VECTOR AND POLLEN TRANSMISSION MECHANISMS FOR THE MAIZE LETHAL NECROSIS DISEASE IN NAIROBI COUNTY, KENYA

PAUL ATENG'A NYAMWAMU (M.Sc. Crop Prot, Virol.)

A THESIS SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY CROP PROTECTION IN THE SCHOOL OF AGRICULTURE AND ENVIRONMENTAL SCIENCES (PATHOLOGY OPTION) OF KENYATTA UNIVERSITY

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DECLARATION

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

Signature..... Date 25/11/2024

Final Ateng'a Nyamwamu

University supervisors

We confirm that the work reported in this thesis was carried out by the candidate under our supervision and has been submitted with our approval as University supervisors.

Signature..... Date 25/11/2024

Prof. Maina Mwangi

Department of Agricultural Science and Technology

Kenyatta University

Signature..... Date 25/11/2024

Dr. Ruth Kahuthia-Gathu

Department of Agricultural Science and Technology

Kenyatta University

Signature..... Date 25/11/2024

Prof. Douglas W. Miano

Department of Crop Sciences and Crop Protection

University of Nairobi

DEDICATION

I dedicate this academic work to my wife Fridah Masese. She accorded me invaluable support and sacrifice during my study. I also dedicate the work to my children Velerie Ogake and Harriet Bonareri. I extend my dedication to my mother, Joyce Gesare for her loving care and instilled the virtue of hard work. Lastly, this work goes to my siblings Pamela Kwamnoka, Ronald Orare, Tom Onsare, and Charles Nyarang'o for their unwavering inspiration and encouragement.

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LIST OF ACRONYMS AND ABBREVIATIONS

AAP	Acquisition access period
ACP	Antigen coated plate
ANOVA	Analysis of variance
ArcGIS	Aeronautical reconnaissance coverage geographic information system
AUDPC	Area under disease progression curve
AYRV	Artichoke yellow ring spot virus
BBLMV	Blueberry leaf mottle virus
BlShV	Blueberry shock virus
BOLD	Barcode of life data
BYDV	Barley yellow dwarf virus
cDNA	Complementary Deoxyribonucleic Acid.
CIMMYT	International Maize and Wheat Improvement Center
CLRV	Cherry leaf roll virus
COI	Mitochondrial cytochrome oxidase 1
contig	Continuous DNA segment
CRD	Completely randomized design
DAS ELISA	Double Antibody Sandwich Enzyme linked immunosorbent assay
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotide triphosphate
DRC	Democratic Republic of Congo
EB	Elution buffer
ECA	East and central Africa
ELISA	Enzyme linked immunosorbent assay
FAO	Food and Agriculture Organization
FAO-STAT	Food and Agriculture Organization statistics
GAIN	Global Agricultural Information Network
GAP	Good Agricultural Practices
GAP	Good Agricultural Practices

Ha	Hectares
IAP	Inoculation access period
ICIPE	International Centre of Insect Physiology and Ecology
IgG	Immunoglobulin G
IgG-AP	Immunoglobulin G alkaline phosphatase
ITS	Internal transcribed spacer
JGMV	Johnson grass mosaic virus
KALRO	Kenya Agriculture and Livestock Research Organization
kDa	Kilodalton
KEPHIS	Kenya Plant Health Inspectorate Service
Kg	Kilogram
KU	Kenyatta University
LSD	Least significant difference
Mab	Monoclonal antibody
MaYMV	Maize yellow mosaic virus
MCMV	Maize chlorotic mottle virus
MDMV	Maize dwarf mosaic virus
MLN	Maize lethal necrosis
MLND	Maize lethal necrosis disease
MoA	Ministry of Agriculture
MSV	Maize streak virus
mtDNA	Mitochondrial deoxyribonucleic acid
MYDV-RMV	Maize yellow dwarf virus
NACOSTI	National council of science, technology and innovation
nM	Nano meters
NRF	National Research Fund
ORF	Open reading frame
PBS	Phosphate Buffered Saline
PBST	Phosphate Buffered Saline Tween
PCR	Polymerase chain reaction.
RT-PCR	Reverse transcriptase Polymerase chain reaction
PCR-RFLP	PCR-restriction fragment length polymorphism
PDV	Prune dwarf virus

PNP	Purine nucleoside phosphorylase
PNRSV	Prunus necrotic ringspot virus
RAPD – DNA	random amplified polymorphic DNA
RBDV	Raspberry bushy dwarf ideaovirus
RBDV	Raspberry bushy dwarf
RNA	Ribonucleic acid
RPM	Revolutions per minute
RT-PCR	Reverse transcription-PCR
SCMV	Sugarcane mosaic virus
SE	Standard error
SoMV	Sowbane mosaic sobemovirus
SrMV	Sorghum mosaic virus
SSA	Sub Saharan Africa
SSR-PCR	simple sequence repeat PCR
TAE buffer	Tris-acetate buffer
TAS-ELISA	Triple-Antibody-Sandwich - ELISA
TSV	Tobacco streak virus
USA	United States of America
USDA	United States Department of Agriculture
WB	Washing buffer
WSMV	Wheat streak mosaic virus

ABSTRACT

Maize (*Zea mays* L.) is a major staple food crop for over 100 million people in sub-Saharan Africa where it is produced on 35 million hectares annually. The maize yields have stagnated at 1.75 t/ha against the potential of 6t/ha due to pests and diseases. Maize chlorotic mottle virus (MCMV) and Sugarcane mosaic virus (SCMV) induces the maize lethal necrosis (MLN) disease which threatens maize production. The disease reduces yields by 90-100%. Effective management of the disease is impended by gaps in the knowledge on MCMV and SCMV transmission mechanisms. *Frankliniella occidentalis* (Pergande) and *F. williamsi* (Hood) transmission efficiency of MCMV remains unclear. Knowledge on the genetic diversity of *F. occidentalis* and *F. williamsi* transmitting the virus has not been conclusive. The role of these thrips on MLN disease spread was determined through vector-virus assay tests. The genetic diversity of thrips infesting maize affected by MLN disease was determined. Colonies of vector thrips; *F. occidentalis* were reared on *Phaseolus vulgaris* pods and *F. williamsi* on maize stalk cuttings. Virus transmission efficiency of vector thrips was compared at 0.5, 1.0, 1.5, and 24-hour virus acquisition access periods (VAAP). Transmission competence of MCMV by vectors was compared at 1, 3, 24, and 48-hour inoculation access periods. Persistence transmission of MCMV by vector thrips was investigated at retention periods of 1, 2, 3, and 4 days. Rates of new plant re-infections (4, daily cycle) was determined. Larvae thrips transmission of MCMV was tested. Virus transmission via pollen was tested through mechanical inoculation and by vector thrips fed on infected pollen. Thrips species and their biotypes involved in MCMV epidemiology were identified by analysis of mitochondrial cytochrome c oxidase COI-5′ (DNA barcodes). Sixty-four plants were each inoculated, placed in 16 wooden cages covered with nylon-mesh (114 µm) in a complete randomized design arrangement with four replicates. Virus transmission was tested by ELISA and PCR. Maize lethal necrosis induced disease severity (5.00 ± 0.1) than MCMV and SCMV (4.2 ± 0.4, 4.1 ± 0.3). *Sugarcane mosaic virus* induced leaf symptom severity similar to that of MCMV. Transmission efficiency of MCMV by *F. occidentalis* was highest at 24 hour AAP (81.2 %) while 0.5 AAP had the least (6.2 %); an indication of transmission without a latent period. *Frankliniella occidentalis* had higher (51.6 %) MCMV transmission rates than *F. williamsi* (26.6 %). Maize chlorotic mottle virus transmission rate of *F. occidentalis* rose while that of *F. williamsi* decreased after 24hour IAP. Means of infected plants by both thrips were significantly different (t = 2.77, DF = 362.97, *P* = 0.006). Plants inoculated by *F. occidentalis* had higher symptom severity than those inoculated by *F. williamsi* (0.27, 0.19), with 1.17 mean difference (higher by 17.6 %). Plants re-infected by thrips were significantly different (F = 10.27, DF = 2, 17, *P* = 0.001). *Frankliniella occidentalis* re-infected more plants than *F. williamsi* (0.32 ± 0.04, 0.27 ± 0.04) at 0.12 LSD. Plants inoculated by *F. williamsi* after day 1 post acquisition period were significantly less from those inoculated by *F. occidentalis* (F = 5.66, DF = 2, 17, P = 0.013). Both thrips transmitted MCMV in a non-persistent pattern and thus should be targeted in IPM strategies for MCMV control. *Maize chlorotic mottle virus* was mechanically transmitted and detected in 3.12 % of the total plants tested while SCMV was not detected in any of the leaf samples. None of the thrips transmitted the viruses from infected pollen. About 95% of the sampled thrips were of *Frankliniella* genus with a similarity range of between 80.04 - 81.98%. *Frankliniella* genus is a major vector for MCMV spread and therefore IPM control strategies should target the thrips for effective MLN management.

CHAPTER ONE: INTRODUCTION

1.1 Maize Production and Consumption

Maize (*Zea mays* L.) crop is the third most important worldwide cereal for over 4.5 billion people who depend on it as a staple food and animal feed (FAO, 2016). Maize is an important staple crop in sub-Saharan Africa (SSA) where the crop is grown on thirty-five million hectares producing over seventy million metric tons of grain annually (FAO, 2018). Maize feeds over 100 million people and is the third most important cereal crop after wheat *Triticum aestivum* L., and rice *Oryza sativa* L. (Khalili *et al*., 2013). Developed countries produce maize mainly for livestock feed, whereas in Africa, it is a major food crop largely produced by small scale farmers (Onasanya *et al*., 2009). Maize cereals have a high nutritive value and are rich in vitamins, carbohydrates, protein dietary fiber, essential and minerals (Mghenyi, 2006). In sub-Saharan Africa, the average the maize yields have remained below 2 t/ha, with many countries achieving 1-2 t/ha. This low productivity is due to high incidences of pests, declining soil fertility, unreliable rainfall, weeds, diseases, low access to fertilizer and quality seed (Shiferaw *et al*., 2011).

Kenya is ranked seventh in Africa on maize production and despite the crop's importance, previous reports indicate drastic yield reduction (Murenga, 2014). Its productivity has remained low at an average yield of 1.7 t/ha, (FAO-STAT, 2021) against 10.5 t/ha in the United States, the world leading producer (Table 1.1). In 2020, Kenya produced 3.78 million tons of maize, valued at US$ 65 billion (FAO-STAT, 2020). Kenya's maize consumption is estimated at 103 kg/person/year (CIMMYT, 2015), with an annual maize demand of 52.8 million bags. However, maize output has been in deficit throughout the years. The low productivity and high yield losses of maize are mainly attributed to abiotic and biotic constraints (Ajala *et al*., 2010). Pests and viral diseases especially maize lethal necrosis disease (MLN) disease also contributes to a reduction its production (Morais & Pinheiro, 2012).

Table 0.1: Kenya's Maize Production Data Against Top World Producers In 2020

Country	Production (metric tons)	Harvested	
		Area (ha)	Yield (tons/ha)
United States of America	347,047,570	32,950,670	10.53
China	260,957,662	41,309,740	6.32
Brazil	101,138,617	17,518,054	5.77
Argentina	56,860,704	7,232,761	7.86
Ukraine	35,880,050	4,986,900	7.19
Indonesia	30,693,355	5,644,775	5.44
India	27,715,100	9,027,130	3.07
Mexico	27,228,242	6,690,449	4.07
Romania	17,432,220	2,681,930	6.50
Canada	13,403,900	1,451,200	9.24
Kenya	3,897,000	2,196,136	1.77

Source: FAOSTAT, 2021

Insects cause more damage in farmers' fields in the tropic and sub tropic regions where over a half of the crop area is infested (Granados *et al*., 2000). Yield reduction due to MLND was estimated at 250,000 metric tons in 2012 (MoA, 2014); which affected farmer's livelihoods as well as the overall farm gate prices in Kenyan markets (Oscar, 2009). The disease affected 22% of the total maize production in 2013, which translated to 186.5 million US dollars in losses (De Groote *et al*., 2016). In 2014, MLN caused yield losses estimated 10%, valued at 50 million US dollars (USDA Gains report, 2015). In Kenya, before 2018, all commercial maize varieties were susceptible to MLN, under artificial and natural transmission (Marenya *et al*., 2018).

Later, second-generation MLN-resistant varieties; Bazooka and H6506 were developed and for commercialized in East Africa (Boddupalli *et al*., 2020). Maize is an important food crop in Kenya as it plays an integral role in national food security. The crop supplies 40 - 45% of calories (FAO, 2012). The crop grows in all agro-ecological zones of Kenya, ranging from highlands to semi-arid areas and humid coastal lowlands (Nyoro, 2002).

Kenya produces maize mainly in parts of the Rift Valley region, Western Kenya particularly Trans Nzoia and Uasin Gishu counties. Maize production in Kenya has been fluctuating since 2000 to 2020 with yields remaining low since 2012 after the MLN disease pandemic (FAO, 2019), (Figure 1.1) The production of maize in Kenya was severely threatened by the fast spreading maize lethal necrosis disease (Gachenge, 2012) leading to drastic decline in yields.

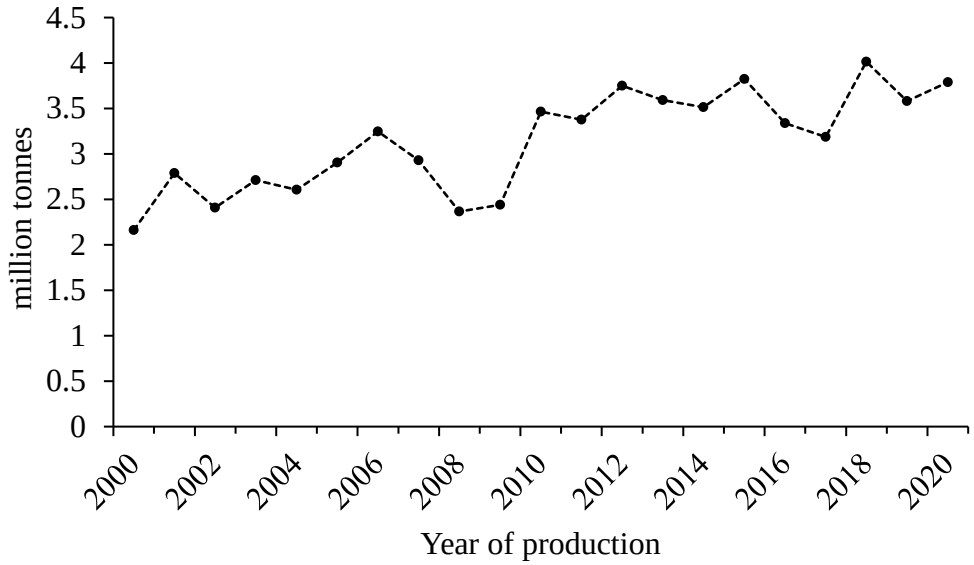


Figure 1.1: Maize Production Trend in Kenya from 2000 to 2020.

Source: FAOSTAT, 2021

The first report of maize lethal necrosis disease in Africa was in Bomet County within the rift valley region of Kenya in 2011 (Wangai *et al*., 2012). The disease spread further into Narok south, Chepalungu, Naivasha, Sotik, Meru central, and Embu west. In 2012, the disease was reported in the Central region of Kenya; Kirinyaga, Murang'a, and Nyeri. The disease then spread to Trans-Nzoia, Uasin Gishu, and Busia (Wangai *et al*., 2012). The disease rapidly spread into all of the Eastern Africa, including the Democratic Republic of Congo (DRC), Rwanda, South Sudan, Tanzania, and Ethiopia (Gitonga, 2014; Wangai, 2012; Miano 2013; Isabirye, 2016), (Figure 1.2). The disease therefore raised a major concern in the entire Eastern Africa because of the low yields due to plant death and insufficient knowledge on its management. Sugarcane mosaic virus is one of the MLN disease virus components that has existed in Kenya since the

1976 (Louie, 1980). Maize chlorotic mottle virus is new in Africa and only reported in Kenya in 2011. Maize chlorotic mottle virus was first reported in 1973 in Peru, later spread into the USA, Latin America, then into China (Xie *et al*., 2011).

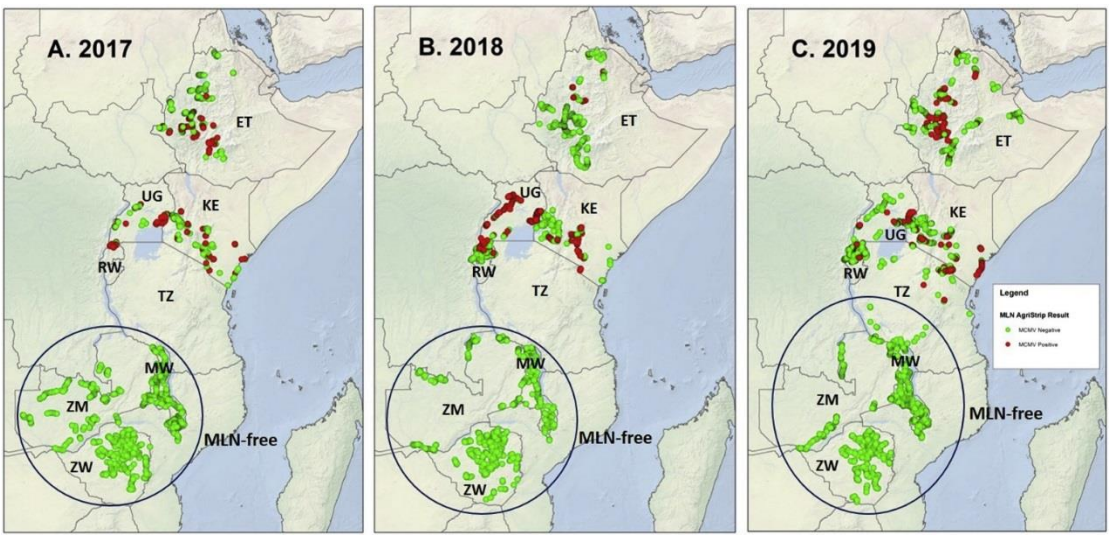


Figure 1.2 The spread of MLN Disease Incidence in Eastern and Central Africa

Source: (Boddupalli *et al*., 2020)

The Kenya Agricultural and Livestock Research Organization (KARLO) International Maize and the Wheat Improvement Center the conducted studies that confirmed the vulnerability of all commercial maize varieties to MLN disease in 2012. The maize varieties became infected both under artificial inoculation and natural disease pressure (Onsando, 2013). According to Prasanna (2015), MLN disease epidemic was exacerbated by the susceptibility of the maize varieties to MLN and the continuous maize cultivation in Eastern Africa. These conditions contributed to the rapid build-up of the virus inoculum and transmission by insect vectors.

1.2 Problem Statement

The MLN disease has a major impact on smallholder farmers' food and nutrition security, with some losing up to 100% of their harvest (Frenken, 2012). When the MLN pandemic occurred in 2011, 16,000 hectares of maize had been affected, with 80% of the crop lost (FAO, 2012). The prospect for huge maize devastation increases concerns about the MLN disease's impact on food prices and inflation in Kenya. In 2012, the disease impacted 60,000 hectares of maize, resulting in a 10% yield drop in 2013

(MoA). Maize output has dropped by more than 90% in some of the most severely impacted areas. Kenya's corn shortfalls lead to increasing imports, which raises food prices even further. Maize lethal necrosis disease causes a major impact on maize yields within the eastern Africa region (Marenya *et al*., 2018). In 2013, the average yield loses in Kenya were estimated to range between 23-100% which translated to 0.5 million tons and valued at 180 million US$ (De Groote *et al*., 2016). The disease also impacted sales of commercial maize seeds during the MLN epidemic which affected medium and small enterprise seed companies within the Eastern African region (Boddupalli *et al*., 2020). The disease still presents a major threat to maize production in many regions of east Africa (Isabirye & Rwomushana, 2016). The mechanisms for the fast spread of MLN disease within eastern Africa is not been well understood.

Insect transmission of MLN disease causing viruses has not been well documented in Kenya (Mahuku *et al*., 2015a). However, neither the competency of vector thrips *Frankliniella* spp., in the spread of MLN nor their genetic diversity within the maize agro ecosystem had been determined. In addition, the rate of seed transmission of MCMV is very low, 0.04 % in the USA (Jensen *et al*., 1991). Recent studies have shown a seed contamination rate of 4.9 - 15.9 % and a transmission frequency of 0.025 - 0.17% (Kimani *et al*. (2021). It was therefore necessary to explore alternative MLN transmission pathways via pollen, which had not been documented. Further vector-virus transmission bioassay involving MLN disease causing viruses was necessary to unravel their transmission mechanisms in order to understand the epidemiology of the disease.

1.5 Justification

The MLN disease caused an epidemic in Kenya in 2011 which rapidly spread into the Eastern Africa region. The cause of the early rapid spread of MLN in Kenya and mechanisms of MCMV spread over a wide geographic area was a mystery and thrips were suspected to be responsible vectors (Mahuku *et al*., 2015). Knowledge on the transmission mechanisms of MLN causing viruses via maize infesting thrips and pollen was critical in unravelling the disease epidemiology. Confirmation of vector thrips that contributed to the spread of the MLN disease epidemic would guide current strategies towards the management of the disease and contribute to food security in Eastern

Africa. Therefore, an understanding of the transmission competence, efficiency and persistent transmission of MLN by *F. occidentalis and F. williamsi* thrips, was necessary.

1.6 Objectives

1.6.1 The General objective

The study sought to determine the mechanisms of maize lethal necrosis disease transmission so as to contribute to the effective management of the disease for the increased and sustainable production of maize.

1.6.2 Specific objectives

i. To determine the efficiency of *Frankliniella occidentalis* and *F. williamsi* in MCMV transmission
ii. To evaluate the mode of transmission of MCMV by *F. occidentalis* and *F. williamsi* thrips
iii. To determine the role of maize pollen in MLN disease transmission
iv. To characterize thrips infesting MLN affected maize in Murang'a County of Kenya

1.6.3 Hypotheses

i. Vector thrips *Frankliniella occidentalis* and *F. williamsi* have a low MCMV transmission efficiency.
ii. The transmission of MCMV by *F. occidentalis* and *F. williamsi* thrips is not persistent.
iii. Maize pollen has no role in MLN disease transmission.
iv. There is low genetic variation among thrips species transmitting MLN causing viruses in Murang'a County of Kenya.

1.7 Conceptual Framework

The interrelationship between the independent, dependent, and the intervening variables for the maize lethal necrosis disease is provided in Figure 1.2. The environmental conditions influence the agricultural practices which introduces MLN disease. The practices may also favour insect vector reproduction, thereby exacerbating

disease transmission. A widespread of the disease that causes high yield losses warrants the introduction of its control.

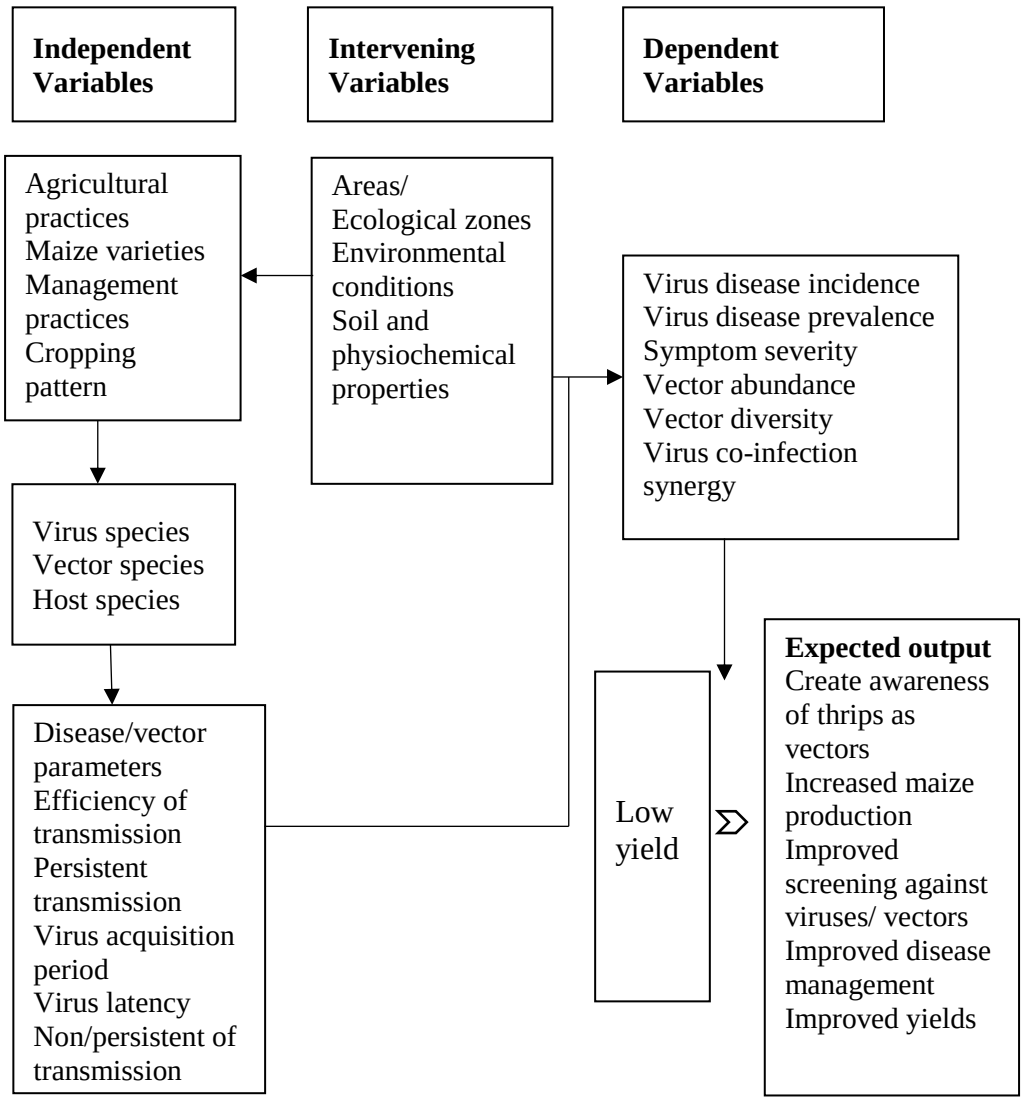


Figure 1.3: Conceptual Framework for the MLN disease Occurrence and Transmission

→ Direction of influence

1.8 Significance of the study

The study will provide insights into the transmission patterns of MLN disease transmission which will guide its control thereby minimizing its impact on smallholder farmers' food and nutrition security. Effective control measures will enable the fast spread of the disease into the potential MLN pandemic regions thereby reducing the hectares affected by the disease within East and central Africa. The huge maize

devastation occasioned by the MLN disease's impact on food prices and inflation in Kenya will be minimal with its effective management. The yield losses occasioned by the maize lethal necrosis disease causes within the eastern Africa region would be reduced. New maize varieties that are resistant to the MLN disease causing viruses would be developed which would enhance medium and small enterprise seed companies within the Eastern African region. The knowledge on insect transmission of MLN disease causing viruses in Kenya would guide would be useful in guiding studies on transmission of other maize infecting viruses in the region. Knowledge on pollen virus transmission would guide similar studies on pollen as alternative pathways for virus disease transmission.

CHAPTER TWO: LITERATURE REVIEW

2.1 Origin and Description of Maize Crop

The cultivated maize originated from teosinte, *Zea Mexicana* and its domestication began over 6,000 years ago (Galinat, 1988). The Europeans (Matsuoka *et al*., 2002) introduced the crop into the temperate and tropical regions in the 15th Century. The crop belongs to the grass family, *Poaceae.* Maize is an annual crop with a thick single stem that grows up to 3 meters high. The maize plants are mono-ecious consisting one or more tillers. The female and male inflorescences are borne on separate flowers within a maize plant.

A maize plant bears panicle tassels and pistils on leaf axils. The spikelets occur in 8 - 16 rows of approximately 30 cm long, on thickened, almost wood-like axis (cobs). Ears are enclosed in foliaceous bracts with masses of long styles (silks) protruding from the tips that resemble silky threads (Hitchcock & Chase, 1971). Staminate inflorescence forms the pollen while pistillate inflorescence produces the ovules. Both self and cross pollination can occur on maize through wind and the shed pollen remains viable within 10 - 30 minutes or longer under favorable conditions (Coe *et al.*, 1988). `

2.2 Importance of Maize Production in Kenya

Kenya produces 43.3 million 90 kg bags against the national demand of 52 million 90 kg bags (Ministry of Agriculture, 2019). Maize is the most important staple for calorie nutrition in Kenya with a per capita annual consumption of between 88 -103 kg (Abate *et al*., 2015). The crop provides 30% of the total dietary calorie intake and 70% of the daily cereal consumption for 85% of the population (Kariuki *et al*., 2020). Maize is consumed in the region either as githeri, ugali, or porridge.

The majority of the Kenyan population prefers maize cereals as their main staple food and any shortage in its supply is synonymous with food insecurity (Nyameino *et al*., 2003). The production of maize and its development has a positive impact on rural incomes, poverty reduction, and food security (Kwach, 2009). Maize production in Kenya is concentrated in the Rift Valley and western regions; Sub-counties of Trans Nzoia East and West, Wareng, Eldoret East and West, Nakuru, Kwanza and Bungoma

regions (Kwach, 2009). About 90% of the population in Kenyan depends directly or indirectly on maize for food, income, and labor (Makone *et al*., 2014).

