BIOCHEMICAL ALTERATIONS IN MAIZE PLANTS INDUCED BY VIRUSES CAUSING MAIZE LETHAL NECROSIS AND THEIR RELEVANCE FOR INSECT VECTORS

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DECLARATION AND RECOMMENDATION

DECLARATION

This thesis is my original work and has not been submitted or presented for examination in any institution.

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RECOMMENDATION

This thesis has been submitted with our approval as supervisors for examination according to Egerton University regulations.

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ABSTRACT

Maize lethal necrosis (MLN) - a big threat to maize production and food security in Kenya, is caused by co-infection of maize with *Maize chlorotic mottle virus* (MCMV) and *Sugarcane mosaic virus* (SCMV). In severely affected maize fields, MLN destroys the crop completely with a devastating impact on food security of smallholder households. Since the disease was only recently documented in Kenya, there is limited information on its pathogenesis due to SCMV and MCMV interaction, the effect of the disease on host plant, and consequently on insect vectors. Therefore, this study aimed to generate baseline information on critical biochemical changes in maize plants infected by viruses causing MLN and the consequence of some of these changes to insect vectors (thrips). This included determination of symptom progression and the rate of viral multiplication in single and dual infection by SCMV and/or MCMV and profiling of changes in volatiles from MCMV- and co-inoculated maize plants. Behavioral responses of selected thrips species towards headspace volatiles from MCMV and SCMV/MCMV co-inoculated maize plants were also determined. The experiments were conducted in insect-proof screenhouses and laboratories at *icipe*, Nairobi. Maize plants were artificially inoculated by respective viruses and disease severity scored on a scale of 1-5. Virus titers on inoculated plants were determined by Double Antibody Sandwich-Enzyme Linked Immunosorbent Assay (DAS-ELISA), followed by quantification using real-time Quantitative Reverse Transcription PCR (RT-qPCR). Volatiles were collected by headspace method using portable air entrainment kit and then chemically characterized. On the other hand, behavioral response assays of two species of thrips to the headspace volatiles from both infected and non-infected plants was carried out in a Four-arm olfactometer. All statistical analyses were done using R software version 3.2.3. Results indicate that disease severity in MCMV and SCMV co-infected plants increased concomitantly with MCMV accumulation. Coupled GC-mass spectrometry (GC-MS) analysis of the volatiles compounds showed that uninfected plants produced richer volatile profiles, mainly comprising terpenoids, C5-C6 alcohols, aromatic and aliphatic compounds than infected plants (both singly and co-infected). Additionally, MCMV inoculated plants emitted VOCs more attractive to *Frankliniella williamsi* and *Thrips tabaci*. The study provides new information on multi-trophic plant-MLN-vector interactions. Clear understanding of these interactions has the potential to improve integrated disease management strategies against MLN.

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LIST OF ABBREVIATIONS AND ACRONYMS

BBCH	Biologische Bundesanstalt, Bundessortenamt und CHemische Industrie
*c*DNA	Complementary deoxyribonucleic acid
CI	Cytoplasmic inclusion protein
CIMMYT	International Maize and Wheat Improvement Centre
CMV	*Cucumber mosaic virus*
CP	Coat protein
DAP	Days after planting
DAS-ELISA	Double antibody sandwich enzyme linked immunosorbent assay
DMNT	(E)-4,8-dimethyl-1,3,7-nonatriene
DPI	Days post-infection
DSMZ	Deutsche Sammlung Microorganismen Zellkulturen
FAO	Food and Agricultural Organization
HC-Pro	Helper component proteinase
IIAT	International Institute of Tropical Agriculture
MCMV	*Maize chlorotic mottle virus*
MDMV	*Maize dwarf mosaic virus*
MeSA	Methyl salicylate
MLN	Maize lethal necrosis
NIa	Small nuclear inclusion protein
NIb	Large nuclear inclusion protein
P3	Proteinase 2 (third protein)
PET	Polyethylene terephthalate

PI	Proteinase 1 (first protein)
PTFE	Polytetrafluroethylene
qRT-PCR	Quantitative reverse transcription Polymerase chain reaction
RM-ANOVA	Repeated Measures analysis of variance
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
SCMV	*Sugarcane mosaic virus*
SSA	Sub-Saharan Africa
TMTT	(E,E)-4,8,12-trimethyltrideca-1,3,7,11-tetraene
VOC	Volatile organic compound
WPI	Weeks post inoculation
WSMV	*Wheat streak mosaic virus*
ZYMV	*Zucchini yellow mosaic virus*

CHAPTER ONE

INTRODUCTION

1.1 Background information

Maize, *Zea mays* L., is third most grown cereal crop in the world and the most important staple food for more than 1.2 billion people in Latin America and sub-Saharan Africa, supplying 50% of the calorie intake in these regions (Oluwafemi *et al*., 2013). In the sub-Saharan region, particularly in eastern Africa, maize is mainly grown by millions of resource-constrained smallholder farmers especially in warm tropical and sub-tropical areas with high rainfall, since it requires warm soils to develop optimally (De Groote *et al*., 2013). Over 90 % of the Kenyan population depend on the crop directly or indirectly in terms of food, employment and income (Ouma and De Groote, 2011).

Maize production is limited by several biotic and abiotic factors which contribute significantly to yield losses estimated at 30% annually (Kainyu, 2014). Despite these losses, maize production in the country over the years has been on the increase until recently when maize lethal necrosis (MLN) (also known as corn lethal necrosis disease) was reported (Wangai *et al*., 2012b). The disease is believed to be the most devastating in Kenya and other maize growing areas in eastern Africa. It occurs as a result of co-infection with *Maize chlorotic mottle virus* (MCMV) and *Sugarcane mosaic virus* (SCMV) (Wangai *et al*., 2012b; Mahuku *et al*., 2015b). The disease was first reported in September 2011 in the Longisa Division of the Bomet County but has since spread to other maize growing counties in Kenya and a number of countries in eastern and central Africa and recently in Ecuador, South America (Isabirye and Rwomushana, 2016; Quito-Avila *et al*., 2016). According to Mahuku *et al*. (2015a), highly affected areas experience massive yield losses of over 90%, impacting negatively on total maize yield in SSA region.

Co-infection of maize with MCMV and SCMV synergistically intensifies systemic symptoms (as compared to single infection with any of the viruses) ranging from severe necrosis of newly emerging leaves, chlorotic mottling, stunting, premature aging and eventually death of the entire maize plant (Mahuku *et al*., 2015a).

The aggravated symptom expression in plants is usually associated with interference of important physiological processes such as RNA silencing and persistent virus-induced host gene down regulation; since virus infection of cells leads to a general inhibition of cellular macromolecular synthesis i.e the shut-off process (Havelda *et al*., 2008). In many synergies of MCMV with other potyviruses, the concentration of MCMV is always increased more than that of the potyvirus (Goldberg and Brakke, 1987;Vance, 1991). However, there are few other reports where the interaction was mutual or even accumulation of potyvirus observed (Scheets *et al*., 1998; Karyeija, *et al.,* 2000). The viruses causing MLN not only attack all maize varieties grown in Kenya, but also other crops in the family gramineae, such as sugarcane and finger millet (Kusia *et al*., 2015). As such, the disease poses a threat to the entire maize sub-sectors and other important cereal crops across the region that can be potentially affected by it.

Transmission of insect-vectored diseases involves complex interactions among pathogens, hosts and vectors. *Maize chlorotic mottle virus* and SCMV, like other viruses are capable of altering the biochemical and phenotypic properties of their hosts in ways that may influence the frequency and nature of interaction between maize plant and the virus vectors. Transmission of MCMV occurs mechanically, by insect vectors such as chrysomelidae beetles, corn rootworms, a number of thrips species and via seed at very low rates (Zhao *et al*., 2014) while SCMV is mainly transmitted by aphids (Hassan *et al.,* 2003). *Sugarcane mosaic virus,* first reported in Kenya in 1980 (Louie, 1980) and it has been present since then with no reports of MLN. It's until 2011 when MCMV and consequently MLN were reported (Wangai *et al*., 2012b) and later spread to other parts of the region (Mahuku *et al*., 2015b). Thus, we hypothesized that the interaction between maize, MCMV and thrips may have resulted in the widespread distribution of the virus and consequently MLN in the region in the short span of time. The transmission of these and other viruses may be largely promoted by infective vector preference for non-infected hosts while acquisition may be promoted by preference of non-infected vector for infected plants. These behavioral changes of the vectors when it comes to host selection and settlement are often mediated by the effect of the virus on the plant and vectors which are usually influenced by visual and olfactory cues such as leaf colour or/and volatile organic compounds from the plant host (Mauck *et al*., 2014). The volatile compounds play an important ecological role mediating a range of interactions including multi-trophic interactions such as: plant-plant, plant-microbe, plant-herbivore and plant-virus-insect interactions (Dudareva *et al*., 2006).

The increased incidence of viruses causing MLN, especially MCMV (Isabirye and Rwomushana, 2016) and their thrips vectors in the field, highlights the need for additional efforts toward elucidating MCMV/SCMV, host plants, and their vectors relationship which is central to MLN epidemiology. This is crucial in developing effective and sustainable mitigation strategies for virus control.

1.2 Statement of the problem

Maize lethal necrosis disease leads to an estimated yield loss of more than 90% in severely affected areas. Currently, there are different management options recommended and are being adopted by farmers in an attempt to mitigate the spread of MLN. These include foliar pesticide application in hot spots and neighbouring farms to reduce the vector population, thus protecting the rest of the crop; phytosanitation and keeping close surveillance on adjacent farms for any MLN outbreak. Despite these efforts, MLN is still spreading at an alarming rate in SSA region and farmers in the worst hit areas are shifting their attention from maize farming to other crops. This is negatively impacting on the already constrained maize production. Therefore, proper management strategies of MLN are needed, but prior to this, there is need for more information i.e a clear understanding of how the two viruses (SCMV and MCMV) interact as well as the interaction of insect vectors, host plant and the viruses. Hence, this study aimed at determining symptom progression in conjunction with the rate of viral replication in SCMV-, MCMV- or co-inoculated maize plants, behavioral responses of selected species of thrips (*Frankliniella williamsi* and *Thrips tabaci*) towards headspace volatiles from MCMV- or co-inoculated maize plants and lastly profiling and chemical characterization of VOCs from maize plants infected with MCMV and/or SCMV.

1.3 Objectives

1.3.1 General objective

To generate baseline information on the biochemical changes in maize plants infected with viruses causing maize lethal necrosis and their relevance for their insect vectors.

1.3.2 Specific objectives

1. To determine the symptom progression and the rate of viral multiplication in single and dual infection by SCMV, and/or MCMV under different concentrations.
2. To profile changes in volatile organic compounds from MCMV- and co-inoculated maize plants.
3. To determine behavioral responses of selected thrips species towards headspace volatiles from MCMV and MCMV/SCMV co-inoculated maize plants.

1.4 Hypotheses

1. There is no difference in symptom expression and the rate of viral multiplication in single and dual infection by SCMV and/or MCMV under different concentration ratios.
2. There is no difference in volatile organic compounds' profile of MCMV and MCMV/SCMV co-inoculated plants.
3. The behavioral responses of thrips towards headspace volatiles from MCMV and MCMV/SCMV co-inoculated maize plants are not variable.

1.5 Justification

Maize lethal necrosis is currently among the important diseases that constrain maize production in the Kenya. The disease is new in Africa hence there is little information available on its epidemiology and management. Due to its complexity, it's difficult to control using the available proposed management strategies and therefore, in order to prevent further spread of MLN in maize growing regions in the country and to mitigate the catastrophic effects on maize productivity, comprehensive management strategies need to be developed and implemented. However, development of effective management strategies requires that critical knowledge gaps in maize-pathogen-vector interactions be filled. Hence this study aimed to fill some of the knowledge gaps, firstly by studying the symptom progression and the rate of MCMV and SCMV replication under different concentration ratios. An understanding of the interaction of the two viruses and their effect on each other is needed for the establishment of viral pathogenesis, evolution and consequently for the development of efficient and stable control strategies. Secondly, the behavioural responses of thrips to headspace volatiles from MCMV and SCMV+MCMV infected plants and profile of volatile organic compounds (VOCs) produced in uninfected and MLN infected maize plants are unknown.

These behaviour modifying semiochemicals influence host-vector interaction and therefore, discovering the mechanisms mediating vectors, host plants and virus interaction will provide useful insights not only to better understand the vector ecology and MLN disease epidemiology but also ways of designing suitable disease mitigating strategies.

CHAPTER TWO

LITERATURE REVIEW

2.1 Maize production and constraints

Maize, *Zea mays* L. (Poaceae), is a major staple and cash crop for over 300 million people in Sub-Saharan Africa (SSA), mainly grown by small holder farmers covering production area of over 27 M ha (Sileshi *et al.*, 2010; Cairns *et al*., 2013). In Kenya over 90 % of the population depends on the crop directly or indirectly in terms of food, employment and income (Ouma and De Groote, 2011). The production of maize is limited by several biotic and abiotic factors, which may result in total or partial crop losses. Abiotic factors including: drought, low soil fertility, low rates of adoption of new technology such as use of certified seeds, contribute to low yields (Faruq, 2008; Karaya *et al*., 2012). On the other hand, biotic factors such as: insect pests especially maize stemborers (Kifr *et al*., 2002) and armyworms, weeds, diseases such as grey leaf spot, northern leaf blight, rusts, rots, smut and, maize streak also add to the losses (Martin *et al*., 2001).

Among the most important diseases in SSA currently is a viral disease termed as maize lethal necrosis, MLN (Mahuku, *et al*., 2015a). In Kenya and eastern Africa in general, MLN has been reported to occur as a result of co-infection of maize with *Maize chlorotic mottle virus* (MCMV) and *Sugarcane mosaic virus* (SCMV) (Wangai *et al.*, 2012b; Mahuku *et al*., 2015b). However, interaction of MCMV with any other cereal viruses in the *Potyviridae* group, mainly *Wheat streak mosaic virus* (WSMV) or *Maize dwarf mosaic virus* (MDMV) leads to MLN (Louie, 1980; Scheets *et al*., 1998). The co-infection of MCMV and SCMV synergistically leads to exacerbated symptoms that eventually cause premature plant death (Adams *et al*., 2013). In Kenya, the disease was first observed in September 2011 in the low altitude zones of Longisa division, Bomet County and later spread to other neighbouring maize growing counties (Wangai *et al*., 2012b; Makone *et al*., 2014). Following the spread in Kenya, MLN and its constituent viruses have been reported in Rwanda, Tanzania, Uganda and Democratic republic of Congo, South Sudan, Ethiopia (Mahuku *et al*., 2015b; Isabirye and Rwomushana, 2016). Maize lethal necrosis has been termed as a 'disease without boarders' and it's since been reported in Ecuador, South America (Quito-Avila *et al*., 2016).

2.2 Symptom expression of MLN

Among the diverse range of symptoms expressed by plants infected with MLN, leaf chlorosis, severe mottling and necrosis are the key symptoms associated with this disease. Thus, infected maize plants show chlorotic mottle on the leaves, usually starting from the base of the young leaves in the whorl and extending upwards toward the leaf tips (Plate 1a**),** mild to severe leaf mottling, dwarfing and premature aging of the plants (Plate 1b); necrosis of young leaves in the whorl before expansion leading to a 'dead heart' symptom and drying up of whole plant (Plate1c) (Wangai *et al*., 2012a).

