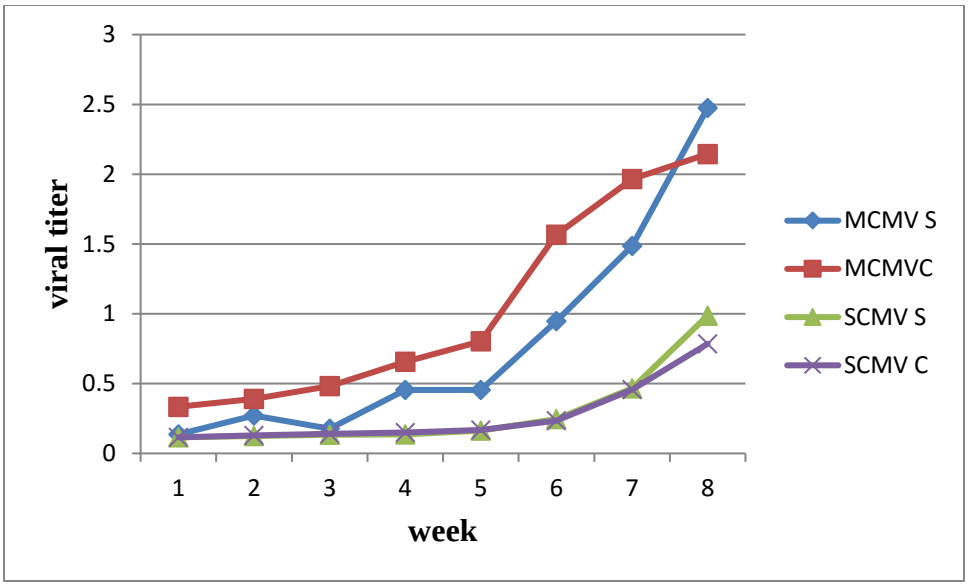


s=single c=co infection

Figure 10: SCMV and MCMV viral titer in CML445

6.2.8 MCMV and SCMV viral titer and weeks of inoculation in Melkassa 1

In Melkassa 1, there was a steady increase in MCMV titer in genotypes co-infected with SCMV and MCMV, and in those infected with SCMV. However, the increase in MCMV was higher in MCMV and SCMV co-infected genotypes than those infected with MCMV alone until week eight. In contrast, SCMV titer in both SCMV single, and SCMV and MCMV co-infected genotypes was comparable from week one to week seven. From week seven to week eight, however, there was a slight increase in SCMV titer in SCMV single (week seven=0.99, week eight=0.79) infected genotypes than those infected by both SCMV and MCMV genotypes (week seven=0.47, week eight=0.46) (Figure 11).



s=single c=co infection

Figure 11: SCMV and MCMV viral titer in Melkassa 1

6.3 Symptom severity in SCMV and MCMV co-infected maize genotypes

All genotypes except MH138, Melkassa 1and Melkassa 4 showed symptom at week one. MH 130 produced chlorotic mottling (score 3) at week one than the other genotypes. In contrast, Jibat, MH 140 and CML 445 were at score 2 (fine chlorotic) at week one. At week three, the highest symptom severity was observed in MH140 followed by Jibat, Melk 4, MH138 and CML 445. Severity remains at 4 from week three to week four in MH138, Melkassa 1, Melkassa2 and MH 130. From week four to week eight, Jibat and Melkassa 2 were at score five, and score five was recorded at week eight in MH 140 MH 138 and MH 130. Melkassa 1 remains at a score 3 from week three to week six, and reached to a score of five in week seven and eight (Figure 12 and 13).