2.3 Constraints to Maize Production in Kenya

The challenges facing maize producers in Kenya are both technological, policy-based, socioeconomic, or due to abiotic and biotic factors (Oscar, 2009).

2.3.1 Social Economic

The socio-economic, policy and technological maize production limitations facing maize producers in Kenya include; the use of uncertified seeds, population pressure and land sub-division. Other limitations include high costs of farm inputs and poor state of infrastructure (GoK, 2010). Other social economic factors include high postharvest losses, poor market structures, expensive land operations, and inefficient fertilizers subsidies. For instance, there was an estimated postharvest loss of 4.5 million bags of maize in 2017 in Kenya. The major causes of loss are the weevils (53.1%), rodents (30.3%), aflatoxins (8.6%), and theft (8.0%), (CIMMTY, 2010).

2.3.2 Abiotic Constraints

Low maize yields in Kenya at 1.6 t/ha against a potential of 6t/ha (Jaetzold, 2006) is due to impoverished soils and unreliable rainfall (Miano, 2013). These, among other constraints, present a serious threat to livelihoods and food security in the region (Mbure *et al*., 2010). Abiotic constraints include drought, low use of farm inputs (fertilizers), low soil fertility, low adoption of new technologies and in-appropriate agronomic practices.

2.3.3 Biotic Constraints

Biotic factors include weeds (especially *striga* spp) and arthropod pests like maize stalk borers *Buseola fusca* (Fuller) (Lepidoptera: Noctuidae) soil pests like termites *Ancistrotermes* cavithorax (Sjoestedt) (Isoptera: Macrotermitinea), nematodes *Trichodorus* (de Man) (Triplonchida: Trichodoridea) *Pratylenchus* zeae (Graham) (Tylenchida: Pratylenchidae), cutworms *Agrotis ipsilon* (Hufnagel), (Lepidoptera: Noctuidae) and chaffer grubs *Rhizotrogus majalis* (Razoumowsky), (Coleoptera: Scarabeidae) (Midega *et al.*, 2010). Maize diseases include Grey leaf spot (*Cercospora zeae-maydis)*, Northern leaf blight (*Setosphaeria turcica*), rusts (*Physopella zeae*

Cumm), rots (root, stalk and ear) smuts (*Sporisorium reilianum*), *Maize streak virus* and MLND greatly contribute to reduction in maize yields.

Maize lethal necrosis is among the most devastating maize foliar diseases contributing to high yield losses. Areas highly affected with the disease experiences massive losses in yield of over 90% which affects the total maize produced in the country (Ochieng *et al*., 2012). Maize yields are prone to frequent fluctuations more than other cereal crops due to the vulnerability and sensitivity of maize to disease attacks (Kodhek, 2005). The MLN disease epidemic placed the entire maize sub-sector at a greater risk across the country especially its role in food security (Wangai *et al*., 2012).

Maize production is also affected by grass weeds like bermuda grass *Cynodon dactylon* L., (Poaceae); couch grass *Digitaria abyssinica* (Hochst. ex A. Rich.) Stapf; velvet fingergrass, *D. velutina*, (Forssk.) P. Beauv. (Poaceae); goose grass, *Eleusine indica* L., (Poaceae); *Setaria spp* (Poaceae); wild oat, *Avena fatua* L., (Poaceae) and *Dactyloctenium aegyptium* L., (Poaceae). These weeds provide alternative hosts for corn thrips which are associated with MCMV spread (Nelson *et al*., 2011).

2.4 Viruses that Infect Maize

The main viruses that are a problem on maize include; Maize streak virus (MSV), Maize dwarf mosaic virus (MDMV), Maize chlorotic mottle virus (MCMV) and Sugarcane mosaic virus (SCMV, formerly MDMV-B), (Niblett and Claflin 1978; Uyemoto *et al*., 1980; Goldberg & Brakke 1987). These viruses can cause synergistic reactions in coinfections with MCMV to cause MLN disease. These viruses are widely distributed in eastern Africa (Redinbaugh & Zambrano-Mendoza 2014; Mahuku *et al*., 2015).

2.4.1 Maize Streak Virus

The disease was first reported in East Africa in 1901 and has since extended to other African countries (Magenya *et al*., 2008, Martin & Shepherd, 2009). Severe epidemics occurred in Kenya between 1988-1989 (Njuguna *et al*. 1990), mainly due to alternate and successive cropping of maize and the presence of other wild grass hosts (Mesfin *et al*., 1995). The virus is transmitted by Leafhoppers *Cicadulina spp*., *C. mbila* (Naude) is the most prevalent vector, and transmits the virus for most of its life after feeding on

an infected plant (Magenya *et al*., 2008). Early disease symptoms begin within a week after infection and consist of very small, round, scattered spots in the youngest leaves. The spots enlarge parallel to the leaf veins and become more profuse at leaf bases but are particularly conspicuous in the youngest leaves. Fully elongated leaves develop a chlorosis with broken yellow streaks along the veins, contrasting with the dark green color of normal leaves (Plate 2.1). Severe infection causes stunting, and plants die prematurely or become barren (Bosque-Perez & Nilsa, 2000). Many cereal crops and wild grasses serve as reservoirs for the virus and vectors. Infection of the crop with MSV at seedling stage results in no ear formation with 100% yield loss, but late infection leads to undersized and poorly filled ears (Kaitisha, 2003).

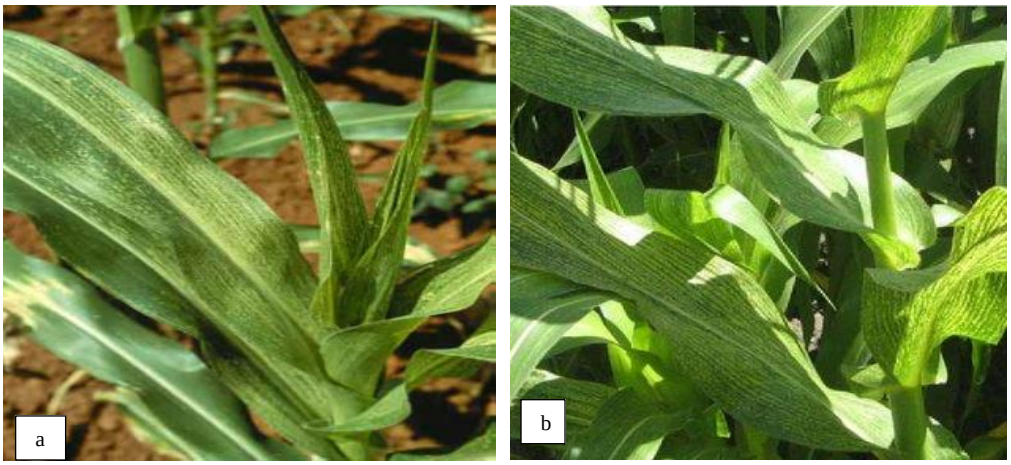


Plate 2.1 Plate 0.1: Pictures of maize plants with *Maize streak virus* symptoms

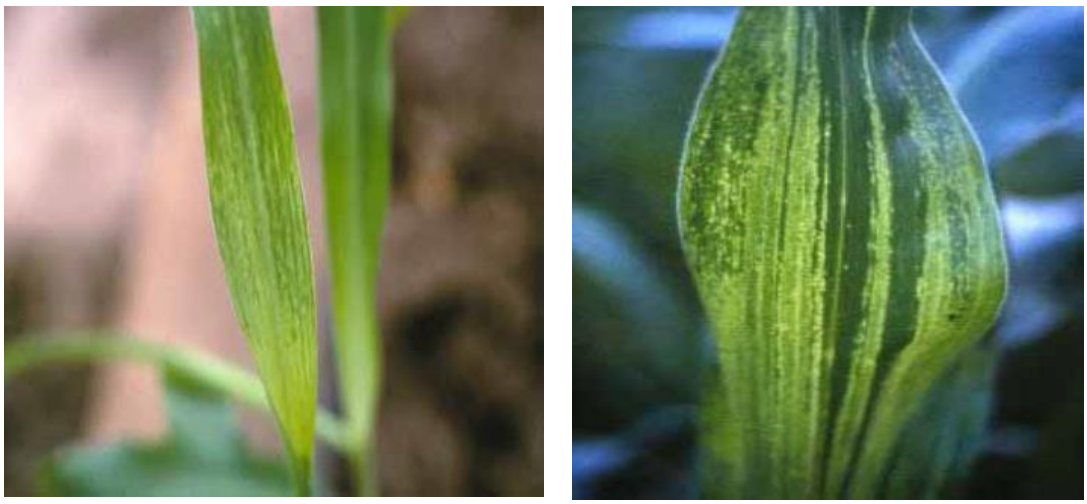
(a) Stunted maize plant with leaf streak, (b) Severe leaf streak symptoms

Source: Charles K., (2014).

2.4.2 Maize Dwarf Mosaic Virus

The virus is transmitted by several genera and species of aphids, including *Rhopalosiphum maidis* (Fitch) and also through seeds (Ford *et al*., 2004). The aphid can immediately transmit the virus after feeding on an infected plant. This pathogen can infect other grass and cereal hosts, such as sorghum, Johnson grass, and sugarcane (Gell *et al*., 2010). No infection occurs in broad-leaf plants. Infected plants develop distinct mosaic irregularities in the distribution of normal green color - on the youngest leaf bases. Sometimes the mosaic appearance is enhanced by narrow chlorotic streaks

extending parallel to the veins (Signoret & Lapierre, 2004). Later on, the youngest leaves show a general chlorosis, and streaks are larger and more abundant (Plate 2.2). As plants approach maturity, the foliage can turn purple or purple-red. Depending on time of infection, there may be severe stunting of the plant while plants infected early may become totally barren. In China, SCMV has been reported to significantly reduce maize production by up to 10 - 50% annually (Li *et al*., 2013).



a b

Plate 2.2 Pictures of maize leaves with Sugarcane mosaic virus disease symptoms

Source: Charles K., (2014).

2.4.3 Other Viruses Infecting Maize

Recent studies have identified other infective viruses on maize plants. Metagenomic analysis of viruses associated with maize lethal necrosis in Kenya by Wamaitha *et al*. (2018), indicated the presence of four main viruses: maize chlorotic mottle virus (MCMV), sugarcane mosaic virus (SCMV), maize streak virus (MSV) and maize yellow dwarf virus-RMV (MYDV-RMV). Other viruses also detected include; Hubei Potylike virus 1, Barley virus G, Scallion mosaic virus and Johnson grass mosaic virus (JGMV). Of these viruses, Hubei Poty-like virus 1, Scallion mosaic virus, JGMV, and Iranian JGMV are potyviruses while barley virus G is a polerovirus. The study concluded that these viruses constitute the virus complex infecting maize in Kenya and that their genetic composition was distant from isolates described before. Since maize infecting poleroviruses are widely distributed in Rwanda (Adams *et al*., 2017) and in

Kenya, there could be a possibility of a synergistic interaction between MCMV and a polerovirus to cause maize lethal necrosis disease. This synegistic interaction could also be responsible for the variation in symptoms in the maize fields (Wamaitha *et al*., 2018). Moreover, maize yellow mosaic virus (MaYMV) was detected in maize plants showing lethal necrosis-like symptoms in Rwanda (Adams *et al*., 2017).

2.5 Maize Lethal Necrosis Disease

The MLN disease was first identified in the USA in 1976 (Niblett & Claflin, 1978). The disease developed when two viruses occurred within in the same maize plant; one virus being maize chlorotic mottle virus (MCMV). Either maize dwarf mosaic virus-strain B (MDMV) also known as sugarcane mosaic virus or wheat streak mosaic virus WSMV, can serve as the second virus in MLN disease complex. Maize chlorotic mottle virus induces mild symptoms when in single infection; however, infection with the second virus on the same maize plant induces rapid synergistic reactions, resulting in serious damage to the plants. Wamaitha *et al*. (2018) also identified Scallion mosaic virus, Hubei Poty-like virus 1, and JGMV as potyviruses contributing to the MLN disease epidemic. Of these potyviruses, only JGMV was previously reported to co-infect with MCMV and cause maize lethal necrosis disease (Stenger *et al*., 2007). The transmission patterns of Scallion mosaic virus, Hubei Poty-like virus 1, and JGMV and their role in the maize lethal necrosis disease complex remains unclear. Gitonga (2014) noted that some maize producing counties had significant yield reductions due to MLN disease (Figure 2.3). Counties of Makueni, Nyamira and Laikipia had higher significant yield reductions than Kilifi Siaya and Kisumu.

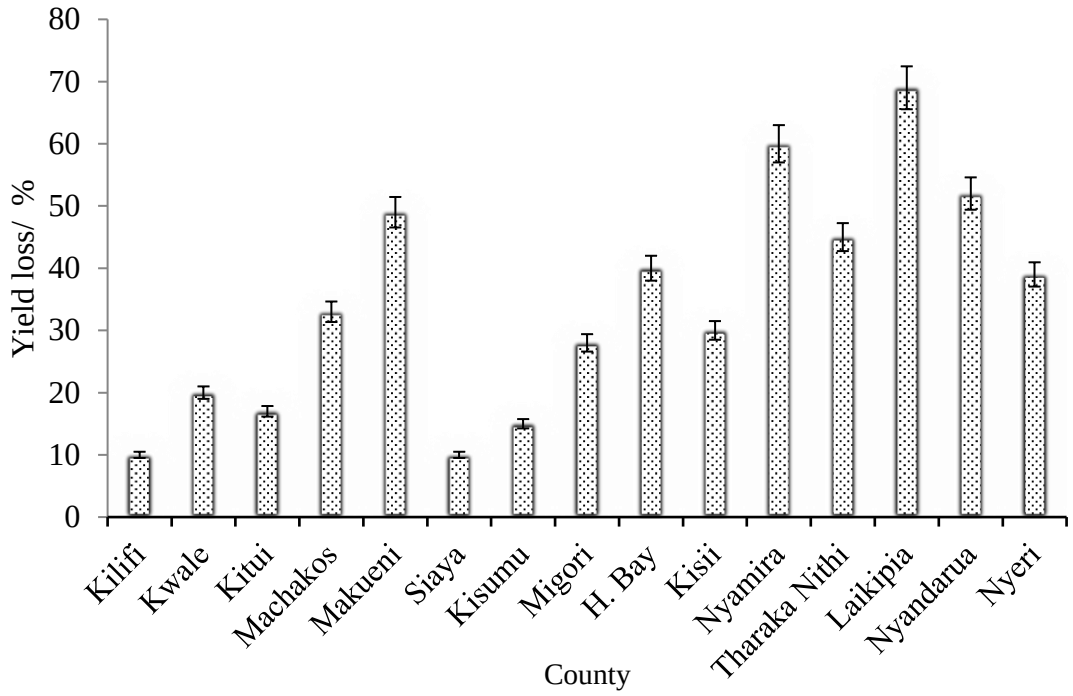


Figure 2.2: Yield losses in Kenya due to MLND in 2014

Source: Gitonga and Snipes, 2014.

There is an urgent need for the development of an effective management strategy of this disease. An effective management strategy of a disease would require rapid diagnosis of its causal agent(s), (Adams *et al*., 2012). The traditional approach to plant virus diagnostics requires bioassays and the use of indicator plants. It also involves symptomatology the determination virus particle morphology (size and shape), vector relations and host range. A combination of more than one diagnostic test provides adequate information on the identity of a virus as compared to a single assay method. The use of multiple, specific, sensitive, and inexpensive diagnostic assays provides more accurate results (Naidu & Hughes, 2003).

2.6 Maize lethal necrosis disease symptoms

A single virus infection with either MDMV or (MCMV) induces a light-greenish mottling (alternating dark green and light portions) leaf symptoms. Often there occurs little obvious damage, and the symptoms may be masked or disappear. However, when both viruses co-infect the same plant, a bright green-yellowish mottle develops on the leaves (Plate 2.3).

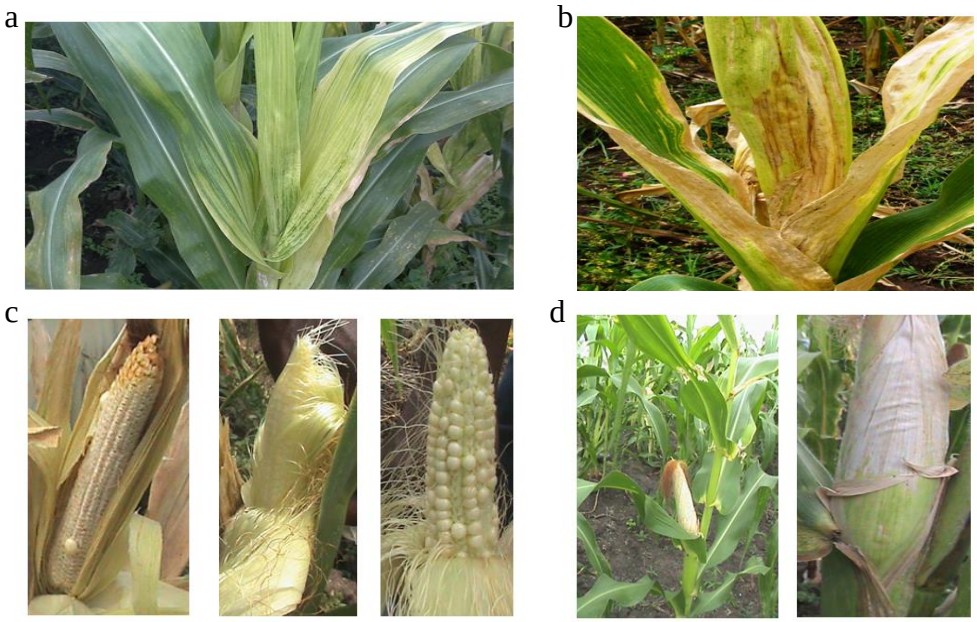


Plate 2.3 Maize lethal necrosis disease symptoms on leaves and ear cobs

(a) Infected leaves showing chlorosis and leaf mottling, (b) Infected plant showing leaf necrosis and dead heart symptom, (c) Ear cobs with no grain and poorly filled kennels, and (d) Maize plants infected MLN showing pre-maturely dried ear cobs. Source: Miano *et al*., (2013).

Unlike in single plant infections where the often outgrows the mosaic symptoms, the MLN induced bright greenish-yellow mottling may persist till the end of the growing season (Ministry of Agriculture, 2012). Leaves may become inwardly necrotic toward the end of the season starting from the margins causing eventual plant death that starts from the top down (Miano *et al*., 2013). Necrosis of young leaves occur inside the whorl of the plant before expansion that leads to a "dead heart" symptom. The ears are often small and distorted, may have few or no kernels formed. Infected plants are usually barren, especially in early plant infections. Symptoms will occur on young leaves that emerge after successful virus infections. Plants infected late in the season may form poorly filled ears coupled with premature drying of the husks without clear mosaic leaf symptoms (Makubi *et al*., 2012).

2.7 The maize Lethal Necrosis Disease Etiology

The disease manifests on maize whenever MCMV together with SCMV or any cereal infecting potyvirus like MDMV, or WSMV infects a plant. Maize chlorotic mottle virus or SCMV single infections induce mild symptoms but rapidly triggers a synergistic reaction that seriously damages or kills infected plants when they occur in combination in the same plant. Recent findings have indicated that MCMV is a threat on its own. The virus induces significant yield losses even in non-synergistic co-infection with other viruses (Mahuku *et al*., 2015). Infected maize leaf samples from Kenya tested positive for MCMV and SCMV which confirmed that these viruses were responsible for the MLN disease epidemic (Wangai *et al*., 2012).

2.7.1 Maize Chlorotic Mosaic Virus

The virus was initially discovered on maize (*Zea mays*) in Peru (Hebert and Castillo, 1973). The virus later spread into Kansas and Nebraska of the United States of America (Doupnik, 1979). The virus associates either with MDMV or WSMV to cause MLN disease (Jiang *et al*., 1990; Uyemoto *et al.*, 1980; Niblett and Clafin, 1978). The virus belongs to the Machlomovirus genus, in the family Tombusviridae, (Nelson *et al*., 2011). The virus virions are composed of isometric single-stranded RNA, with single-component particles being either smooth, spherical, or hexagonal in shape (Scheets, 2010). The MCMV-P (Peru) and MCMV-K (Kansas) are the only genetically and geographically existing distinct virus strains (Nyvall, 1999).

According to recent studies, MCMV isolates in Kenya are related closely with those from China and have low genetic variation (Braidwood *et al*., 2017; Wamaitha *et al*., 2018). Maize chlorotic mottle virus particles are isometric, non-enveloped, and are 28 - 34 nm in diameter. The virus particles are composed of 180 polypeptides of which two-thirds of its 4437 nt viral RNA encode 5' sense strand proteins (Nutter et.al., 1989). The first open reading frame (ORF) encodes a 32 kDa protein, p32 which is unique to MCMV. The p50 together with its read through (rt) protein p111 are related to the highly conserved RNA dependent RNA polymerases (RdRps) encoded by Tombusviridae viruses. The p50 resembles an ORF_1 of the Panicum mosaic virus (48 kDa), a *Panicovirus*. The unique p50 amino acid terminal sequence is related to the p32 ORF overlap (Figure 2.4).

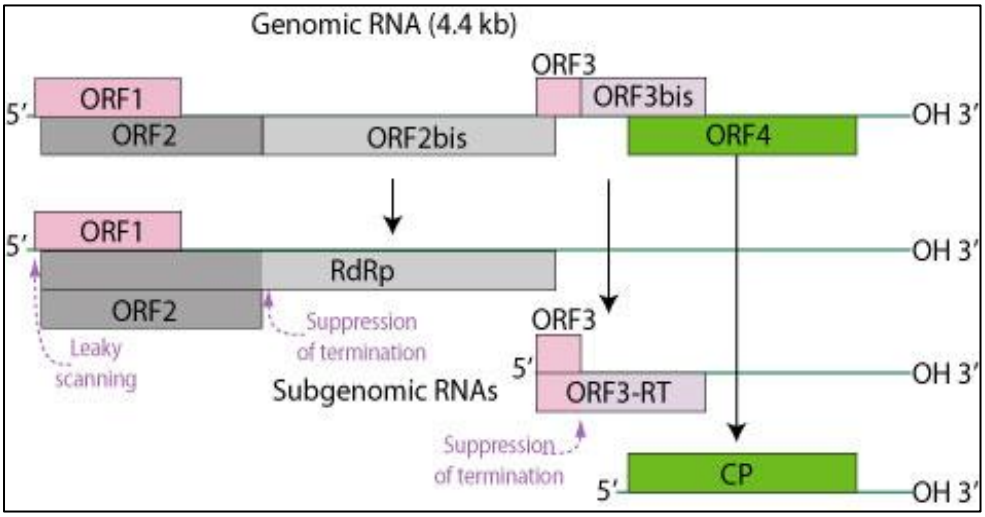


Figure 2.3: The genomic assembly for Maize chlorotic mottle virus.

Linear, ssRNA (+) genome of 4-5.4 kb, which lacks a cap structure and a poly(A) tail. Source: Swiss Institute of Bioinformatics, https://viralzone.expasy.org/10.01.2022

The sub genomic RNA_1 transcribes the 3' viral genome ORFs with the ORF_1 encoding a p7a peptide (7.2 kDa). The suppression of its stop codon produces a 31 kDa protein, p31 (Scheets, 2000). The carboxy-terminus of a p7a has sequences similar to that of movement protein 1 (MP_1) found in the tombusvirid genera that encodes two or more small MPs, (Rochon et. al., 2011). A small ORF without methionine codons (~8 kDa coding capacity) after the p7a ORF encodes a peptide similar to a panicovirus MP_2 (Riviere & Rochon, 1990; Scheets *et al*., 2015).

The MP_2 and MP_1 both facilitate movement between cells in tombusvirid viruses (Scheets *et al*., 2015). The 31 kDa protein has a unique carboxy terminal extension and the second AUG of the sub genomic RNA_1 starts 25 kDa coat protein ORF, (Scheets, 2000). In infected plants, there is an accumulation of the 337 nt non-coding sub-genomic RNA_2 within the protoplasts (Scheets, 2000). Unlike in red clover necrotic mosaic virus whose accumulated non-coding sub-genomic RNA_1 is a degradation product, that of MCMV has not been differentiated from true sgRNA (Iwakawa *et al*., 2008).

2.7.2 Host Range for Maize Chlorotic Mottle Virus

Maize is the only known natural plant host for MCMV. Other hosts are limited to plants in the *Poaceae* family (grasses) where the virus has been experimentally transmitted (Scheets, 2004). Amongst these grasses, only 73 plant species within 35 genera are susceptible to MCMV-P or MCMV-K, or both virus strains. Hosts of MCMV include the following plant species; fall panic grass guinea grass *Panicum maximum* Jacq., *P. miliaceum* L. *Panicum dichotomiflorum* Mich*x*., and maize, *Zea mays* (Nelson *et al*., 2011). The status of other *Panicum* grasses as hosts of MCMV strains either in experimental or natural conditions is unknown.

2.7.3 Sugarcane Mosaic Virus

Sugarcane mosaic virus (SCMV) is an important viral pathogen of maize, sorghum, and sugarcane (Teakle *et al*., 1989). The virus was initially reported on maize in the USA (Brandes, 1920). The virus later spread to Eastern Africa where it was detected in sugarcane and maize (Hansford, 1935; Riley, 1960). The virus became endemic to all major maize producing regions of Uganda, Tanzania, and Kenya (Louie, 1980; Kulkarni, 1973). The virus is described as the main potyvirus in the MLN disease complex in eastern Africa where the virus frequently occurred in co-infection with MCMV (Wamaitha *et al*., 2018; Mahuku *et al*., 2015 and Adams *et al*., 2014). *Sugarcane mosaic virus* is as a *Potyvirus* of the family Potyviridae (Zhu *et al*., 2014). The SCMV virions exist as single-stranded positive-sense RNA genomes of an approximate length of 9.6 kb (Figure 2.5).

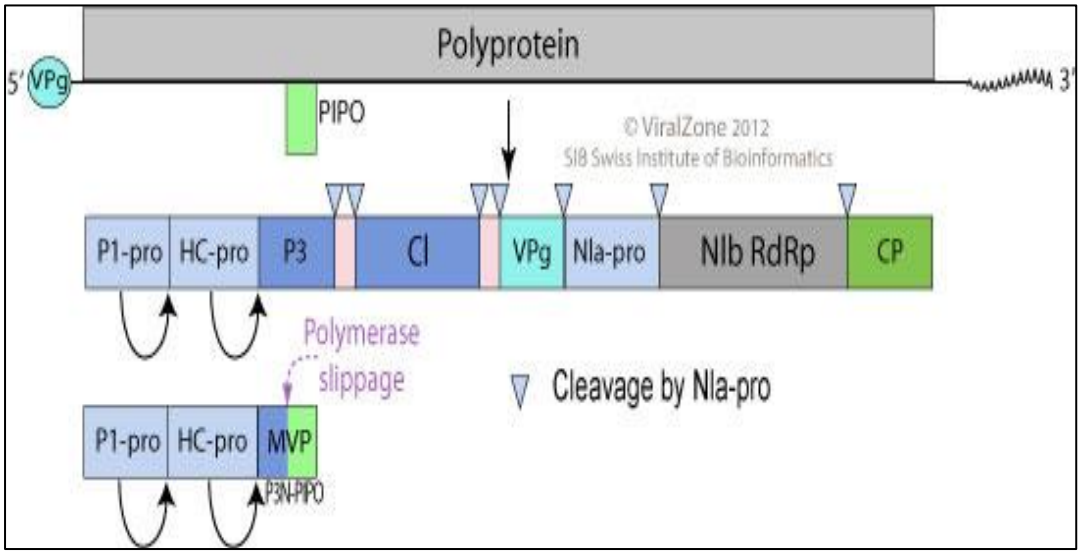


Figure 2.4: Genome Organization of Sugarcane Mosaic Virus

A linear, ssRNA(+) genome of 10 kb in size. 3' terminus has a poly (A) tract. 5' terminus has a genome-linked protein (VPg).

Source: Swiss Institute of Bioinformatics, https://viralzone.expasy.org/10.01.2022

A covalent link joins the genomic proteins on the 5′ termini and a poly-A protein on the 3′ termini (Gell, *et al*., 2015). Virus genomes encode polyproteins that are subsequently cleaved into the ten mature proteins (NIa-Pro, P1, P3, HC-Pro, 6K2, 6K1, CI, NIa-VPg, NIb, CP) by three self-encoded proteinases (Urcuqui Inchima, *et al*., 2001). The virus has numerous strains that replicate in a complex and dynamic pattern that form swarms of mutant virions (Padhi and Ramu, 2011), (Figure 2.4). This occurs because its RNA-dependent RNA polymerase has a weak proofreading activity, thus the virus easily mutates within short generation times, and leads to large populations (Li, *et al*., 2013).

There is a wide genetic variation in the SCMV genome with sequence phylogeny of sugarcane and maize isolates clustered by host and geographic origin (Li *et al*., 2013). The virus is as the primary potyvirus agent associated with MLN disease outbreak in East Africa. The virus was reported in Kenya (Louie, 1980), and in South Africa (Handley *et al*., 1998) but has spread widely in Africa. Together with MDMV, SCMV these viruses are responsible for the most worldwide spread of viral diseases on maize (Redinbaugh and Zambrano, 2014).