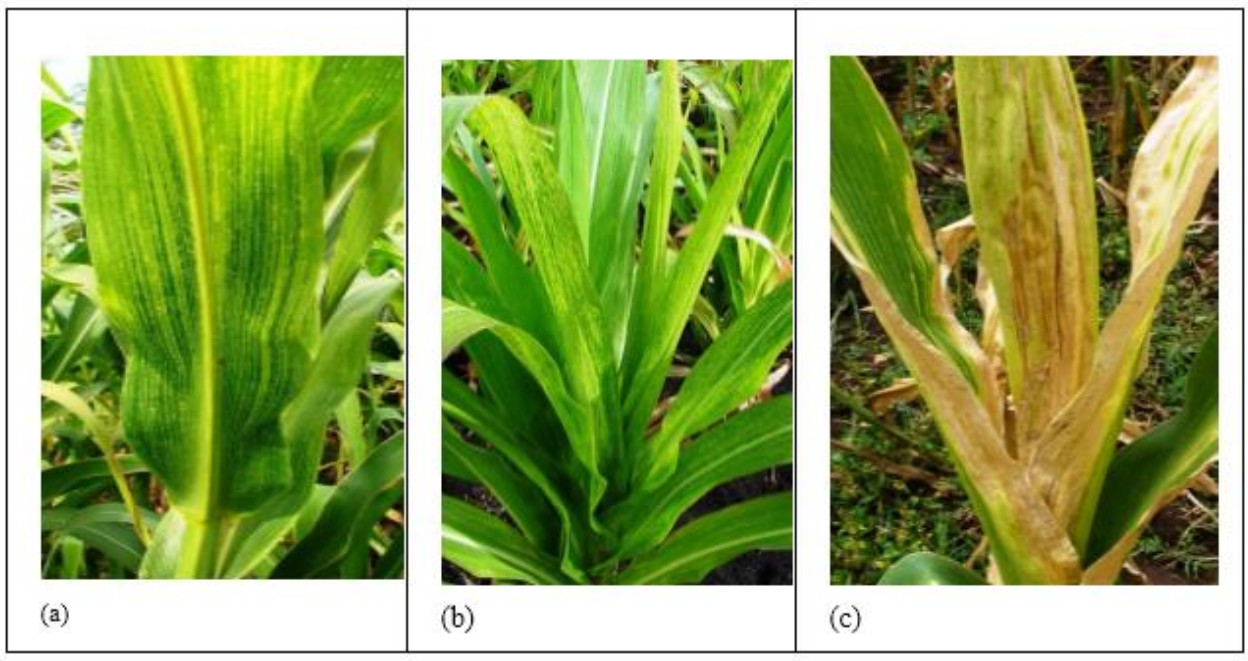

Plate 1: MLN symptoms in infected maize, (a) chlorotic mottle on the leaves (b) dwarfing and premature aging (c) 'dead heart' symptom (Wangai et al. (2012a).

Later during crop growth, chlorosis, necrosis with leaf reddening, discoloration of internodes and fungal symptoms such as brownish white moldy growth on rotting cobs are noted (Plate 2a). Ear bracts dry when the rest of plant is still green (Plate 2b). Partial grain or no grain filling (Plate 2c and d) and all infected plants may die giving blighted appearance of the crop. (Uyemoto 1981; Wangai *et al*., 2012a).

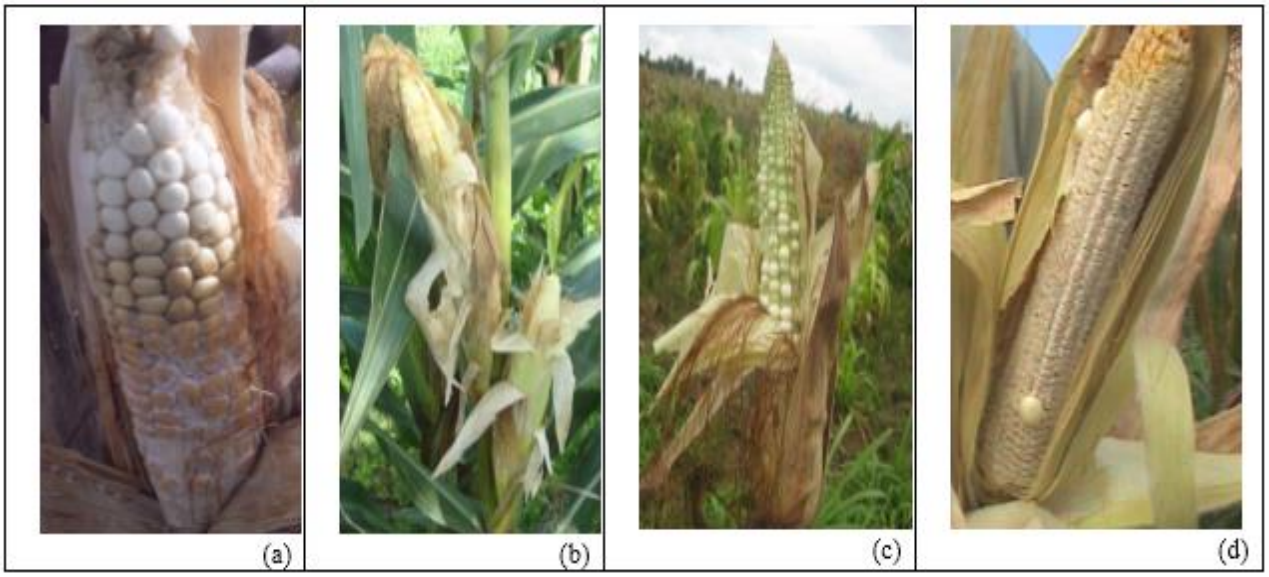

Plate 2: Symptoms of MLN on maize cobs; (a) brownish white mouldy growth (b) dry ear bracts (c) & (d) Partial or (d) no grain filling (Wangai *et al.* 2012a).

2.3 Viruses causing maize lethal necrosis

In Kenya and SSA in general, MLN has been reported to occur as a result of co-infection of maize with *Maize chlorotic mottle virus* (MCMV) and *Sugarcane mosaic virus* (SCMV) (Wangai *et al.,* 2012b; Mahuku *et al* 2015b). *Maize chlorotic mottle virus* (MCMV, the only member of genus *Machlomovirus,* family Tombusviridae) (Rochon *et al.,* 2011) has icosahedral virions of 30-nm in diameter containing a single stranded plus-sense RNA of approximately 4.4-kb (Scheets, 2004). The viral genome encodes six overlapping open reading frames (ORFs), five of which are required for replication and movement in the plant (Stenger and French, 2008). MCMV is restricted to the *Poaceae* family, maize being its main natural host though sorghum, wheat, sugarcane and finger millet, *Eleusine coracana* are also reservoirs of the virus (Wang *et al*., 2014; Kusia *et al*., 2015). The virus causes an array of symptoms in maize ranging from mild chlorotic mottling to severe mosaic and stunting, yellowing and necrosis, premature plant death, shortened male inflorescence with few spikes, or shortened, malformed, partially filled ears, depending on the plant's genetic background, its developmental stage at the time of infection, and prevailing environmental conditions (Mahuku *et al.*, 2015a). Yield losses range between 10 to 15% in natural infections and upto 59% inoculated maize plots (Uyemoto *et al.*, 1983).

The other virus that has been implicated in MLN manifestation is *Sugarcane mosaic virus.* SCMV is a member of the genus *Potyvirus*, family *Potyviridae* with flexuous, filamentous particles of about 12 by 750 nm long, which contain a single positive strand of RNA about 9.3 kb in size and has poly A tail and a genome with an open reading frame (ORF) encoding more than seven functional proteins (Adams *et al*., 2005). SCMV is closely related to *Maize dwarf mosaic virus* (MDMV) from the same genus *Potyvirus*, however, other members in this genus include *Johnsongrass mosaic virus* (JGMV) and *Sorghum mosaic virus* (SrMV) (McKern *et al*., 1991). The virus occurs wherever sugarcane is grown but can also infect corn, sorghum, wheat, rye, pearl and other Poaceae hosts (Hassan *et al*, 2003). In the mid-altitude regions of eastern Africa, SCMV is considered as an important plant virus and was first reported in Kenya in 1980 (Louie, 1980). Maize plants infected with SCMV show leaf mosaic, irregular chlorosis, mottle, striping, and a tendency to develop ring-like flecks symptoms. Susceptible varieties may show extreme distortion and stunted growth (Mahuku *et al*., 2015a).

2.4 Viral synergism in relation to MLN

Disease synergism refers to a situation in which mixed infection of a plant with two (usually unrelated) or more viruses results in increased multiplication of one or both viruses, and viral partners interacting with each other induce symptoms more severe than would be expected if they interacted in an additive manner (Syller, 2012). The synergistic interaction of MCMV+SCMV is what exacerbates symptoms hereafter leading to MLN. The mechanism of MCMV+SCMV synergistic interaction is yet to be fully understood however, there are different hypotheses that have been put forward. This could be due to a number of factors including higher concentrations of virus in each infected cell or due to infection of a higher proportion of cells than in a singly infected plant. Similarly, in a mixed infection one virus may facilitate the movement of the other virus from cell to cell and by so doing increasing the number of infected cells in dually infected plants (Taiwo *et al*., 2007). Another scenario is when some viruses for example potyviruses, encode for viral suppressor proteins that function to interfere and suppress RNA silencing mechanism that normally regulates the expression of endogenous genes and counteract invading nucleic acids, including viruses (Lewsey *et al*., 2010; Pallas and Garcia, 2011). Hereafter, the replication and pathogenicity of the pair virus increases.

Interaction of genetic elements of two heterologous viruses in one host may alter accumulation and pathogenicity of one or both of the co-infecting viruses thereby impacting on viral replication and disease robustness (Pruss *et al*., 1997). In most cases of synergism involving potyviruses, symptoms are more pronounced and the non-potyvirus is usually the beneficiary of the synergism, accumulating to higher titres when the potyvirus is also actively replicating in the tissue. In co-infections of MCMV with MDMV-B, WSMV or SCMV, MCMV shows a marked increase in concentration as compared to MCMV concentration in single infections (Goldberg and Brakke, 1987; Scheets, 1998; Xia *et al*., 2016). Therefore, in such a case, intensification of disease symptom is often hypothesized to be due to among other factors, accumulation of non-potyvirus component (Goldberg and Brakke, 1987; Vance, 1991; Taiwo *et al*., 2007). However, not all potyvirus related synergism follow this pattern since viral synergistic interactions is host specific. Mutual interaction of both members and also enhancement of portyvirus has been reported. For example, in *Maize chlorotic mottle virus* (MCMV)/ *Wheat streak mosaic virus* (WSMV) synergism, interaction was described to be mutually beneficial to both members of synergistic viral pair (Scheets *et al*., 1998; Tatineni *et al*., 2010).

2.5 Transmission of viruses causing MLN

Maize lethal necrosis causing viruses have been reported to be transmitted through various means including: insect vectors, mechanically and by seed, but at very low rate. The largest class of plant virus-transmitting vectors are insects but other vectors include mites, nematodes and chytrid fungi, which transmit viruses through different modes. These modes of viral transmission by vectors include non-persistent, semi-persistent and persistent, whereby the transmission window to disseminate the virus to a new host plant after feeding on an infected plant by the vector lasts from seconds to minutes, hours to days, or days to weeks, respectively (Dietzgen *et al*., 2016).

MCMV is transmitted mechanically and in a semi-persistent manner by a number of vectors including: Chrysomelidae beetles, and corn rootworms (*Diabrotica sp*) (Nelson *et al*., 2011), corn thrips, *Frankliniella williamsi* (Cabanas *et al*., 2013) and western flower thrips, *Frankliniella occidentalis* (Zhao *et al*., 2014). In eastern Africa, onion thrips (*Thrips tabaci*) and pale form of common blossom thrips (*Frankliniella schultzei*) are also known to transmit MCMV (Mahuku *et al*., 2015a; Nyasani *et al*., 2015).

The virus can also be transmitted through seed at rate of 0 to 0.33%, soil and infected plant debris since the virus can survive in plant residues for some time (Mahuku *et al*., 2015a). Therefore, continuous maize production in a field greatly increases the incidence of the viruses and vectors.

On the other hand, SCMV is transmitted in a non-persistent manner by several species of aphids including *Rhopalosiphum maidis, R. padi, Myzus persicae, Schizaphis graminum*, and *Aphis craccivora* (Hassan *et al*, 2003; Brault, 2010). The virus can also be seed transmitted with transmission rates of 0.4 to 4.8% depending on the maize plant genotype (Li *et al*., 2007).

2.6 Effects of virus infection on plant host and vectors

Biochemical effects of virus infection are not only confined within the host but also have been reported to modify the behaviour of the potential vectors. When plant viruses infect susceptible plant host cells, they exploit host resources. Their viral components, host proteins and other cellular structures directly interact and they multiply in the infected plant cells (Pallas and Garcia, 2011). Consequently, there are alterations of the whole-plant and leaf morphology, metabolite profiles and plant responses to biotic and abiotic stresses (Xu *et al*., 2008). Many plant defence pathways can also be activated or suppressed by virus infection, depending on the compatibility of the virus–host interactions (Lewsey *et al*., 2010). Virus infection has furthermore been shown to influence the production of the key plant hormone ethylene (Chaudhry *et al*., 1998) and may also have effects on precursors to defence pathways, such as membrane fatty acids, through modulation of organelle and plastid membranes to become virus replication scaffolds (Whitham and Wang, 2004).

Some of biochemical changes due to vector-borne pathogens are capable of altering the phenotypes of the host plants in ways that influence the frequency and nature of interactions between hosts and vectors. Studies by Mauck *et al*. (2014) showed that *Cucumber mosaic virus*, CMV (affects cultivated squash, *Cucurbita pepo*) infection disrupts levels of carbohydrates and amino acids in leaf tissue (where aphids initially probe plants and acquire virions) and in the phloem (where long-term feeding occurs) in ways that reduce plant quality for aphids. CMV infection has also been shown to cause constitutive up-regulation of salicylic acid, alters herbivore-induced jasmonic acid biosynthesis as well as the sensitivity of downstream defenses to jasmonic acid and elevates ethylene emissions and free fatty acid precursors of volatiles.

Plant virus can also directly alter host selection and feeding behavior by its insect vector. Stafford et al. (2011) demonstrated that Tomato spotted wilt virus (TSWV) infected male thrips spent more time feeding than that of non-infected thrips. This attraction of vectors to infected plants is often mediated by both visual and olfactory cues such as leaf colour or emission of volatile organic compounds (VOCs) (Mauck *et al.*, 2014). However, VOCs emitted from virus-infected plants, differ from healthy plants, and are important mediators in the attraction and decision-making of insect vectors when selecting a host plant (De Vos and Jander, 2010). Generally, Viruses may induce changes in plant hosts and vectors to enhance their transmission.

2.7 Plant semiochemicals and their utilization in pest management

The influence that plant semiochemicals have on the behaviour of receptor organisms can be exploited from the perspective of their potential in a sustainable pest management. Semiochemicals also known as behaviour-modifying chemicals, are natural organic compounds that transmit chemical messages from one organism to another (Maffei *et al*., 2011). They are emitted by one individual and cause a behavioral response in another without having direct effect on physiology of the receiving organism other than interacting with sensory systems (Howse *et al*., 1998). The emission of these substances is not necessarily related to abiotic or biotic environmental stress, since intact plants, which are under no stress constitutively emit chemicals (McAuslane and Alborn, 1998). These chemicals, produced from flowers, leaves, stem or roots can be volatile or non-volatile organic compounds (Knudsen *et al*., 1993). Volatile organic compounds (VOCs) are synthesised and emitted by plants from vegetative plant parts, flowers as well as roots and they are perceived through olfaction while non-volatile ones are perceived through contact chemoreception (Steeghs *et al*., 2004; Rasmann *et al*., 2005).