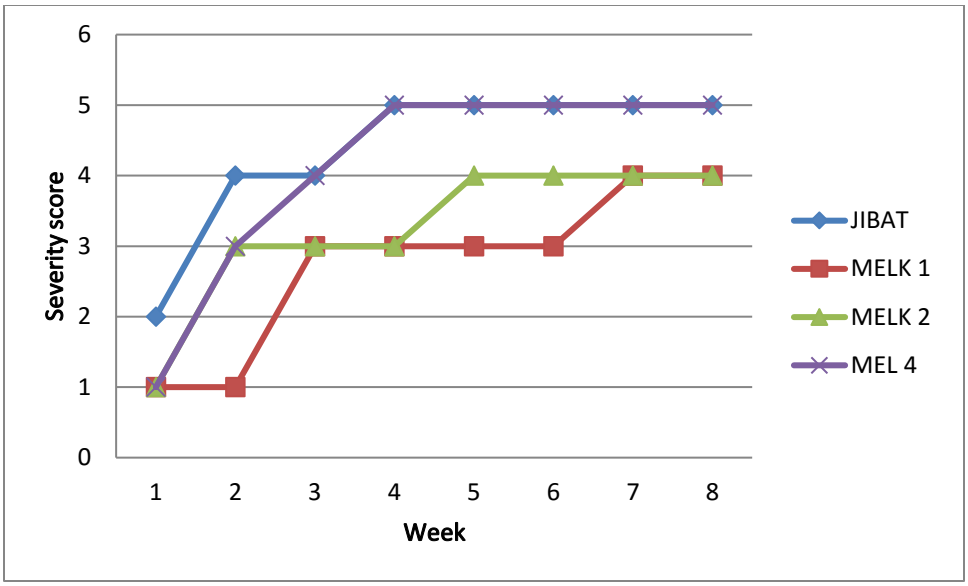


Figure 12: Symptom severity of co- infected(Jibat, Melkassa 1,Melkassa2 and Melkassa 4)genotypes.

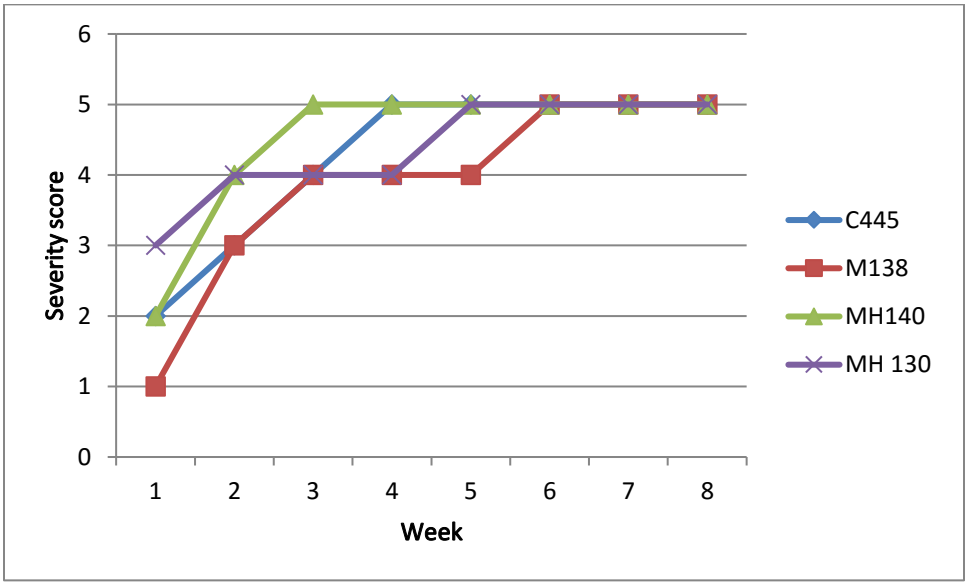


Figure 13: Symptom severity of co infected (CML 445, MHQ138, MH140 and MH130)genotypes.