Sugarcane mosaic virus is easily detected by DAS-ELISA using a polyclonal antibody raised against the virus. This antibody reacts with only SCMV but not with sorghum mosaic virus, SrMV; maize dwarf mosaic virus, MDMV-A; and Johnson grass mosaic virus, JGMV; all of which are *potyviruses* with a similar host range with SCMV (Shukla *et al*., 1992). The strains of SCMV include B, D, and E (Grisham and Pan, 2007). Strains A, H, I, and M were reported in Louisiana, but they were later classified as sorghum mosaic virus (SrMV), (Yang and Mirkov, 1997). The details about occurrence of these strains and their extent of interaction with MCMV to cause MLND in Kenya remain unclear.

2.7.4 Host Range for Sugarcane Mosaic Virus

Several crops and non-crop plants can act as primary inoculum sources in the field. Noone *et al*. (1994) found that transmission rates from sweet corn and sugarcane to maize were 12-16% and 2-5%, respectively. Srisink *et al*. (1993) detected natural infections of SCMV strains in wild sorghum, *Sorghum verticilli florum* (Steud.) Stapf and hairy arm grass, *Brachiaria piligera* (F. Muell. ex Benth.) Hughes. Garud *et al*. (1991) found that SCMV spread faster into sorghum planted in close proximity to sugarcane fields other plants.

2.8 Epidemiology of Maize Lethal Necrosis Disease

Maize lethal necrosis disease rapidly spread into the eastern and central regions of Africa after its first incidence in Kenya (Wangai *et al*., 2012). The disease was detected in Tanzania in 2012 around the Lake Victoria region of Arusha (CIMMYT Periodic Newsletter, Dec 2012). Around the same period, the disease was also detected in areas along the Kenya border districts of Busia and Tororo in Uganda (CIMMYT Periodic Reports Newsletters, Dec 2012). The disease spread further into Iganga and Mbale districts of Uganda; (Kagoda *et al*., 2016). In 2013, MLN was detected in Rwanda, where it was prevalent in all maize-growing districts (Adams *et al*., 2014). The MLN disease continued its rapid spread further into the western regions of south and north Kivu provinces of the Democratic Republic of Congo (DRC) where it was reported in 2014 (Lukanda *et al*., 2014). Within the same period, the disease spread into Ethiopia, where MLN symptomatic maize plants were sighted in 2014 which prompted surveillance that led to its first report (Mahuku *et al*., 2015b). The disease was also

reported in Southern Sudan (Mahuku *et al*., 2015a, b) and in Burundi. The rapid spread of MLN disease prompted an investigation of the underlying mechanisms of its transmission which could guide in developing control strategies in east Africa. Vectors are required to transmit and spread MCMV from plant to plant, then from field to field (Makubi *et al*., 2012). Both MCMV and SCMV can be mechanically transmitted and through seed (Cabanas *et al*., 2013). In addition, MCMV is experimentally transmitted by *F. williamsi* thrips and beetles *Oulema melanopa* L., Coleoptera, Chrysomelidae), (Plate 2.4a) while maize aphids transmit SCMV, (Cabanas *et al*., 2013). However, the rate of virus transmission via fertilization and seed is low (0.04%), (Jensen *et al*., 1991).

a b c d

Plate 2.4 Vectors that spread Maize lethal necrosis disease

(a) A cereal leaf beetle; *Oulema melanopa*, (b) The spotted cucumber beetle; *Diabrotica undecimpunctata*, (c) Southern corn rootworm, and (d) Maize infesting thrips

Source: Miano *et al*., 2013

Sugarcane mosaic virus is also spread through seed at a rate of 0.4 to 3.9% depending on the genotype (Li, *et al*., 2011). Nonetheless, low rates of virus transmission through seed are epidemiologically important. Since maize is propagated entirely through seed,

invasive plant viruses can be introduced into new areas through this mode (Mahuku *et al.*, 2014). The low rate of virus transmission via seed can also result into an epidemic through secondary spread of the invasive virus via insect vectors (Maule & Wang, 1996). Research under greenhouse conditions demonstrated that maize chlorotic mottle virus is transmitted by maize rootworm beetles *Diabrotica undecimpunctata* Horwardi (Coleoptera: Chrysomelidae) (Figure 2.7b), however, this has not been demonstrated in the field. The virus is also mechanically spread through soil and infected plant debris where it survives on plant residues (Nyvall, 1999). The soil-borne transmission mechanisms of MCMV in the field, remains undetermined. Thrips remain the primary vectors for the non-persistent transmission of the virus.

Maize (*Z. mays* L.) is the most preferred host for infectious thrips but they also infest cassava *Manihot esculenta* (Crantz), beans *Phaseolus vulgaris* L., sorghum *Sorghum* spp, onions *Allium cepa* L., various grasses *graminea family*, rice *Oryza sativa* L, peppers *Capsicum annuum* L, coriander *Coriandrum sativum* L., and peas *Pisum sativum* L. The thrips also infest the following weedy species; black jack *Bidens pilosa* L., and mexican sunflower *Tithonia diversifolia* (Hemls.), (Capinera, 2008). Green bugs and maize leaf aphids vector maize dwarf mosaic virus-strain B while wheat curl mites transmit wheat streak mosaic virus.

In maize, the MCMV is detectable in pollen, inflorescences, leaves, cotyledons, and seeds (endosperm, ear husks, pericarps, embryo, and cotyledons), (Scheets, 2004). Loss of plant turgor due to water stress increases sap viscosity which makes feeding difficult for vectors (Miles *et al*., 1982). Phloem amino acids fluctuation due to water stress also induces behavioral changes of many vector species (White, 1984). The continuous production of maize in a given field greatly contributes to an increased viruses and vectors incidence. Since MCMV colonizes pollen and with maize varieties being susceptible, from seedling stage to near maturity, the role of pollen and thrips in MLN disease epidemiology remains critical.

2.9 Vector Transmission of MLN Causing Viruses

Insect vectors such as beetles and thrips transmit MCMV while maize aphids spread SCMV (Wangai *et al*., 2012; Brunt *et al*., 2000). Insect vectors transmit potyviruses

such as SCMV, WSMV, and MDMV in a non-persistent pattern. The virus attaches to the tip of an insect stylet from which it inoculates onto the next plant the insect feeds on. Cereal leaf aphids *Rhopalosiphum maidis* (Fitch) (Homoptera: Aphididae), *Sitobium avenal,* and *Rhopalosiphum padi* transmit *Sugarcane mosaic virus* (Pemberton & Charpentier, 1969) while the wheat curl mites; *Aceria tosichella* Keifer (Acari: Eriophyidae), transmits WSMV (Slykhuis, 1955). Mites acquire the virus during feeding and their efficiency of virus transmission varies between the different stages of growth. Immature stages have higher transmission efficiency than adults (Siriwetwiwat, 2006).

The mites acquire the virus within 15 min acquisition access period (Orlob, 1966). A broad range of aphids transmit MDMV, these include; the pea aphid, *Acyrthosiphon pisum* Harris (Hemiptera: Aphididae), the corn root aphid, *Aphis maidiradicis* Forbes (Hemiptera: Aphididae) and, the Green bug aphid *Schizaphis graminum,* Rondani (Hemiptera: Aphididae). Others include, the cotton aphid *A. gossypii* Glover (Hemiptera: Aphididae), black bean aphid *A. fabae* Scopoli (Hemiptera: Aphididae), Cowpea Aphid *A. craccivora* Koch (Hemiptera: Aphididae) and the alfalfa aphid *Therioaphis maculate* Buckton (Hemiptera: Aphididae) (Sharma, and Misra, 2018). The green peach aphid *Myzus persicae* Sulzer (Hemiptera: Aphididae), grain aphid *Rhopalosiphum padi* L. (Hemiptera: Aphididae), corn leaf aphid *R. maidis* Fitch (Hemiptera: Aphididae) and the grass aphid *Rhopalomyzus poae* Gillette (Hemiptera: Aphididae) also transmit the virus. The apple grain aphid *R. fitchii* (Hemiptera: Aphididae), cabbage aphid *Brevicoryne brassicae* L. (Hemiptera: Aphididae) and potato aphid *Macrosiphum euphorbiae* Thomas (Hemiptera: Aphididae) also spread the virus. The vector aphids have short virus acquisition and inoculation periods (Ford *et al*., 2004). Of these aphids, only *Schizaphis graminum* R. have a latent period of about 20 hours, (Berger *et al*., 1987). The maize dwarf mosaic virus is also spread via maize seeds at transmission rates ranging from 0.007 - 0.4% (Ford *et al*., 2004).

2.9.1 Thrips as Vectors of Plant Viruses

Thrips belong to the order *Thysanoptera* and are characterized with distinctive long hairs around the wing margins, a mandible used to punch holes in plants to suck the cell sap using a stylet, and a bladder like appendage for clinging onto plant surfaces. An

adult body size range from 0.5 - 5 mm and usually have four slender wings, (Plant Health Australia, 2011), (Plate 2.5). Many thrips species have been grouped into nine families of which 95% belong to the *Thripidae* and *Phlaeothripidae* families (Jones, 2005). The species that are virus vectors are members of the *Thripidae*. Both larval and adult stages of thrips actively feed on virus infected host plants. However, it is only early, late larval instars and adults that transmit plant viruses (Persley *et al*., 2006). Thrips are an economic problem in a wide range of crops that includes maize, vegetables, ornamentals, strawberries, and grapes.

a b

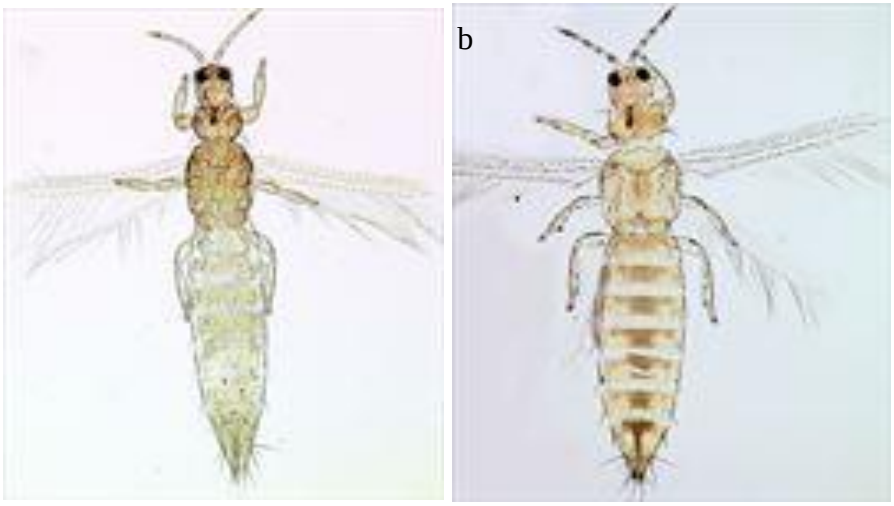


Plate 2.5 Images of thrips that vector maize chlorotic mottle virus

(a) An image of *F. williamsi*, (b) an image of *F. occidentalis* mounted on microscope slide. Source: Mound and Tree (2020) .

Grass thrips *Frankliniella williamsi* Hood (Thysanoptera: Thripidae) found in maize are minute (about 1/16th inch) elongate, yellow insects. The adults have slender fringed wings with long hair (Heming, 1993). The immature ones are wingless and yellow orange in colour. Both adults and immature thrips may occur on maize at any given period during the growing season. Eggs are oviposited in plant tissues where they hatch within 5 days' period. The immature stage takes 5 to 7 days to completely develop (Mound, 1997). Thrips population increase in warm dry periods which increase the activity of the insects and the spread of the disease. The thrips damage the maize plants while rasping the leaf tissues and sucking the plant sap exudates from the ruptured cells. They feed deep in whorls, underneath the leaf sheaths and on the under-side of lower

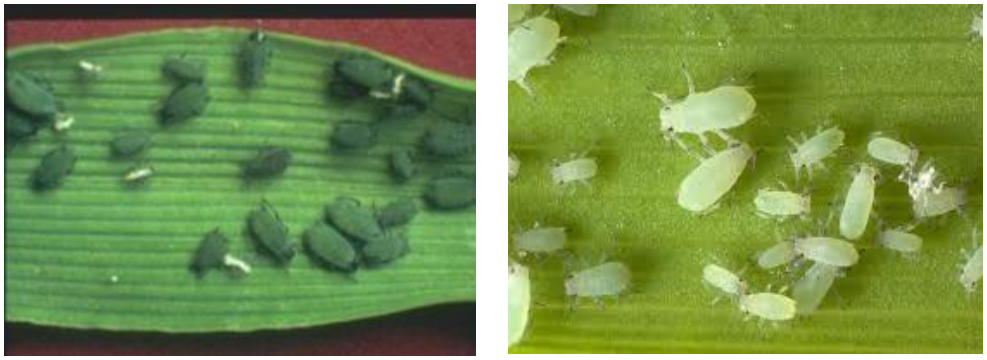
leaves, on floral parts such as petals and pollen, however when this injury is extensive and the maize plants are stressed, leaves and ear husks appears silvery (Childers & Bullock, 1999). Feeding by thrips has never been an economic problem.

Thrips are most noticeable with a great concern on young seedling plants and at ear formation stage during the growing season. On young seedlings their feeding makes the plants wilt and can cause stunting. Heavy infestation of thrips may cause distorted leaves with the edges cupped upwards that turn brownish (Childers & Achor, 1995). Despite infestation by the thrips the plants are able to tolerate the attack. At ear formation, thrips may cause mechanical damage to developing kernels that provides entry points for *Fusarium* spp leading to Fusarium ear rot disease infections (Parsons & Munkvold, 2010). Maize thrips are reported to spread MCMV which in combination with any potyvirus (ScMV or MDMV) cause MLND (Ministry of Agriculture, 2013), while Western Flower Thrips; *F. occidentalis* transmits MCMV in Thailand (Zhao *et al*., 2014). The role of maize infesting thrips in pollen mediated virus transmission has been a subject of speculation.

2.9.2 Maize aphid Rhopalosiphum maidis

Maize aphids *Rhopalosiphum maidis* (Fitch) (Homoptera: Aphidae) transmits *Sugarcane mosaic virus*; a potyvirus in the MLN disease complex. The adults are may either be winged or wingless and they are usually less than $1/25^{th}$ inch in length. They are green in colour, have black antennae, legs and tail pipe (cornicles) (Plate 2.6). The nymphs are similar to adults in appearance, are light green, smaller, and wingless. The soft bodied insects colonize the tassels and the upper leaves of the maize plants.

The feeding behavior of the aphids' causes' injury, deprives plants nutrients and while the excess sap is secreted as honey dew that may cover the tassels and the leaves. Colonies of aphids may be found in central leaf whorl (Nalam et al., 2019). The insect feed on the inflorescence and young leaves especially during dry periods causing yellow mottling of leaves (Ministry of Agriculture, 2013). The maize aphids transmit barley yellow dwarf virus (BYDV) and MDMV.



a b

Plate 2.6 Maize aphid *Rhopalosiphum maidis* on a maize leaf

(a) Immature forms of maize aphids and (b) Mature forms of the aphids.

Source: (Prasanna, 2014)

2.9.3 Scarab beetle larvae Phyllophaga *spp*

The larvae/grubs of Scarab beetles are white in colour. The hind body is shiny and smooth with their dark body contents usually appearing through the skin. The fully grown grubs are slightly longer than an inch and curl into a "C" shape appearance (Plate 2.7 b). The grubs have a 3-year life cycle. They eat roots and severely cause wilting in which the center leaves of the young maize plant die. During the feeding, the grubs are reported to transmit MCMV that is acquired from plant debris on soil surfaces when maize is off season (Ministry of Agriculture, 2013).

a b

Plate 2.7 An image of a Scarab beetle that transmit maize chlorotic mottle virus

Source: https://www.alamy.com/stock-photo/african-black-beetle.html.

2.10 Pollen Mediated Transmission of Plant Viruses

Pollen transmit a variety of viruses, including *Ilarvirus, Nepovirus, Sobemovirus, Idaeovirus, Potyvirus*, and others. Pollen-transmitted viruses include at least 45 from

16 genera and five viroids (Bhat and Rao, 2020). Pollen from some virus-infected plants can cause natural infection and virus transfer to other plants. Humans, the wind, or insects can all carry virus-infected pollen. Virus transmission via pollen can occur vertically or horizontally. The transmission of plant viruses through pollen dates back to the 1940's, and since then, more than fifty viruses have been associated with pollen mediated transmission (Cooper *et al*., 1988). Based on the mode of pollen transmission, the transmission can either be vertical or horizontal. In vertical transmission, a virus is inherited by an offspring during fertilization (Cooper *et al*., 1988). On the other hand, horizontal transmission occurs when a virus is spread between plants via pollen (Mink, 1993). Three nepoviruses; blueberry leaf mottle virus (BBLMV), artichoke yellow ringspot virus (AYRV), and cherry leaf roll virus (CLRV), are transmitted horizontally through pollen. Other viruses spread horizontally via pollen include; Ilarviruses; tobacco streak virus (TSV), blueberry shock virus (BlShV), PDV, PNRSV and sowbane mosaic sobemovirus (SoMV). Raspberry bushy dwarf ideaovirus (RBDV) is also spread in the same manner (Mink, 1993).

Horizontal pollen-transmission of plant viruses plays a critical role in the epidemiology of a viral disease (Mandahar, 1984). Pollen from one infected plant can transport a virus to many healthy plants. The secondary infected plants produce more virus-infected pollen that will move from infected-pollen to healthy plants during pollination and fertilization. Sdoodee and Teakle (1987) first reported that TSV-contaminated pollen could be carried by onion thrips *Thrips tabaci* (Lindeman) and then transmitted to leaves of the experimental host plant, tree spinach *Chenopodium aranticolor* (Coste & Reyn.) via wounds caused by thrips. Also, Greber *et al*. (1991) demonstrated that the presence of both thrips and weed pollen resulted in higher TSV incidences on test plants. Thus, the interaction of thrips *Microcephalothrips abdominalis* (Crawford 1910*)* and pollen of the blue billgoatweed *Ageratum houstonianum* (Mill.*)*, the most common wild host of TSV in that area, caused the disease epidemic.

Maize chlorotic mottle virus can be detected on pollen, shed from an infected maize plant. The virus also be detected on other plant parts; both female and male inflorescences, ear husks, cotyledons, seeds (pericarps, cotyledons, endosperm, and embryo), (Scheets, 2004). Montenegro and Castillo, (1996) also detected MCMV on

pollen, leaves, inflorescences, ear sheaves and bracts, and in seed (cotyledons, embryo, pericarp and endosperm). The highest concentration of the virus was detected in pollen, endosperms, and pericarps. Li (2004), confirmed that SCMV also enters maize seeds during fertilization through infected pollen and flowers of infected maternal plants after conducting cross-pollination experiments. Li *et al.* (2006) also conducted pollen-anther transmission tests which demonstrated that surface contamination of pollen with SCMV can lead to transmission of the virus via pollen. However, there is no conclusive evidence demonstrating that pollen horizontally transmits MCMV during fertilization, or through wounds initiated by feeding thrips. Thus, it was prudent to conduct tests on the possibility of pollen-mediated horizontal transmission of MCMV and the role thrips play in this mode of transmission.

2.11 Virus Disease Diagnosis and Virus Detection

Rapid and accurate diagnosis of a disease causing agent (s) is the first and critical step for its control. It forms the basis for the deployment of effective control measures such as those targeting the control of insect vectors like pesticide application (Adams *et al*., 2012). Maize lethal necrosis disease causing viruses can be identified through various methods. Accurate symptom examination is essential for field observations. The most critical MLN disease symptoms scouted for are; leaf mosaic, mottling and necrosis. However, symptomatology alone can be unreliable since symptom expression can be influenced by other factors like infestation of sucking insect pests, plant genotype, environmental conditions, and plant-water relations (Green, 1991). Molecular-based laboratory techniques and several serological methods have been developed to complement symptomatology in plant virus identification (Lima *et al.*, 2012). Plant samples can be taken from leaves, pollen, female, and male inflorescences, ear husks, cotyledons, and seeds (pericarps, endosperm, cotyledons, and embryo) of maize. These plant parts have previously shown to harbor the maize chlorotic mottle virus (Scheets, 2004).

2.11.1 Virus Detection by Serological Methods

Enzyme linked immunosorbent assay (ELISA), is a serological procedure that utilizes antisera prepared targeting a specific virus antigen. A virus antigen is inoculated into a rabbit for antibodies to form before harvesting antiserum from the blood (Clark and

Adams, 1977). The Double Antigen Sandwich-Enzyme Linked Immunosorbent Assay, (DAS-ELISA) is a common procedure used in the serological detection of plant viruses. This method differentiates viruses based on viral protein characteristics. However, the serological methods, the triple antibody sandwich (TAS-ELISA), is the most efficient as it employs the monoclonal antibodies that target viruses like those causing MLN disease (Credi *et al*., 1989). Kusia *et al*., (2015) diagnosed SCMV and MCMV on MLN disease infected finger millet in Kenya using DAS - ELISA. Lukanda *et al*., (2014) diagnosed MCMV on maize in DR Congo using antigen coated plate, ACP - ELISA. Effective serological detection using poyclonal antisera depends on the particular antiserum used and the specific viral strains present.

2.11.2 Molecular Virus Detection

Molecular diagnostics targets the specific proteins or the genetic composition of a given virus. Polymerase chain reaction (PCR) was developed in 1984 by an American biochemist, Kary Mullis, and has been widely adopted in disease diagnosis (Bartlett and Stirling, 2003). Kusia *et al*., (2015), adopted RT-PCR to detect SCMV and MCMV on finger millet, Adam *et al*., (2014) diagnosed MLN disease causing viruses in Rwanda using real time PCR, while Lukanda *et al*. (2014) used RT PCR to report MCMV in Congo, and Canabas *et al*. (2013) employed real time PCR in detecting MCMV viruliferous corn thrips (*Frankliniella williamsi*). Wangai *et al*. (2012) also diagnosed MLN disease in Kenya using RT-PCR.

Viral genome sequencing has enabled the development of primers that are reliable in distinguishing virus strains during disease diagnostics. It involves generation of unique sequences that identify a viral pathogen through comparison with similar viruses documented in the GenBank database (Adams *et al*., 2012). For instance, MCMV isolates collected in 2012 from Bomet and Naivaisha in Kenya (Wangai *et al*., 2012) had 99% sequence identity to 12 MCMV isolates in the GenBank and with four Rwandan isolates (Adams *et al*., 2014; Adams *et al*., 2013). However, these isolates were 99% similar to those from Yunnan and Sichnan, China; and 97% similarity to isolates from Nebraska and Kansas in the United States of America. The MCMV isolates from Tanzania were 99% similar to those from Rwanda, DR Congo, and Kenya (Mahuku *et al*., 2015). Wangai *et al*., (2012), compared the SCMV genomes of Bomet

and Naivasha isolates with those in the Genbank. Only eight contigs generated according to Stewart *et al*. (2014) matched with SCMV genome sequences from Rwanda and China.

Three Rwandan isolates were 90% identical to isolates from Ohio (Adams *et al*., 2014). Since the first report of MLN disease in Kenya in 2011, research on virus diagnostics, possible vectors, alternative hosts, and management was initiated. Efforts on disease awareness to farmers, epidemiology, and screening for resistance, in Kenya and other ECA countries (Miano, 2013) are underway. In Kenya, H6506 hybrid which is both MLN and drought tolerant variety has been found suitable for commercialization (Boddupalli *et al*., 2020). This study sought to inform on the diversity and diversity of thrips vectors on the spread of MLND in the region, the role of thrips on disease virulence and pollen disease spread. Therefore, in order to effectively manage this disease, the role on pollen transmission in spreading the disease was important. Additionally, the efficiency of thrips in transmission of MCMV which is a major virus in this disease, was investigated.

2.12 Management of Maize Lethal Necrosis Disease

All economically important plant viruses like those involved in MLN disease complex require arthropod vectors to transmit them. The integration of cultural practices with host tolerance and insecticides effectively controlled MCMV in Hawii, (Nelson *et al*., 2011). Adoption of rotation programs for crops also effectively reduced the MCMV incidences in the United States (Uyemoto, 1983). Effective combinations of agronomic practices, vector management, and host resistance for effective management MLN disease among smallholder farmers in Eastern Africa is yet to be established (Mahuku *et al*., 2015). These integrated cultural and vector management approaches could be beneficial to commercial maize seed production regimes. In Eastern Africa, the smallholder farmers often practice relay maize planting with other intercrops. These farmers have little or no knowledge on virus vectors as well as cultural management methods (Mahuku *et al*., 2015).

The knowledge on survival of plant viruses within the farming systems is critical in developing their effective management strategy. Viruses that cause MLN disease can survive in different ways other than on maize plants. These viruses can survive either

in seeds, plant debris alternative hosts or in vectors (Miano, 2013). In 2011, the Ministry of Agriculture recommended uprooting and destruction of infected maize and planting non-host crops in 2-3 consecutive seasons in Kenya as an attempt to control the spread of the disease. Moreover, it was the continuous maize production within fields that increases the MCMV incidence, (Nelson *et al*., 2011). The regular scouting for disease symptoms emergence and insecticide application against thrips, aphids, and maize beetles is also necessary since these insects spread the disease. Seed treatment can also contribute to vector control at the seedling stage of the maize crop. Several natural enemies can effectively control maize aphids. They include ladybird beetles (Coleoptera), lacewing (Neuroptera), and parasitic wasps (Hymenoptera). Frequent use of chemicals to manage insect pests is discouraged as it will also threaten the natural enemies leading to a resurgence of the insect population. Field hygiene, closed season, promotion of Good Agricultural Practices (GAP) and crop rotation are important methods of control of MLN. Since thrips populations survive on weeds, it is important to control them before emergence of maize seedlings in order to reduce their buildup. The removal of weedy areas after maize germination may increase thrips problems as they will migrate from the weeds to the nearby maize plants. Parasitoid wasps especially *Aphidius colemani*, is key natural enemy in East Africa; (Woolley et al., 2022) are important in the control of many insect pests. Moreover, rapid and sensitive diagnostic tools are critical for surveillance, early warning, and rapid implementation of prevention strategies (Mahuku *et al*., 2015).

2.12.1 Characterization of Virus Vectors

Traditionally, identification of thrips utilized diagnostic aids like dichotomous keys (Moritz *et al*., 2005) which were guided by the external insect morphology, like body colour, length and shape (Mound & Kibby, 1998). Later, matrix based keys with pictorials and information provision were adopted. More modern and computerized keys were later developed. These include; pest thrips of the world, thrips of California and thrips of North America (Moritz *et al*., 2004; Hoddle *et al.*, 2008; Moritz *et al*., 2007). They use interactive computer systems and taxonomic features with images that enable rapid identification of thrips such as the Lucid Keys of pest thrips of East Africa (Moritz *et al*., 2013) that identifies over 100 thrips.

2.12.2 Morphological Characterization of Thrips

The traditional methods for the identification of thrips were based on external morphological features of the body (Mound & Kibby, 1998; Moritz, 1994). Morphological identification of thrips uses dichotomous keys as diagnostic aids (Moritz *et al*., 2005). Several modern and computerized dichotomous keys have been developed. For instance, it became easier to identify over 100 pest thrips in east Africa using the Lucid Key developed for pest thrips of East Africa (Moritz *et al*., 2013). Key morphological features that can distinguish species within a genus include arrangement of the ocellar setae within the anterior margins of the hind ocelli, presence of the metanotal campaniform sensillae and the presence of postero-marginal comb of microtrichia of a targeted abdominal tergite (Mound & Sartiami, 2013). These taxonomic features were applied in this study for the biological characterization of thrips.

2.12.3 Molecular Characterization of Thrips

Molecular techniques have been developed for a more accurate identification of adult and immature thrips up to the species level (Farris *et al.*, 2010). The most common molecular techniques are Polymerase chain reaction (PCR) based. These PCR techniques are useful in both species diagnostics and population studies (Farris *et al*., 2010). They include; real-time PCR (Walsh *et al.*, 2005), DNA sequencing (Brunner *et al.*, 2002), random amplified polymorphic DNA (RAPD) (Fang *et al*., 2005), PCR-restriction fragment length polymorphism (PCR-RFLP) (Rugman-Jones *et al.*, 2006), and simple sequence repeat (SSR-PCR) (Brunner & Frey, 2004). Various molecular tools have been employed in thrips identification, (Inoue and Sakurai, 2007). The DNA barcoding is one of them and can also identify other insect species. Deoxyribonucleic acid (DNA) barcoding technique utilizes short genetic markers (Waugh, 2007). The mitochondrial cytochrome oxidase 1 (COI) gene is the most commonly used barcode loci for the identification of insect (Macharia *et al*., 2015). The internal transcribed spacer (ITS) ribosomal DNA region can also be used for insect identification (Moritz *et al*., 2001a; Farris *et al.*, 2010). In this study the mitochondrial cytochrome oxidase 1 (COI) gene marker was used.