There exists variability in the quality and quantity of volatiles emitted between and amongst plant genera. Some volatiles are commonly produced by all species while others are specific to only one or a few related taxa (Pichersky and Gershenzon, 2002). This includes a large variety of different terpenes, fatty acid derivatives, benzenoids, phenylpropanoids, and amino-acid-derived metabolites (Pichersky and Gershenzon 2002, Pichersky *et al*., 2006).

These volatile chemicals play an important ecological role, mediating a range of multi-trophic interactions such as plant-plant, plant-microbe, plant-herbivore and inter-trophic interactions between the plant and organisms acting beneficially for the plant like pollinators and herbivores' natural enemies (Langenheim, 1994; Dudareva *et al*., 2004). Similarly, organisms including herbivores in these inter-trophic interactions are able to detect specific semiochemicals or specific ratios of these semiochemicals, which induce behavioural modification of the perceiving herbivores, for example attraction or repellency (Pickett *et al*., 2006; Bruce and Pickett, 2011).

The chemical composition of plant-emitted volatile blends and their intensity can carry information about the plants' nutritional and physiological status, and the stresses they have been subjected to (Dudareva *et al*., 2006). This is because volatile blend from intact, herbivore attacked or pathogen infected plants are all qualitatively and quantitatively different (Dudareva *et al*., 2006; De Vos and Jander 2010). For example; Green leaf volatiles are indicative of any mechanical damage and could provide early signals to receiving plants while Methyl salicylate (MeSA) in leaf tissue plays a role similar to that of gaseous MeSA in the pathogen-induced defense response and in response to aphid feeding damage (Mann *et al*., 2012). Methyl salicylate is also emitted by tobacco in response to infection by the *Tobacco mosaic virus* (Shulaev *et al*., 1997; Vlot *et al*., 2008). Thus, volatile cues induced by infection with vector-transmitted viruses can have great influence on virus lifecycle as well as virus-host-vector interaction. For these reasons, researchers have utilized these behaviour modifying semiochemicals released by intact plants to develop habitat diversification crop protection strategies against injurious herbivorous pests, some being vectors of phytopathogens. Such novel crop protection approaches include the stimulo-deterrent diversionary or "push-pull" strategy for cereal stemborer control in East Africa (Pickett and Khan, 2016).

The use of semiochemicals in pest control is usually encouraged because of the environmental benefits associated with them, in contrast to conventional insecticides. Semiochemicals such as pheromones and kairomones influence insect behaviour and for that reason, they have been employed to control thrips.

Pheromones can be divided in sex, aggregation and alarm pheromones and they attract the opposite sex, both sexes and causes avoidance or dispersal in conspecifics, respectively (Cook *et al*., 2006). Kairomones on the other hand are produced and emitted by the organisms to mediate interspecific interactions which benefit the receiver but not the emitter (Cook *et al*., 2006). For example, in controlling *F. occidentalis*, alarm pheromone decyl and dodecyl acetate was shown to reduce oviposition (Teerling *et al*., 1993), induce larvae to fall from plants, increase take-off and decrease landing rates (MacDonald *et al*., 2002). The aggregation pheromone has also been added to blue sticky traps which showed an increasing capture of *F. occidentalis* up to 3 times compared to traps without the pheromone (Davidson *et al*., 2007). Therefore, combination of both mechanical and biological control such as parasitoids, together with use of semiochemicals can be applied in crop protection or defence strategies against thrips vectors of MCMV. However, for efficient use of semiochemicals, olfaction information by the vectors is needed.

CHAPTER THREE

Symptom Progression and Viral Quantification in *Sugarcane mosaic virus* (SCMV)-, *Maize chlorotic mottle virus* (MCMV) – and Co-infected Maize Plants

3.1 Introduction

Co-infection of maize plants with *Maize chlorotic mottle virus* (MCMV) and *Sugarcane mosaic virus* (SCMV) synergistically intensifies systemic symptoms as compared to single infection of individual virus, leading to premature aging and eventually death of the entire maize plant (Mahuku *et al*., 2015a). This symptom development is correlated with plant growth stage, the age of plants at the time of infection and environmental conditions (Scheets, 2004; Makumbi and Wangai, 2013). The aggravated symptom development in co-infected plants is hypothesized to be as a result of a number of factors including arm race between plants RNA silencing defence mechanism and activity of viral suppressors of RNA silencing (VSR) (Waterhouse *et al*., 2001). In a number of synergistic interaction of MCMV with other potyviruses, the concentration of MCMV has always been shown to increase more than that of the potyvirus (Goldberg and Brakke, 1987; Vance,1991; Xia *et al*., 2016). However, synergy patterns may vary depending on virus strain, host species and even host plat cultivar (Taiwo *et al*., 2007; Wintermantel *et al*., 2008). There are also few reports where the interaction was mutual or even accumulation of portyvirus was observed (Scheets *et al*., 1998; Karyeija., 2000) but there is no information on the degree of synergism of MCMV and SCMV when the starting (inoculum) concentration ratios are different.

Therefore, to fully examine the relationship between virus concentration and symptom progression (severity), as well as how interactions between viruses co-infecting common host plants may influence virus emergence and dominance in an infected plant, we directly compared titre levels of MCMV and SCMV with symptom progression in maize hosts during single and mixed infections under different starting concentration ratios. We determined virus concentrations in single and double infections using both ELISA and qRT-PCR. A clear understanding plant-MLN viruses interactions may be of crucial significance for the understanding the pathogenesis of MLN and consequently provide a technical platform for development of efficient and stable antiviral strategies in maize plants.

3.2 Materials and methods

3.2.1 Plants and virus culture

Maize seeds (H6210) were planted in pots (21cm in height, 20 cm diameter) with sterile soil and then the uninfected (healthy) seedlings for raising a culture of either SCMV or MCMV maintained in three separate insect-proof screenhouses. Maize leaves from plants previously confirmed by RT-PCR to harbor only SCMV or MCMV were used in inoculum preparation for artificial inoculation of the healthy maize seedlings. A culture of either virus was raised in separate screenhouses. The first, second and third screenhouses had healthy maize plants inoculated with SCMV, MCMV and SCMV+MCMV, respectively. A fourth screenhouse had uninfected maize plants, which acted as controls. To ensure purity of the viruses, random samples of plants were tested for the presence of major maize viruses using antibodies from Deutsche Sammlung Microorganismen Zellkulturen (DSMZ), Germany.

3.2.2 Virus inoculum preparation and inoculation

Maize leaves from plants raised for the different viruses were harvested, placed in sample bags and transported to the laboratory for blending. The infected maize leaves were weighed and placed in a kitchen blender and consequently homogenized (1:10, wt/vol) with 0.01 M potassium phosphate buffer, pH 7.0. The leaves were blended for 5 minutes and the crude extract from the blended leaf tissue was sieved into a sterile beaker to serve as virus inoculum. To prepare mixed inoculum, the homogenate inocula of MCMV and SCMV were mixed in four different ratios of MCMV: SCMV (V/V) as follows; 1:1, 1:3, 1:5 and 1:7. The virus inocula i.e MCMV, SCMV and MCMV+SCMV (Four different ratios) generated were then used to artificially inoculate the first two primary leaves of the test plants, 14 days after planting (at phenological developmental stage 1 (BBCH)), as described by Taiwo *et al.* (2007). The plants were inoculated by rubbing the inoculum homogenate using sterile inoculum-soaked cheesecloth pads onto carborundum-dusted leaves. Control plants were inoculated with phosphate buffer. In all experiments, each treatment had 10 replicates arranged in a completely randomized design. Known Kenyan virus isolates of MCMV and SCMV were used in these experiments (Wangai *et al.*, 2012b). Samples for ELISA and RT-qPCR were collected from the first systemically infected leaves weekly starting from 7 days post inoculation (dpi).

3.2.3 Assessment of disease severity

Disease symptom development on inoculated plants was observed on a daily basis for six weeks and observations made were recorded as well as photographs of infected plants taken. Disease severity was rated on a scale of 1 to 5 (Makumbi and Wangai, 2013): (scale 1=No observable symptoms, the plant still green; scale 2= ~25% of plant leaves showing mild mottling and chlorotic spots; scale 3= ~50% of the leaves showing mild mottling, necrosis and yellowing of the leaves; scale 4= ~75% leaves showing severe mottling and necrosis/leaf turned yellow; scale 5 = ~100% of leaves showing severe mottling and necrosis and the entire plant has begun drying up. Local lesions were distinguished from systemic symptoms and the disease severity means at each week were used to generate disease progression curves.

3.2.4 Determination of virus titres

Virus titers on the test plants were determined by Double Antibody Sandwich- Enzyme linked Immunosorbent Assay (DAS-ELISA) using standard methods modified from Clark and Adams (1977) using antisera raised to MCMV and SCMV (DSMZ, Germany). Briefly, purified IgG was diluted in coating buffer (recommended dilution) then 100µl added to each well of a microtitre plate. The plate was incubated at 37^{o}c for 2-4 hours then washed with PBS-Tween using wash bottle, soaked for a few minutes and washing repeated two times. The plate was blotted by tapping upside down on a paper towel. After washing, 100µl aliquots of the test sample (extracted in sample extraction buffer) were added to duplicate wells, then incubated overnight at 4^{o}c. The plate was washed three times, 100µl anti-virus conjugate in conjugate buffer added to each was and incubated at 37^{o}c for 2-4 hours. After incubation, the plate was washed three times, 100 µl aliquots of freshly prepared substrate (10mg p-nitrophenyl phosphate [Sigma, Fluka] dissolved in 10 ml of substrate buffer) added to each well and then incubated at room temperature for 30-60 minutes, or as long as necessary to obtain clear reactions. The absorbance of the colour developed was read at 405 nm in an ELISA reader EPOCH™ microplate spectrophotometer (Chaves-Bedoya *et al*., 2011). Positive controls of SCMV or MCMV were used in each test. The plates were loaded in duplicate and samples considered positive when the absorbance value (at 405 nm) was at least twice that of the mean for the negative controls.

3.2.5 Total RNA extraction and cDNA synthesis.

Total RNA was extracted with RNeasy Plant Mini Kit™ (Qiagen, Germany) according to manufacturer's instructions. The concentration of each RNA samples was measured using a NanoDrop2000/2000c Spectrophotometer (Thermo Scientific); only the RNA samples with a 260/280 ratio between 1.9 and 2.1 and a 260/230 ratio greater than 2.0 were used for further analysis. The integrity of the RNA samples was assessed by 1.2% (w/v) agarose gel electrophoresis with Ethidium bromide staining. Before use in RT-PCR reactions, 1µg sample RNA was treated with RNAse-free DNAse I (Bioline) at 37°C for 30 min to remove any potential contaminating genomic DNA. The RNA was incubated at 65°C for 10 min to inactivate DNAse I and then the samples were used as template for first-strand cDNA synthesis (with random heximer primers), using a high capacity cDNA reverse transcription kit (Applied Biosystems), in a total volume of 10 µl. Five biological replicates per treatment were used for qRT-PCR to measure virus (RNA).

3.2.6 Quantitative Real-time RT-PCR (qRT-PCR).

Primers for MCMV, SCMV and 18S rRNA as reference gene were used for relative quantification of SCMV and MCMV by qRT-PCR (Table 1). Primer validation experiments to demonstrate that the amplification efficiencies of target primers (MCMV and SCMV) were approximately equal to the efficiency of the endogenous reference primer (18S rRNA) were performed. Briefly, qRT-PCR was done with fivefold serial dilutions of a cDNA sample using MCMV, SCMV and 18S rRNA primers. Once the respective quantification cycle, Cq (also known as threshold cycle (Ct) were obtained at different dilutions, the ΔCq (Cq of target minus Cq of reference) was calculated and plotted against the logarithmic value of input cDNA concentrations (Bajaji *et al*., 2003).

Using the Stratagene Mx3005p Sequence Detection system (Aqilent technologies), qRT-PCR reaction was done in a total volume of 12.5 µl that contained 6.25µl Maxima SYBR Green/ROX qPCR Master Mix (2X) (Applied Biosystems), 1µl primer pair mix of 10µM primer pair stock (Table 1), and sterile nuclease-free water to a final volume of 12.0µl. Finally, 0.5µl diluted (1:5) cDNA was added to this mixture. Negative controls consisted of sterile water replacing the template (no template control, NTC) and no reverse transcriptase (NRT) samples.

Table 1: Primer name and sequences for RT-qPCR

Target	Primer sequence (5'-3')	Amplicon size (bp)	Source
18S rRNA	Forward: GAT TCC GGT CCT ATT GTG TTG	125	Margaret G. Redinbaugh
	Reverse: TTT CGC AGT TGT TCG TCT TT		
SCMV-Nib	Forward: CCA GGC CAA CTT GTA ACA AAG C	76	(Adams *et al*., 2012)
	Reverse: CAT CAT GTG TGG ATA AAT ACA GTT GAA		
MCMV-CP	Forward: CCG GTC TAC CCG AGG TAG AAA	68	(Adams *et al.*, 2012)
	Reverse: TGG CTC GAA TAG CTC TGG ATT T		

Abbreviations: 18S rRNA, 18S ribosomal RNA; SCMV-Nib, Nuclear inclusion b (larger) protein of SCMV; MCMV-CP, Coat protein of MCMV.

The PCR reaction was initiated by denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15s, 50°C for 30s annealing and extension at 72°C for 30s/cycle. Immediately after the final PCR cycle, a melting curve analysis was done to determine primer specificity by incubating the reaction at 95°C for 15 s, annealing at 50°C for 20 s, and then slowly increasing the temperature to 95°C over 20 min. The Cq used in the qRT-PCR quantification is defined as the PCR cycle number that crosses a user or instrument chosen signal threshold in the log phase of the amplification curve. The Cq is inversely proportional to the log of initial copy number. Each cDNA was loaded in triplicate in an Optical 8-Tube Strips (0.2 ml, MicroAmp, Applied Biosystems). Relative quantification was measured using the comparative Ct ($2^{-\Delta\Delta Ct}$) method (Livak and Schmittgen, 2001). Here, the change in amount of the target RNAs (SCMV and MCMV) was normalized to the expression of the 18S rRNA gene. The $2^{-\Delta\Delta Cq}$ data analysis is where ΔΔCq = (Cq of test sample – Cq of 18S rRNA) – (Cq of calibrator sample – Cq of 18S rRNA).

In our experiments, calibrator sample was the experimental control samples for each week. Therefore, the expression of the target RNA in all the samples was expressed as increase or decrease relative to that of the calibrator.

3.3 Data analyses

Statistical analyses were performed using R statistical software, version 3.2.3 (R Core Team, 2015). Data on disease severity and titre levels by ELISA over time were analysed using repeated measures analysis of variance (RM-ANOVA), after subjecting the data to Shapiro-Wilk normality test (Logan, 2010). Pairwise comparison of means was performed using holm's paired t-test method. The mean disease severity for each week was used to generate disease progression curves using GraphPad Prism version 7 for Windows, GraphPad Software, La Jolla California USA (www.graphpad.com). Relative quantification by qRT-PCR was determined using the comparative Cq ($2^{-\Delta\Delta Cq}$) method (Livak and Schmittgen, 2001). The statistical significance of qRT-PCR changes in viral quantities over time in the four different ratios were determined by RM-ANOVA. All tests were performed at 5% significance level.