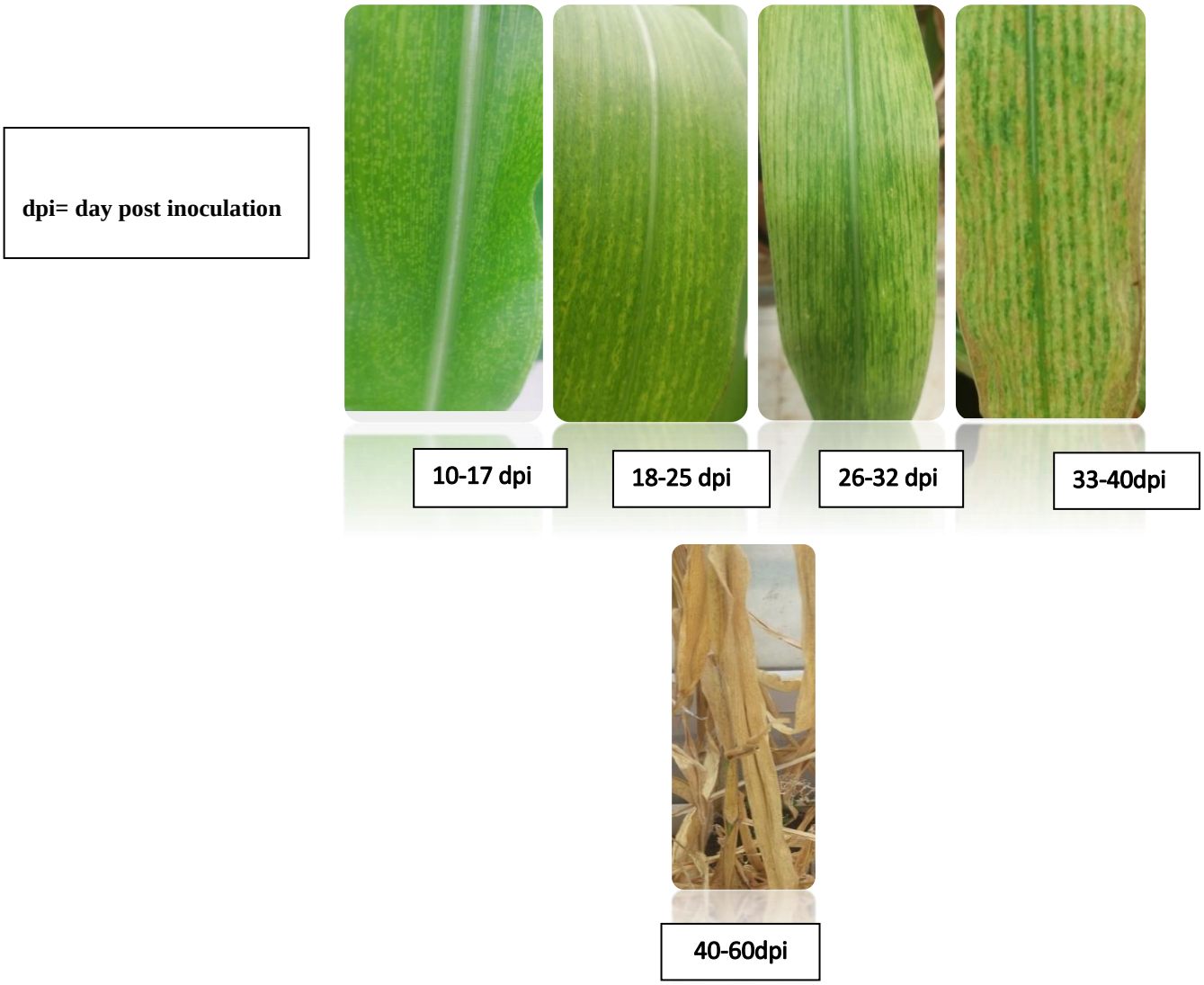


Figure14: Symptom severity of co-infected plant in different time interval (MH 130)

Figure15: Sugar Cane Mosaic Virus

6.4 Difference in mean MCMV viral titer among the different maize genotypes

The mean MCMV titer was higher in all of maize genotypes infected by both MCMV and SCMV than those infected by MCMV alone except in Melkassa1. In CML 445, there was significantly higher viral titer than single MCMV infected samples. In Melkassa 4, there was higher mean MCMV titer in MCMV and SCMV co-infection than those infected by MCMV only and the difference in viral titer between MCMV single and MCMV-SCMV co-infected was statistically significant (P=0.033).Similarly, a significant difference in MCMV viral titer was observed in MCMV single and MCMV-SCMV co-infected ((Mean (±SD) 2.4, (0.51)) genotypes of Jibat (P =0.005).In contrast, there were no significant differences in mean MCMV viral titer between genotypes infected by MCMV and those infected by both MCMV and SCMV in Melkassa 1 (P=0.860), Melkassa 2(P= 0.572) and MHQ138 (P=0.734)(Table 2).