2.12.4 Thrips Characterization Using DNA Barcoding

The DNA barcode is a molecular identification tool that utilizes the mitochondrial DNA sequences of the Cytochrome C oxidase I gene. These nuclear DNA sequences are highly conserved and thus are used to group unknown specimens. The technique relies on sequence similarity, whereby an organism is identified based on the previously defined similar taxonomic entities. It involves matching the DNA sequences with an organism's taxonomic relatives after an initial morphological classification. Unmatched sequences are either grouped with existing taxa in the database or taken as new based on a given threshold of sequence similarity. This is because variation among species is usually lower than interspecies (Hajibabaei *et al*., 2006). Therefore, DNA barcoding technique has been widely adopted in molecular taxonomy and identification of insects (Galtier *et al*., 2009). Cytochrome C oxidase I (COI) being a subunit of a trans-membrane protein within the mitochondrion is highly conserved across all the species (Waugh, 2007). It is present in the mitochondrial DNA maternal inheritance which qualifies it an appropriate molecular marker for biodiversity. *Frankliniella occidentalis* which transmit MCMV, is among the thrips species that have been documented using COI gene markers (Macharia *et al.*, 2015). The COI sequences suggest possible genetic differences in the *F. occidentalis* population. The cytochrome C oxidase I (COI) gene marker helped to characterize thrips species infesting MLN affected maize in Kenya.

CHAPTER THREE: MATERIALS AND METHODS

3.1 Description of the study area

The research study was carried out at Kenyatta University (KU) farm and at the International Centre of Insect Physiology and Ecology(ICIPE), the National museums of Kenya within Nairobi County between October, 2016 and November, 2019. Samples of thrips were collected from maize fields of Bomet and Kisii counties of Kenya that lie between 0 °90‘55‘‘S, 35 °40‘98‘‘E / 0°90‘68‘‘S, 35 °41‘63‘‘E at an altitude of 1900 - 2100 m above sea level. Kenyatta university is located at 1.183056° S, 36.926111° E at 2,200 m above sea level (asl). Nairobi county lies within a tropical climate that receives bimodal rainfall with an average annual rainfall of 1,000 mm with an average mean day temperature of 24 - 28 ºC. These parameters provide suitable conditions that influence vector - pathogen - host interaction. The study sites have been illustrated in Figure 3.1 below.

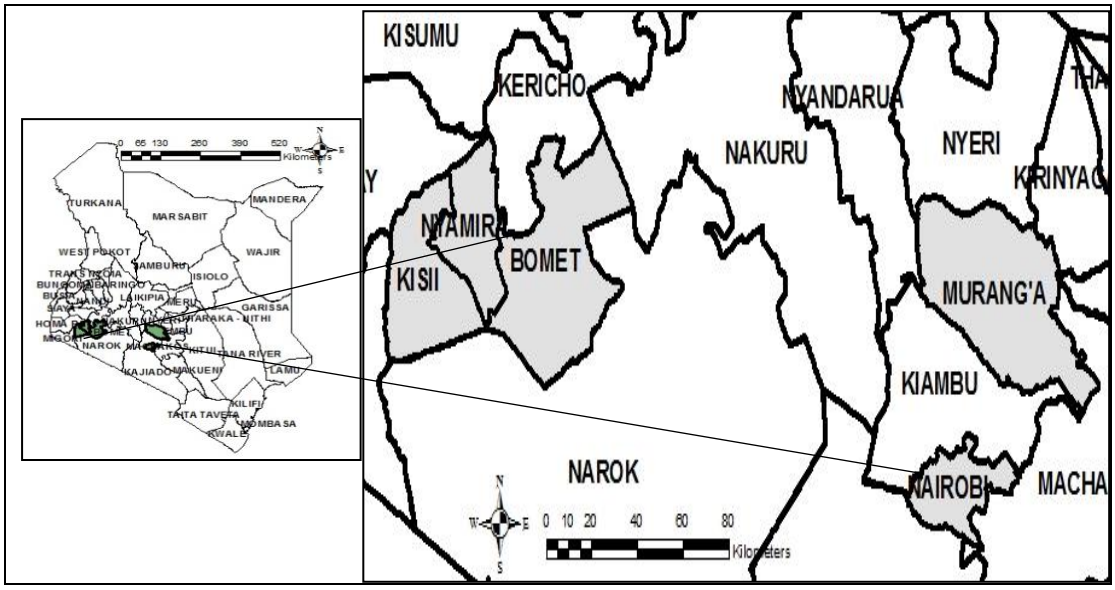


Figure 3.1: Map of Kenya Indicating Study Site and Sample Collection Counties

3.2 Biological characterization of MLN causing viruses

Prior to transmission tests of maize lethal necrosis disease inducing viruses (MCMV and SCMV), the viruses were assayed on potted maize seedlings of cultivar Duma 43. The viruses were sap inoculated onto maize seedlings and disease severity monitored over an eight-week period. The DAS ELISA and PCR techniques were used to detect

the presence of the two viruses in leaf samples. The knowledge on the symptom progression characteristics of the viruses was useful in understanding the virus – vector – host plant interaction in subsequent transmission tests.

3.2.1 Inoculum Preparation and Inoculation of MLN Causing Virus Isolates

The virus inoculum was sourced from Kenya agricultural research and livestock organization (KARLO) Nairobi in May 2019 where pure isolates of SCMV and MCMV were cultured on maize seedlings under quarantine conditions. The virus isolates were maintained on potted maize seedlings of cultivar Duma 43, raised under greenhouse conditions (26 ± 2 °C, 63 ± 2% RH). The Duma 43 maize variety was randomly selected among the susceptible varieties for MLN disease bioassays. Being a short variety, Duma 43 was the ideal choice for greenhouse bioassay virus-vector bioassays. The variety also showed distinct symptoms of single and mixed infections during preliminary inoculations.

Five grams of MCMV infected maize leaves were crushed using a mortar and pestle. Twenty milliliters of 50 mM Potassium phosphate buffer solution of pH 7.5 was added in a 1:1 volume ratio before the extracts were homogenized. The leaf extracts were vortexed at 400 rpm for ten minutes, sap collected and stored on ice. The SCMV inoculum was prepared with the same procedure. The maize seedlings were raised each on eight-inch diameter plastic pots filled with sterilized soil mixed with compost manure in a 2:1 ratio and gm. of Di-ammonium Phosphate fertilizer. Virus inoculations were done at four leaf-stage, 14 days after planting and the seedlings were maintained under greenhouse at average day temperature of 20 °C, with and an average of 12 h both light and night.

The double inoculation of the two viruses (in order to cause MLN disease) was done by mixing MCMV and SCMV leaf sap extract in a volume ratio of 1:2 as described by Gowda *et al*. (2015) mechanical inoculation protocol for MLN. The protocol also prescribes maintenance of the culture and the optimized combination of SCMV and MCMV virus inoculum. Sap extracts were held on ice during the inoculation period so as to preserve virus infectivity. Each treatment comprised of 64 plants arranged in a complete randomized design. The leaves of maize plants were dusted with

carborundum-mesh powder before cotton wool swabs soaked in inoculum-buffer mixture was rubbed gently to inoculate the virus. Three leaves were targeted for inoculation on each plant while the flag leaves were omitted. The carborundum mesh was washed off the leaves with clean water upon drying of the inoculum using a wash bottle. The treated plants were observed on a weekly basis and disease incidence and symptom severity recorded.

3.2.2 Determination of Disease Severity and Incidence

The infection of maize plants with MCMV and SCMV was observed at weekly intervals for a period of 8 weeks on Duma 43 maize (var.) after virus inoculation. A disease severity scale of 1 to 5 adopted from a protocol previously used by Gowda *et al*. (2015), also described in plate 3.1 was used to examine the inoculated plants for the presence of virus disease symptoms where 1 = no symptom, 2 = fine chlorotic streaks, 3 = whole plant chlorotic mottling, 4 = excessive chlorotic mottling and necrosis of newly emerging leaves (dead ear), and 5a = complete plant chlorosis and plant death, 5b = complete plant chlorosis and necrosis and leaf margin necrosis.

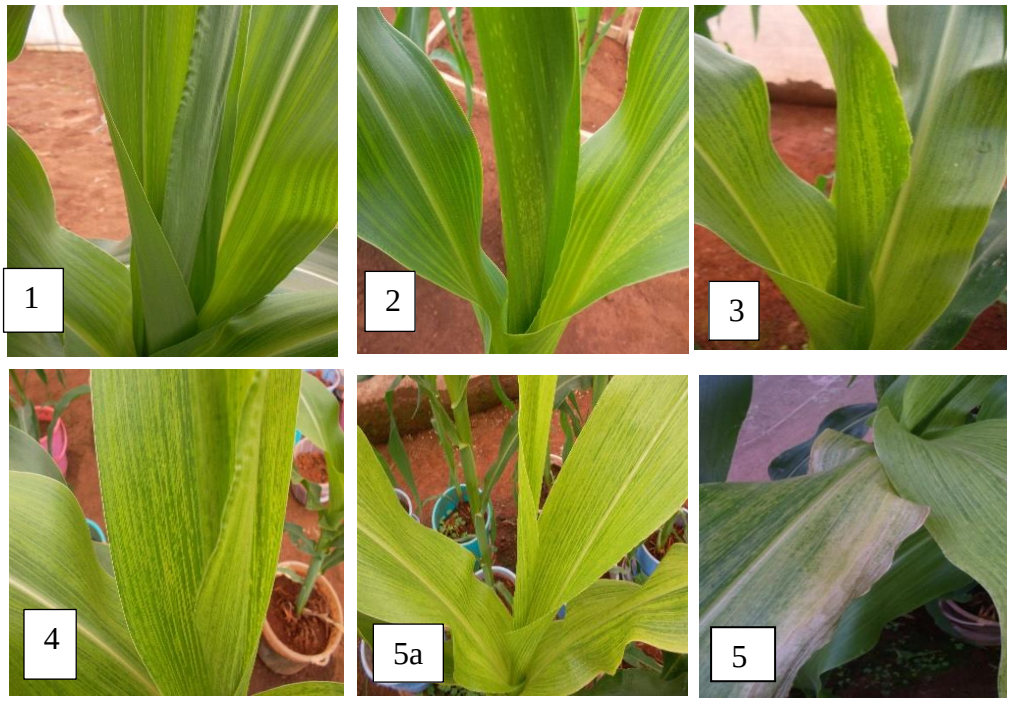


Plate 3.1 The symptom severity scale showing 1-5 scores for MLN infected plants

Plants with severe symptoms scored 5, and 1 for mild or no symptoms.

Source: Photos were taken by the author during this study.

The number of symptomatic plants was recorded against the inoculated plants for an eight-week period. Leaf samples for subsequent determination of the presence of virus by DAS ELISA were collected. The viral disease incidence was taken as the proportion of infected against the inoculated plants with virus symptoms according to James (1974).

$$\text{virus incidence} = \frac{\text{infected plants}}{\text{inoculated plants}} \text{ x100}$$

3.2.3 Detection and Confirmation of Virus Infection

Virus infection was analyzed in leaf samples from inoculated plants by ELISA and RT-PCR techniques. A hundred and eight leaf samples were collected and analyzed using RT-PCR for virus infection.

3.2.4 Serological Detection of Viruses

Virus infection was detected by DAS - ELISA using antisera raised against maize chlorotic mottle and sugarcane mosaic viruses. The DAS - ELISA procedure was performed according to method described by Clark and Adams (1977) with modifications. The sample analysis was done at the Plant Molecular Laboratory of the University of Nairobi, Kabete Campus. An Agdia (USA) ELISA kit was used following the manufacturer's instructions and the Nunc polysorp 96 well plates. The specific coating antibodies (IgG) for MCMV and SCMV were each diluted with coating buffer at 1:1000 according to the manual kit. About 100 µl of the IgG dilution were added to wells of the ELISA plates. The upper and lower wells were left out to prevent the border effects during washing of the plates. The coated well plates were sealed using parafilm, incubated for 2 hours at 37 °C and then washed three times with washing buffer (PBS).

The plates were tap-dried using paper towels before 100 µl of each leaf sap extract (sample antigen) was added in triplicate to the wells. The leaf sap extracts were obtained from 0.05 g of fresh leaf samples crushed in plastic bags containing 2500 µl extraction buffer using a pestle. The positive and negative controls provided in the extraction kit were also included in each plate for comparison the tested samples. The ELISA plates were incubated at 4 °C overnight after the wells being sealed with parafilm. The plates were washed thrice with washing buffer at 5 minute intervals, tap dried using a paper

towel, and 100 µl of specific enzyme conjugate solution added to each well. The specific enzyme conjugated (IgG-AP) solution was diluted in conjugate buffer solution at 1:1000 dilution volume ratio. The plates were incubated for 2 hours at 37 °C. The plates were washed, tap dried on paper towels, 100 µl of Sigma PNP substrate solution added, and incubated at 21 °C for an hour. Then substrate solution was prepared by dissolving the Sigma PNP in substrate buffer. The ELISA plate wells were visually assessed for any color development. An ELISA plate reader (model Human, Germany), was used to measure the well absorbance readings at 405 nm (A_{405} nm). The sample absorbance readings were compared to those of negative and positive controls. Positive samples developed clear yellow colors and their average absorbance readings exceeded double the mean of the negative controls.

3.2.5 Molecular Detection of Viruses Causing MLN Disease

Total RNA of the samples was extracted using Inclone biotech RNA Mini Extraction Kit. RNA extraction was performed at National Agricultural Research Laboratories, KALRO, Nairobi, using the protocol described by López *et al*. (2003). Fresh leaf samples were put in crushing bags and 50 mg of $CaCl_2$ (Silica gel) and 1500 µl of Lysis (Extraction buffer) were added. The samples were grinded using a mortar and pestle to obtain an extract which was then centrifuged at 12,000 rpm for 2 min. The supernatant was put into micro-centrifuge tubes and 500 µl of Chloroform added. The mixture was centrifuged at 12,000 rpm for 10 min. About 300 µl of the aqueous phase were transferred into the spin columns, 400 µl of Isopropanol were added and then centrifuged.

The laysates were discarded, and the columns washed using washing Buffer 1 (WB1). The columns were centrifuged for 60 seconds, washed using washing Buffer 2 (WB2), and centrifuged for 60 seconds. The columns were transferred onto a new RNase free micro centrifuge tubes and 50 µl aliquot of Elution Buffer (EB) was added. The spin columns were incubated for one minute at 21 °C room temperature. The columns were centrifuged at 12,000 rpm for one minute and the RNA extract was stored at -20 °C. The quality of the total RNA extracted was visualized on 1.5% (w/v) agarose gel through UV light as earlier described by (Wamaitha *et al*., 2018). The gel was prepared by adding 2 g agarose powder into 100 ml TAE buffer.

The mixture was then heated in microwave and melted agarose at 95 °C and poured gently into a gel cast tray at 45 °C before it was allowed to solidify at room temperature of 21-23 °C. About 2 µl of extracted RNA were gently loaded into the gel comb pots. The RNA samples were electrophoresed for 30 min at 135 V on gels submerged in 0.5 x TAE buffer mixed with safe gel stain. A Bio-Rad gel doc system was used to visualize and capture gel cast images. The images with good bands indicated good quality RNA of adequate quantity.

3.2.6 Reverse Transcriptase Polymerase Chain Reaction

The RT-PCR was employed to detect SCMV and MCMV viruses. The PCR procedure was done according to Wangai *et al*. (2012). The PCR master mix of about 25 µl reaction volume was of 1µl RNA template, 1 µl of each primer, 12.5 µl of 2 x reaction mix, 1 µl of enzyme, and 8.5 µl of distilled water was prepared. A one step RT-PCR program was used to amplify RNA target codons using an Applied Biosystems; 96 well Thermal Cycler.

The initial cDNA synthesized at 42 °C for 50 min. The reverse-transcriptase enzyme was then deactivated with one cycle of 95 °C for 12 min. This followed the denaturation step of 30 seconds at 95 °C. The annealing was for 30 seconds at 55 °C and an extension of one min at 72 °C. These steps were repeated in 35 cycles, with a 10 min extension at 72 °C and final holding at 10 °C. The PCR amplification was optimized for all primers through adjustment of the reaction mixtures. The primer concentration, the RNA and reaction mixes were varied with the same PCR program. The primers used to detect SCMV and MCMV are given in table 3.1.

Table 3.1: Sets of Primers Used for Viral Genome Amplification

No	Primer	Sequence (5′→ 3′)	Loci	Band Size
1	MCMV-F	GTCCTGGCCTCAGTGGTTAAGG	3226-3247	478 bp
2	MCMV-R1	CGCACAGAGTTGAACACAA TTGT	3703-3681	
3	SCMV-F1	AGCTAAGAGAAGCCCACATGCAG	9169-9190	319 bp
4	SCMV-R	AGAAGACACTGGGTCCAACCCTG	9487-9465	

The amplified PCR products were electrophoresed on 1.5 % agarose gel submerged in 0.5 x TAE buffer containing safe gel stain. The positive and negative controls for both SCMV and MCMV were loaded accordingly in 3 µl PCR product with 2 µl loading dye mixtures. A 100 bp DNA ladder, used as a standard molecular weight marker was to compare the amplicons with their expected base pair sizes. The agarose gel electrophoresis was run at 135 V for 30 minutes.

3.3 Identification and Rearing of Thrips for Virus Transmission

Frankliniella occidentalis and *F. williamsi* culture colonies were initiated from adult thrips samples collected on maize plants from the field. The samples were processed and identified according to Mound *et al*. (2009). *Frankliniella occidentalis* adults were collected from maize ears by gently tapping the silks onto collecting trays. *Frankliniella williamsi* adults were collected from underneath the leaf sheaths along the maize stalks. The sheaths were gently opened and the thrips sucked using an aspirator. The collected thrips were transferred into labeled vials, taken into the laboratory and identified according to Mound *et al*. (2009) using the head, wings, thorax and abdominal characters indicated in Table 3.2. Identified thrips were laboratory cultured and their colonies used for bioassay studies. Prior to culturing, twenty of each species were separated based on body colour and size.

Table 3.2: Character combinations of head, thorax and abdomen for identification of maize infesting thrips; *Frankliniella occidentalis, Frankliniella schultezei* and *Frankliniella williamsi*

Character combination	Thrips identity		
	F. williamsi	*F. schultzei*	*F. occidentalis*
Body colour Antennal segment colour	Yellow VII-VIII brown	Yellow or brown brown III-V yellow	Yellow or brown III-V yellow VI-VIII brown
Forewing colour	Pale	Pale with dark setae	Pale with dark setae
Antennae segments	8 III & IV with fork sense cone	8 III & IV with fork sense cone	8 III & IV with fork sense cone
Antennae VIII longer than VII.	-	VII shorter than VIII	VII shorter than VIII
Head	wider than length	wider than length	wider than length
Ocellar seta	3 pairs	3 pairs	3 pairs
Ocellar setae pair III	arise just within ocellar triangle margins	arise close together within margins	arise on anterior margins
Post-ocular setae pair I	Present	Absent	present
Pronotum	5 pairs of major setae	5 pairs of major setae	5 pairs of major setae
Antero-marginal setae	Similar length with anteroangulars	shorter than anteroangulars	shorter than anteroangulars
Minor setae of posteromarginal submedian setae	a pair	a pair	a pair
Metanotum	2 pairs of setae on anterior margin	2 pairs of setae on anterior margin	2 pairs of setae on anterior margin
Companiform sensilla	Present	Absent	present
Forewing setae	2 complete rows of veinal-setae	2 complete rows of veinal-setae	2 complete rows of veinal-setae
Tergite ctenidia	Laterally on V-VIII, anterolateral of VIII spiracle	Laterally on VI – VIII, anterolateral of VIII spiracle	Laterally on V-VIII, anterolateral of VIII spiracle
Postero-marginal comb	on VIII, with long regular microtrichia	on VIII, not developed	on VIII, with short slender microtrichia
Discal setae	Present on sternite II	Absent	absent

These thrips were transferred into culture boxes for each species. Another twenty thrips of each species were transferred into vials with AGA (60% ethyl alcohol, glycerine and acetic acid in a 10:1:1 ratio) for identification. They were transferred into 60% alcohol for 24 hours in a watch glass. The 60% alcohol was then replaced with fresh 60% alcohol mixed with 5% NaOH for 30 minutes to 2 hours until the abdomen colour turned from dark to pale. During this process the abdomen were macerated using a fine needle and then gently massaged to clear the contents of the abdomen. The body appendages except the wings were spread out before draining NaOH. Ten milliliters of distilled water were added followed by 30 ml of 50% ethanol gradually added. The mixture was replaced with 60% ethanol and thrips left for 24 hours. The macerated thrips were then dehydrated in 70%, 80%, 95% and absolute ethanol for 20, 10, and 5 minutes respectively. The dehydrated and macerated thrips were placed in clove oil for 30 minutes before they were mounted onto microscope slides using Canada balsam then observed under compound microscope.

Colonies of *F. occidentalis* and *F. williamsi* populations were reared according to Degraaf and Wood (2009) with slight modifications. The colonies were maintained in cages made from modified 5-liter cylindrical plastic boxes sealed and covered with insect-proof nets at an average diurnal temperature of 28 ± 2 °C, a 60 – 80 % relative humidity, and a 12-hour night and day photoperiod (12 L: 12 D). Relative humidity was regulated by rectangular windows (12×7 cm), cut out on the 2 opposite sides of the boxes and covered with insect-proof clothe secured with glue. The cages were placed in a well-ventilated area near the windows. Water was provided using water wick bottles placed inside the culture boxes. The water also served to increase and maintain relative humidity at between 50 - 65 %. The water wick was a modified 20 ml plastic bottle covered at the top with an insect proof net with cotton wool as the wick into the bottle with clean water. The water wick was washed and water replaced after every two weeks.

3.4 Selection of Substrates for Rearing Thrips

The growth of *F. occidentalis* cage colonies was tested on French bean (*Phaseolus vulgaris* L.) pods, courgettes (*Cucurbita pepo* L.) and Cucumber (*Cucumis sativus* L.), (Plate 3.2 a, and b).

Plate 3.2 Cage Set Up for Thrips Oviposition and Colony Rearing

a- cylindrical plastic cage boxes with nylon clothe cover (114 µm) with paper towels placed at the base for pupation, b- cylindrical plastic box with cloth-ventilated side openings, c- washed and sterilized fresh French bean (*Phaseolus vulgaris*) pods, used for rearing *F. occidentalis*, and d- maize stalks cut at internodes with leaf sheaths split open, for culturing *F. williamsi*

The French bean pods, cucumbers and courgettes used in this set up were commercially obtained from an organic producing farm in Murang'a County. The substrates were surface sterilized in 5% Sodium hypochlorite solution for 5 minutes, and then rinsed thrice in water. After washing, the substrates were rubbed with paper towels to dry before they were put vertically on top of the paper towel platforms. The substrates remained in the cages for 10 days before they were replaced with fresh ones. Previous reports indicated that maize seedlings could be used to raise *F. williamsi* inside PVC ventilated cages (Cabanas, 2013) but preliminary tests were unsuccessful in this study.

Three substrates were therefore tested for rearing of *F. williamsi*; French bean (*P. vulgaris*) pods (Figure 3.2 c), maize seedlings and young maize stalks cut at internodes, washed and replaced after 14 days. The leaf sheaths on the maize stalks were split open into quarters using a sterile scalpel blade (Plate 3.2 d). The stalks and the pods were also sterilized for 5 minutes in 5% Sodium hypochlorite solution. They were rinsed twice in tap water, wiped using paper towel, and placed vertically inside the culture boxes. Larval populations and emerging adults were monitored at laboratory conditions; 20 ± 4 ºC and 65±5% relative humidity (R. H) for an 8-week period. A completely randomized design (CRD) was used with 4 replicates. Average numbers of adult and larval generations developed on different food substrates were recorded. The best substrate was selected for rearing adult colonies used in MCMV transmission experiments.

Oviposition cages of 5 L, 15 cm radius, cylindrical and clear plastic boxes (Figure 3.2 a) were set up and five adult thrips were introduced into each box. *F. occidentalis* was raised on fresh French bean pods, provided weekly and sterilized in 5 % Sodium hypochlorite solution for 10 minutes, rinsed twice with tap water, dried using paper towels, and placed vertically upright on the paper towels. The bean pods remained in the cages and got replaced with new ones at 14 day intervals. *Frankliniella williamsi* was raised on young maize stalks cut at internode supplemented with immature maize cobs that were both replaced at 14 day intervals. The stalks were washed and tap dried on paper towels as had been done with cucumbers for *F. occidentalis*. The maize stalk leaf sheaths were slit into quarters using a sterile scalpel blade. The stalks were placed vertically upright inside the culture boxes after surface sterilization. Both populations were reared for two months, regularly monitored and sampled for identification to detect any contamination before transmission tests.

3.5 Determining Thrips Transmission Efficiency of Maize Chlorotic Mottle Virus

Sixty-four potted maize plants (cv. Duma 43) were maintained in insect proof cage nets in a greenhouse and inoculated using *F. occidentalis* adult thrips at different virus vector acquisition and also inoculation access periods. Virus acquisition period was the time the virion took to attach to the receptor protein of the vector while inoculation access period was the time for the virion took to attach to the receptors of the host plant

cells. A set up for the transmission experiments was designed following the insect vector transmission procedures described by Wijkamp *et al*. (1995). Prior to the transmission tests, leaf samples of the test plants and thips vectors were tested for virus detection by DAS ELISA to confirm their healthy status. The experiment was set up in a completely randomized design (CRD) with four replicates. *Frankliniella occidentallis* were fed on MLN affected leaf discs placed inside universal bottles for 24 hours to acquire the virus (Plate 3.3 b). The leaf discs of 2 x 5 cm were cut from symptomatic maize leaves sourced from mechanically inoculated plants. Prior to exposure to infected leaf discs, the thrips were starved for 3 h so as to increase the chances of feeding to acquire the virus. Healthy leaf tips of two week old maize seedlings were inserted into the same bottles, now with viruliferous thrips.

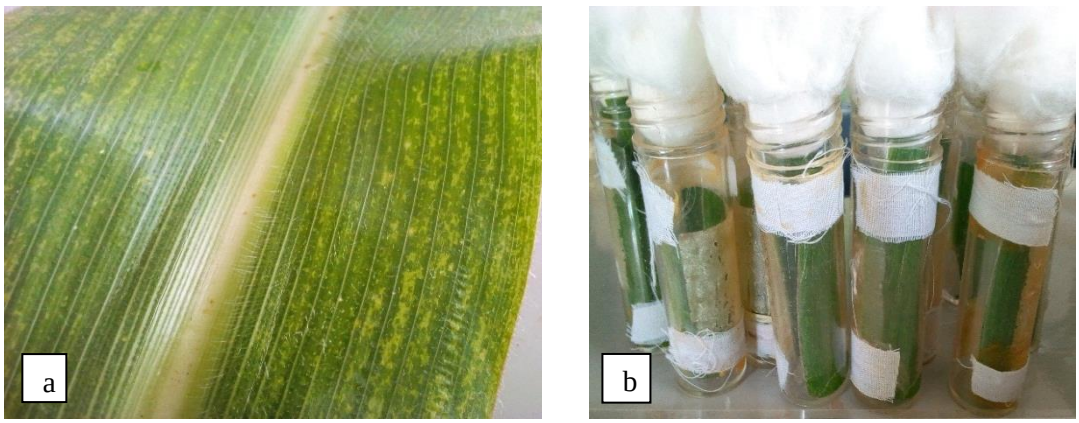


Plate 3.3 Laboratory universal bottle set up and leaf discs for virus vector inoculation

a- a symptomatic maize leaf infected with maize chlorotic mottle virus showing chlorosis and mottling symptoms; b- infected maize leaf discs inside modified universal bottles containing *F. williamsi* and *F. occidentallis* for virus acquisition

The efficiency of the adult thrips to transmit MCMV was tested on a 0.5, 1.0, 1.5, 24-hour virus acquisition access periods (IAPs) with 72-hour inoculation access period. The thrips were allowed to feed on infected leaf discs at the different acquisition periods and a subsequent exposure of 72 hours onto healthy plants. The thrips' transmission rate of the virus was also tested on a 0.5, 1.0, 1.5, 24-hour inoculation access periods

(IAPs). The same procedure was used to test the transmission efficiency of *F. williamsi* adults.

The universal bottles containing *F. williamsi* adults were covered with wet cotton wool. As controls, healthy plants were exposed to thrips fed on healthy maize leaf discs. The inoculated plants were treated with a carbosulfan insecticide (Marshal 250 EC®) after the expiry of the inoculation access period. The inoculated plants were covered in insect-proof cages placed inside a greenhouse for symptom observation. The number of infected symptomatic plants was recorded per treatment. A DAS-ELISA was done on leaf samples to confirm successful transmission of MCMV.

3.6 Determining Transmission of Maize Chlorotic Mottle Virus by larval thrips

Tests to determine transmission of MCMV by different larval stages of *F. occidentalis* were done using potted maize plants raised in insect proof cage nets in a greenhouse according to Wijkamp *et al*. (1995). First, second and third instar larvae of *F. occidentallis* were allowed 24 hour AAP and 72 hour IAP. The larvae were exposed to MLND infected leaf discs placed inside universal bottles after 3-hour starvation as described by Cabanas *et al*. (2013) with modifications. The bottles were covered with cotton wool. Leaf tips of two-week old test maize plants were inserted into these bottles for virus transmission. The MCMV transmission by *F. williamsi* larvae was tested with the same procedure as with *F. occidentalis* but the universal bottles were covered with wet cotton wool. For control, healthy plants were exposed to larvae fed on leaf discs from healthy maize plants. The inoculated plants were sprayed with an insecticide after termination of inoculation access period. The test plants were maintained in insect-proof cages placed in a greenhouse for symptom observation. The proportion of symptomatic plants was recorded for each treatment and successful MCMV transmission was confirmed by DAS-ELISA.