3.4 Results

3.4.1 Severity of MLN as influenced by different concentration ratios of MCMV and SCMV.

There was a significant interaction between treatment and time post inoculation on severity in both trials, by RM-ANOVA (Trial 1:$F_{25, 270} = 8.384$, $P<0.001$; Trial 2: $F_{25, 270} = 6.37$, $P<0.001$). Symptom expression in maize co-inoculated with MCMV and SCMV in 1:1, 1:3, 1:5 and 1:7 mixture ratios was 1.38, 1.50, 1.50 and 1.58 times higher, respectively in trial one compared to single inoculation with MCMV (Figure 1A). Similarly, in trial two symptom expression in mixed infections was 1.43-1.55 times higher compared to single inoculation with MCMV (Figure 1B). Symptom expression in co-inoculated maize plants was 1.33-1.53 times higher in trial one (Figure 1A) and 1.38-1.49 times higher in trial two (Figure 1B) as compared to single infection with SCMV.

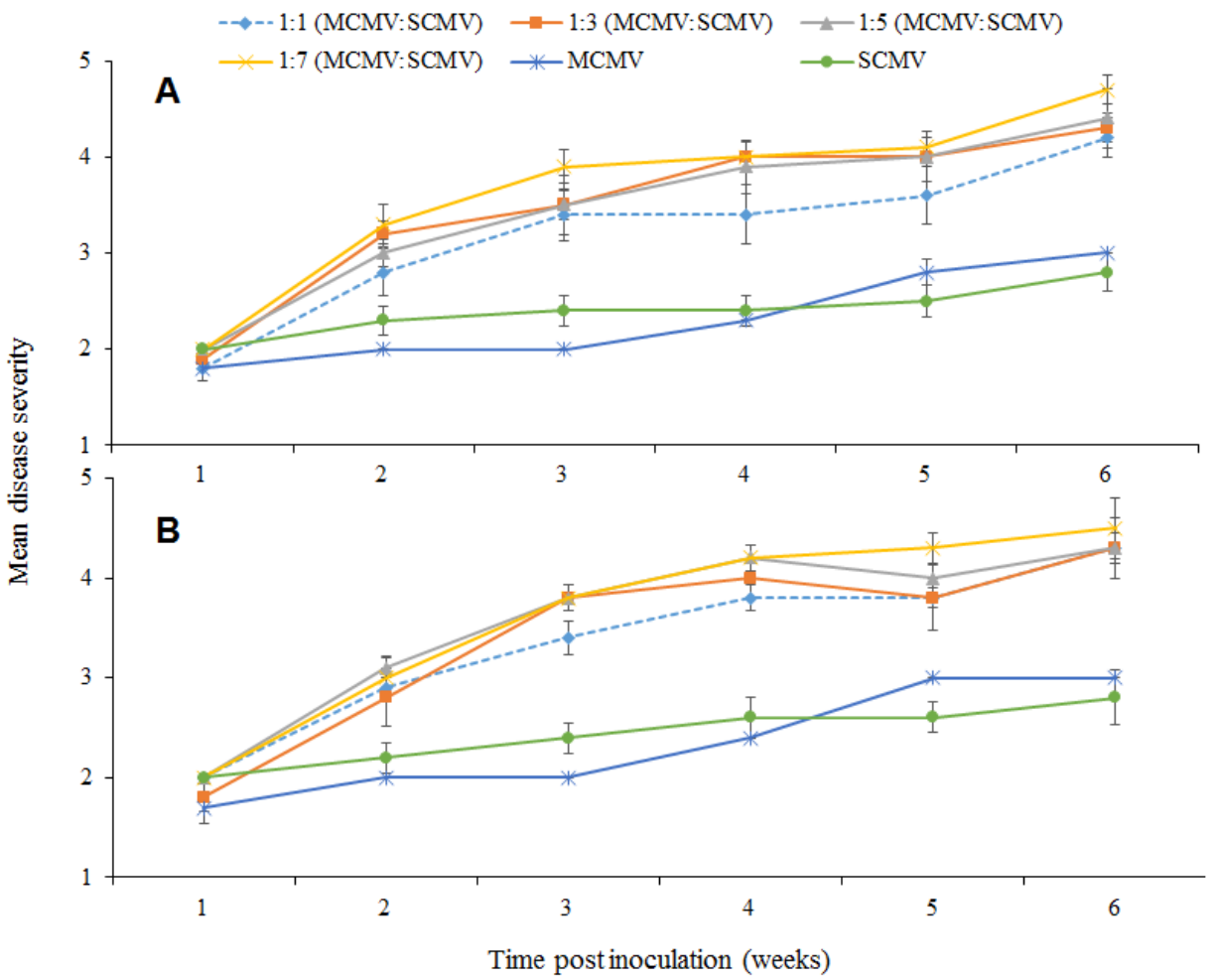

Figure 1: Disease severity on maize plants as influenced by different treatments of *Sugarcane mosaic virus* (SCMV) and *Maize chlorotic mottle virus* (MCMV) over time in trials one (A) and two (B). A 1-5 scale was used where; 1= absence of symptoms; 2 = ~ 25% of the leaves showing mild mottling, chlorotic spots; 3 = ~50% of the leaves showing mild mottling, necrosis and yellowing of the leaves; 4 = ~75% of the leaves showing mottling, necrosis and yellowing of the leaves; 5 = 100% leaves showing severe mottling and necrosis with symptom and the entire plant has begun drying up.

Symptom expression in co-inoculated and SCMV singly inoculated plants started showing by 5-7 dpi while in MCMV singly inoculated plants, symptoms were visible by 7-9 dpi (Figure 2). The systemic symptoms induced by MCMV at this stage were milder compared to those induced by SCMV and all the four ratios of MCMV/SCMV. These symptoms included irregular chlorosis and mottling spots.

However, there were no distinguishable symptoms in dually inoculated plants and those plants inoculated with SCMV alone in first week post inoculation (wpi). The symptoms became more pronounced in co-inoculated plants as the time progressed. The chlorotic mottles later joined to form stippling and streaks on the leaves, with necrosis on the tips and margins of the newly forming leaves in the whorl. At 4 wpi, all dually inoculated plants (in all four ratios) had a severity greater than three with necrosis on more than 50% of the leaves. The plants were generally stunted, with most leaves becoming brittle and had started drying up and falling off (Figure 2).

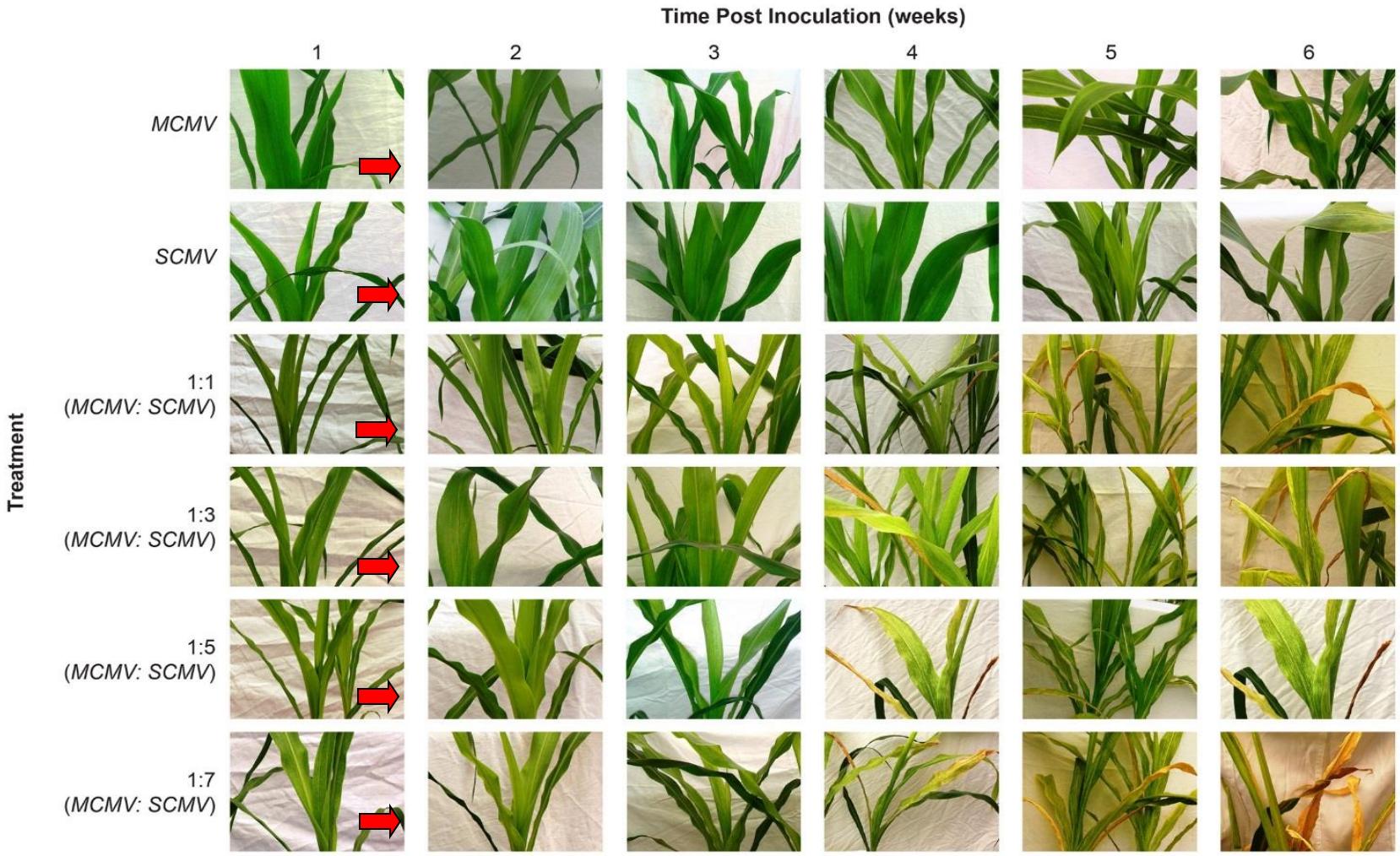

Figure 2: Disease symptom progression in maize plants inoculated with MCMV, SCMV and four ratios of MCMV: SCMV (1:1, 1:3, 1:5 and 1:7). The arrows (➡) indicate onset of symptom expression in various treatments.

3.4.2 Titre levels in SCMV-, MCMV- and co-inoculated plants

There was no significant interaction between treatments and time post inoculation on SCMV titre concentration in both trials, by RM-ANOVA (Trial 1: $F_{20, 225} = 0.47$, $P = 0.975$; Trial 2: $F_{20, 225} = 1.102$, $P = 0.348$). Titre concentrations of SCMV in maize co-inoculated with MCMV and SCMV in 1:1, 1:3, 1:5 and 1:7 mixture ratios was 0.82, 0.89, 0.79 and 0.72 times higher respectively in trial one compared to single inoculation with SCMV (Figure 3A). Similarly, in trial two titre concentration in mixed infections was 0.90-0.91 times higher compared to single inoculation with SCMV (Figure 3B). Therefore, the titre levels of SCMV over time were not significantly different in various treatments in the two trials (Trial 1: $F_{4, 45} = 1$, $P = 0.418$; Trial 2: $F_{4, 45} = 1$, $P = 0.837$). That's, in both trials, increase in titre concentrations of SCMV in all doubly inoculated maize with MCMV+SCMV in 1:1, 1:3, 1:5 and 1:7 mixture ratios was not significantly different to the titre levels of SCMV in singly inoculated plants 6 weeks post inoculation (Figure 3).

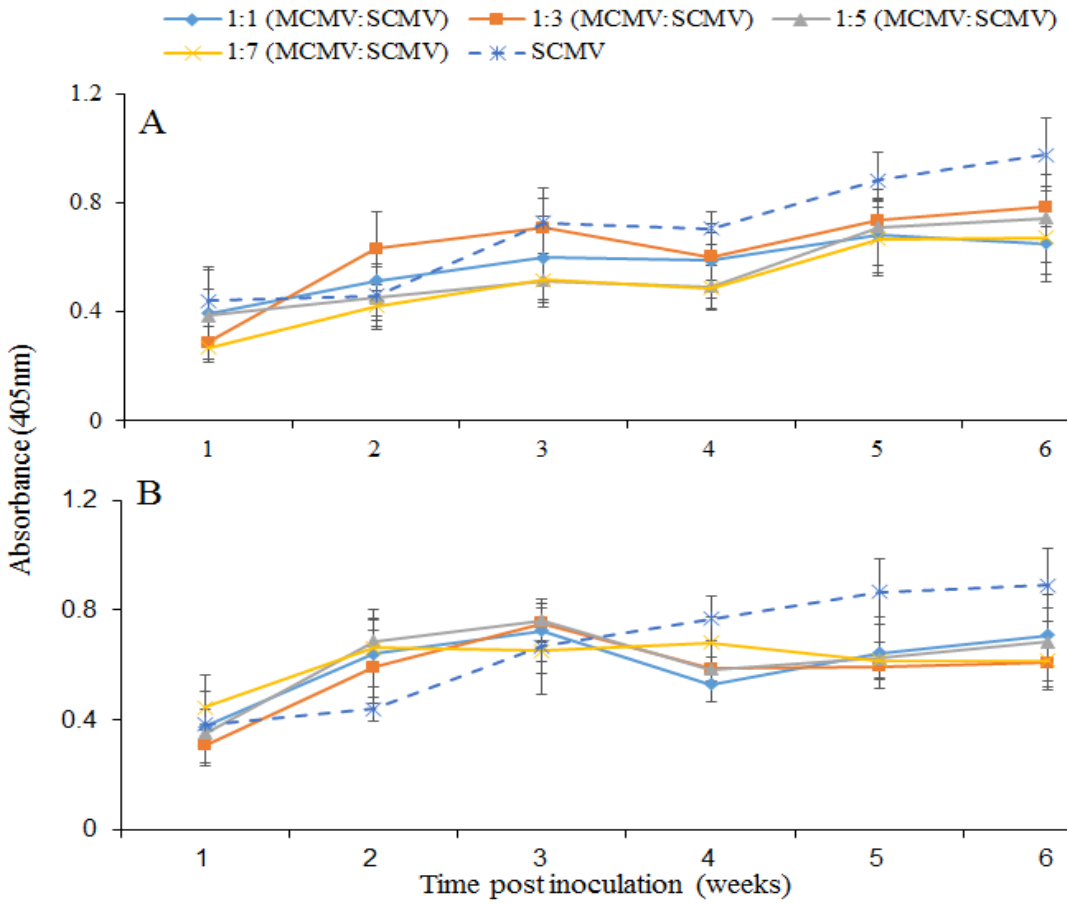

Figure 3: Titre concentrations of SCMV over time as influenced by different treatments in trials one (A) and two (B).

There was a significant interaction between treatments and time on MCMV titre concentration in both trials (Trial 1: $F_{20, 225} = 0.972$, $P < 0.001$; Trial 2: $F_{20, 225} = 2.044$, $P < 0.01$). Titre concentrations of MCMV in maize co-inoculated with MCMV and SCMV in 1:1, 1:3, 1:5 and 1:7 mixture ratios was 1.41, 1.15, 1.12 and 1.06 times higher respectively in trial one compared to single inoculation with MCMV (Figure 4A). Similarly, in trial two titre concentration in mixed infections was 1.31-1.03 times higher compared to single inoculation with MCMV (Figure 4B). Titre concentrations of MCMV in doubly inoculated plants with MCMV+SCMV in 1:1, 1:3, 1:5 and 1:7 mixture ratios in both trials increased significantly with time compared to the increase of MCMV in singly inoculated plants (Figure 4).