Table 2: Difference in MCMV viral titer between maize genotypes infected by MCMV and both MCMV and SCMV.

Variety	Mean (SD) [x]	P -value[†]
MHQ 138		
MCMV	0.71 (0.62)	0.73
MCMV + SCMV	0.81 (0.53)	
Melkassa 2		
MCMV	0.79 (0.64)	0.57
MCMV + SCMV	0.99 (0.72)	
Melkassa 4		
MCMV	0.77 (0.66)	0.033*
MCMV + SCMV	1.42 (0.41)	
MH 130		
MCMV	0.86 (0.66)	0.035*
MCMV + SCMV	1.81 (0.92)	
MH 140		
MCMV	0.94 (0.82)	0.007*
MCMV+ SCMV	2.02 (0.51)	
Jibat		
MCMV	1.1 (1.05)	0.005*
MCMV + SCMV	2.4 (0.51)	
CML 445		
MCMV	0.73 (0.74)	0.042*
MCMV + SCMV	1.6 (0.76)	
Melkassa 1		
MCMV	0.29 (0.31)	0.860
MCMV + SCMV	0.27 (0.24)	

[x] Values shown in bracket are the ± standard deviations; †ANOVA; * Significant difference (<0.05)

6.5 Difference in mean SCMV viral titer between different maize genotypes

Although higher mean concentration of SCMV titer was found in all genotypes infected by SCMV than those infected by both SCMV and MCMV, the difference was not statistically significant ($P>0.05$ for all).The mean SCMV concentration in SCMV infected Melkassa 4 is relatively higher than genotype infected by both SCMV and MCMV. Similarly, as compared to those infected by both SCMV and MCMV, there were higher mean SCMV load in SCMV infected Jibat ,MHQ 138, Melkassa 2, MH 130,CML 445and Melkassa 1than the same genotypes infected by both viruses(Table 3).

Table 3. Difference in SCMV viral titer between maize genotypes infected only by SCMV and those infected by both MCMV and SCMV.

Variety	Mean (SD) [x]	P- value†
MHQ 138		
SCMV	0.61 (0.75)	0.17
MCMV + SCMV	0.22 (0.14)	
Melkassa 2		
SCMV	0.58 (0.61)	0.64
MCMV + SCMV	0.45 (0.51)	
Melkassa 4		
SCMV	0.47 (0.51)	0.28
MCMV + SCMV	0.25 (0.17)	
MH 130		
SCMV	0.44 (0.49)	0.20
MCMV + SCMV	0.20 (0.10)	
MH 140		
SCMV	0.49 (0.6)	0.18
MCMV + SCMV	0.19 (0.09)	
Jibat		
SCMV	0.58 (0.70)	0.18
MCMV + SCMV	0.22 (0.12)	
CML 445		
SCMV	0.55 (0.79)	0.39
MCMV + SCMV	0.30 (0.23)	
Melkassa 1		
SCMV	0.29 (0.3)	0.86
MCMV + SCMV	0.27 (0.23)	

[x] Values shown in bracket are the ± standard deviations; †ANOVA; * Significant difference (<0.05).

7. DISCUSSION

In this study the suspected MLN-infected plants were clearly observed using ELISA and RT-PCR tests. Similarly, in Ethiopia Mahuku *et al*. (2015a) and Mengistu *et al*. (2017) have indicated that MLN is caused by mixed infection of MCMV and SCMV. Maize lethal necrosis disease (MLND) is reported to be caused by co-infection by any of the cereal infecting viruses in the family *Potyvirideae* such as SCMV, MDMV or WSM and MCMV (Niblett and Claflin, 1978). Viruses from other families, including *Maize rayadofino virus* (*MRFV*), family *Tymovirideae*, genus *Marafivirus*, *Maize chlorotic dwarf virus* (*MCDV*), family *Secovirideae*, genus *Wakavirus* and *Maize mosaic virus (MMV)*, family *Rhabdovirideae*, genus *Nucleorhabdovirus*, can also cause synergistic reactions in co-infection with MCMV.