3.7 Persistence Transmission Tests for Maize Chlorotic Mottle Virus

Potted maize plants (var. Duma 43) maintained in insect proof cage nets in a greenhouse were exposed to *F. occidentalis* larvae whose adults were exposed to infected leaf discs. The transmission tests were carried out following Wijkamp *et al*. (1995) vector inoculation protocols. The inoculated maize seedlings were arranged in a completely randomized design (CRD having four replicates (Appendix 4). The first instar and third

larval instars of *F. occidentallis* were allowed to feed on MLND infected leaf discs placed inside universal bottles after 3 h starvation. The bottles were covered with cotton wool. The viruliferous larvae were allowed to develop into adults while feeding on honey and maize pollen. The emerged adults were then exposed onto healthy leaf tips of two-week old test maize plants (var. Duma 43) inserted into universal bottles for virus transmission. Adult thrips were also exposed to MLN diseased leaf discs, allowed to oviposit eggs and their emerging first instar larvae exposed to healthy leaves for virus transmission. *Frankliniella williamsi* larvae transmission of MCMV was tested with the same procedure as that followed with *F. occidentalis* but the universal bottles were covered with wet cotton wool. Healthy plants were exposed to larvae and emerged adults exposed to healthy maize leaf discs as controls. The inoculated plants were hand-sprayed with an insecticide after termination of inoculation access periods. The inoculated plants were maintained in insect-proof cages inside a greenhouse for symptom observation. A record of plants developing symptoms was maintained for each treatment. Leaf samples were then subjected to DAS-ELISA to confirm successful transmission of MCMV.

3.8 Effect of Vector Retention Period on Virus Transmission

Persistence of transmission of an MCMV Kenyan isolate by *F. occidentalis* and *F. williamsi* adults was tested on Duma 43 maize cultivar raised in plastic pots (2 kg with sterilized soil, organic manure and 5 g DAP fertilizer). The plants were placed in nylon covered-wooden cages (1 x 1 x 2 M) inside a greenhouse. Symptomatic leaf discs (2 x 5 cm) from mechanically inoculated plants served as a source of inoculum. Transmission tests were done based on; 1, 3, 6, and 24-hour virus inoculation access periods, and persistence inoculations on 1, 2, 3, and 4 sets of plants. The same batches of thrips exposed to plants on the first day were transferred to new sets of plants at 24 hour intervals of four days.

Inoculation access period was based on the time the thrips took to inoculate MCMV, latent period as the time thrips remained viruliferous while persistence of transmission was the repeated virus inoculations onto new plants after virus acquisition. Ten adult thrips were starved for 24 hours inside modified 50 ml plastic universal bottles (nylon covered 2 x 1 cm side openings, cotton wool top cover). Two wet leaf discs (5 x 2 cm)

from symptomatic leaves were inserted into the universal bottles for thrips to feed. The discs were removed after 48 hours using a pair of forceps. Thrips were starved for 4 hours before healthy maize leaf tips (64 plants, 14 day old seedlings) were each inserted into individual bottles and sealed with cotton wool. The thrips used for testing virus retention period were fed on honey and maize pollen after starvation before being sequentially introduced to new seedlings. The thrips were then introduced onto healthy seedlings at intervals of 24, 48, 72 and 96-hour retention periods. To test persistence of transmission (new infections), thrips exposed to healthy seedlings in day 1 were re-introduced to a new set of plants daily in a 4-day cycle. Control plants were exposed to thrips fed on discs from healthy asymptomatic plants. Positive controls consisted of mechanically transmitted plants using a leaf sap extract from a 5 g infected leaf sample crushed in 0.05 M Phosphate buffer solution (pH 7.2).

The virus coat protein was preserved at the neutral pH of 7.2 during virus extraction. The extracted leaf sap was rubbed directly onto leaves of 3-leaf stage maize seedlings with carborundum mesh abrasive. A total of 64 plants arranged in a completely randomized design (CRD) were inoculated in each experiment. The plants were covered with nylon-mesh wooden cages inside a greenhouse. The plants were observed daily for symptom development for an 8-week period. Reverse transcriptase polymerase chain reaction, and ELISA confirmed the transmission of the virus in leaf samples after inoculation. The virus transmission efficiency by *F. occidentalis* and *F. williamsi* was determined based on the proportion of MCMV infected plants against the total inoculated according to Verbeek *et al*. (2010).

3.9 Effects of Vector Intensity on Virus Disease Severity

The effect of vector intensity on MCMV disease severity was tested on Duma 43 maize cultivar raised in plastic pots (2 kg with sterilized soil-manure mixture and 5 g DAP fertilizer). The vector intensity was quantified as the vector abundance according to Moreno *et al*. (2007). The plants were placed in nylon covered-wooden cages (1 x 1 x 2 M) inside a greenhouse. Symptomatic leaf discs (2 x 5 cm) from mechanically inoculated plants served as a source of inoculum. Transmission tests were done based on 5, 10, 15, and 30 vector intensity levels with a 24 and 72-hour virus acquisition and inoculation access periods respectively. The thrips were starved for 24 hours inside

modified 50 ml plastic universal bottles (cotton wool top cover). Two wet leaf discs (5 x 2 cm) from symptomatic leaves were inserted into the universal bottles for thrips to feed. The discs were removed after 24 hours using a pair of forceps. Thrips were starved for 4 hours before maize leaf tips (64 plants, 14 day old seedlings) were each inserted into individual bottles and sealed with cotton wool.

Disease severity was monitored over an eight-week period. The severity scores were recorded and the area under disease progress curve determined using the trapezoidal method according to (Nainwal et al., 2020) and compared with vector intensity.

3.10 Role of Pollen Grains in MLN Disease Spread

To analyze the contribution of maize pollen in individual and combined spread of MCMV and SCMV, an artificial inoculation method was used according to Goldberg *et al*. (1987). Cultivar Duma 43 maize plants of 3-4 leaf stage were used. One hundred and sixty potted maize plants were inoculated. Control plants were inoculated with pollen from healthy maize plants. The emerging tassels of inoculated and symptomatic plants were covered by pollination bags to trap the shedding pollen. Fresh pollen was collected at mid-morning by gently tapping maize tassels. Collected pollen was poured into clean trays and labeled.

The inoculation sap from pollen was prepared using K_2HPO_4 buffer and 10-gram pollen from DAS-ELISA positive maize plants. The pollen was crushed in a mortar using a pestle. About 10 ml buffer was added to the pollen before crushing. The carborandum mesh powder was added into the crushed pollen before inoculation. The inoculation was repeated after a week to increase transmission efficiency. Semarate inocula were prepared using pollen samples collected from MCMV, SCMV, and MCMV+SCMV infected plants. Fourty plants were inoculated for each treatment. Symptoms development was observed on the inoculated plants at weekly intervals of eight weeks. Each leaf of the inoculated plants was observed for the induced individual or synergetic co-infection symptoms. Virus infection was tested on leaf samples using DAS-ELISA.

3.11 Vector Mediated Pollen Transmission of MLN Causing Viruses

To study the role of vectors in pollen mediated virus transmission, thrips inoculation method was used according to Cabanas *et al*. (2013). Individual and combined

transmission of MCMV and SCMV was tested. *F. occidentalis* was raised on French bean pods while *F. williamsi* was raised on young maize stalks. Modified water wicks were introduced into the rearing cages. Vector mediated transmission of MCMV via pollen was tested on sixty-four potted Duma 43 maize plants raised in insect-proof cage nets (Plate 3.4 b). The vectors were used to inoculate maize chlorotic virus at the 3-4 leaf stage. The plants were arranged in a 4 **x** 4 pot arrangement per treatment.

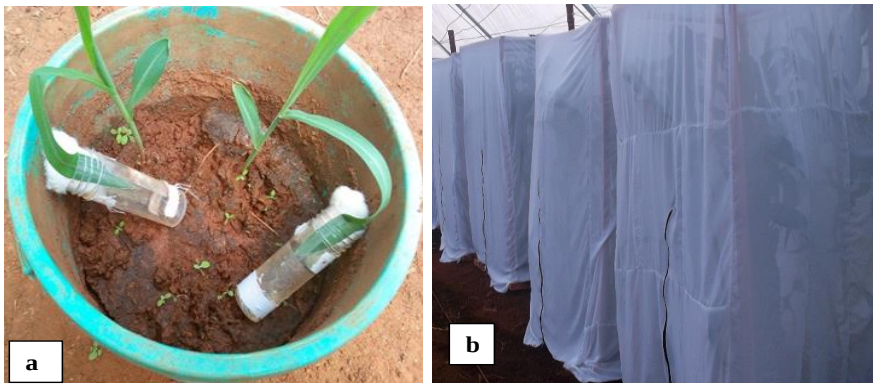


Plate 3.4 Greenhouse cage set up and maize seedling vector virus inoculation

a- modified universal bottles with thrips exposed to maize leaf tips and covered with cotton wool for virus inoculation; b- cages covered with insect proof nets for maintaining inoculated maize plants inside a greenhouse.

Five non-viruliferous thrips were transferred into 50 ml laboratory universal plastic bottles using an aspirator. Each bottle was covered with clean cotton wool and thrips starved for three hours. To regulate relative humidity, temperature and air circulation, 2 side openings of 2 x 1 cm were provided on each bottle. The openings were sealed with pieces of insect proof clothes using an adhesive. A portion of 2-3 grams of freshly collected pollen from symptomatic plants was transferred into the universal bottles. The thrips were allowed a 24 hour feeding time to acquire the virus from pollen. Leaf tips of 14 day old seedlings were inserted into the universal bottles containing thrips sealed with cotton wool (Plate 3.4 a). The thrips were allowed a 72 hour feeding period so as to transmit the viruses. Control plants were exposed to thrips that were fed on pollen from healthy maize plants. *Frankliniella williamsi* was used to inoculate a similar number of maize plants using the same procedure as with *F. occidentalis*. However, the universal bottles for *F. williamsi* had wet cotton wool covers to increase relative

humidity. After the treatment, the test plants were maintained inside a greenhouse for symptoms observation on a weekly basis over an eight-week period. Leaf samples of the test plants were subjected to DAS – ELISA at week 10 post inoculation to confirm virus transmission.

3.12 Molecular Characterization of Maize Infesting Thrips

Three hundred and twenty specimen thrips were collected from MLN infected maize farms in Murang'a County, Central Kenya. The insect DNA was isolated, amplified at the 5' end of the COI barcode region and sequenced. The sequences were analyzed for species identification using BLAST online searching tool and a phylogenetic tree was also constructed.

3.12.1 Collection of thrips Samples

Thrips were collected from maize infected with MCMV at agro-ecological zones classified based on altitude according to Corbett (1998). Three hundred and sixty-seven samples were collected from low, mid and high altitude regions as shown in figure 3.2.

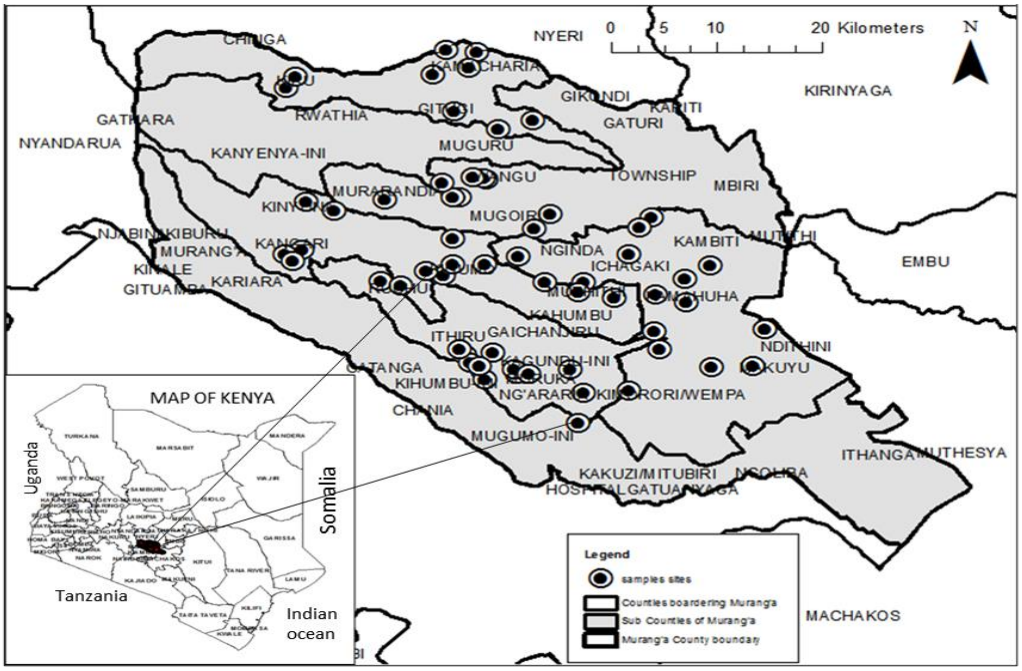


Figure 3.2: A map of Murang'a County showing points where thrips were collected

The map was generated with GPS coordinates using an *ArcGIS* 10 software.

The farms located below 1000 m elevation were considered low altitude, 1000 - 1799 m mid altitude and 1800 m or more as high altitude agro-ecological zone. Thrips were collected from 51 localities in Murang'a County (Fig 3.5) from MLN symptomatic plants following standard protocols (Bradley and Mayer, 1994). Adults and late instar larvae were collected at fifty-one randomly selected sites in Murang'a County according to Lokeshwari *et al*. (2015). Collection sites were considered at a minimum distance of 5 km difference to avoid collecting samples with similar parthenogenetic lineage. Thrips were aspirated mechanically into universal laboratory bottles and 10 samples randomly picked and transferred into each 2 ml plastic vials containing 96% ethanol using a moistened camel brush. The genotyping of the thrips specimens was done at the Natural Resources Institute laboratory at Greenwich, United Kingdom.

3.12.2 Extraction of thrips DNA

The total genomic DNA of individual specimen thrips was extracted using the Chelex 100 resin based protocol as described by Boonham *et al*. (2012) with slight modifications. Thrips were pippeted onto a paper towel and the residual ethanol allowed to dry leaving the exposed specimen thrips. Single specimen thrips were put into 0.5 mL eppendoff micro tubes using a fine pair of forceps. A 50 µL aliquot of nuclease free water mixed with Chelex 100 resin slurry (Biorad Laboratories, Hercules, CA USA) in a 1:1 ratio was added. The thrips were crushed by grinding them in the eppendoff tubes using plastic pestles. The tubes were incubated for 15 minutes at 56 °C. Another incubation was done at 96 °C for 5 to denature the proteins. The tubes were centrifuged for 5 minutes at 13000 rpm. The supernatant was put into new micro centrifuge tubes, stored at -20 °C before amplification by PCR.

3.12.3 Amplification and Sequencing of thrips COI

Double stranded *COI* DNA templates were generated using universal mtDNA *COI* primers; Forward 5' - LCO1490 and HCO2198 reverse primers (Folmer *et al*., 1994). A set of primers; MTD7.2F and MTD9.2R, which were specific to thrips (Brunner *et al*., 2002) were also used. The PCR reaction volumes were 50µL of 0.2 mM dNTPs, 0.2µM for each primer, 2.5mM $MgCl_2$, 1x buffer, 2 U/µl Bio-X-nAct Long DNA polymerase (Bioline London UK). A 2720 thermo cycler (Applied Biosystems, Califonia USA) was used to amplify the DNA segments with similar cycling program

set for each primer pair. The initial denaturation was set at 94°C for 3 minutes, then followed the amplification of 35 cycles at 94°C for 20 secs, 52°C for 20 secs, 72°C for 1 min followed by an extension of 72°C for 5 min. PCR products were analyzed on 1% agarose Easy Cast mini gel electrophoresis system (Fisher Scientific Inc.). The amplified products were purified using a PCR purification kit (GeneJET® ThermoFisher Scientific, Waltham, Massachusetts, USA).

A nanodrop spectrophotometer (Thermo Scientific© Nanodrop 2000™) was used to measure the concentration of the purified DNA at a 260:280 wavelength ratio of light. Both PCR product strands per sample were sent to the Source Biosciences, Cambridge UK for sequencing. Total DNA was isolated by chelex method and amplified using universal barcoding primers targeting the 5' end of the COI barcode region. The PCR products were purified before they were matched against known DNA markers using on 1% agarose Easy Cast mini gel electrophoresis system.

3.13 Data Analysis

The GenStat 15th Edition software was used to analyze the data. The symptom severity of the mechanically inoculated plants was compared among the viruses. The leaf severity of mechanically inoculated plants was log transformed then tested for symptom homogeneity between MCMV, SCMV and MLN infected plants by Chi square test. The area under disease progression (AUDP) curve was plotted after the eight week upon inoculation. A one-way ANOVA was used to compare the means of the induced disease severity of the viruses. Fisher's LSD at 0.05 significance level separated the significantly different means.

The morphological data of thrips was used to identify the collected specimen thrips. The Morphological images of specimen thrips were compared with the online thrips identification keys published by Mound & Kibby (1998). The colour of the body and the antennal segments, the arrangement of the ocellar setae pair III were compared with online keys. Other characters were; the presence of the post-ocular setae pair I, the shape of the antero-marginal setae, and the presence of the companiform sensillea. The shape of the lateral ctenidia on tergite V, the anterolateral spiracle on tergite VIII, posteromarginal comb on tergite VIII, and the discal setae on sternite II were also compared. Some of the images of the identified specimens were taken. The number of

larvae and adults on the different test substrates were inverse transformed and compared to determine vector host preference. The means of *F. occidentalis* larvae and adults emerging on *P. vulgaris* pods, *C. pepo* and *C. sativus* substrates were tested for homogeneity by a Chi square test at P = 0.05 significance level. The means of *F. occidentalis* adult populations on the tested substrates were plotted against time over week rearing period.

A one-way analysis of variance was done on the means of *F. williamsi* larvae on maize seedlings, maize stalk cuttings, and *P. vulgaris* pods. Fisher's least significant difference test separated the means. The means of *F. williamsi* adults on the tested substrates were subjected to Chi square test and a graph plotted of means against rearing period in weeks. The regression graphs indicating the effect of temperature and relative humidity on the number of emerging larvae and adults for both *F. occidentalis* and *F. williamsi* were plotted. The proportion of infected plants through vectors was subjected to a t- test to determine the efficiency of virus transmission between *F. occidentalis* and *F. williamsi* at various virus acquisition periods. A regression curves indicating MCMV transmission rates of transmission against acquisition feeding time for the vectors were plotted.

The proportion of plants transmitted with MCMV by thrips at different inoculation access periods was compared to determine the difference in the rate of virus transmission**.** The means of plants inoculated with MCMV by *F. williamsi* and *F. occidentalis* at different inoculation access periods were subjected to a t-test which indicated the difference in transmission competence between these vectors. The proportion of plants transmitted with the virus by larval thrips was compared against the total inoculated plants. The number of plants infected inoculated by different stages of larvae were recorded. The data was tabulated in excel sheets and averages and percentages tabulated. The data on persistence in transmission of *Maize chlorotic mottle virus* was compared between the vector thrips. The proportions of infected plants were tabulated into means and percentages.

The means of infected plants infected by *F. occidentalis* and *F. williamsi* were plotted against the different sets of plants transmitted after virus acquisition. The data on effect of retention period on virus transmission was compared between the vectors. The means

of infected plants at different inoculation times by *F. williamsi* and *F. occidentalis* were subjected to an analysis of variance. The means of plants inoculated in day one to four, post virus acquisition were compared. The means were separated by least significant difference at 0.05 significance level. The severity of infected plants by different amounts of thrips was compared to determine the effect of vector intensity on symptom severity. The mean severity of MCMV infected plants via different number of vectors was compared by ANOVA.

The means of infected plants were plotted against the number of thrips and the proportion of plants transmitted by *F. occidentalis* compared to those transmitted by *F. williamsi* by a t-test. The proportion of plants transmitted with the virus via pollen was compared with the total plants inoculated. The means of plants mechanically infected by *Maize chlorotic mottle virus* via pollen were tabulated into percentages. The plants infected by SCMC were compared with those infected by MCMV in tabulated tables.

CHAPTER FOUR: RESULTS AND DISCUSSION

4.1 Biological characterization of MLN causing viruses

The viruses causing MLN, that is MCMV and SCMV, induced a wide range of symptoms on assayed maize plants. Maize lethal necrosis disease affected plant growth and vigour (Plate 4.1 a). The disease affected ear and leaf formation (Plate 4.1 b), cob size and plant height. Plants infected with the combination of SCMV and MCMV developed severe symptoms than those inoculated with either virus (Plate 4.1 c).

Plate 4.1 Infected plants showing maize lethal necrosis symptoms on Duma 43 cav

a- MCMV symptoms; chlorotic streaks and mottling, b- SCMV symptoms; yellowish and mosaic patterns, c- MLN symptoms; severe leaf chlorosis, and deformed ear, d- healthy plant with no viral symptoms

Plant infection with either SCMV or MCMV alone induced mosaic and mottling symptoms respectively. The reduction of plant growth was moderate. The combined infection by both viruses; SCMV and MCMV induced initial symptoms of mosaic, mottling, and severe chlorotic streaks. The plants later developed necrotic leaf margins, stunting, dead hearts and others dried prematurely. In mixed infections with both viruses the synergism induced stunting of plants, general leaf chlorosis, bleaching, and necrosis. These symptoms were unique to MLN as indicated by Wangai *et al*., (2012). Plants that reached maturity, had dried husks, the tassels became sterile, and some developed deformed ears which later became rotten. The combined infection with both viruses induced more distinct disease symptoms.

It caused more severe symptoms to mature maize plants like premature death, tassel sterility, and ear rot. These severe conditions are responsible for the high yield losses of between 50-90% (Niblett and Claflin, 1978). These maize lethal necrosis disease hinders chlorophyll formation and also denatures enzymes responsible for maize to produce, which further reduces maize yields (Wangai *et al*., 2012). Plants inoculated with SCMV showed yellowish streaks on leaves (Plate 4.1 c). The plant leaves developed leaf mosaic patterns of light green to yellow contrasting with dark green patches. The green patches had diffuse irregular margins. The plants infected with SCMV appeared yellowish and pale than healthy plants. These plants developed more distinct symptoms especially in young growing leaves but the symptoms faded gradually as the leaves matured.

The mosaic patterns on the leaves persisted to the tasseling stage and the infected plants formed small ears with fewer kernels (Plate 4.2 d). Infected plants formed small ears with limited kernels while maize plants that become infected early at seedling stage would produce nubbins or were totally barren (Thorat *et al*., 2015). As with other plant viruses that have shown to cause near-asymptomatic infections while others become infective but asymptomatic Lima *et al*., (2012)., SCMV remained asymptomatic in some inoculated plants.

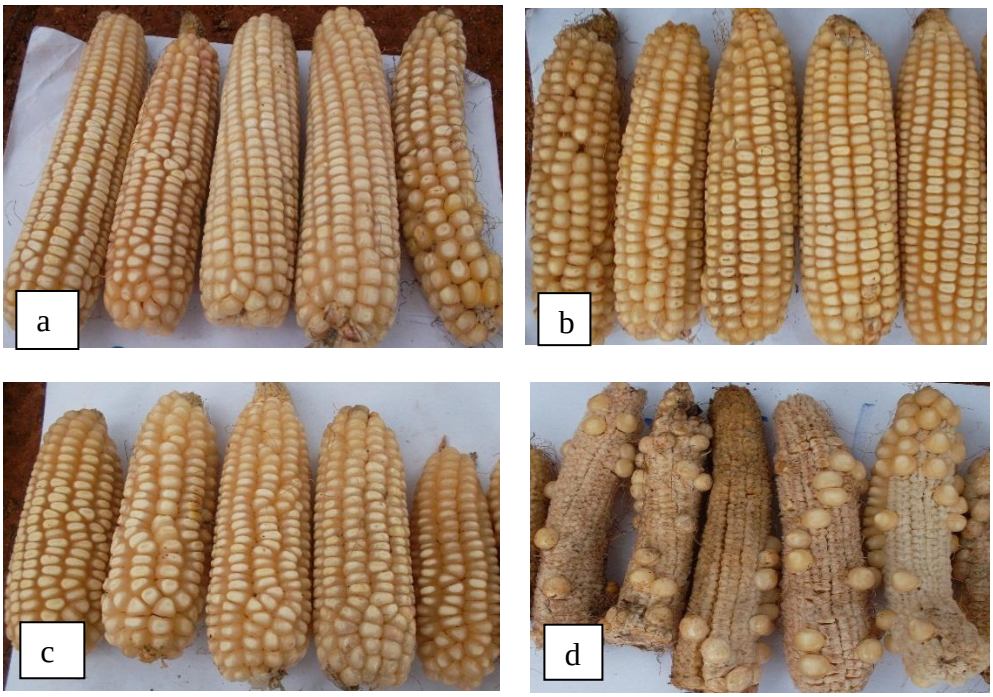


Plate 4.2 Images of Maize cobs Harvested from Duma 43 Maize Infected with MLN Disease

a- cobs from healthy maize, b- cobs from SCMV infected maize c- cobs from MCMV plants, and d- cobs from MLN infected maize plants

In order to verify the transmission of MCMV and SCMV, leaf samples from inoculated plants were tested using RT-PCR and DAS-ELISA. The DAS ELISA tested one hundred and six samples of which 60% (14/30) of them tested positive for MCMV, 86.7% 26/30 for SCMV and 76.7% 23/30 for the two viruses. All of the leaf samples from the healthy controls tested negative (Plate 4.3).

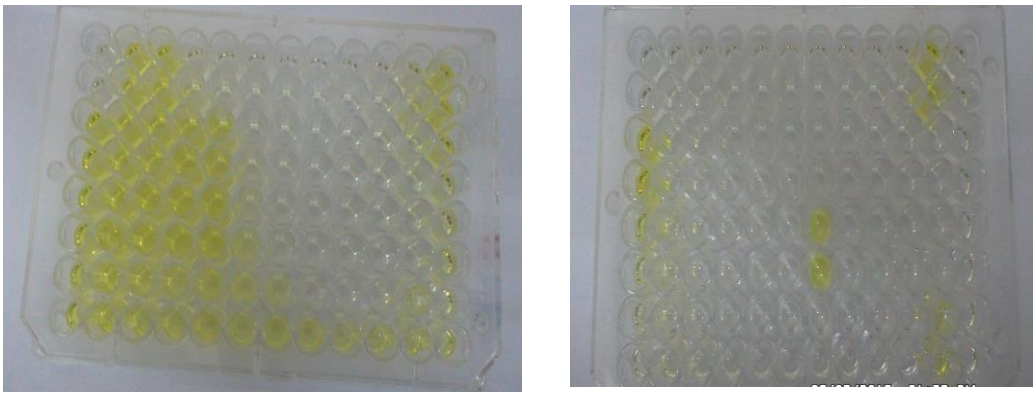


Plate 4.3 The ELISA plates indicating yellow wells for positive virus infection in maize leaf samples

Wells with yellow colouration indicates positive samples

Plants infected with MCMV exhibited clear chlorotic mottle leaf symptoms while others had light green mottling (alternate light and dark green) which became mild six weeks after inoculation. The masking of MCMV symptoms as infected plants mature would complicate MCMV control if the virus is undetected and could provide the inoculum for further transmission of the virus. Moreover, mild virus infections are often overlooked more so if infected plants look asymptomatic (Mathews, 2009). Whenever two unrelated viruses infect a single plant, the viruses can induce a synergistic infection through an interaction that either increase or decrease the amount of either virus or no effect either virus. Scheets (1998) reported that MCMV interacted with WSMV to increase both viral concentrations that caused corn/maize lethal necrosis. Maize plants inoculated with MCMV, SCMV and both SCMV and MCMV were all asymptomatic at week one after inoculation but developed mild symptoms after two weeks. The SCMV + MCMV induced higher initial disease symptom severity (0.92) than SCMV (0.11) and MCMV (0.043). The SCMV + MCMV symptom severity remained high in the eight weeks' post-inoculation period. Although MCMV and SCMV had a relatively lower mean severity than MLND, MCMV induced higher severity than SCMV at weeks four and seven.

4.1.1 Severity of Symptoms on Plants Inoculated with MLN Causing Viruses

The maize plants inoculated with MCMV developed clear leaf mosaic and necrosis symptoms. The virus also induced leaf chlorotic mottling that initially appeared at bases

of younger leaves but later progressed up to the tips. Some infected plants developed light green leaf mottling of alternating pale and greenish portions. Plants infected with MCMV had no clear leaf damage, and the symptoms faded with aging of the plants and became almost asymptomatic after week six of inoculation. Only plants infected with both SCMV and MCMV exhibited clear mosaic and leaf necrosis (Plate 4.1 c). Plants inoculated with MCMV + SCMV had the least mean symptom severity score (Table 4.1).

Table 4.1: The mean disease symptom severity induced on MCMV, SCMV, and MLN inoculated maize cv. Duma 43 at Kenyatta University Greenhouse farm in 2019

Week after inoculation.	*Maize chlorotic mottle virus*	*Sugarcane mosaic mottle virus*	MCMV + SCMV
1	0.05 a	0.10 a	0.90 a
2	1.55 b	0.25 a	1.70ab
3	2.30 c	1.10 b	2.60 b
4	2.75 cd	3.25 c	3.90 c
5	3.35 d	3.55 cd	4.00 cd
6	4.10 e	3.90 de	4.25 cd
7	4.10 e	4.40 ef	4.85 cd
8	4.35 e	4.50 f	5.00 d
P value	<0.001	<0.001	<0.001
SE	0.621	0.583	0.060

***** Means within the same column followed by the same letter are not significantly different after separation by LSD at P=0.05 significance level.