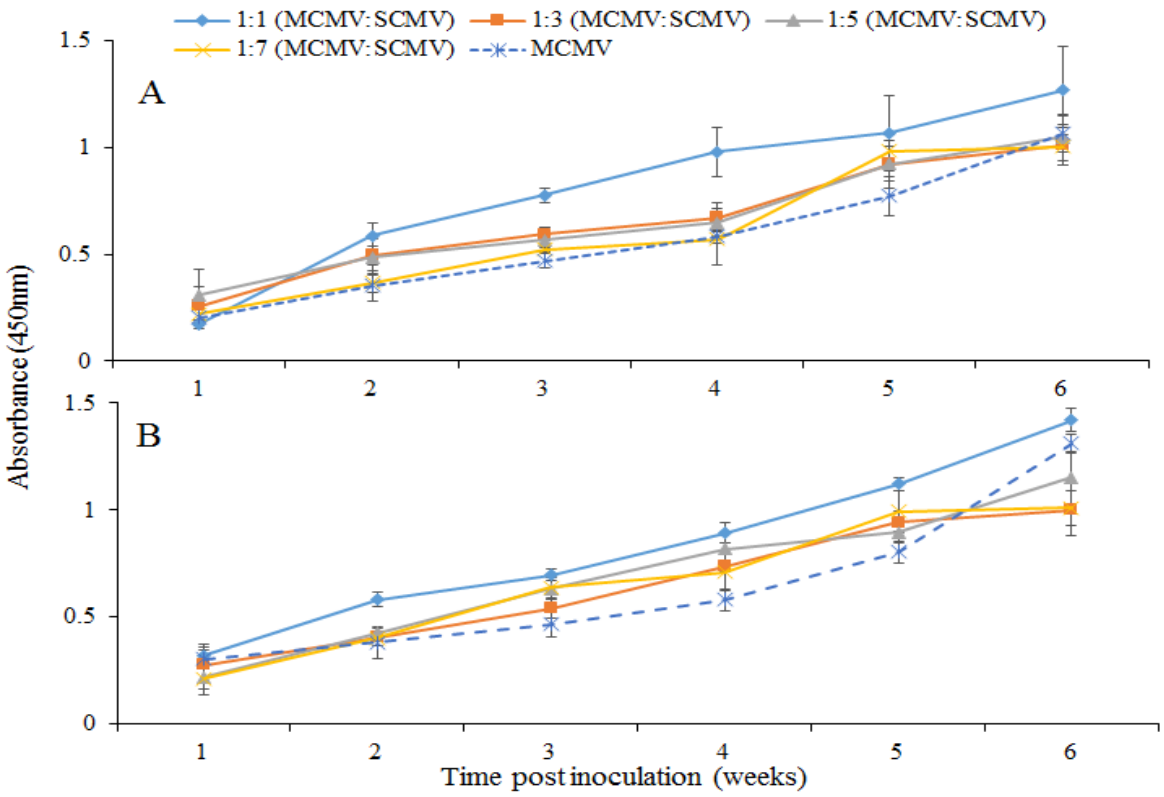

Figure 4: Titre concentrations of MCMV over time as influenced by different treatments in trials one (A) and two (B).

3.4.3 Quantification of SCMV and MCMV titre levels by qRT-PCR.

3.4.3.1 Amplification efficiency and specificity of SCMV and MCMV primers

Utilizing the comparative Cq ($2^{-\Delta\Delta Cq}$) method for relative quantification of viral RNAs required that the SCMV and MCMV primers be validated with respect to the endogenous control primer, 18S rRNA (Livak and Schmittgen, 2001). The absolute value of the slope of the plot for each primer set was determined to be less than 0.1 (Figure 5A and B), which indicated that the amplification efficiencies of MCMV, SCMV, and 18S rRNA primers were approximately equal.

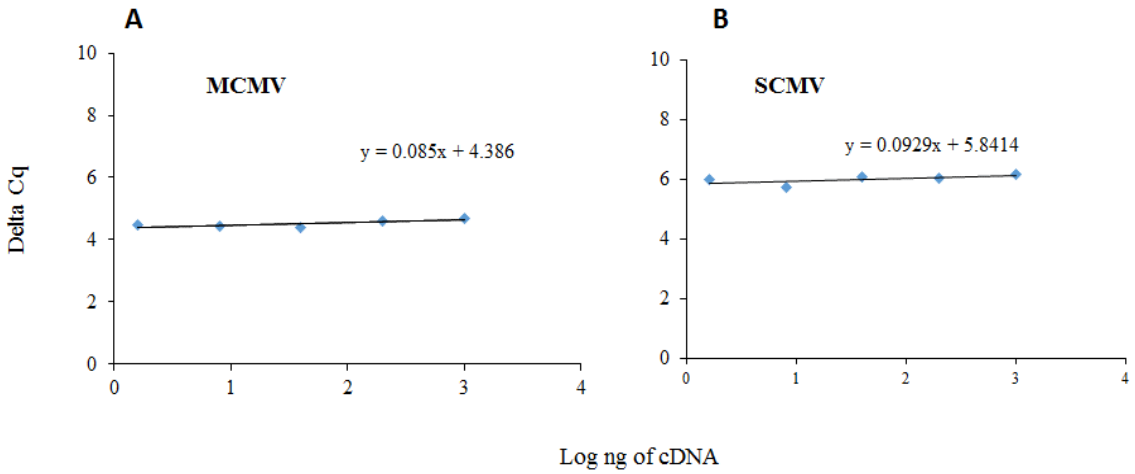

Figure 5: Primer efficiency validation curves. Primer efficiency validation was determined using fivefold serial dilutions of cDNA that were amplified by qRT-PCR using MCMV (A) and SCMV (B) specific primers. The 18S rRNA primers were used as an endogenous internal control.

Dissociation curve analysis demonstrated that each of the primer pairs tested (Table 1) amplified a single PCR product with a distinct melting temperature (*Tm*); each double-stranded DNA product has its own specific *Tm* (Figure 6, A and B). Because 18S rRNA, SCMV, and MCMV primer pairs each amplified products that had the same *Tm*, nonspecific products or primer dimers were not observed in the experiments. This demonstrated that the primers for SCMV and MCMV were appropriate to use in real-time detection and quantification of respective viruses in plants. The Cqs for SCMV, MCMV and 18S rRNA were used to monitor and quantify SCMV and MCMV replication in co-inoculated plants relative to their expression in singly inoculated plants.

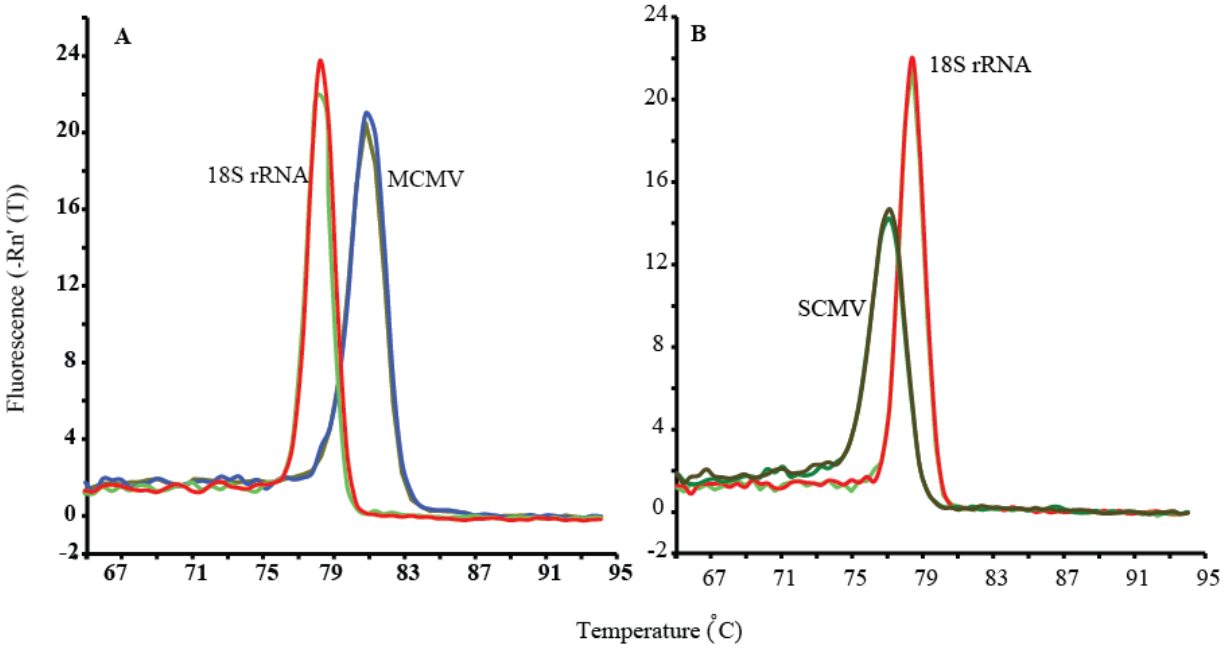

Figure 6: Representative dissociation curve analysis of MCMV (A) and 18S rRNA amplicons and SCMV (B) and 18S rRNA amplicons in maize plants after virus inoculation. All of the MCMV, SCMV, and 18S rRNA dissociation curves are grouped at a common melting temperature (*Tm*) for each amplicon.

3.4.3.2 Evaluation of SCMV and MCMV quantities in co-inoculated maize plants by qRT-PCR

The expression of SCMV and MCMV RNAs was analysed in singly and in all the four different ratios of 1:1, 1:3, 1:5 and 1:7, MCMV:SCMV co-inoculated plants. There was no difference in the relative expression of SCMV RNA between singly and co-inoculated plants, in all the four mixture ratios (Figure 7). There was no significant difference in terms of fold changes among the four treatments ($F_{3, 80} = 0.2716$, $P = 0.8457$)

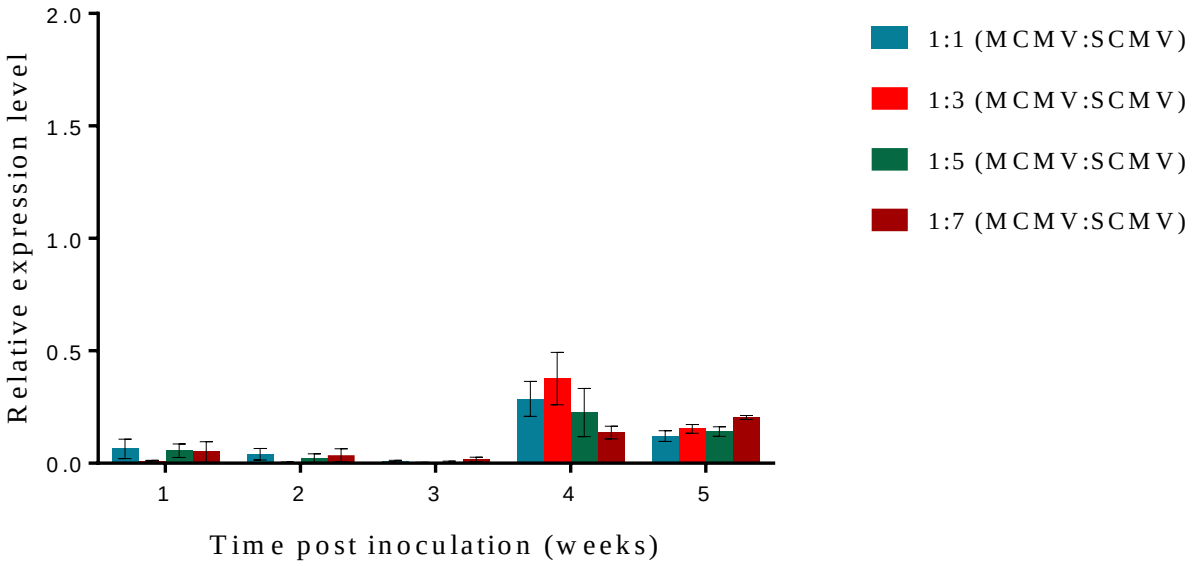

Figure 7: Relative expression levels of SCMV RNA over time, in MCMV/SCMV co-inoculated plants. The relative quantity of SCMV RNA was calculated using comparative Ct ($2^{-\Delta\Delta Cq}$).

There was difference in the relative expression of MCMV between singly inoculated and co-inoculated plants, in all the four ratios (Figure 8). The first and second week post inoculation (wpi), had low relative expression levels (averagely, 0.2 folds) of MCMV co-inoculated. However, at week 3, 4 and 5-post inoculation, there was a substantial increase in the relative levels of MCMV in doubly inoculated plants from 0.4 to 8.4 folds. There was no significant difference in terms of fold changes among the four treatments ($F_{3, 80} = 0.1548$, $P = 0.9263$).

At 6 wpi ELISA results indicated that MCMV titres had increased 1.4 times in dually inoculated plants whereas qRT-PCR results showed that MCMV RNA increased upto 8.4 folds in co-inoculated plant at only 5wpi.

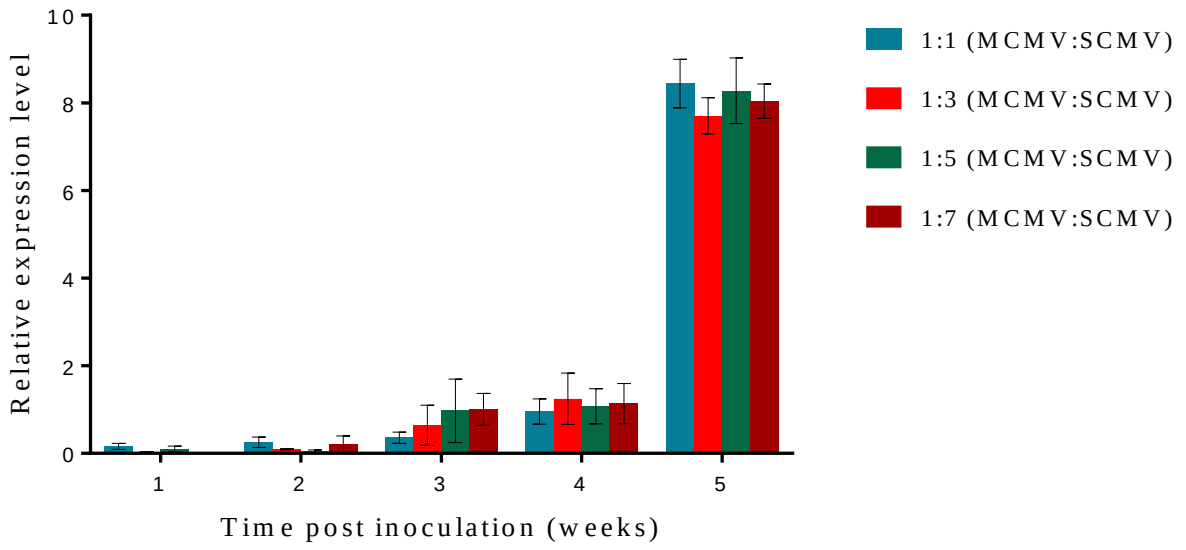

Figure 8: Relative expression levels of MCMV RNA over time, in MCMV/SCMV co-inoculated plants. The relative quantity of MCMV RNA was calculated using comparative Ct ($2^{-\Delta\Delta Cq}$).