There was a relatively higher MCMV titer in all genotypes co-infected with MCMV and SCMV than those infected with MCMV except in Melkassa1**.** In contrast, SCMV titer was lower in SCMV and MCMV co-infected genotypes than those infected with SCMV and MCMV. It has also been reported that the titer of MCMV in plants infected with both MCMV and potvirus is higher than in plants infected with MCMV alone (Goldberg and Brakke, 1987). Similarly, in the MLN, concentration of the *Potyvirus* in a single infection is similar whereas the concentration of MCMV is increased markedly (Xie *et al*., 2016).

Although several reasons were suggested for the increased concentration of MCMV in SCMV and SCMV co-infected plants, the role of HC-Pro of *Potyviruses* in suppressing antiviral defense mechanism and PTGS are the major once. It has been reported that HC-Pro of *Potyviruses* involve in viral vascular movement and suppression of an antiviral defense mechanism in plants which will result in the increment of other co-existing virus (Savenkov and Valkonen, 2001). Therefore, this phenomenon may be the cause for the observed higher MCMV in MCMV and SCMV co-infected maize genotypes used in this study and previous studies.

In this study various symptoms were observed among the different maize genotypes inoculated with SCMV, MCMV or both viruses. it has also observed that symptoms like fine chlorotic streaks mostly on older leaves, chlorotic mottling throughout the plant,

excessive chlorotic mottling on lower leaves and necrosis of newly emerging leaves and complete plant necrosis. Intense susceptibility to MLN and various disease symptoms have also been reported in East Africa (EA) among the pre-commercial and commercial maize varieties (Jumbo *et al*., 2015; Semagn *et al*., 2015).

Fine chlorotic, chlorotic mottling and excessive chlorotic are some of the MLND symptoms observed in this study. Spotting (early-stage'), streaking ('mid-stage') and necrosis on the margin ('late-stage') was also observed in a study conducted in Kenya (Adams *et al.*, 2012). Maize lethal necrosis disease (MLND) symptoms such as mottling, mosaic, necrosis, leaf deformation and local lesions were also reported by other studies (Incarbon 2013; Wangai *et al*., 2012). Moreover, symptoms like streaks may coalesce to create chlorotic mottling, chlorotic mottling followed by leaf necrosis were also reported in study conducted in other part of Ethiopia (Mengistu *et al*., 2017; Makone *et al.*, 2014). Diversion of assimilates from the host plant resulted from interactions of the host with the virus favors the virus cellular processes like its replication and multiplication and cause various symptoms (Revers *et al*., 1999). This process, therefore, may be responsible for the various symptoms observed in our study and previous studies.

All the maize genotypes used in this study were observed to be infected with SCMV and MCMV. The interactions of these two viruses were also observed to cause MLND with varied levels of severity among the genotypes used. However, among the genotypes used in this study, Melkassa 1, 2 and MHQ 138 were found to be relatively resistant than the other genotypes. Similarly, Jacob, (2015) has conducted in Tanzania where landrace genotypes were better adapted against MLND than either improved varieties or inbred lines. The observed partial diseases resistances in the current study and previous studies have indicated imply an incompatible interaction between the host and the virus which could have been stimulated by the rapid necrosis at the foci of virus entry preventing its further spread (Ronde *et al*., 2014). Validation through similar repeat experiments over several seasons with reliable inoculations may facilitate the use of resistant genotypes obtained in this study to boost maize production.

In this study, MLND severity ranges from one to five with the highest score in six of the eight genotypes (Melkassa 4, Jibat, MH 140, MH 130 , MH 138 and CML 445). The observation that MLN disease severity score varies according to maize genotypes is also in agreement with a study conducted in Kenya (CYMMIT,2012) that 2.0, 2.0 and 2.5 and 2.9 MLND severity score were reported in CKH10767, CKH114272 and CKH101509 maize varieties.. Low MLN severity ranging from 2-3 were also reported in seven maize genotypes (MLN1, MLN15, MLN16, MLN17, MLN19, MLN18 and MLN12) studied in Kenya (Sitta, 2017). Though no logical reasons were given for the observed variation in MLN severity between the different maize genotypes investigated in this study and previous studies, the observed difference in diseases severity may be due to variation in gene content between the different genotypes used.