These viruses could have genes that affect the replication and spread of either virus in the MLN disease complex. High virus titer of MCMV and SCMV which induces high disease severity is responsible for yield losses of between 50 - 90% (Niblett and Claflin,

1978). An increase in disease severity leads to a reduction in the size of ear cobs formed. The disease also induces male sterility, poor tasseling, reduced grain formation on the cob, malformed and rotten cobs (Stewart *et al*., 2017). *Maize chlorotic mottle virus* had lower severity than SCMV + SCMV but recent findings have also shown that MCMV alone is a threat that can cause significant yield losses (Miano, 2013).

4.1.2 Symptom Progression on MCMC and SCMV Inoculated Plants

Maize plants inoculated with SCMV + MCMV had moderate to high symptom severity while those of single virus infection of these viruses ranged from low to moderate. All negative control plants remained asymptomatic. There was a significant difference between the symptom severity induced by SCMV, MCMV and SCMV + MCMV on maize plants in the area under disease progression (AUDP) curves at week eight after mechanical inoculation χ^2 (3, N = 40) = 59.67, $p < 0.001$, Bartlett's test) at $P \leq 0.05$ significance level (Figure 4.3). Maize lethal necrosis was more severe and had higher area under disease progression than MCMV. MCMV and SCMV co-infection induced the highest disease severity (5.00) and also had the highest rate of disease progression compared to the MCMV and SCMV inoculated maize plants (severity of 4.35 and 4.45, respectively), (Figure 4.1).

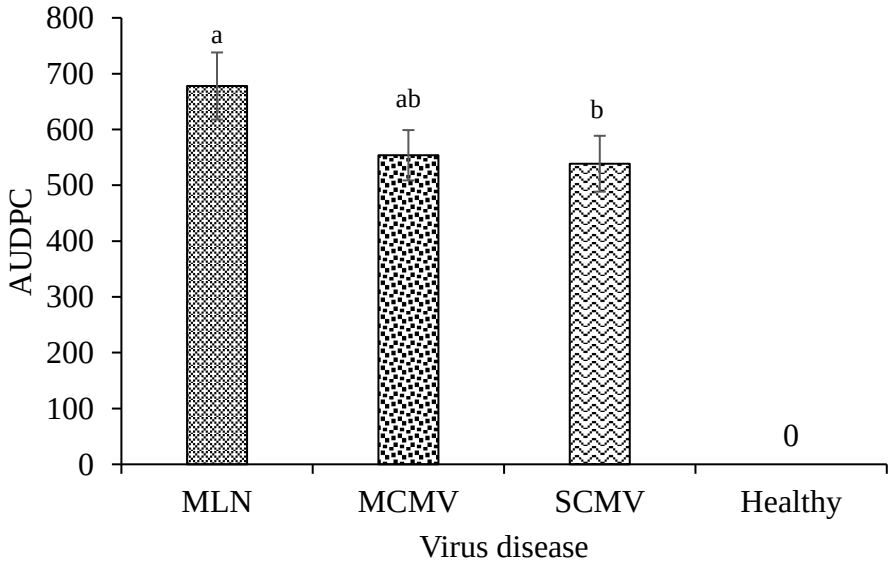


Figure 4.1: Area under disease progression curves of maize plants cv. DUMA 43 infected with SCMV, MCMV, and MLN at week 8 after inoculation. Error bars indicates standard errors

A one-way ANOVA also revealed significant differences F (2, 57) = 84.0, p <0.001 on the mean disease severity between SCMV, SCMV and MLN disease inoculated plants. A post-hoc analysis using Fisher's LSD indicated that MLN disease symptom severity was significantly higher (2.24±0.18) than in MCMV (1.83±0.12) and SCMV (2.12±0.14) inoculated plants. Disease severity between SCMV and MCMV inoculated plants was also significantly different t (20) = 8.96, p <.001). Symptom severity on SCMV infected plants was lower than MCMV but not significantly different but their area under disease progression curves changed over time. However, low symptom severity of single virus infections in the field can easily be undetected especially if infected plants look asymptomatic or have mild symptoms (Mathews, 2009). In addition, the level of SCMV and MCMV symptom severity on maize is influenced by prevailing environmental conditions, the genetic background and developmental stage of the plants (Mahuku *et al*., 2015). Thus, MCMV or SCMV infections inducing mild symptoms may can act as viral reservoirs that facilitate further spread of the viruses by insect vectors.

All the virus treatments had symptomatic plants after mechanical inoculation. This shows that both SCMV and MCMV, could be transmitted mechanically in the field whenever plants are bruised thus spreading the disease. The observed symptoms development and ELISA confirmation of viral infection concur with the results of Wang *et al*. (2017). Mild leaf symptoms appeared on the second week after mechanical inoculation in line with an earlier report by Scheets (1998) who indicated that initial MLN symptoms appeared nineteen days' post-inoculation.

Since maize is susceptible to MLN disease in all stages of development, the virus was easily transmitted at the seedling stage. Plants inoculated with both viruses had more symptomatic leaves than those infected with either MCMV or SCMV, which indicates a synergistic interaction between MCMV and SCMV. Moreover, synergism involving a potyvirus dramatically increases the non-potyvirus component while the potyvirus concentration remains unchanged in doubly inoculated plants (Xia *et al*., 2016). A recent study on the occurrence and genetic diversity, of maize lethal necrosis disease causing pathogens in Kenya revealed more virulent strains of MCMV compared to American and Asian strains (Mwatuni *et al*., 2020).

4.2. Vector thrips Identification and Rearing

The slide-mounted specimens of thrips showed an asymmetric colour arrangement of antennal segments ranging from yellow to pale brown, (Plate 4.4 a, and b).

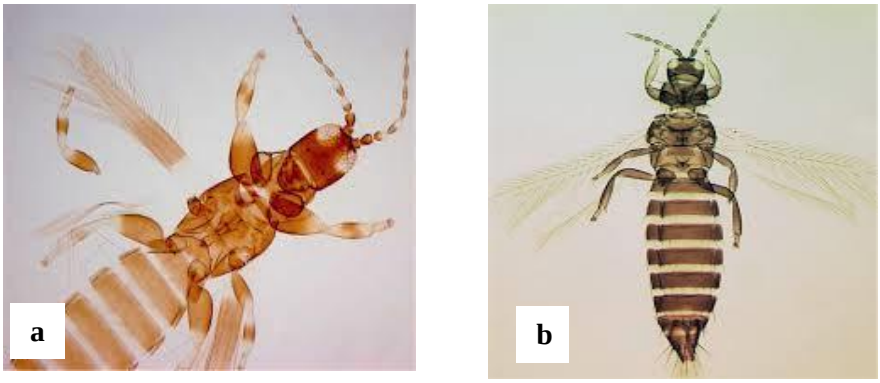


Plate 4.4 Images of slide-mounted thrips

a- Frankliniella williamsi, b- *Frankliniella occidentalis*.

The morphological features and the setal pattern combinations examined on the specimens (Table 4.2).

Table 4.2: Morphological features used to distinguish the identity of *Frankliniella williamsi, F. schultzei,* and *F. occidentalis*

Morphology	Identity		
	F. williamsi	***F. schultzei***	***F. occidentalis***
Yellow coloured body	X	XO*	XO
Brown colour of antennal segments	XO	XO	XO
Ocellar setae pair III arising within ocellar triangle	X	O	O
Presence of post ocular pair I setae	X	O	X
Antero-marginal setae length shorter than anteroangulars	O	X	X
Companiform sensillea absent	O	X	O
Lateral ctenidia on tergite V and anterolateral spiracle on tergite VIII	X	O	O
Posteromarginal comb on tergite VIII	O	X	O
Discal setae on sternite II present	X	O	O
Total of each combination (n=114)	16	37	51

Specimens examined: *F. williamsi* = 16; *F. schultzei* = 37 and *F. occidentalis* = 51

*xo- indicates colour combination of both brown and yellow. 0- Indicates presence and, x- absence of morphological features.

The morphological characteristics identified three major species infesting maize within the regions sampled. *Frankliniella occidentalis* is a slender, narrow insect with a flattened body of approximately 2 mm long. It is yellow or pale brown in colour, has a dark strip on its abdomen. The insect has a pair of 8-segmented antennae with two, black banded end narrow segments (Plate 4.5). The insect has forked sensorium on segment III & IV. Its head has three pairs of ocellar setae. The ocellar setae pair III arises within the ocellar triangle. The insect also has five pairs of post-ocular setae located next to the compound eyes.

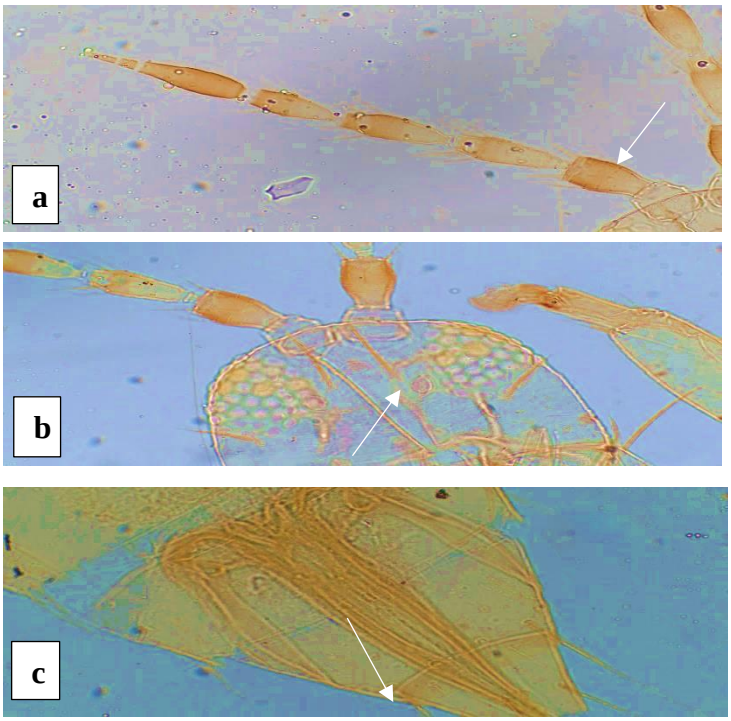


Plate 4.5 Images of *Frankliniella occidentalis* morphological features

(a- Forked dark brown antennae segments, b-ocella setae within ocella triangle, and c- complete comb on tergite VIII, (x400 mg)

The post-ocular setae IV are similar in thickness and length to the ocellar setae pair III. A pair of antero-marginal setae on the pronotum is equal in length to the antero-angular setae, and the two pairs of postero-angular setae. The forewings have a pair of complete row venal setae. The dorsal side of the abdomen has dark markings as well as a black tip. A median pair of metanotal setae arises from the margins of the metanotum. The

insect metanotum has companiform sensilla arranged in a reticular pattern. The abdominal tergite VIII has a complete postero-marginal comb.

The slide-mounted thrips samples from Murang'a region of Kenya showed an asymmetric colour arrangement of antennal segment ranging from yellow to pale brown. Sixteen specimens were *F. williamsi*, 37 were *F. schultzei,* and 51 were *F. occidentalis*. The presence of the postero-marginal combs on abdominal tergite VIII and the absence of companiform sensillea distinguished *F. schultzei* from *F. williamsi* and *F. occidentalis.* The presence of the lateral ctenidia on tergite V and anterolateral spiracle on tergite VIII as well as the presence of the discal setae on sternite II distinguished *F. williamsi* from *F. occidentalis* (Moritz *et al*., 2001b). Moreover, *F. williamsi* sternites III–VII has small oval pore plates while sternite VII has toothed craspedum on posterior margins (Cavalleri & Mound, 2012).

The morphological identification of thrips grouped specimens up to presumptive species. Only 34 specimens were identified to genus and family level. Identification of thrips to species level was based on expert knowledge of the thrips genera (Hoddle and Mound, 2003). Moreover, an accurate examination of minute morphological details was achieved through the use of cleared, slide-mounted specimen thrips. Collected adult thrips were confirmed as *F. williamsi* and *F. occidentalis* (Mound & Kibby 1998). Only adult thrips were identified since larval specimen identification to species level without the presence of adults could be impossible (Brunner *et al*., 2002). These difficulties of morphological thrips identification could lead to inaccurate vector thrips identification, deficient control as well as a viral disease pandemic like MLND.

Frankliniella williamsi has a head that is wider compared to its length. The head has three pairs of ocellar setae. Ocella setae pair III arise within the lateral margins, is longer than the ocellar triangle margin. Ocella setae pair IV is equal in length with the distance between the hind ocelli. The antennae of these vectors are 8-segmented with forked sensori on segment III–IV and segment VIII is twice long as VII. The pronotum has 5 pairs of major setae with the antero-marginal setae being equal in length to the anteroangulars. They have a pair of minor setae located medially between the posteromarginal submedian setae. The metanotum has a campaniform sensilla and 2

pairs of setae within the anterior margin (Plate 4.6b). Their forewing has 2 complete sets of veinal setae. Abdominal tergites V–VIII have a pair of ctenidia, anterolateral to the spiracle on tergite VIII. They have complete posteromarginal combs on tergite VIII with long, regular microtrichia. Sternites III–VII lack discal setae and sternite II has one or two medial discal setae. Sternites III–VII has small oval pore plates while sternite VII has toothed craspedum on posterior margins.

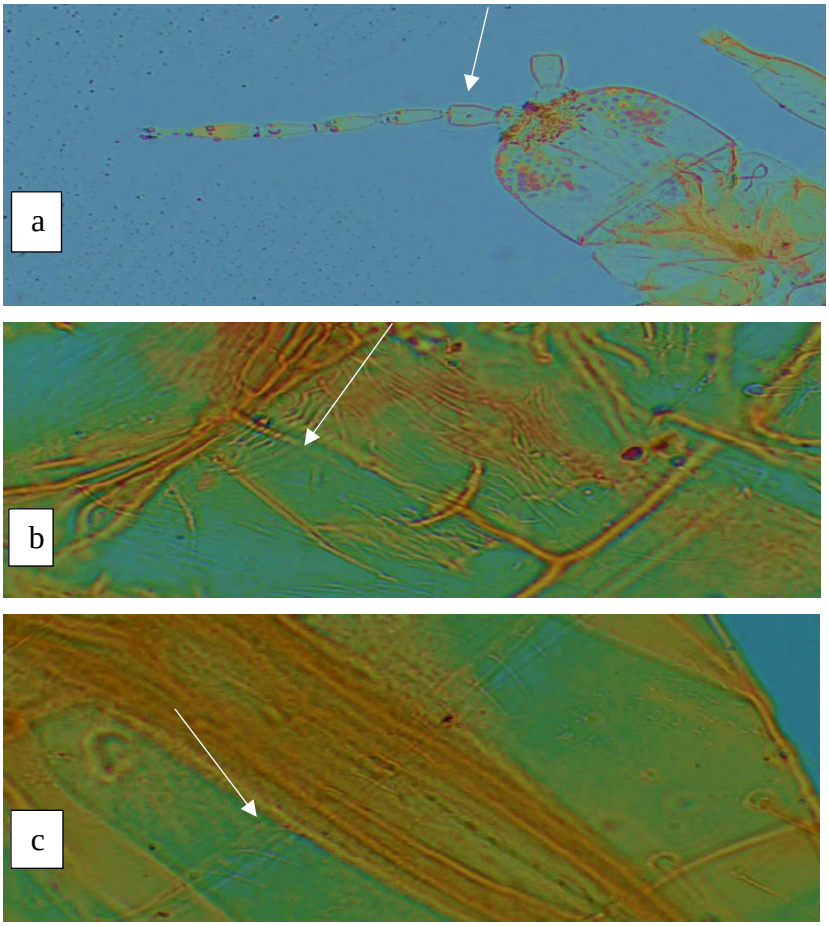


Plate 4.6 Images of *Frankliniella williamsi* showing Morphological Features

a- Pale antennae segments, *b-metanotal sculpture, and c- complete comb on tergite VIII,* (x400 mg)

Maize thrips (*F. williamsi*) and Western flower thrips (*F. occidentalis*), are known to transmit MCMV (Zhao *et al*., 2014; Cabanas, *et al*., 2013). *Frankliniella schultzei, Thrips tabaci, T. pusillus* and *Scirothrips spp.* are widely distributed in the MLN endemic East African region (Riley *et al*., 2011). However, their competence for MCMV transmission is yet to be established. The objective of the activity was first to

determine the best substrate of raising colonies of *F. williamsi* and *F. occidentalis* and then use the raised colonies in subsequent bio assay experiments on the biological interactions of MCMV and adult cultures of *F. occidentalis* and *F. williamsi*. *Maize chlorotic mottle virus* is associated with MLN epidemics in Eastern Africa (Adams *et al*. 2013), and both *F. williamsi* and *F. occidentalis* are known to transmit the virus (Zhao *et al*., 2014; Cabanas *et al*., 2013). Unraveling a transmission pattern of a virus and its vectors presents an important step for control of the disease. Knowledge of the interaction can help in developing disease control measures through reduction of viral inoculum and limit its spread via vectors (Lecoq *et al*., 2004).

In vitro culture of insect vectors provides an understanding of their feeding behaviour, preference of substrate and micro-climate requirements. Bioassays for evaluating thrips transmission of plant viruses require consistent supply of adult thrips. Earlier methods for rearing *F. occidentalis* used whole or parts of a plant as substrates inside cages (Mortazavi *et al*., 2015). Common substrates in earlier studies were French bean *Phaseolus vulgaris* plants, bean pods, bean leaves (Steiner and Goodwin, 1998), germinated beans, and bean cotyledons (Murai and Loomans, 2001). Cabanas *et al*. (2013) raised *F. williamsi* adult and larval colonies on maize seedlings inside cylindrical mesh ventilated Polyvinyl chloride (PVC) cages. However, there was no evidence of colony development on seedlings in our preliminary tests. Different food substrates were tested and appropriate substrates selected for rearing *F. occidentalis* and *F. williamsi*. Adult colonies were used in MCMV transmission bio assays.

4.2.1 Effect of Substrates on Thrips Population Growth

The number of *F. occidentalis* larvae was monitored on *P. vulgaris* pods, *C. pepo* and *C. sativus* rearing substrates. There was a significant difference in the number of *F. occidentalis* larvae among the *P. vulgaris* pods, *C. pepo* and *C. sativus* substrates, χ^2 (2, N = 24) = 2.48, p = 0.029 Bartlett's test) at P = 0.05 significance level. Between week 2-5 *C. pepo* was higher than *P. vulgaris* and then reduced. These substrates had a varied effect on the population of larval that emerged after oviposition. However, the larval population was highest on *Phaseolus vulgaris* pods (106±3.4) followed by Courgettes (54±2.3). The least larval population (46±3.7) was recorded on *Cucumis sativus* substrate (Figure 4.2).

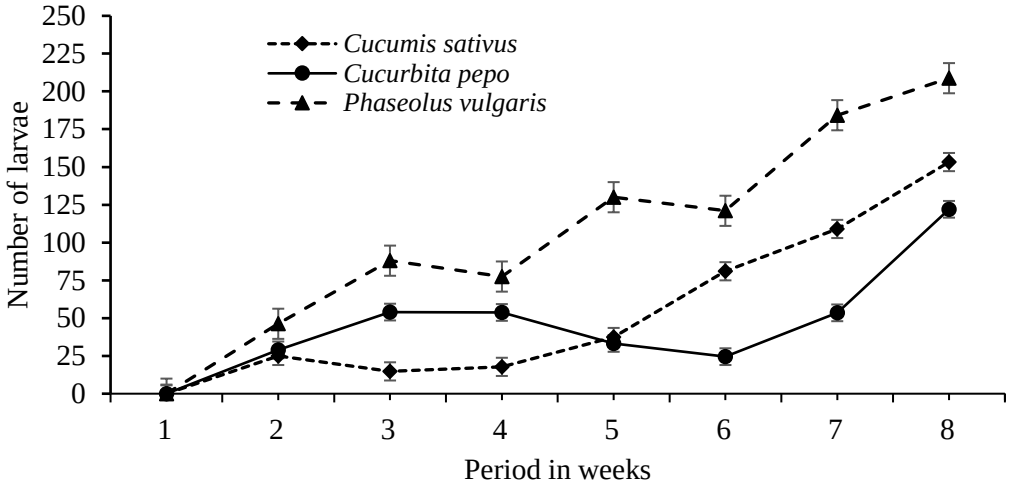


Figure 4.2: Mean number of *Frankliniella occidentalis* larvae populations recorded on *Cucumis sativus, Cucurbita pepo* and *Phaseolus vulgaris* over an 8 week rearing period.

Error bars indicates standard errors

French bean pods had the highest number of larvae and were thus used to rear *F. occidentalis* thrips. Lewis (1997) had identified sterilized French bean *Phaseolus vulgaris* pods to be ideal food and oviposition sites for *F. occidentalis*. However, French bean pods as a substrate will require an efficient rearing method with minimal mortalities in order to increase the number of larvae (Lewis, 1997). Moreover, use of cages with sufficient ventilation, temperature and moisture regulation provides an ideal rearing microclimate (Mcdonald *et al*., 1998). In this research, nylon clothe covers for the 5 L cylindrical cages were placed adjacent to windows provided sufficient ventilation that regulated moisture. In addition, the cotton water wicks placed inside the cages for water provision to the thrips but also increases cage moisture.

The pods were obtained easily from French beans produced in a greenhouse which presented a low risk of contamination with other species of thrips (Mortazavi *et al*., 2015). Courgettes *Cucurbita pepo* had more larvae than Cucumber *Cucumis sativus* but Courgettes had frequent fungal and bacterial attack even after surface sterilization using 5% Sodium hypochlorite. Courgette and Cucumber (*Cucumis sativus*) substrates presented a slight reduction in insect fecundity and longevity. Insects reared in artificial conditions can become sensitive to food substrates because of restrictions in diet, space,

temperature and humidity variation (Grundy *et al*., 2000). However, these challenges are minimized by selecting the best rearing substrates (Mortazavi *et al*., 2015). A study by Nyasani *et al*. (2013) on feeding, oviposition, and host preference of *F. occidentalis* in Kenyan French bean fields identified courgettes, (*Cucurbita pepo*) and French bean (*Phaseolus vulgaris*) as highly preferred oviposition hosts. The number of *F. occidentalis* adults were significantly different (χ^2 (2, N = 24) = 8.12, p = 0.017) among *C. sativus*, *C. pepo* and *P. vulgaris*. *Phaseolus vulgaris* pods recorded an exponential growth in adult population after the third week of rearing. On *C. sativus* and *C. pepo*, the number of adults grew exponentially after the fifth week of rearing (Figure 4.3). Thus, *P. vulgaris* pods supported a larger colony of *F. occidentalis* compared to *C. sativus* and *C. pepo* over the same rearing period. Since French bean pods recorded a higher number of adults than Courgettes and Cucumber, they offered a suitable substrate option for rapid *in vivo* rearing of *F. occidentalis*.

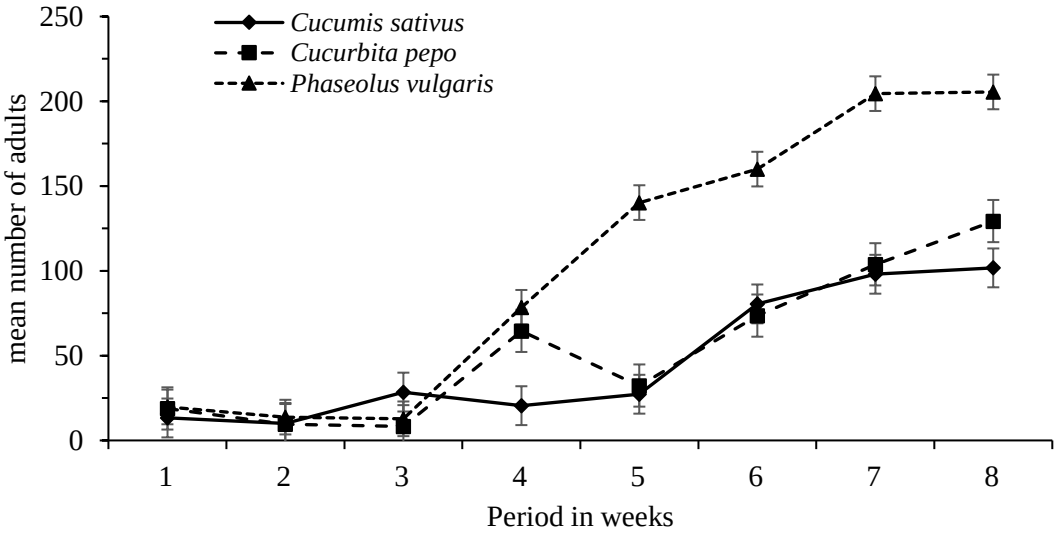


Figure 4.3: Mean adult population of *Frankliniella occidentalis* reared on substrates *Cucumis sativus, Cucurbita pepo* and *Phaseolus vulgaris* over an eight week period

Error bars indicates standard errors

A one-way analysis of variance showed a significant effect F (2, 14) = 8.65, p = 0.004 of the choice of substrate on the population of *F. williamsi* larvae. The number of larvae on maize seedlings, maize stalk cuttings, and *P. vulgaris* pods were significantly

different. A post-hoc analysis using the Fisher's least significant difference test for significance indicated that the average number of larvae was significantly lower on maize seedlings (6.62±2.26) and *P. vulgaris* pods (1.12±0.26) than on the maize stalk cuttings (28.72±5.56). Maize seedlings had larvae only in week one while *P. vulgaris* pods had no larvae (Figure 4.4). Maize stalks with slit leaf sheaths had the highest means of larvae (32±0.32). No larvae were observed on maize seedlings beyond week two after emergence of the first generation.

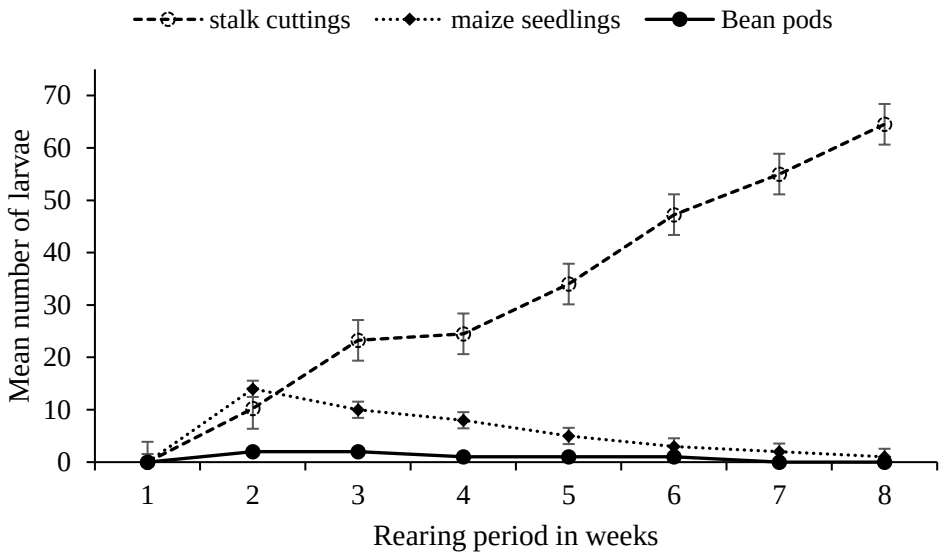


Figure 4.4: Mean number of *Frankliniella williamsi* larvae recorded on maize seedlings (*Zea mays*), maize stalk cuttings and bean (*Phaseolus vulgaris*) pods as rearing substrates over an eight week rearing period

Error bars indicates standard errors

Maize stalks were the best substrates for *F. williamsi* larvae but required regular replacement after 7-10 days. They provided suitable oviposition sites inside slit leaf sheaths. Moreover, under field conditions, *F. williamsi* predominantly reside under leaf sheaths of maize. *Frankliniella williamsi* prefer to feed and oviposit eggs under leaf sheaths where larval predation is minimized (Beres *et al*., 2013). Cabanas *et al*. (2013) previously used maize seedlings to raise *F. williamsi* colonies inside confined PVC cages. However, the seedlings were not able to support larval or adult population build up in nylon-mesh ventilated cages. The small size of the cages (5 L, 15 cm radius,

cylindrical boxes) with nylon net covers may have hampered sufficient microclimate regulation which was necessary for the seedlings and thrips development (Mortazavi *et al*., 2015). Moreover, proper ventilation, with sufficient moisture and temperature constitute an ideal microclimate necessary for thrips development (Katayama 1797).

The rearing substrates significantly affected the number of *F. williamsi* adults χ^2 (2, N = 24) = 8.67, p = 0.013. The preference of *F. williamsi* adults for maize seedlings (*Zea mays*), maize stalk-cuttings, and bean *P. vulgaris* pods as food substrates were not equally distributed. While the adult population increased on maize stalk cuttings, it declined on *P. vulgaris* pods and maize seedlings (Figure 4.5). *Phaseolus vulgaris* pods did not support any new generation of *F. williamsi*. The initial adult population sharply declined in the second week of rearing, beyond which they were only observed on maize stalk cuttings.