3.5 Discussion

Plants co-infected with MCMV and SCMV showed a starting ratio-independent disease synergism with a significant increase in titre of MCMV as compared to SCMV that remained equivalent to that in singly infected plants. Regardless of increasing concentration of SCMV in three of the four concentration ratios of MCMV:SCMV (1:1, 1:3, 1:5 and 1:7) in the starting material, severity and concentration of MCMV increased concomitantly over time, an indication that initiation of MCMV/SCMV interaction is qualitative but not quantitative. However, this was lower in singly infected plants. This is a clear sign of synergistic interaction of MCMV and SCMV (Wangai *et al*., 2012; Makumbi and Wangai, 2013) which started as early as 9 dpi and generally increased with dpi. The quick manifestation of symptoms of irregular chlorosis and mottling spots in SCMV and all co-infected maize plants but not in MCMV singly infected plants, in the first week post inoculation (wpi), shows that SCMV invade cells more rapidly and more extensively facilitating fast symptom development compared to MCMV, which had only mild mottling. This could also mean that, symptoms observed on newly forming leaves in first week post inoculation in dually inoculated plants are probably caused solely or largely by SCMV because they were not different from those of SCMV singly inoculated plants.

Tatineni et al. (2010) demonstrates that the concentrations of individual viruses in mixed infections should reach a certain threshold level in order to elicit synergistic interaction. In the current study, the first noticeable difference between singly and doubly infected plants (in the four ratios), and thus the first indication of synergism, was mosaic or prominent mottling with large and a higher density of chlorotic spots and streaks on the newly forming leaves in the whorl, in the second week post inoculation (9-10 dpi). This was followed by necrotic areas especially on the tips of both old and newly forming leaves. This implies that the concentrations of the interacting viruses had reached threshold levels of eliciting synergistic interaction. However, this concentration was not determined by the starting concentration of the pair virus in an inoculum but probably by the rate of multiplication of the virus as observed in the current study. The results corroborate earlier studies involving a potyvirus and other viruses, whereby, there was always an increase in severity in dually inoculated plants as compared to singly inoculated plants (Uyemoto *et al*., 1983; Scheets *et al*., 1998; Taiwo *et al*., 2007).

In a synergistic interaction, the virus titre of both, one, or neither virus may be enhanced and, as a consequence, the rate of disease spread may be affected (Syller, 2012). To examine differences in viral titres of MCMV and SCMV, we used, both DAS-ELISA and qRT-PCR. Both the two methods did not reveal any significant change in titres of SCMV between singly and dually infected plants. However, MCMV titre levels were significantly increased, up to 8.4 folds higher in co-infected plants compared to that in plants infected with MCMV only. This shows that MCMV/SCMV synergism is unilateral and that SCMV, either directly or indirectly aids the infection of MCMV in maize plant by increasing its replication hence its pathogenicity. These results corroborate previous reports on synergism between MCMV and a potyvirus, where concentrations of MCMV is always increased more than that of the potyvirus (Goldberg and Brakke, 1987; Xia *et al*., 2016). The increase in severity and concentration of MCMV in the presence of potyviruses is likely to be as a result of molecular interactions and this could be attributed to a number of mechanisms among them, the highly effective and non-specific silencing suppressor, Helper component protein (HC-pro) i.e Silencing suppression could be involved in the development of MLN (Xia *et al*., 2016). Potyviruses encode for viral suppressors for RNA silencing (VSRs) that suppress a conserved surveillance mechanism in plants known as RNA silencing (Incarbone and Dunoyer, 2013).

This is sequence-specific RNA degradation mechanism that is mediated by small interfering RNAs and functions as an adaptive immune response, which is used by plants to restrict the accumulation or spread of inducing viruses. (Waterhouse *et al*., 2001). Therefore in this compromised defence system due to presence of suppressor proteins encoded by SCMV, MCMV is be able to replicate fast and accumulate in the plant leading to severe disease symptom observed in this study. This replication may be facilitated by p32 protein in MCMV genome, that enhances accumulation in maize protoplasts and is responsible for enhanced symptoms and accumulation of MCMV in dually infected plants and, p31 that is required for efficient systemic movement (Scheets, 2016). However, Stenger et al. (2007) demonstrated that WSMV lacking HC-Pro was competent to produce disease synergism in double infections with MCMV. This implies synergism between a potyvirus and a non-potyvirus may not be entirely due to HC-Pro.

In addition, MCMV and SCMV co-infection has been shown to induce deleterious changes in cell structure and organelles (Wang *et al*., 2017). This is because, chloroplasts of cells co-infected with SCMV and MCMV exhibit smaller starch grains than mock or MCMV infected cells, suggesting that photosynthesis is reduced during co-infection thus the severe chlorosis exhibited (Makumbi and Wangai, 2013). Similarly MCMV/SCMV co-infection leads to disruption of cells, severe enough to cause leaking of mitochondrial content. The early severe damage of mitochondria could also explain the accelerated damage to plants affected by MLN (Wang *et al*., 2017). However, our findings contrast with interactions of MCMV/WSMV in maize and WSMV/TriMV (*Triticum mosaic virus*) in wheat where there was an increase in concentration of both interacting viruses (Scheets, 1998; Stenger *et al*., 2007; Tatineni *et al*, 2010). This is probably so because, synergy patterns have been demonstrated to vary depending on virus strain, host species and even host plat cultivar (Taiwo *et al*., 2007; Wintermantel *et al*., 2008).

The exacerbated symptoms in co-infected plants, an indication of synergism started as early as 9-10 dpi and generally increased with dpi, implying that, under field conditions, detrimental effects from co-infection of a maize crop by both viruses are likely to be aggravated especially when the crop is attacked at principal phenological growth stage 1 i.e. leaf development stage (Lancashire *et al*., 1991). This may definitely lead to reduced or complete loss in yields.

Numerous factors including, vector transmission efficiency of a virus, efficiency of accumulation in host plants, and potential interactions resulting from related viruses co-infecting a plant, contribute toward epidemiology, emergence and dominance of the virus (Wintermantel *et al*., 2008). The significant accumulation of MCMV in the co-infected plants is likely to increase its acquisition by the vector (thrips) in the field and consequently its transmission rate. This is because vector transmission of plant viruses, in general, is directly related to virus concentration in the source plants (Wintermantel *et al*., 2008) i.e there will be increased potential of host plants to be sources of inoculum for MCMV. Similarly, the semi persistent transmission mechanism of MCMV by its vector thrips could also be highly favored by this increased concentration of the virus in co-infected plants (Ng and Falk, 2006; Nelson *et al*., 2011), however, factors like visual and olfactory cues also determine how thrips visits and interacts with a plant (Stafford *et al*., 2011; Abdullah *et al*., 2015).

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CHAPTER FOUR

Behavioral responses of thrips to volatiles induced by MCMV and SCMV/MCMV Co-infected maize plants.

4.1 Introduction

The maize chlorotic mottle virus (MCMV) (Tombusviridae: *Machlomovirus*), one of the causative agents of MLN, has been predicted to continue spreading to maize production regions across SSA (Isabirye and Rwomushana, 2016). The virus is transmitted mechanically and in a semi-persistent manner by a number of vectors including, maize thrips (Cabanas *et al*., 2013), Chrysomelidae beetles (*Oulema melanopa*) and corn rootworms (*Diabrotica sp*) (Nelson *et al*., 2011). In eastern Africa, onion thrips (*Thrips tabaci*) and pale form of common blossom thrips (*Frankliniella schultzei*) are also known to transmit MCMV (Mahuku *et al*., 2015a; Nyasani *et al*., 2015). However, the range of vectors for MCMV in Africa is not known, although thrips have been observed in high densities in fields affected by MLN- and MCMV (Wangai *et al*., 2012; Mahuku *et al*., 2015a). The attraction of thrips and other insect vectors to virus infected or intact plants is fully or partially mediated by visual cues such as leaf color from a distance and/or volatile olfactory cues (VOCs), which play a crucial role in both pre- and post-alighting stages of host selection (Koschier *et al*., 2000; Mauck *et al*., 2014; Abdullah *et al*., 2015). Volatiles emitted from virus-infected plants differ from healthy plants and this could influence the preference of vectors such as thrips to infected plants (De Vos and Jander 2010).

Studies have shown that viruses could manipulate their vectors behavior via host plant nutrients and volatiles to enhance their transmission and spread (Mauck *et al*., 2014; Shalileh *et al*., 2016). However, there is scarcity of information on multi-trophic interaction between disease vectors (Thrips), host plant (maize) and viruses (MCMV) involved in inducing MLN. In this regard, we investigated behavioural response of two species of thrips, *i.e.* maize thrips (*F. williamsi*) and onion thrips (*Thrips tabaci*), to volatiles induced by MCMV infected maize plants. The VOCs from healthy and virus-infected plants were further analyzed by coupled GC-mass spectrometry (GC-MS). Discovering the mechanisms mediating vector thrips, maize plants and MCMV interaction will provide useful insights not only to better understand the vector ecology and MLN disease epidemiology but also ways of designing suitable disease management strategies.

4.2 Materials and methods

4.2.1 Plants and virus inoculation

Disease-free maize seeds of variety H6210 were planted singly in pots (21cm in height, 20cm diameter) filled with sterilized (autoclaved) soil in an insect-proof screen house at the International Centre of Insect Physiology and Ecology (*icipe*), Duduville campus, Nairobi. Three weeks-old plants, at principal phenological growth stage one (BBCH-scale, Lancashire *et al*., 199l) were used in experiments. The plants were artificially inoculated with either MCMV or MCMV/SCMV (1:1) inoculum consisting of infected leaf sap in 0.01 M potassium phosphate buffer (PH 7.0) and carborundum 100 mesh grit (Wangai *et al*., 2012). Application to the host plant was done using a soft finger-rubbing technique, i.e. by dipping cheesecloth-tied fingers in the inoculums, and gently rubbing the maize plant leaves and later incubating in a separate screen house for one week before use in the experiments. Concurrently, control plants were treated the same way, but without virus inoculum

4.2.2 Insects

Adult maize thrips, *F. williamsi* and onion thrips, *T. tabaci* were obtained from thrips cultures maintained at Thrips IPM program lab at *icipe*, Duduville campus. The thrips culture was originally initiated with adults that were collected from maize and onions fields in Central Kenya. The field collected insects were reared on baby corn, *Zea mays* and snow peas, *Pisum sativum*, respectively as described by Nyasani et al. (2013) and maintained in ventilated plastic jars (17 cm in height, 8cm diameter) at 25±1°C, 50–60% relative humidity (RH) and 12L: 12D photoperiod. The laboratory-reared adult thrips used in various behavioral assays were reared for more than 30 generation in the lab with intermittent infusion of field collected thrips. Identification and separation of male and female adult thrips was based on visible external morphological features under a stereomicroscope (Moritz *et al*., 2013). Female and male thrips of each species were aspirated and transferred separately into ventilated plastic jars.

4.2.3 Collection of plant volatiles

Volatiles from seedlings of virus infected and healthy controls were collected by headspace sampling (Tamiru *et al.,* 2011) (Plate 1). Individual maize plants were placed in odourless polyethyleneterephthalate (PET) bags (volume 3.2L, ~12.5 mm thickness) heated to 100 ^{o}C for 1 hour before use and fitted with Swagelock inlet and outlet ports.

The bottom of each bag was tightened around the plant stem and the upper bag opening closed with a twist-on seal. Charcoal-filtered air was pumped constantly at 600 mL min^{-1} through the inlet port for 24 hours. Headspace volatiles were simultaneously collected at room temperature for 24 hours on Porapak Q (0.05 g, 60 ⁄ 80 mesh; Supelco) filters inserted in the outlet port through which air was drawn at 300 mL min^{-1}. After entrainment, volatiles retained by the Porapak Q filters were desorbed with 0.5mL dichloromethane. Each sample was stored at -20°C in individual small glass vials (1.5 mL) with polytetrafluoroethylene (PTFE) lined screw cap until analysis by GC and GC-MS.

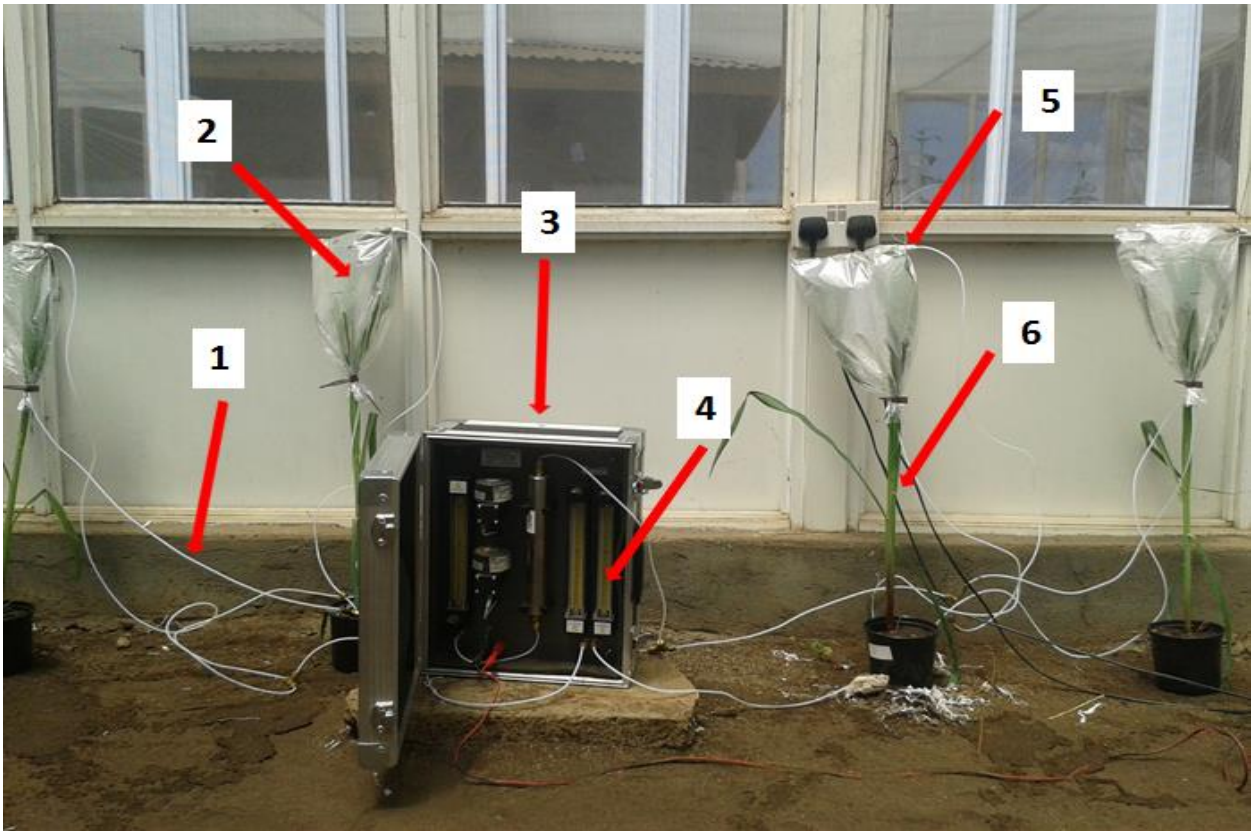

Plate 1: Headspace sampling set-up for volatile collection from either healthy control plants, MCMV-, SCMV- or co-inoculated maize seedlings. The labels represent (1) Ethylene terephthalate tubes transporting air to/from the pump, (2) Polyethyleneterephthalate (PET) bags enclosing maize leaves, (3) Portable air entrainment kit, (4) Flow-metre controlling air flow rate, (5) Porapak Q tubes trapping volatiles, (6) Maize seedling from which volatiles are collected.