This study also showed that there is higher MCMV viral titer in SCMV-MCMV co-infected maize plants compared with that in MCMV infected maize plants. Furthermore, the viral titer of MCMV increases more in plants that are co-infected with SCMV-MCMV than plants infected by MCMV alone. Similarly, Drake *et al*. (2007), reported that MCMV titer was greater in plants infected with both SCMV and MCMV than those infected with MCMV alone.

It has been demonstrated that HC-Pro, the silencing suppressor encoded by *Potyviruses*, could enhance the pathogenicity and accumulation of other heterologous viruses (Syller *et al*., 2012; Pruss *et al*., 1997). This finding may explain why MCMV alone causes only mild disease symptoms in the infected plant, and the systemic necrosis and the increased MCMV RNA accumulation that result from the synergism between MCMV and SCMV in co-infected plants may be due to the presence of SCMV HC-Pro protein. However, Scheets, (1997) has shown that although WSMV increases MCMV concentration in maize coinfected with MCMV and WSMV plant, the synergistic infection of WSMV and MCMV was independent of WSMV HC-Pro, which was not a silencing suppressor (Pruss *et al*., 1997; Stenger *et al*., 2007). Similarly, Goldberg and Brakke (1987) have observed that where SCMV concentrations remain the same in SCMV -MCMV and those infected with SCMV alone.

The study also investigated variation in MCMV and SCMV viral titer with weeks of inoculation to examine if there is difference in MCMV or SCMV resistance between the genotypes used. Generally, all of the maize genotypes used in this study have early increase in MCMV than SCMV titer. MHQ 138, Melkassa 1 and Melkassa 2 have lower SCMV and MCMV viral titer than the other genotypes.

8. CONCLUSION

On the basis of viral titer and symptom severity used to determine disease response, Melkassa 1 and Melkassa 2 have high resistance to MLND. MH 138 was also found to have higher resistance to increase in viral titer than the other maize genotypes used in this study. This study also found increase in both SCMV and MCMV titer as week of inoculation increases. Using the identified resistant genotypes in this study as markers is, therefore important for further investigation of resistant maize genotypes in Ethiopia and increase maize production in the country.

9. RECOMMENDATIONS

- It is recommended that farmers in Ethiopia use Melkassa 1, Melkassa 2 and MHQ 138 than the other maize genotypes investigated in this study.

- Similar to studies conducted in other parts of the world, this study report almost the same MLND symptom characteristics. This report may, therefore, be very useful in early MLND identification in Ethiopia for researchers and extension workers.

- It is also recommended that the Government of Ethiopia establishes MLND diagnostic laboratories in different parts of the country for early identification and intervention before it progressed to sever MLND.

- This study didn't consider all maize genotypes found in Ethiopia. It is, therefore, recommended that other studies should screen the available maize genotypes in Ethiopia to obtain more MLND resistant genotypes.

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11. APPENDICES

Appendix 1. Double Antibody Sandwich ELISA (DAS-ELISA)Steps

- Dilute specific coating antibody (IgG) in coating buffer; i.e. 20µl in 20ml buffer at a recommended dilution of 1:1000. Add 100 µl to each well of the microtiter plate.
- Cover the plates and incubate at 37^0C for 2-4 hr.
- Wash the plates with PBS-Tween using wash bottle, soak for a few minutes and repeat washing two times. Blot plates by tapping upside down on tissue paper.
- Add 100 µl of aliquots of the test sample (extracted fresh leaf samples in Sample extraction buffer 1:20 weight/volume) to duplicate wells.
- Cover the plates and incubate overnight at 4^0C.
- Wash three times as in step 3.
- Dilute specific enzyme conjugate (IgG-AP) in conjugate buffer; i.e. 20µl in 20ml buffer at a recommended dilution of 1:1000. Add 100µl to each well of the microtiter plate.
- Cover the plates and incubate at 37 ^{0}C for 2-4 hr.
- Wash three times as in step 3.
- Add 100µl aliquots of freshly prepared PNP substrate to each well.
- Cover the plates and incubate at 37 ^{0}C for 30-60 min, or as long as necessary to obtain clear reactions.
- Assess results by: Visual observation Spectrophotometer measurement of absorbance at 405 nm

<u>Buffers used in ELISA prepared from buffer packs:</u>

- Coating Buffer

  Empty the contents of two coating buffer capsules in 200 ml of deionized water and dissolve. Do not dissolve complete capsules! This will yield 200 ml of 0.05M carbonate-bicarbonate coating buffer, pH 9.6 which is sufficient for 10 plates.