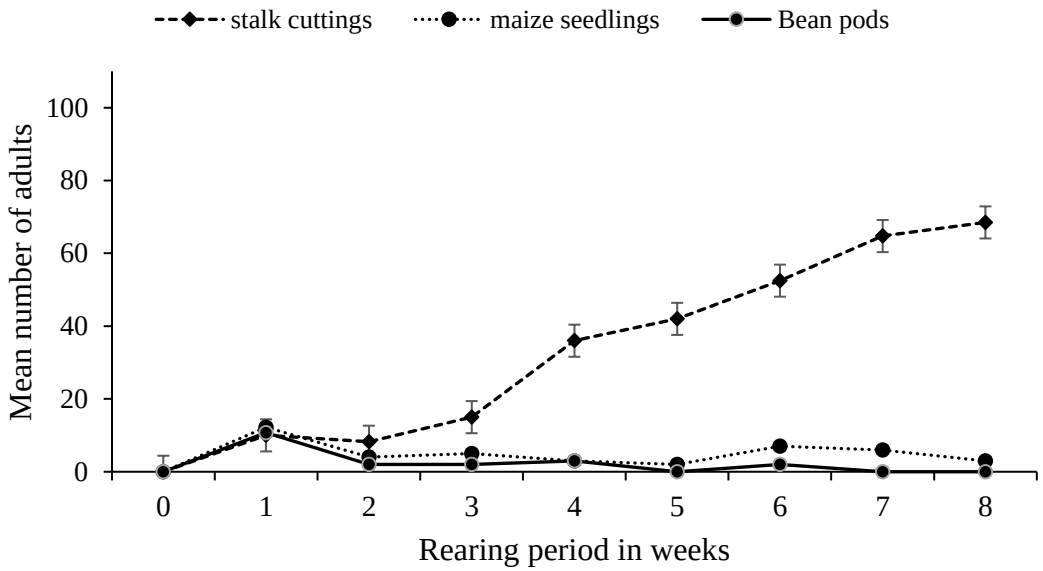


Figure 4.5 Mean number of *Frankliniella williamsi* adults recorded on rearing test substrates (maize stalk), maize seedlings and French bean pods) over an eight week rearing period

Error bars indicates standard errors

There were more *F. williamsi* adults on maize stalks than French bean and maize seedlings. Thus, maize stalk cuttings from plant parts raised inside a greenhouse are better substrates for rearing *F. williamsi*. In Kenya, these thrips infest maize, French

bean and onions (Otipa *et al*., 2018; Moritz *et al*., 2013). However, in this study *F. williamsi* had little preference for French beans as substrates for oviposition and reproduction. French bean crops could be serving as alternative hosts for *F. williamsi* in the field which can complicate MCMV vector control. Therefore, the role of French beans and other plants hosting *F. williamsi* within maize fields in Eastern Africa need further research. Thrips often feed on plants on which they may not reproduce (Paini *et al*., 2007). *Frankliniella occidentalis, F. fusca* (Hinds) and *F. tritici* (Fitch) all attack tomato (*Solanum lycopersicum* L.) in Florida, but only *F. occidentalis* is able to reproduce on tomato (Salguero - Navas *et al*., 1991).

Wild plants and weeds host thrips when crop hosts are unavailable or not flowering. *Frankliniella bispinosa* (Morgan) uses two plant species, as reproductive hosts from May to August inn North Florida. *Ligustrum sinense* Lour and *Lagerstroemia indica* L serves as reproductive hosts for *F. bispinosa* during May to August (Paini *et al*., 2007). Cockfield *et al*. (2007) also noted that the native weeds around the apple orchards supported *F. occidentalis* colonies whenever apple trees had no flowers. Selection of an appropriate substrate for rearing particular species of thrips is important for the success of any *in vitro* culturing of thrips for bio assay experiments.

4.2.2 Influence of Temperature on survival of Frankliniella occidentalis reared on Phaseolus vulgaris pods

Larvae emerged on bean pods 4 days after oviposition with 1st instars taking 2-3 days to form 2nd instars which took 3 days to form pre-pupa. The 1st instars were pale in colour, the 2nd and 3rd instars were pale - green while the pre-pupa and the pupae were yellow to orange. Pupation occurred below the folded paper towels and took 4 days for adults to emerge. Larvae population remained low (141 - 161) during the months of June and July, a period which experienced low mean day temperatures (19.1 - 19.9 °C) and high relative humidity (79 – 78%). The number of adults was high (351 - 394) between September to December when mean day temperature ranged between 21.8 – 22.7 °C and 66.6 – 68.3 % RH. A colony of *F. occidentalis* was raised on French bean pods using rearing cages adopted from Steiner and Goodwin (1998). *Frankliniella williamsi* was raised on maize stalk cuttings inside clothe ventilated PVC cages as adopted from Cabanas *et al*. (2013). Thrips population build up was monitored in

different months of the year, a period with fluctuations in temperature and relative humidity; factors that determine population abundance of thrips (Waiganjo *et al*., 2008). Thus, these factors are responsible for a spike in vector population and the rate of MCMV spread. Rearing temperature affects thrips longevity which directly influences the thrips population. *Frankliniella occidentalis* has a greater longevity at an optimum temperature of 27.2 °C which is almost triple than at higher temperatures above 30 °C (Robb and Parella, 1991). At this optimum temperature, thrips have a shorter development period with low mortality rates Robb (1989). Previous studies agree with results of this study on the optimum temperature for *F. occidentalis* of 27-28 °C. At this favorable temperature range, the high rate of oviposition and the rapid increase in *F. occidentalis* population could have a significant effect on the abundance of this vector which could complicate MCMV control.

The number of adults was low in July to November during which the mean day temperatures were relatively higher (19.9 - 22.7 °C). The population of *F. occidentalis* larvae on bean pods increased in direct proportion to increase in the mean daily temperature (Figure 4.6). Every increase in day degree temperature led to an increase in larval population of 0.9 larvae per cage. The peak larval emergence was at 22.5 °C beyond which it remained unchanged. An increase in temperature resulted into a proportionate increase in adult population. The adult population increased by 3.0 for each degree increase in day temperature. Adult population gradually increased at temperatures 19 – 21 °C. Temperatures of between 23.7 - 24.9 °C favorably increased adult emergence. The optimum temperature for adult and larval emergence ranged between 22 - 23.5 °C (Figure 4.6).

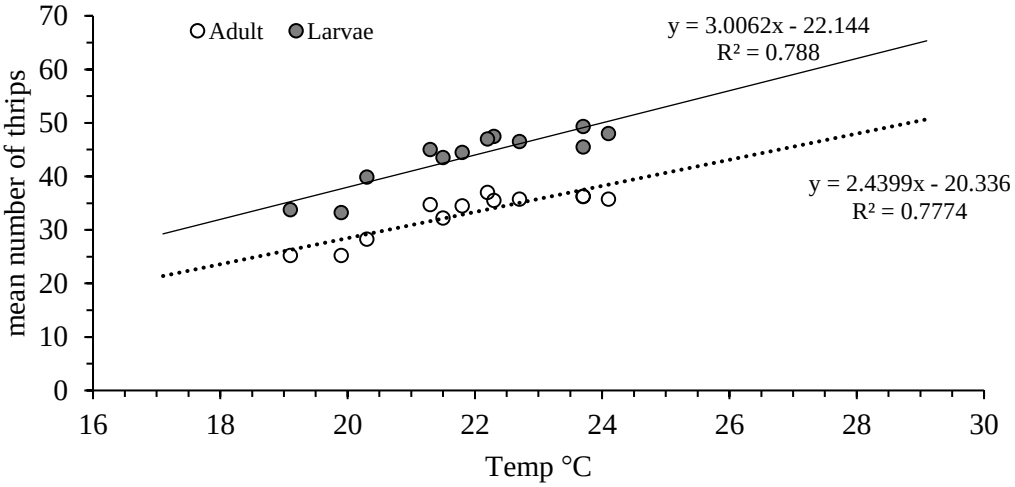


Figure 4.6: Regression of mean number of larval and adult populations of *Frankliniella occidentalis* reared on *Phaseolus vulgaris* pods and rearing temperature.

4.2.3 Influence of Relative Humidity on Survival of Frankliniella Occidentalis

The population of *F. occidentalis* larvae declined with an increase in relative humidity (Figure 4.7). The results showed a direct linear negative relationship between an increase in relative humidity and population of larvae on bean pods. There was a decline in larval population for each 1% increase in relative humidity from 65-80 %. An increase in % RH also resulted in a decline in the adult population. Each 1% increase in RH translated into a decline in the adult population mean by 1.2. The optimum % RH for adult and larval population ranged between 64 – 68% (Figure 4.7).

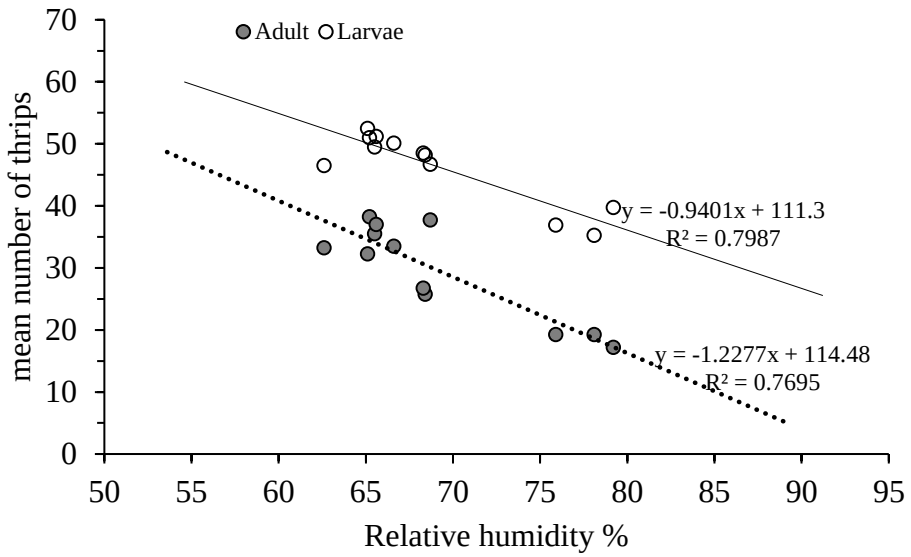


Figure 4.7: Regression of mean number of larval and adult populations of *Frankliniella occidentalis* thrips reared on *Phaseolus vulgaris* pods against % relative humidity

Data showed that cage humidity varied within the rearing months of the year. June and July had the highest relative humidity (79.2 and, 78.1% respectively) while December had the least (62.6 %). The variation in relative humidity was attributed to the changes in room temperature, air circulation inside the rearing cages and the difference in the transpiration activity of newly introduced bean pods (Fatnassi *et al*., 2006). Larval and adult emergence declined with an increase in relative humidity from 62-70% but was slightly affected by increase of RH between 71-79% which was the point of least larval emergence.

The optimum RH for larval and adult emergence and larval mortality was between 62-65%. High RH could be achieved at high room temperatures which hindered larval emergence that was critical for thrips population build up. Steiner *et al*. (2011) illustrates that all stages of *F. occidentalis* are sensitive to humidity and thus affects its population build up. In their study, a humidity range of 65-85% was unfavourable to the larvae but was more favourable to the pupae and adults. In this study, the results showed an optimal humidity of 65% for both larvae and adults.

4.2.4 Effects of Temperature on Frankliniella williamsi Population growth on MaizeSstalk Cuttings

Larval population was higher during the cooler months of the year; May (20.3 °C, 94), June (19.1 °C, 111) and July (19.9 °C, 101, and lower in the warmer months of February (24.1 °C, 42) and October 22.3 °C, 36). Larval and adult populations declined at higher relative humidity and low daily temperature. The developmental period from egg hatching to emergence of adults was about 9-11 days and the mortality was above 50% at RH above 66%. Temperature greatly influenced the survival of thrips inside the cages. An increase in temperature from 19.1 to 24.1 °C significantly affected larval and adult populations. Larval population increased by 0.26 units while adults declined by 1.1 unit for each degree increase in temperature (Figure 4.8). The optimal temperature for both larvae and adult population growth ranged between 21-23 °C.

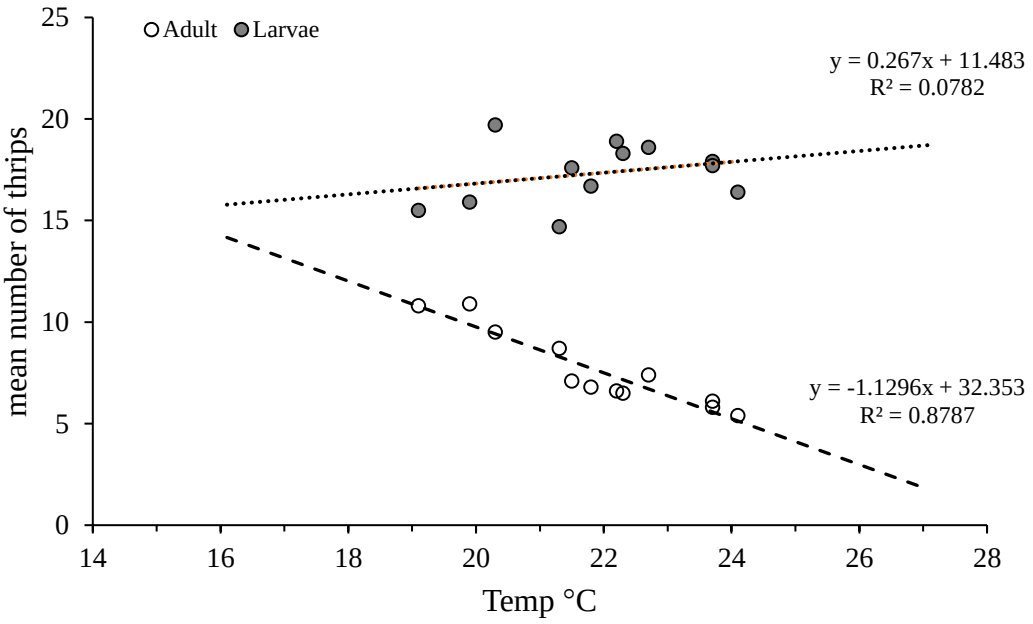


Figure 4.8: Relationship between rearing temperature and population of *F. williamsi* at larval and adult stages at temperatures between 19.1 to 24.1 °C

Maize stalks cuttings were the best substrates for *in vitro* rearing of *F. williamsi* because of their ease of handling, are from host plants and stayed fresh for longer periods; all being desired qualities of a substrate (Loomans and Murai, 1997). Thrips population varied between months of the year as for reared chilli thrips *Scirtothrips dorsalis*

(Hood) by Latha *et al*. (2015). The peak population was in March, May, June, and July; a period characterized with low daily temperatures but with high humidity. Low adult mortality was in January, February, and December. Vector thrips populations could hike during this period which will exuberate virus spread considering most farmers practice relay planting of maize with intercrops (Mahuku *et al*., 2015). Temperature was responsible for *F. williamsi* population build up as it influenced their survival and longevity of substrate sources.

Temperature affects the lifespan, development, and mortality of an insect but also increases substrate aging (Reiter *et al*., 2015). Adult population declined by 0.4983 units with each additional degree in temperature which was attributed to high rates of substrate deterioration with increasing temperature (Prange, 2004). There was no significant effect in temperature increase on larval population from 19.1 °C to 24.1 °C. Moreover, an increase in temperature decreases fecundity or viability of *Thrips tabaci* (Kaur *et al*., 2017). The occurrence of many insect species in agro systems is influenced by temperature (Cannon, 1998). Insects will also need a threshold temperature to complete their life cycle (Danks, 2000). Their relationship between population development and temperature is essentially linear within favorable temperatures (Stacey and Fellowes, 2002).

4.2.5 Effect of Relative Humidity on Frankliniella williamsi Reared on Maize Stalks

Relative humidity within the rearing cages influenced the *F. williamsi* thrips population abundance Relative humidity significantly affected (r= 0.997) the adult population development ($F_{1, 10} = 43.2$, $P = <.001$, $r^2 = 0.825$). Adult survival improved by 0.31 for each 1 % RH change from 62.6 to 79.2 with a standard error of 0.005 (Figure 4.9).

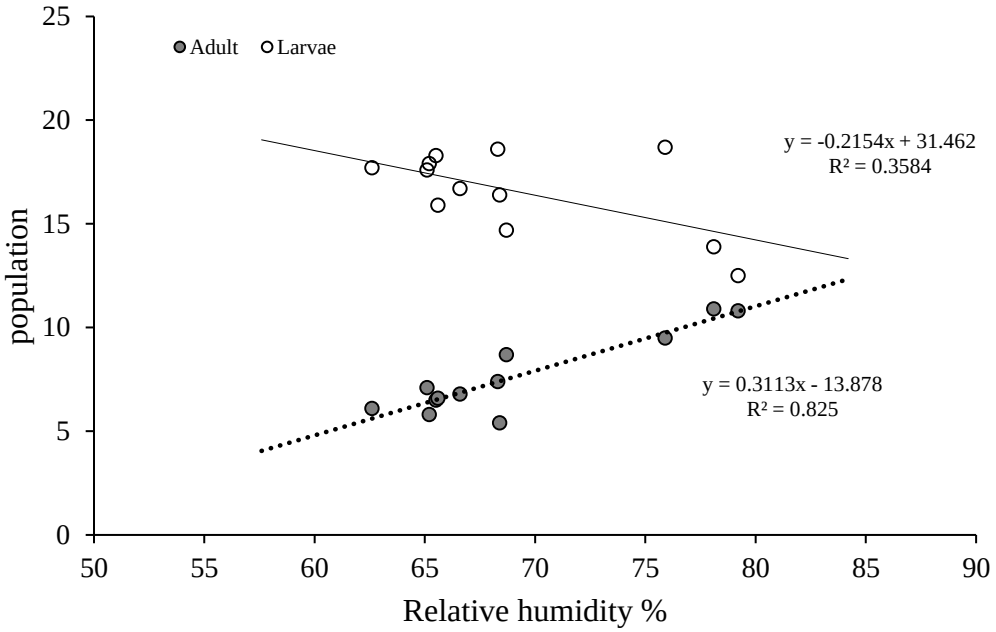


Figure 4.9: Relationship between Relative humidity and *F. williamsi* larval and adult populations reared on maize stalk cuttings

Relative humidity was a critical factor for the positive role in *F. williamsi* population growth. There was a strong positive correlation (r= 0.997) between increase in humidity and population growth. Adult survival improved by 1.038 for each additional % RH from 62.6 to 79.2. Relative humidity is positively associated and correlates with thrips population under field conditions (Khan *et al*., 2008; Gupta *et al*., 1997). High % RH therefore plays a critical role in vector abundance which exacerbates MCMV given that, the virus has a great potential risk for further spread across the warm arid, semi-arid, and the sub-humid tropics, of Eastern and Southern Africa (Mahuku *et al.,* 2015). The study developed efficient rearing techniques for *F. occidentalis* and *F. williamsi* both of which are vectors of MCMV (Zhao *et al*., 2014; Cabanas *et al*., 2013). Successful *in vitro* culturing of these insects presents a major milestone in understanding their feeding behaviour, substrate preference, and developmental process. It will also secure consistent supply of healthy thrips for virus transmission bio assays.

4.3 Thrips Transmission Efficiency of Maize Chlorotic Mottle Virus

Frankliniella occidentalis transmitted MCMV in all acquisition access periods (AAP). The efficiency of transmission varied from 6.2% to 81.2% for 30 minutes and for 24

hours. The virus transmission increases by 1.3 plants for each extra hour of feeding (Figure 4.10 a). The highest proportion of infected maize plants was after a 24 h of VAAP followed by 3 h. The least was after 1.5 hour of VAAP and no virus transmission occurred at 0.5 hour of VAAP. The infected plants became symptomatic and tested positive by ELISA. The negative controls remained asymptomatic and tested negative by ELISA. *Frankliniela williamsi* transmission rate increased by 1.1 for each extra hour of feeding acquisition access period.

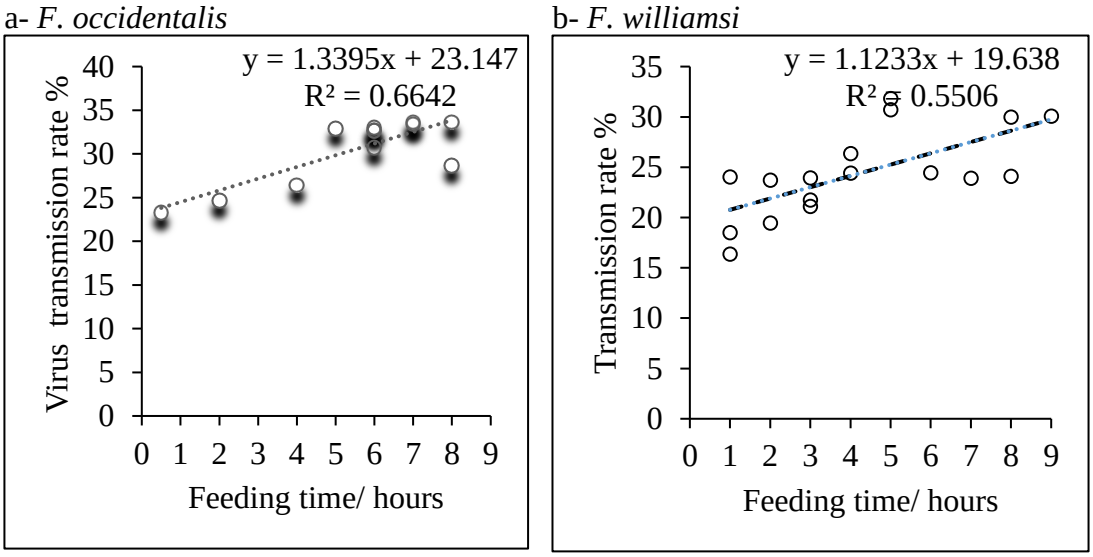


Figure 4.10: Regression analysis of MCMV transmission against acquisition feeding time for *F. occidentalis* and *F. williamsi*

Frankliniella williamsi transmitted MCMV in all the virus AAPs except at 0.5 hour. The efficiency varied from 12.5% for 1.5 hour to 43.7% for 24 hours (Figure 4.10b). The 24 hour VAAP had the highest number of symptomatic and infected plants while 1.5 hour VAAP had the least. Some maize plants were asymptomatic but tested positive for MCMV by ELISA. *Frankliniella occidentalis* had higher transmission efficiency of MCMV than *F. williamsi* (Figure 4.11). There was significant statistical difference observed in transmission rates between *F. occidentalis* and *F. williamsi* adults with an average range of 2.86 and 1.12%, respectively (t =8.38; df = 66; P=0.001).

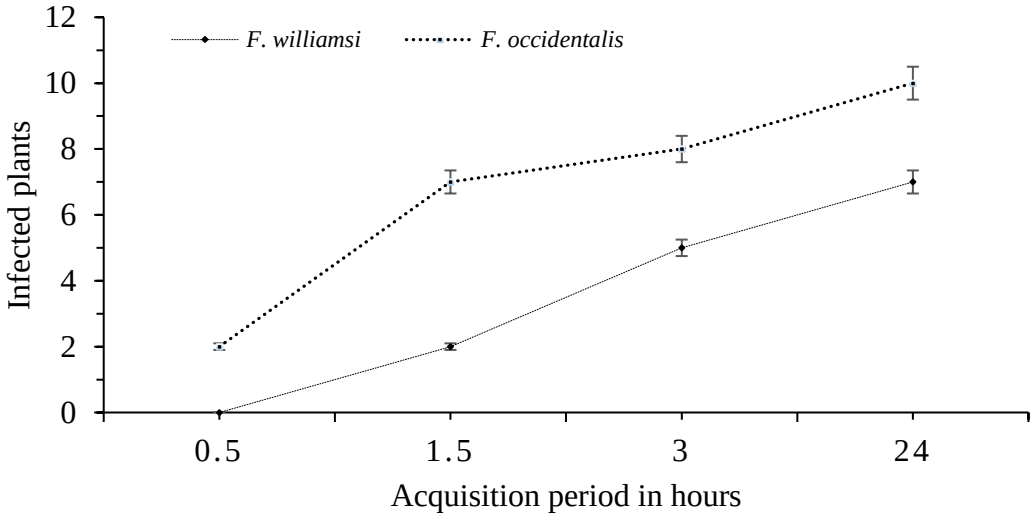


Figure4.11: Transmission efficiency of MCMV by vector thrips *F. occidentalis* and *F. williamsi* adults at different virus acquisition access periods

Error bars indicates standard errors

The rate of virus transmission by *F. occidentalis* was highest at 24 hour AAP (81.2 %) while 0.5 AAP had the least (6.2 %); a confirmation that *F. occidentalis* transmits MCMV. However, virus transmission after short AAPs (0.5 hour) indicates lack of a latent period; a characteristic of a non-persistent virus (Andret-Link and Fuchs, 2005). *Frankliniella occidentalis* transmits MCMV after 30 minutes of exposure after 48h starvation period (Zhao *et al*., 2014). However, 3 hours' starvation period was sufficient for thrips to acquire and transmit the virus. This rapid acquisition and transmission of MCMV soon after exposure without a latent period was similar to that of *F. williamsi* and western corn rootworms (Cabanas *et al*., 2013).

Frankliniella williamsi transmitted MCMV in a similar pattern like *F. occidentalis* but did not transmit the virus at 0.5 hour AAP. This was likely due to the differences in probing and feeding behavior between these thrips species. *Frankliniella occidentalis* produces non-ingestion, short, and long ingestion probes (Stafford *et al*., 2011), but those of *F. williamsi* are yet to be illustrated. Thrips salivate before and during the non-ingestion probing which is associated with inoculation of propagative viruses, such as *Tomato spotted wilt virus* (TSWV). Understanding the feeding behavior of *F. williamsi* on maize may be useful in characterizing the transmission biology of MCMV (Cabanas *et al*., 2013). *F. williamsi* and *F. occidentalis* belong to the same taxa (*Frankliniella*),

and they transmit MCMV in a similar pattern which indicates that these thrips play a part in MCMV epidemiology in maize growing areas. Moreover, taxa specific vectors spread plant viruses within a genus (Nault, 1978; Ng and Falk, 2006). *Frankliniella williamsi* transmission rate of MCMV was relatively higher than that of *F. occidentalis* in all the inoculation access periods (1, 2, 3, 24, and 48). Transmission rate of these thrips increased steadily after 24 hours and was highest after 48 hours of inoculation access period (Figure 4.12). The transmission rate by *F. occidentalis* was relatively uniform in all the inoculation access periods investigated but slightly increased at 24 hours.

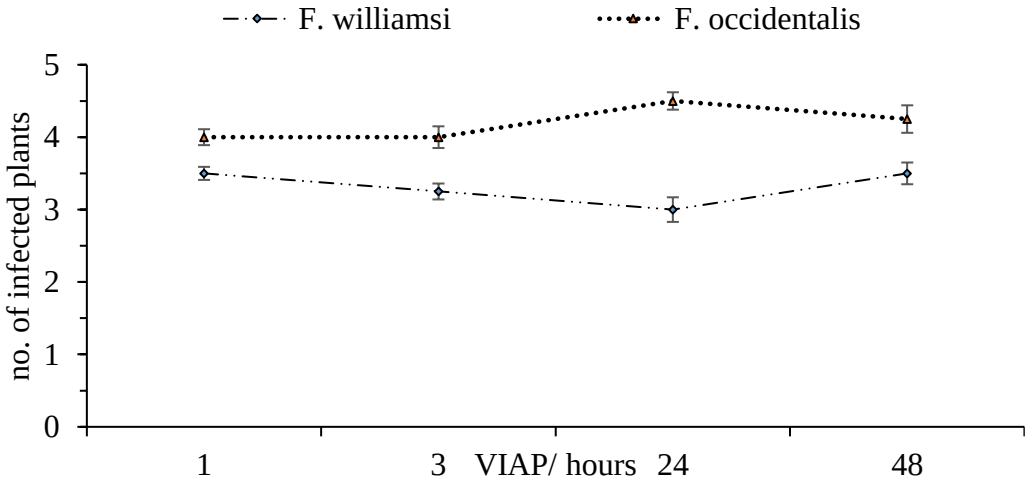


Figure 4.12: Mean (±SE) number of MCMV infected maize plants following exposure to ten thrips of *Frankliniella williamsi* and *F. occidentalis* adults.

Error bars indicates standard errors

There were significant differences between mean number of plants inoculated with MCMV by *F. williamsi* and *F. occidentalis*, (t = 2.77, df = 362.97, P = 0.006). Plants inoculated with the virus by *F. williamsi* vectors had higher (0.27) mean disease severity than those inoculated by *F. occidentalis* inoculated plants (0.20). The disease severity for *F. williamsi* inoculated plants was 17.6 % higher than plants inoculated by *F. occidentalis. Frankliniella occidentalis* had higher mean transmission rates than *F. williamsi* (51.5, 25.6 %) with mean difference of 25.9 which may suggest that there exists a variation in virus-vector specificity among these thrips. This variation could be

due to variations in molecular determinants that guide virus acquisition by a set of specific vectors (Hogenhout *et al*., 2008) and characterize virus transmission (Link and Fuchs, 2005). Unlike in passive virus transmission through infected pollen by insects, non-persistence transmission requires an active association of the virus with the vector (Blanc *et al*., 2011). However, the mechanism of association between MCMV and thrips is not yet understood and therefore requires investigation (Cabanas *et al*., 2013). Some maize plants were asymptomatic but tested positive for MCMV by ELISA. It was confirmed that *F. occidentalis* transmits MCMV. Zhao *et al*. (2014) illustrated that *F. occidentalis* transmits MCMV after 30 minutes of exposure and 48h starvation period, but 3 hours' starvation period was sufficient for thrips to acquire the virus. The rapid acquisition of MCMV soon after exposure was similar to that of *F. williamsi* and western corn rootworms transmission and without latent periods (Cabanas *et al*., 2013).