4.2.4 Olfactometric bioassays

The responses of thrips species (*F. williamsi* and *T. tabaci*) to plant volatiles was tested in a Perspex four-arm olfactometer (Tamiru *et al*. 2011) (Plate 4). A choice-test was carried out to compare insect responses to headspace samples from MCMV, SCMV+MCMV infected, and control plants. Headspace samples (10µl aliquots) was applied using a micropipette (Drummond 'microcap', Drummond Scientific Co., Broomall, PA, USA), to a piece of filter paper (4×25 mm) placed in the inlet port at the end of each olfactometer arm. Two opposing arms held the test stimuli (10µl aliquots of headspace sample) and the remaining two arms held solvent controls. Male and female putatively non-viruliferous thrips of each species were first starved for 24 h and acclimatized to room temperature for 2 h before experiments. One adult was transferred individually into the central chamber of the olfactometer using a soft camel hair brush. Air was drawn through the four arms towards the centre at 260 ml min^{-1}. The time spent by thrips in each olfactometer arm was recorded with 'Olfa'software (F. Nazzi, Udine, Italy) for 12 minutes say maximum time allowed. To avoid directional bias, the position of the treatments was randomly allocated between each replicate and the olfactometer arms were gently rotated 90^{o} after every 3 min during the test. Each olfactometer was used only once per replicate and was scrupulously cleaned and air-dried before the next bioassay run. The experiment was replicated 12 times (each insect representing a replicate). A test insect was discarded when they remain motionless for more than 2 uninterrupted minutes and replaced with a new insect one.

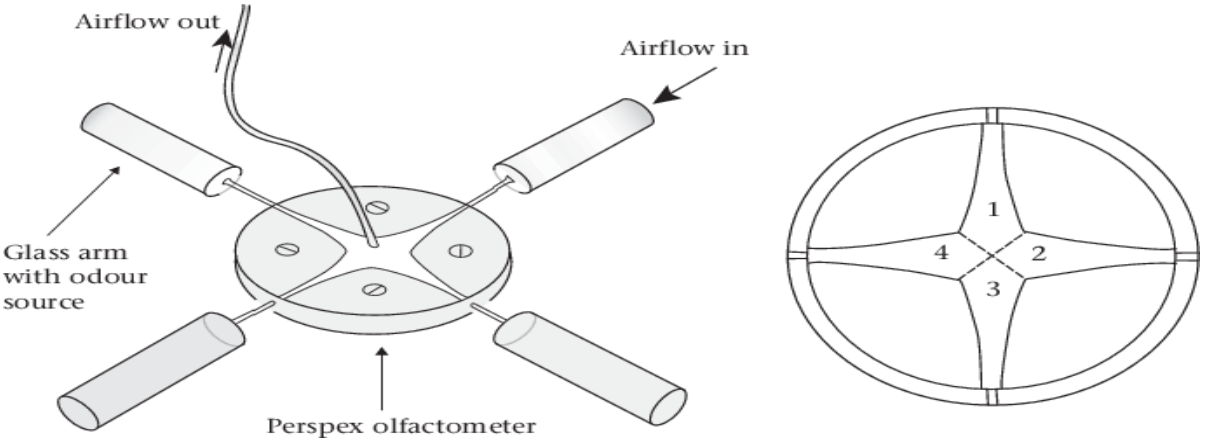

Plate 4: Diagrammatic representation of the four-arm olfactometer (120 mm diameter) with cylindrical glass arms (90 mm × 20 mm internal diameter with 50 mm × 3mm internal diameter connecting arms) used to contain odour sources alongside diagram showing division of regions within the olfactometer. Adopted from Webster et al. (2010).

4.2.5 Coupled GC-Mass Spectrometry (GC-MS) analysis

Headspace volatile samples (1µl) of the plants were analysed in GC-MS equipped with cold on-column injector (VG Autospec, Fisons Instruments, Manchester, UK) operated in electron impact mode (70 eV, 250^0C). To separate the volatiles, non-polar column (HP-1, 50 m, 0.32 mm i. d., 0.52 µm) was used with Helium as carrier gas at constant flow. The oven temperature was maintained at 40ºC for 1 min, then programmed at 5^0C min^{-1} to 250^0C. The volatiles were identified by comparison of spectral data with those of authentic standards.

4.3 Data analyses

Statistical analyses were performed using R statistical software, version 3.2.3 (R Core Team, 2015). Time spent by the thrips in each arm of the olfactometer was compared by analysis of variance (ANOVA) after conversion of the data into proportions of the total time the insect was allowed to make its choice (12 min). This was followed by a log ratio transformation to conform to ANOVA distributional assumptions (Logan, 2010). The mean responses of the insects were separated using Student Neuman Kuel (SNK) procedure. All tests were performed at 5% significance level.

4.4 Results

4.4.1 Changes in volatile profiles in MCMV- and co-infected plants

Gas chromatography analysis of headspace samples revealed qualitative or quantitative variation in the volatile plumes of uninfected (healthy), MCMV-, and co-infected (SCMV+MCMV) maize plants. Most of these compounds belong to the three main classes of compounds: terpenoids, phenylpropanoids/benzenoids, and green-leaf volatiles GLVs (which consists of C6-aldehydes, alcohols, and their esters) (table 2a, b and c). Volatile collection from uninfected plants revealed a wider range of compounds compared to the blend composition of MCMV-, and co-inoculated maize plants (Figure 9). Overall, greater amounts of 10 identified VOCs were released following MCMV- and MCMV/SCMV co-infection (Table 2b and 2c) and more than 15 compounds (Table 2a) from uninfected control plants.

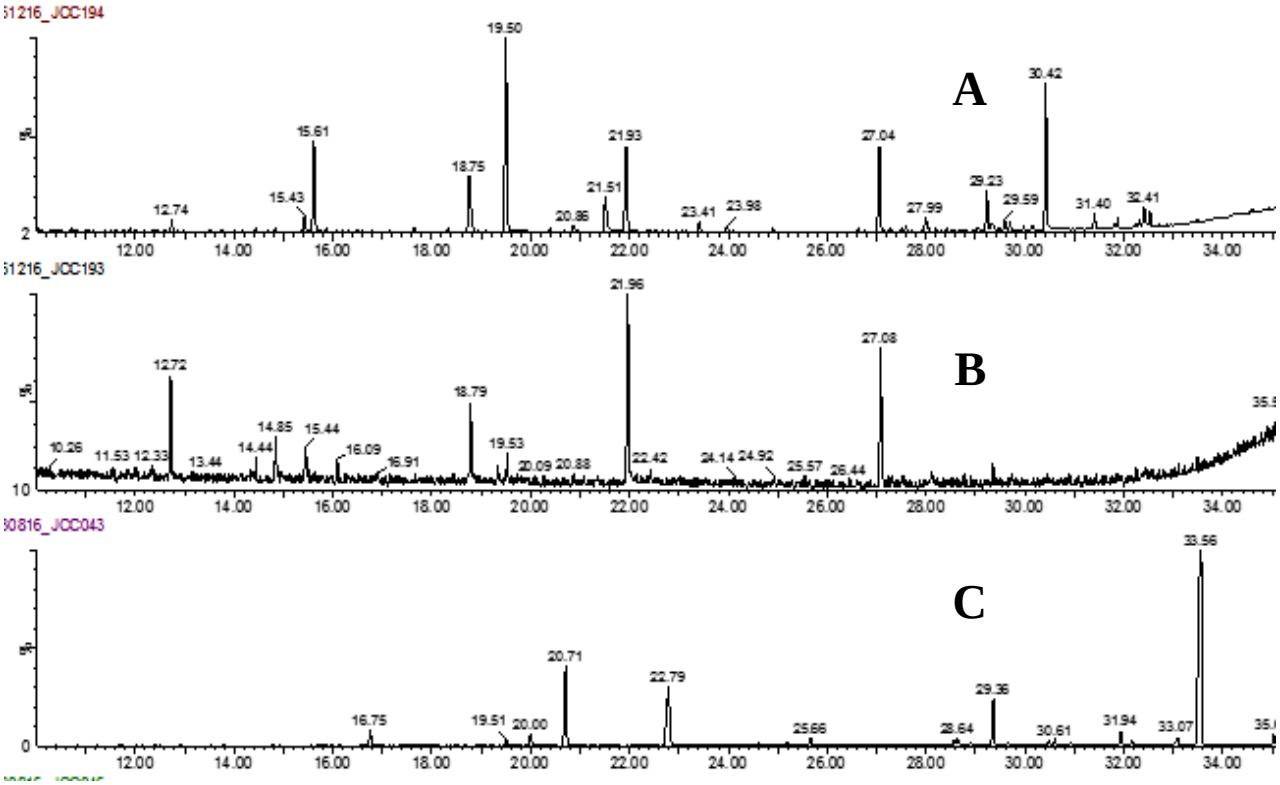

Figure 9: GC-MS profiles of healthy and virus infected plants: A = healthy plants, B = dually infected plants by Sugarcane mosaic virus and Maize chlorotic mottle virus (SCMV+MCMV) and C = MCMV infected plant. Identified compound at each retention time has been listed in the tables below.

Table 2: Volatiles emissions from healthy maize plants (a), dually infected plants by *Sugarcane mosaic virus* and *Maize chlorotic mottle virus* (SCMV+MCMV) (b) and those singly infected plants by *Maize chlorotic mottle virus* (c)

a) Volatiles emitted from healthy maize plants

Retention Time (Min)	Compound	Class of compound
12.74	Nonane	Alkane
14.84	6-methyl-5-hepten-2-one	Ketone
15.43	Octanal	Aldehyde
15.61	3-hexen-1-ol acetate	GLVs
18.34	methyl benzoate	Benzenoid
18.75	Nonanal	Aldehyde
19.50	(E)-4,8-dimethyl-1,3,7-nonatriene (DMNT)	Homoterpene
20.40	3-ethylbenzaldehyde	Benzenoid
21.51	methyl salicylate	Acetate
21.93	Decanal	Aldehyde
24.89	Undecana	Aldehyde
27.04	Cyclosativene	Sesquiterpene
27.59	alpha-curcumene	Sesquiterpene
27.99	beta-copaene	Sesquiterpene
29.23	Unidentified sesquiterpene	Terpene
29.59	cadinene gamma	Sesquiterpene
29.69	cadinene delta	
30.16	Unidentified terpene	Terpene
30.42	(E,E)-4,8,12-trimethyltrideca-1,3,7,11-tetraene (TMTT)	Hormoterpene
31.40	Cadinene	Sesquiterpene

b) Volatiles emitted from dually infected maize plants by *Sugarcane mosaic virus* and *Maize chlorotic mottle virus* (SCMV+MCMV)

Retention Time (Min)	Compound	Class of compound
12.72	Nonane	Alkane
14.85	6-methyl-5-heptene-2-one	Ketone
15.44	Octanal	Aldehyde
16.09	Decane	Alkane
18.79	Nonanal	Aldehyde
19.34	undecane	Alkane
19.53	DMNT	Homoterpene
21.96	Decanal	Aldehyde
27.08	Cyclosativene	Sesquiterpene
29.35	Pentadecane	Alkane

c) Volatiles emitted from singly infected maize plants by *Maize chlorotic mottle virus*

Retention Time (Min)	Compound	Class of compound
16.75	3-hexen-1-ol acetate	GLVs
19.51	methyl benzoate	Benzenoid
20.00	Terpinolene	Monoterpene
20.71	DMNT	Homoterpene
22.79	methyl salicylate	Acetate
25.66	Unidentified monoterpene	monoterpene
28.68	Unidentified sesquiterpene	Sesquiterpene
29.36	Unidentified sesquiterpene	Sesquiterpene
30.46	E-beta-farnesene	Sesquiterpene
30.63	gamma-himacholene	Sesquiterpene
31.94	beta-bisabolene	Sesquiterpene
33.07	TMTT	homoterpene
35.02	Cadinene	Sesquiterpene

4.4.2 Behavioural responses of thrips spp to headspace samples of VOCs.

Frankliniella williamsi: Both female and male *F. williamsi* spent significantly more time in an olfactometer arm containing volatiles from plants inoculated with MCMV in comparison to those from healthy plants and solvent controls (Male: $F_{2,43}$ = 7.67, P = 0.001; Female: $F_{2,43}$ = 13.52, P < 0.001) (Figure 10). The preference of males to volatiles from MCMV infected plants was 1.69 and 1.78 times higher than to volatiles from uninfected plants and solvent control, respectively. On the other hand, females' preference was 1.47 and 2.02 times higher than to volatiles from uninfected and solvent control, respectively. However, there was no significant difference between the time spent by male and female in olfactometer containing VOCs from singly (MCMV) and dually infected (SCMV+MCMV) plants (Figure 10).

Both males and females were attracted to volatiles from SCMV/MCMV co-infected plants and plant control than solvent control. However there was no significant difference between the time spent in olfactometer containing volatiles from co-infected plants and healthy plants (Figure 10). The males and females preferred volatiles from SCMV/MCMV co-infected plants 1.81 and 1.74 times higher, respectively, than solvent control.

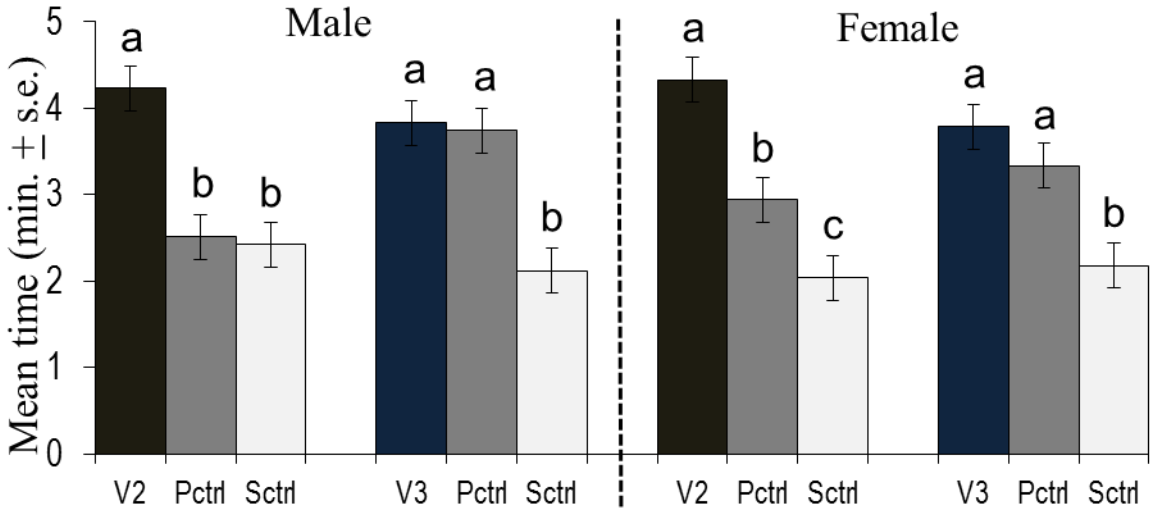

Figure 10: Responses of corn thrips to volatiles from MCMV (V2), SCMV+MCMV (V3) infected plants, uninfected plant (Pctrl) and solvent control (Sctrl) in four-arm olfactometer bioassay. Thrips responses were compared by ANOVA after conversion of data into proportions and by log-ratio transformation. Different letters on the bars indicate a significant difference using Student Neuman Kuel test (P<0.05, N=12)

Thrips tabaci: Male *Thrips tabaci* spent significantly more time in olfactometer arms containing volatiles from MCMV inoculated plants in comparison to those with volatiles from plant control and solvent ($F_{2,43}$ = 3.98, P = 0.027). The preference of males to volatiles from MCMV infected plants was 1.58 and 1.62 times higher than plant and solvent controls, respectively. However there was no significant difference between times spent by females in olfactometer arms containing volatiles from MCMV inoculated plant and both plant control and solvent controls ($F_{2,43}$ = 0.79, P = 0.459). Both male and females could not show any significant preference differences between volatile from SCMV/MCMV co-inoculated plants and plant controls. However, the females preferred SCMV/MCMV volatiles 1.56 higher than solvent control (Figure 11).