- PBS-Tween

Empty the contents of one pouch SIGMA phosphate buffered saline with Tween 20 in 1000 ml deionized water and dissolve. This will yield 1 Lit PBS-Tween wash buffer. This is sufficient for all washing steps of one plate.

- Sample Extraction Buffer

Mix 50 ml of 10x conc. ELISA extraction buffer with 450 ml of deionized water to yield 500 ml sample extraction buffer. This is sufficient for the extraction of all samples of one plate.

- Conjugate Buffer

Mix 2 ml of 10x conc. conjugate buffer with 18 ml of deionized water to yield 20 ml conjugate buffer. This is sufficient for one plate.

- PNP substrate

Mix 4 ml of 5x conc. Substrate buffer with 16 ml of deionized water. Dissolve one SIGMA PNP substrate tablet in these freshly prepared 20 ml of 1x concentrated substrate buffer to yield 20 ml PNP substrate. This is sufficient for one plate.

Appendix 2. RNA extraction kit

Procedure

1. To the ground plant material, immediately add 1 ml of RNA-XPress Reagent and mix thoroughly.
2. Transfer the mixture to a 2.0 ml capped collection tube.

4. Phase separation

Incubate the sample for 5 minutes at room temperature (15-25°C) to permit the complete dissociation of nucleoprotein complexes. Add 200 µl of Chloroform per ml of RNA-XPress Reagent used. Cover the sample tightly, shake vigorously for 15 seconds and allow tostand for 5-10 minutes at room temperature (15-25°C). Centrifuge the resultingmixture at 12,000 x g (»13,000 rpm) for 15 minutes at 4°C. Following centrifugation,mixture separates into lower organic phase (containing protein), an interphase(containing cell debris and DNA) and upper aqueous phase containing RNA.

9. Transfer the aqueous phase containing RNA to a fresh tube (not provided) and add 1 ml of Binding Solution (PBR) (DS0078). Mix thoroughly by gentle pipetting. Transfer the entire solution to a 15 ml tube (not provided).

10. Add 0.5 volumes (usually 775 µl) of ethanol (96-100%) to the above solution, and mix immediately by gentle pipetting. Do not centrifuge. Continue without delay with step 6.

11. Add 700 µl of Prewash Solution (RW1) (DS0041) to HiElute Miniprep Spin Column. Close the tube gently, and centrifuge for 1 minute at ³8000 x g (»10,000 rpm). Discard the flow through.

12. Add 500 µl of diluted Wash Solution (WS) (DS0012). Close the tube gently, and centrifuge for 1 minute at ³8000 x g (»10,000 rpm) to wash the column. Discard the flow-through.

13. Add another 500 µl of diluted Wash Solution (WS) (DS0012) to the HiElute Miniprep Spin Column. Close the tube gently, and centrifuge for 2 minutes at ³8000 x g (»10,000 rpm) to dry the membrane. Spin the empty column for additional 1minute at ³13,000 x g (»14,000 rpm) for drying of the column as it will avoid Wash Solution carryover.

10. **RNA Elution**

Transfer the column to a new uncapped 2 ml collection tube. Add 30-50 µl Elution Solution(RNase-Free Water) directly onto the spin column. Close the tube gently, and centrifuge for 1 minute at ³8000 x g (»10,000 rpm) to elute.

11. If the expected RNA yield is >20 µg, repeat the elution step (step 10) as described, with a second volume of RNase-Free Water. Elute into the same collection tube. Transfer the eluate into fresh capped 2.0ml collection tube for longer storage.

Appendix 3. One-Step RT-PCR protocol

- The first strand of the cDNA was synthesized at 42 ^{0}c for 50 minute
- Adjust one cycle of 95 ^{0}c for 12 minute to inactivate the reverse transcriptase.
- Then the second strand of cDNA and synthesis of DNA was conducted at 95 ^{0}c for 30 seconds of denaturation
- Annealing at 55 ^{0}c for 30 seconds and then extension at 72 ^{0}c for one minute for 35 cycles
- Final extension of 10 minute at 72 ^{0}c with holding at 10 ^{0}c

Appendix 4.Disease Score

1= No visible MLN

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

5 = Complete plant necrosis

Appendix 5.MCMV single and MCMV coinfection viral titer for each week and genotype

MH 138		Melkassa 2		Melkassa 4		MH 130		MH 140		Jibat		CML 445		Melkassa 1	
MCM	MCC	MCS	MCC	MCS	MCM	MCS	MCC	MCS	MCM	MCS	MCC	MCS	MCC	MCS	MCM
0.1075	0.356	0.1275	0.3445	0.143	0.205	0.1055	0.2325	0.11	0.402	0.099	0.2825	0.1135	0.312	0.1365	0.334
0.223	0.394	0.4265	0.354	0.1615	0.3295	0.1775	0.4045	0.214	0.3545	0.139	0.36	0.153	0.473	0.27	0.391
0.375	0.4405	0.1415	0.3805	0.124	0.596	0.211	0.4475	0.117	0.341	0.18	0.329	0.179	0.439	0.1775	0.482
0.383	0.626	0.28	0.5455	0.443	0.644	0.997	0.561	0.767	0.489	0.2135	0.331	0.233	0.4835	0.4535	0.657
0.4975	0.6405	0.909	0.909	0.994	1.1265	0.841	0.647	0.7325	1.4155	1.4765	0.3765	0.5295	0.5295	0.4535	0.8025
0.8675	0.867	1.456	1.356	1.34	1.8957	1.234	1.345	1.456	1.897	1.968	0.867	1.3445	2.123	0.9465	1.567
1.345	1.286	1.786	1.868	1.568	2.145	1.457	1.965	1.987	2..467	2.456	1.575	1.189	2.678	1.486	1.9657
1.8895	1.876	1.2	2.145	1.98	2.245	1.89	2.648	2.145	2.145	1.987	1.985	2.134	3.567	2.475	2.145

Appendix 6.SCMV single and SCMV coinfection viral titer for each week and genotype

MH 138		**Melkassa 2**		**Melkassa 4**		**MH 130**		**MH 140**		**Jibat**		**CML 445**		**Melkassa 1**	
SCMV	SCM	SCM	SCM	SCM	SCM	SCM	SCM	SCM	SCM	SCM	SCM	SCM	SCM	SCS	SCM
0.086	0.106	0.0805	0.119	0.0955	0.111	0.076	0.107	0.0795	0.102	0.0785	0.1095	0.1365	0.1365	0.115	0.115
0.106	0.124	0.119	0.104	0.111	0.115	0.107	0.12	0.085	0.113	0.097	0.118	0.115	0.151	0.125	0.128
0.122	0.1645	0.114	0.106	0.1215	0.134	0.115	0.122	0.102	0.129	0.1095	0.158	0.1225	0.0815	0.133	0.14
0.179	0.1685	0.262	0.109	0.1225	0.1415	0.156	0.1235	0.124	0.154	0.173	0.165	0.139	0.1175	0.136	0.15
0.2055	0.18	0.334	0.136	0.1835	0.1885	0.2045	0.1835	0.2	0.167	0.216	0.1745	0.157	0.2	0.163	0.167
0.745	0.195	0.746	0.589	0.678	0.346	0.564	0.256	0.665	0.235	0.6454	0.24	0.1867	0.546	0.245	0.234
1.345	0.267	1.345	0.985	0.999	0.43	0.897	0.345	0.967	0.276	1.456	0.367	1.4758	0.632	0.465	0.456
2.135	0.567	1.674	1.456	1.456	0.56	1.456	0.38	1.746	0.38	1.876	0.465	2.145	0.567	0.987	0.785