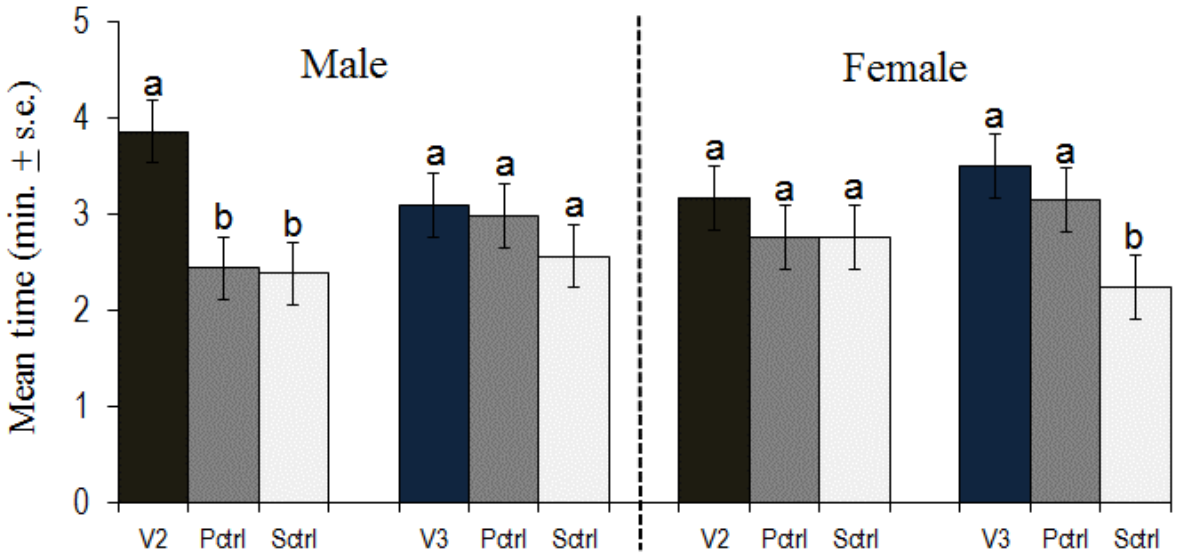

Figure 11: Responses of onion thrips to volatile from MCMV (V2), SCMV+MCMV inoculated plants (V3) and plant (Pctrl) and solvent control (Sctrl) in four-arm olfactometer bioassay. Thrips responses were compared by ANOVA after conversion of data into proportions and by log-ratio transformation. Different letters on the bars indicate a significant difference using Student Neuman Kuel test (P<0.05)

4.5 Discussion

Insects rely on a single compound or a small fraction of VOCs in a particular ratio to locate their feed plants (Birkett *et al*., 2004; Tasin *et al*., 2007; Webster *et al*., 2008). Plants on the other hand constitutively or inductively (when subjected to herbivory or pathogen attack), release VOCs to communicate with neighboring plants, insects and microbes, including pathogens (Dudareva *et al*., 2006; Frost *et al*., 2008). A number of plant viruses have been shown to alter plant volatile plumes that serve as key foraging cues in a way that may positively or negatively affect the attraction of their vectors (Eigenbrode *et al*., 2002; Mauck *et al*., 2014). Our results reveal that infection of maize plants with MCMV induces emission of VOCs that are more attractive to both male and female *F. williamsi*, and male *T. tabaci* than emissions from MCMV+SCMV co-infected plants and uninfected plants. However, the attraction of female *T. tabaci* to MCMV volatile blend was not different from their attraction to MCMV+SCMV co-infected and uninfected plants emissions.

Overall, decreased attractiveness of *T. tabaci* to health and virus infected maize plants were observed compared to Corn thrips. Despite *T. tabaci* being extremely polyphagous (feeding on a range of host plant species), maize is not its primary host (Moritz *et al*., 2013) which could explain this response. Differences in behavioral response among male and female *T. tabaci* were observed in this study and males were more attracted towards MCMV infected plants. Feeding behavior of thrips vectors to tospovirus infected plants are known to differ among the sexes and viruliferous nature of the thrips such as Western flower thrips (WFT), *Frankliniella occidentalis* (Van de Watering *et al*., 1999; Stafford *et al*., 2015). Males of WFT transmit TSWV more efficiently than females, and this is due to more robust virus infection of males and sexually dimorphic feeding behaviors (Van de Watering *et al*., 1999; Rotenberg *et al*., 2009). However, this is the first instance when such differences are present with Thrips –MCMV-Maize interaction. Increased thrips attraction towards MCMV infected plants is likely to enhance acquisition of MCMV and subsequently enhance dispersal by *F. williamsi* and male *T. tabaci*. However, this increased attraction was not significantly observed towards co-infected plants. Conversely, co-infection of MCMV and SCMV in maize plants is known to increase the titre levels of MCMV by more than 5 folds with SCMV remaining significantly same (Goldberg and Brakke, 1987: Xia *et al*., 2016, Mwando *et al*. (*unpublished data*)).

In the MCMV infected plants, higher terpenes were uniquely observed including, terpinolene, DMNT, TMTT, E-beta-farnesene, gamma-himacholene, among others. On the other hand, in co-infected plants, higher alkanes such as decane, undecane and Pentadecane were uniquely observed. These compounds have been reported to elicit biological activities in different insect species including attraction and repellency (Obonyo *et al*., 2008; Tamiru *et al.* 2011) and they could be responsible for difference in the preferential responses of the thrips. However for the biological activity to effectively occur, the compounds must be in the right blend since amount and combination of volatiles also play an important role in feed plant location and judging of nutritional quality of the plant by insects (Bruce and Pickett, 2011).

Exposure of plants to virus infection can alter the preferential behavior of the vector in a way that after nonviruliferous vectors acquire the virus, they immediately switch their olfactory preference after and become attracted to healthy plants (Bosque-Pe´rez and Eigenbrode 2011; Ingwell *et al*., 2012). The viruses also manipulate their vectors behavior via host plant nutrients and volatiles to enhance their transmission and spread (Mauck *et al*., 2014; Shalileh *et al*., 2016). In this context, our findings therefore could imply that MCMV and MCMV/SCMV co-infected plants may manipulate corn thrips and male onion thrips via the maize plant volatile to probably enhance MCMV transmission and spread, as shown for other host–virus–vector systems (Stafford *et al*., 2011; Ogada *et al*., 2013). This consequently promotes its dispersal among host plants.

Terpenes are significant in constitutive direct plant defence against insect attack and virus infection may affect their synthesis with an impact to their vectors. For example, monoterpenes; terpinen-4-ol and 1,8-cineole have been reported to play a crucial role in direct defence by reducing feeding and oviposition rate of *Thrips tabaci* (Koschier and Sedy, 2001). Interestingly, infection of plants with the MCMV may have suppressed the synthesis of herbivore-induced defensive enzymes, especially for terpene synthesis making infected plants more susceptible to *F. williamsi* and male *T. Tabaci*. Terpenes are also known to mask other plant volatiles (Yamasaki *et al*. 1997), and thus reduce their attractiveness to herbivorous insects and parasitoids. Reduction in release of terpenes occasioned by MCMV infection therefore could have led to the thrips attraction to volatiles from MCMV infected plants.

Similar results were observed on maize plants co-infected with MCMV+SCMV that had a significant reduction in the quality and concentration of the volatiles such as beta-bisabolene, terpinolene, TMTT and (E)-beta-farnesene. However, such a reduction in the concentration of terpenes was not associated to any differences in attraction in both *F. williamsi* and *T. tabaci* between co-inoculated plants and healthy plants.

Transmission efficiency of a virus by thrips in the field is determined by among other factors, the number of the viruliferous thrips in a population and their sex ratio (Van de Watering *et al*., 1999). Therefore these factor have to be put into consideration when thinking of an efficient and reliable thrips management strategy. But owing to their small body size, high fecundity, tendency to aggregate, broad host range, cryptic feeding and resistance to insecticides, thrips present a great challenge when it comes to controlling them (Brunner and Frey, 2010; Gao *et al*., 2012). The indiscriminate use of pesticides by farmers for the management of thrips leads to negative effects on non-target organisms, the development of resistance in target pests and harmful effects on the environment (Nderitu *et al*., 2007). Thus, knowledge of semiochemicals that thrips employ to locate host plants could be utilized to design ecologically sound and effective way to control thrips population and by so doing reduce spread of MCMV and MLN diseases.

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CHAPTER FIVE

General Discussion, Conclusions and Recommendations

5.1 General Discussion

Having a clear understanding of virus–virus interactions in plants, symptom development and progression as a result of these interactions is important for the understanding of viral pathogenesis and evolution and consequently for the development of effective disease control strategies. The recent dissemination of MCMV and consequently MLN across the Eastern Africa and SSA presents a major epidemiological problem and a major threat to food security (Isabirye and Rwomushana, 2016). Farmers must understand the disease, what the symptoms look like and what are the potentially devastating implications of having and spreading the constituent viruses. Reports indicate that co-infection of SCMV and MCMV in maize leads to a number of symptoms with chlorotic mottling and necrosis being a key indicator of MLN (Wangai *et al*., 2012b; Mahuku *et al*., 2015a). Conversely, how these symptoms progress in relation to specific virus concentration has not been well highlighted. The current study demonstrates that symptom expression in SCMV and co-infected maize plant begins as early as 5-7 dpi whereas in MCMV infected plants, they start showing 7-9 dpi but are relatively mild. The key symptoms for SCMV infected include irregular chlorosis and mottling, which are so much pronounced especially in the first week post inoculation and later on, leaf mosaic and mild chlorosis on the older leaves persist. MCMV infected plants present mild chlorotic mottling in the first week post inoculation. These symptoms persist and later lead to chlorosis especially in the midrib spreading towards the leaf margin. Both SCMV and MCMV symptoms are more pronounced in co-infected. However, SCMV, MCMV and MLN severity and symptoms vary depending on the plant genotype, age of infection and environmental condition (Mahuku *et al*., 2015a).

This study was also able to demonstrate that symptoms severity in co-infected plants concomitantly increases with increase in MCMV titre levels, implying that increased severity due to MLN is largely contributed by MCMV. However, SCMV being a potyvirus, could be playing a key role in this because potyviruses encode gene silencing suppressor protein (HC-Pro) implicated in the increased replication and pathogenicity non-potyviruses (Kasschau and Carrington, 1998; Pruss *et al*., 1997; Syller, 2012).

SCMV HC-Pro and P1 suppressor proteins could be responsible for increased MCMV levels and severe symptoms in MCMV+SCMV synergistic interactions however, this need to be scientifically investigated.

The best way to manage MLN and other viral diseases would be to prevent the introduction of the virus and vector into an area. There are recommendations and attempts to use pesticides in areas where the vector already exists (Miano *et al*., 2013), however, the indiscriminate and frequent use of these pesticides can result in pest resistance evolution, pollution of environment as well as elimination of natural enemies leading to secondary outbreaks (Desneux *et al*., 2007; Nderitu *et al*., 2008). Additionally, the use of synthetic insecticides for control of thrips and aphids could be uneconomical and impractical for many resource-poor smallholder farmers (Nderitu *et al*., 2008). Moreover, elusiveness and high fecundity of vectors like thrips makes it difficult to control them (Hamilton *et al*., 2005; Brunner and Frey, 2010). Therefore, discovering the mechanisms involved in plant host virus vector interactions and consequently exploitation of these mechanisms to control pests of maize plants which vector SCMV and MCMV presents as an alternative and viable option for management of MLN. Plants constitutively release volatile organic compounds (VOCs) that play an important ecological role, mediating a range of multi-trophic interactions such as plant-plant, plant-microbe, plant-herbivore and inter-trophic interactions between the plant and organisms (Dudareva *et al*., 2004). The constitutive release of VOCs from a plant is altered by phytopathogens including insect vectored ones (McLeod *et al*., 2005) in a way that it may influence the transmission of these insect-vectored phytopathogens because most vectors use visual or/and volatile chemical cues to locate their hosts.

In this study, there was both qualitative and quantitative change in volatile profile upon infection with MCMV. The change in volatile cues in infected plants was attractive to both male and female *F. williamsi* and male *T. tabaci,* which are potential vectors of MCMV (Cabanas *et al*., 2013; Mahuku *et al*., 2015a). Identification and incorporation of the right ratios of these compounds into new integrated pest management strategies targeting these two species of thrips could play a vital role in controlling them and manage the spread of MCMV and MLN. However, a combination of crop rotation, using virus-free 'clean seed', roguing (removing plants

showing disease symptoms) and controlling insect pests could be the best way of controlling MLN.

5.2 Conclusions

There are significant differences in symptom expression and the rate of viral multiplication in SCMV-, MCMV- and co-infected maize plants. In SCMV/MCMV synergistic interaction, the titre levels of MCMV increases while that of SCMV remain unchanged regardless of the initial concentration of SCMV in the starting inoculum MCMV/SCMV ratio. Therefore, the study concludes that the intensification of disease symptom could be due to accumulation of MCMV and that the virus concentration ratios in inoculum for co-inoculation do not affect the respective virus multiplication and concentration over time i.e MCMV/SCMV synergism in maize is qualitative rather than quantitative.

There exists both the inter-specific and intra-specific variability in the behavioral responses of *F. williamsi* and *T. tabaci* towards headspace volatiles from MCMV and MLN infected plants. This is probably due to significant differences in both qualitative and quantitative volatile organic compounds' profile of both MCMV- or co-infected maize plants. Seemingly, MCMV induces changes in host plant volatile to attract vector thrips species. The increased attraction of non-viliruferous thrips towards MCMV infected plants is likely to enhance acquisition of MCMV and subsequently enhance dispersal by *F. williamsi* and male *T. tabaci*. This behaviour has an implication on the virus epidemiology and consequently on MLN transmission.

5.3 Recommendations

Unravelling complex relationships among vectors, plant hosts, and MLN viruses can provide insight on how to manage virus epidemiology in modern agriculture. The study identified the following research gaps, which need further investigations:

1) The effect of SCMV HC-Pro and P1 silencing suppressor proteins on synergistic interactions of MCMV+SCMV.
2) The impact of MCMV on the thrips in terms of behaviour (feeding, locomotion, development and longevity, and preference for infected and healthy plants).

3) Explore metabolome change in maize plants due MCMV- and MCMV+SCMV infection to understand possible affected pathways.
4) Responses of SCMV vectors (aphid *spp*) towards SCMV and infected maize plants.

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