Frankliniella williamsi transmitting MCMV in all the virus AAPs except for 0.5 hour suggested a non-persistent mode of transmission. The transmission of MCMV in a non-persistent manner was similar to that of *F. occidentalis* and western corn rootworm. Zhao *et al*. (2014) reported similar results for *F. occidentalis* of high MCMV transmission rates after a long starvation period (48 hours) with short acquisition exposure period (30 minutes). The rapid acquisition and transmission of MCMV by corn thrips indicates that they play a major part in MCMV epidemiology in maize growing areas.

Frankliniella occidentalis showing higher transmission efficiency than *F. williamsi* suggests that they could play a bigger role in the spread of MCMV. This could also indicate variations in the virus-vector recognition which are often species specific (Hogenhout *et al*., 2008). Unlike passive transmissions through infected pollen by insects (Blanc *et al*., 2011), non-persistent MCMV transmission by thrips requires an active virus - vector association. However, these association mechanisms are currently not understood (Cabanas *et al*., 2013) and therefore require further investigation.

4.4 Transmission Efficiency of Maize Chlorotic Mottle Virus by larval thrips

Results of larvae transmission trials showed that *F. occidentalis* and *F. williasmi* larvae failed to transmit MCMV onto maize plants (Figure 4.13). Leaf samples taken from

maize plants inoculated with the virus using larvae thrips tested negative for MCMV by DAS ELISA. The two larvae populations of thrips did not transmit the virus at the three instar levels.

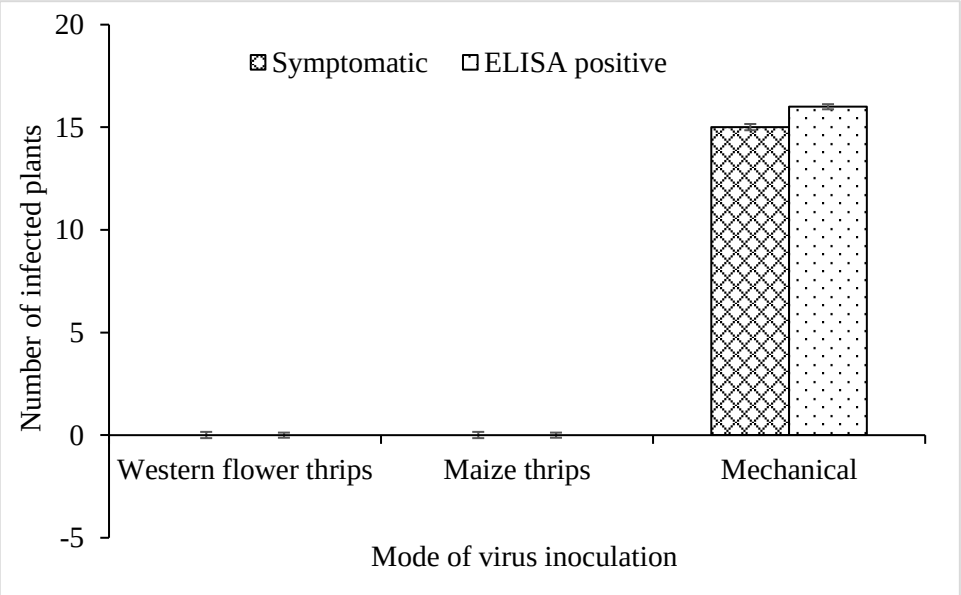


Figure 4.13: The Proportion Of Maize Plants Infected By Maize Chlorotic Mottle Virus Through *F. Occidentalis, F. Williamsi* And Mechanical Modes Of Transmission Via Pollen

Frankliniella occidentalis and *F. williamsi* larvae did not transmit MCMV onto maize seedlings. Larvae from both thrips species could not transmit the virus at the three instar levels. The wide range of potyviruses that could evade detection by antisera generated against the East African MCMV isolate may have led to the failure to serologically detect the virus. In addition, low virus titers as with the MCMV on larval thrips affects serological virus detection (Stewart *et al*., 2017). However, previous studies had shown both larvae and adults of *F. williamsi* as competent MCMV vectors where the adult viral load became six times more than in larvae (Cabanas *et al*., 2013).

Frankliniella williamsi adults and larvae transmit MCMV in a transmission pattern similar to that of Chrysomelid beetles (Nault *et al*., 1978; Gergerich, 2001). The virus retention period for *F. williamsi* is usually short and the virus gets depleted during moulting and development; a unique feature of MCMV transmission pattern of Chrysomelid beetles and corn thrips. Whereas *F. williamsi* larvae lose the virus immediately after feeding on host plants (Cabanas *et al*., 2013), *F. occidentalis* larvae have not been shown to transmit the virus which may require

further research. These results suggest that MCMV is likely spread via adult thrips, and because of the limited dispersal activity, it is likely that larvae are limited in the spread of MCMV. The research investigated the competence of *F. occidentalis* and *F. williamsi* in transmission and spread MCMV. Understanding the efficiency and persistence virus transmission by thrips presents a major step in the management of MCMV spread. Plants exposed to *F. occidentalis* for 3 hours IAP had higher disease severity than those inoculated at 1- and 2-hour IAP, indicating a higher transmission efficiency after 3 hours. Thus, 3 hours was the optimum IAP for MCMV transmission by *F. occidentalis*. This suggests that the thrips required a 1-3-hour time interval between acquisition and transmission to effectively transmit MCMV. Thrips also spend time to explore their food substrates rather than fast feed on introduced food sources (Cabanas *et al*., 2013). However, the time taken by thrips on food exploration has not been established. The least symptom severity recorded was at 48 hours' inoculation access period, an indication of thrips losing ability to transmit or depletion of the virus from thrips. This agrees with Cabanas *et al*. (2013) that MCMV gets depleted from *F. williamsi* 2 days after feeding on maize plants. Symptoms developed 5 weeks after inoculation in all the IAPs which was due to viral translocation mechanisms (Nagata *et al.*, 2002).

Infected mild symptomatic plants could serve as reservoirs for new virus infections within maize fields since they are overlooked (Mathews, 2009). Mechanically inoculated plants had higher severity than thrips inoculated plants, which could be attributed to the differences in virus titres in leaf sap extracts. New MCMV infections with *F. occidentalis* as vectors can induce low symptom severity or asymptomatic plants which could have implications on MLND epidemiology. In East Africa, MCMV was first reported in 2011 while that of *F. williamsi* was in 2009. In addition, all maize hybrids in Eastern Africa were highly susceptible to the virus (Adams *et al*., 2012; Moritz *et al*., 2013; Mahuku *et al*., 2015). These conditions provided suitable conditions that favoured MCMV occurrence and spread (Redinbaugh and Zambrano-Mendoza, 2014). Moreover, emergence of a virus epidemic is associated with the introduction of a non-indigenous pathogen and its vectors into an area where host plants have little or no resistance (Colvin *et al*., 2006; Thresh, 2006).

Frankliniella williamsi transmitted the virus after an acquisition period of 3 hours, without a latent period like beetles, (Mahuku *et al*., 2015). The pattern of virus transmission by *F. williamsi* adults was similar to that of *F. occidentalis*. The capacity of transmission by thrips depends on compatibility with the thrips species, MCMV isolate and plants used for acquisition and inoculation (Tavella *et al.*, 1997). The difference in competence of virus transmission could be related to the differences in virus translocation mechanisms and vector feeding behavior.

Feeding behaviour influences the transmission efficiency of *tospoviruses* by thrips. Thrips puncture the leaves without forming scars or may produce scars that irreversibly damage the cells thereby inhibiting viral infection (Tavella *et al.*, 1997). However, it is not known which feeding behavior is exhibited by *F. occidentalis* and *F. williamsi* while feeding on maize leaves.

4.5 Persistence in transmission of maize chlorotic mottle virus by vector thrips

Real time-PCR confirmed successful MCMV vector transmission (Plate 4.7). *Frankliniella occidentalis* and *F. williamsi* transmitted MCMV after a short retention period post-acquisition. The RT-PCR confirmed that the virus was retained on the stylets by the vector thrips for few days before it was transmitted in a non-persistent manner. Samples were considered positive if the amplified PCR products of the coat protein occurred at 480bp and 900bp fragments and polyproteins for MCMV and SCMV accordingly. For samples with mixed viruses (MCMV and SCMV), both primers were used to amplify the DNA, as with sample two, three, four, and six (Plate 4.7).

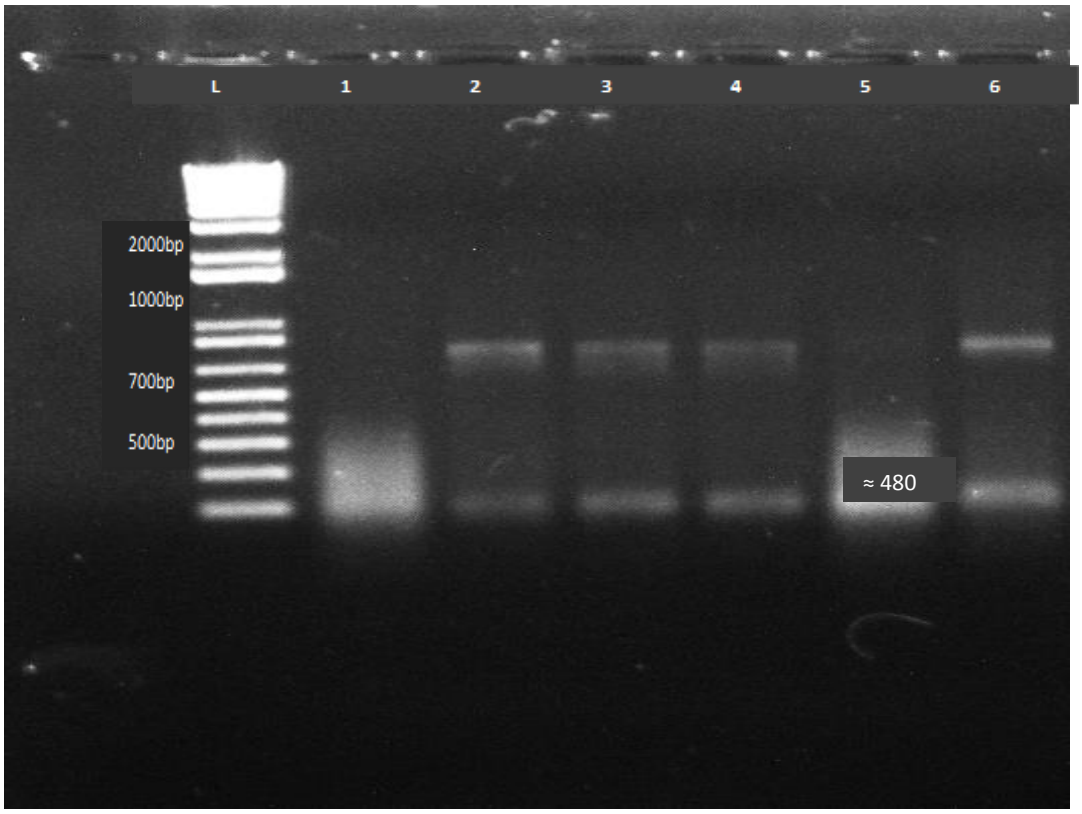


Plate 4.7 One step RT-PCR product bands showing MCMV positive samples
L-marker, 1-Positive control, 2,3,4,5, &6 - from diseased maize plants

Frankliniella occidentalis transmitted the virus to 40.6% of the inoculated maize plants in the first cycle on transmission. None of the plants inoculated in series 2, 3, and 4 set of plants became infected (Table 4.3). *Frankliniella occidentalis* could not transmit the virus persistently to new sets of maize plants even after a 48-hour inoculation access period.

Table 4.3: The percentage of re-infected of plants by *Frankliniella occidentalis* in four inoculation cycles

Inoculations	Plants infected/no, plants inoculated (% infected) per inoculation series					
	Set 1		Set 2		Set 3	
	F. o	*F. w*	*F. o*	*F. w*	*F. o*	*F. w*
1	7/16 (43.6)	5/16 (7.8)	0/16 (0.0)	3/16 (4.7)	0/16 (0.0)	0/16 (0.0)
2	5/16 (31.3)	2/16 (12.5)	0/16 (0.0)	2/16 (11.1)	0/16 (0.0)	0/16 (0.0)
3	6/16 (37.5)	4/16 (25.0)	0/16 (0.0)	1/16 (6.3)	0/16 (0.0)	0/16 (0.0)
4	8/16 (50.0)	3/16 (18.7)	0/16 (0.0)	2/16 (11.1)	0/16 (0.0)	0/16 (0.0)
Total	26/64 (40.6) [c]	14/64 (21.8) [b]		7/64 (10.9)		

The initials *F.o* represents *F. occidentalis* and *F.w*, *F. williamsi*

Frankliniella williamsi transmitted MCMV to 10.9% of the maize plants in the second set of re-inoculation after which there was no re-infection of maize plants in both sets 3 and 4 (Table 4.3). All mechanically inoculated control plants became infected with MCMV and showed initial symptoms during week three after inoculation. *Frankliniella williamsi* transmitted MCMV in cycles 1 and 2 but not 3 and 4 after 48 hours of exposure. Initial symptoms developed at week 5 after inoculation. Disease severity progressed from initial average score of 0.25 to 0.95 at week eight. Average severity score of leaf symptoms of mechanically inoculated control plants ranged between 0.05

at the second week to 4.05 at the eighth week after inoculation. The transmission behaviour of adult populations *F. occidentalis* and *F. williamsi* emerging from larvae exposed to MCMV was determined.

4.6 Effect of virus Retention Period on Vector Transmission

Frankliniela occidentalis retained and transmitted the virus within a day beyond which no transmission of the virus occurred by the same thrips in the second and third set of inoculated plants. However, *F williamsi* retained and transmitted the virus within the first and second day post acquisition (Figure 4.15). *Frankliniella williamsi* retained the virus for a longer period than *F. occidentalis*.

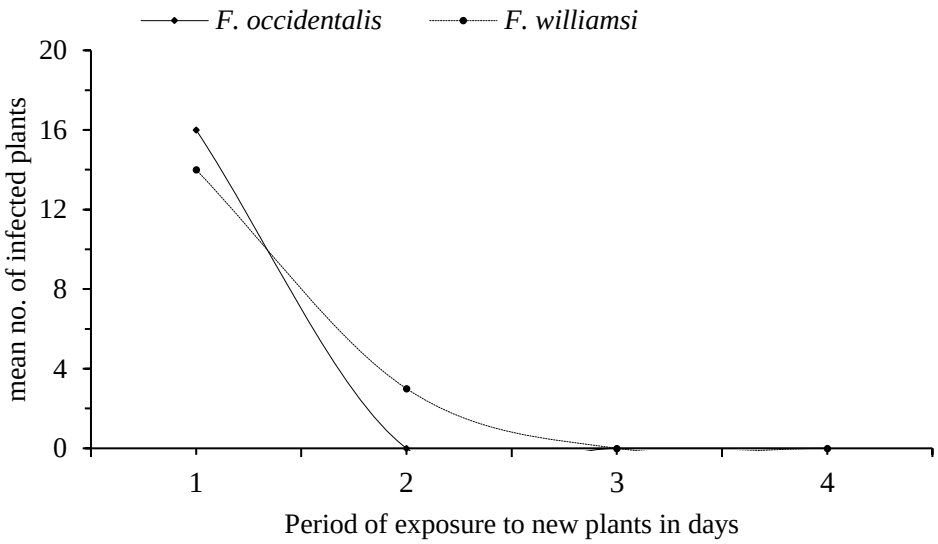


Figure 4.14: The Pattern of *F. williamsi* and *F. occidentalis* Transmission of *Maize chlorotic mottle virus*

There were significant differences (F = 5.66, df = 2, 17, P = 0.013) between mean proportion of plants inoculated by *F. williamsi* and *F. occidentalis* at 1, 2, 3 and 4 days' post acquisition and inoculation retention periods. Plants inoculated by *F. williamsi* after day 2 of acquisition were not significantly different from those infected after day 1 but were different from day 3 (0. 0.402, 0.497) at 0.149 LSD. The retention period of 3 days was sufficient to significantly affect the number infected plants by thrips. Cabanas *et al*. (2013) demonstrated that viral titers in thrips decrease and eventually deplete 2 days of IAP on healthy leaves. *Frankliniella williamsi* and *F. occidentalis*

adults transmitted MCMV in a similar pattern over the 4 days' post-acquisition period because vectors in specific taxa spread plant viruses within a genus (Ng and Falk, 2006; Nault, 1997). *Frakliniella williamsi* adults transmitted MCMV semi-persistently onto sets plants in day one and two after virus acquisition which agrees with Cabanas *et al*. (2013). The inability of *F. occidentalis* to re-transmit the virus indicated that they lost the ability to transmit MCMV after a 24 hour feeding on healthy plants. The PCR also detected a decline in proportion of virus infected plants with an increase in plants inoculated by same vectors. This pattern of MCMV transmission suggests that MCMV is a non-persistent transmitted virus as it was retained by thrips for few hours or 48 hours after acquisition (Link and Fuchs, 2005). However, it has not been demonstrated whether the virus is propagative or not. *Frankliniella williamsi* larvae lose the virus upon feeding on healthy plants and both pupae and adults that emerge from infected second instar larvae are not competent in transmitting the virus (Cabanas *et al*., 2013). In addition, there were no previous reports concerning *F. occidentalis* larval transmission of MCMV or whether the virus persists during thrips development. Therefore, *F. williamsi* and *F. occidentalis* larvae plays a limited or no role in MCMV transmission and spread.

4.7 Effect of Vector Intensity on Disease Symptom Severity

An assessment of the MCMV disease incidence and symptom severity commenced at the fourth week after inoculation. The number of vector-thrips used to inoculate the maize plants with MCMV significantly affected the virus disease symptom severity (Figure 4.16).

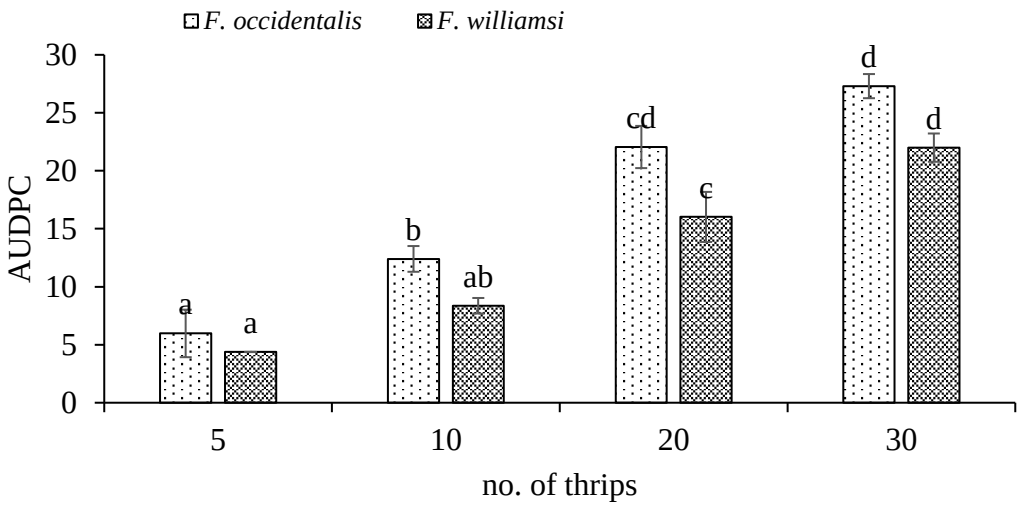


Figure 4.15: Area under disease progress curve (AUDPC) for *Maize chlorotic mottle virus* disease on maize plants inoculated with varying population density of thrips

The severity of MCMV was significantly different among maize plants inoculated by 5, 10, 20 and 30 thrips ($F = 80.91$, df = 3, 4, $P < 0.001$). Plants inoculated using 30 thrips had the highest severity. However, there was no significant difference in symptom severity between plants inoculated by *F. williamsi* and *F. occidentalis*. ($t = 1.66$, df = 3, $P = 0.196$). Results showed an increase in symptom severity with an increase in the number of vector thrips. The virus symptom severity on maize plants infected with the virus after inoculated with multiple thrips was greater than those of plants inoculated by individual thrips. The virus transmission in relation with symptom severity was consistent with semi-persistent viruses (Link and Fuchs, 2005).

The MCMV disease severity was significantly different among maize plants inoculated by 5, 10, 20 and 30 thrips. Plants inoculated using 30 thrips had the highest severity (l.s.d 4.391). However, successful transmission of MCMV by few thrips indicated that viral inoculum released by thrips during feeding is sufficient to infect maize plants and spread the virus. Unlike in MCMV, there was a slight reduction in incidence and severity of *Tomato spotted wilt virus* (TSWV) on peanut *Arachis hypogaea* L., plants with an increase in the number of *F. fusca* from 3 - 10 which was associated with

excessive feeding by a large number of thrips (Shrestha *et al*., 2015). Excessive feeding induces plant cell damage, which hinders virus replication.

Some studies have shown sexual dimorphism in the feeding behavior of *F. occidentalis*. Females feed more frequently for longer periods than males (van de Wetering *et al*., 1999). Moreover, females move less frequently between probes than males thereby producing patches of severely damaged cells that are not viable for virus multiplication (Stafford *et al*., 2011). Thrips used in this study were not segregated into males and females. Knowledge on MCMV transmission efficiency by male and female thrips could provide a better understanding on transmission patterns of this virus. Under field conditions vector thrips density vary between seasons and regions. Seasons with high thrips densities could have higher disease incidence and severities than those with low densities as reported by Todd *et al*. (1995) on *Tomato spotted wilt virus* (TSWV) in peanut crops. This inconsistency in vector density and disease pressure, can interfere with screening for viral disease resistance and undermine its control (Mandal *et al*., 2001). Knowledge on MCMV vector thrips density and disease pressure within maize growing regions and seasons in Kenya will be critical in designing effective MLN management strategies.

4.8 Role of Maize Pollen in MLN Transmission

Maize chlorotic mottle virus was mechanically transmitted onto maize plants through infected pollen. However, the transmission rate of MCMV through pollen was low (0.031) (Table 4.4). There was no incidence of sugarcane mosaic virus on mechanically transmitted plants through pollen. The virus could not be detected in all the leaf samples analyzed by DAS-ELISA. The healthy controls remained asymptomatic while positive controls were symptomatic.

Table 4.4: Mechanical Transmission of Maize Chlorotic Mottle Virus Via Pollen

Virus source	Symptomatic	ELISA positive	Transmission rate
MCMV infected pollen	2/64	2/64	0.031
SCMV infected pollen	0	0	0
Healthy control	0	0	0
Positive control	16/16	16/16	100

Positive control = plants inoculated using leaf sap extracts from symptomatic plants

Maize chlorotic mottle virus has been associated with pollen transmission. Li *et al*. (2006) successfully transmitted MCMV onto anthers of healthy using pollen collected from infected maize plants. However, that successful transmission of the virus through pollen was deemed to be due to surface contamination. However, no conclusive evidence has been demonstrated on whether pollen horizontally transmits MCMV or SCMV through fertilization and wounds initiated by thrips during feeding. Results of low transmission rates of MCMV indicated a possibility a limited role of pollen in MCMV spread between affected maize fields. The differentially low detection of these viruses by DAS-ELISA was likely due to low virus titer and their isolate absorbance (Mahuku *et al*., 2015).

4.9 Vector Mediated Virus Transmission Through Pollen

Leaf samples obtained from the maize plants exposed to *F. occidentalis* and *F. williamsi* fed on infected pollen tested negative for both SCMV and MCMV by DAS-ELISA. The thrips could not transmit MLN causing viruses; SCMV and MCMV via pollen. Inoculated plants remained asymptomatic while positive controls became symptomatic and tested positive for MCMV and SCMV. Despite MCMV and SCMV being associated with surface contamination of pollen (Li *et al*., 2007; Scheets, 2004), there was no horizontal transmission of these viruses by vector thrips via pollen. Pollen-borne viruses are accidentally transmitted mechanically by thrips during feeding (Jones,

2005), but SCMV and MCMV were not transmissible via this mode. There was no transmission of these viruses despite long acquisition access period and inoculation access period.

Furthermore, MCMV that was mechanically transmitted at low rates from pollen did not show evidence of vector mediated transmission, yet pollen-borne viruses are accidentally transmitted mechanically by thrips during feeding (Jones, 2005). This is epidemiologically significant because a pollen-borne virus not transmitted horizontally via pollen raises the question on whether pollen plays a role in MCMV and SCMV epidemiology. It will be important to determine whether pollen is involved in the epidemiology of these viruses in Eastern Africa. Furthermore, in Nebraska and Kansas, MCMV rapidly spread during the growing seasons, which suggested that there could be other sources of virus spread other than vectors (Scheets, 2008).

4.10 Virus Detection by Serological Analysis

In this study, DAS ELISA and RT-PCR detected the MLN associated viruses in the samples from the inoculated plants. Leaf sample analysis confirmed the detection of SCMV and MCMV in mechanically inoculated maize plants. Only MCMV was detected in samples from MCMV inoculated plants via vector thrips. The viruses had earlier been confirmed to infect maize in Ethiopia, Congo, Rwanda, and Kenya by serological tools (Mahuku *et al*., 2015). The DAS - ELISA detected 32 % plants inoculated SCMV. It was possible that plants that tested negative had low concentrations of SCMV viral nucleic acid. The low DNA concentrations could affect detection of the virus in plants samples (Lukanda *et al*., 2014). Moreover, the accuracy of serological methods relies on the quality of antibodies used. *Maize chlorotic mottle virus* was detected in both mechanically and thrips inoculated plants. The virus was more prevalent than SCMV in mechanically inoculated plants, 48 out of 64 (75%) tested positive, an observation that concurs with natural transmissions within maize fields (Mahuku *et al*., 2015).

4.11 Virus Detection by Molecular Sample Analysis

The RT-PCR tool was more accurate than DAS-ELISA in the detection MCMV in leaf samples. *Maize chlorotic mottle virus* was detected in 52 of 64 plants inoculated by *F.*

occidentalis and in 31 of the same number of plants inoculated by *F. williamsi*. Three samples became RT-PCR positive but ELISA negative while two samples became ELISA positive but RT-PCR negative. The PCR method is the best confirmatory and reliable diagnostic test and has been employed before in the rapid detection of MCMV in maize seeds and leaves (Zhang *et al*., 2011; Adams *et al*., 2014). In addition, RT-PCR has been widely used to detect SCMV and, MCMV in maize and sugarcane (Wangai *et al*., 2012; Wang *et al*., 2014).

4.12 Molecular Characterization of Thrips Vectoring MCMV

Thirty-five analyzed individual samples produced clear bands of 700 bps that matched with the fragment sizes from the markers. Three main species, was detected by PCR, using extracted DNA. An additional weak band appeared occasionally, but did not affect identification of the three species. (Figure 4.8). The mtDNA COI universal primers; HCO2198 and LCO1490 were initially employed to amplify the COI region of specimen thrips. Another set of primers MTD7.2F and MTD9.2R that were specifically designed for thrips identification were also tested. However, MTD7.2F and MTD9.2R primers failed to develop clear DNA bands as with the universal primers. As identified morphologically, the COI were successfully amplified in all the 35 specimens of thrips.

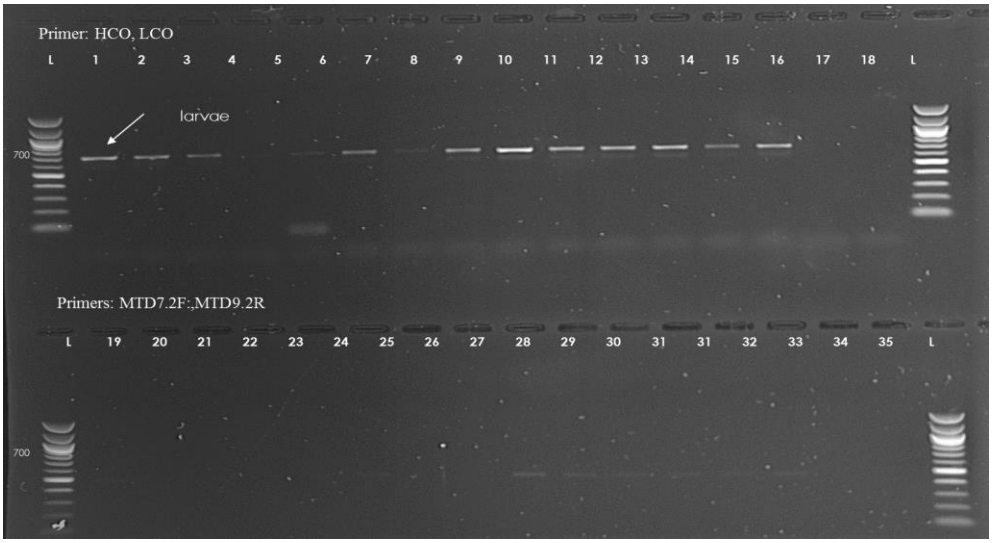


Plate 4.8 Electrophoretic banding patterns of PCR products from various developmental stages (sample 1-3) and different populations (samples 4-35) of *F. occidentalis*. L: 100 bp marker

The PCR generated amplicons fragments of approximately 700 bp which were cloned and sequenced (Plate 4.9).

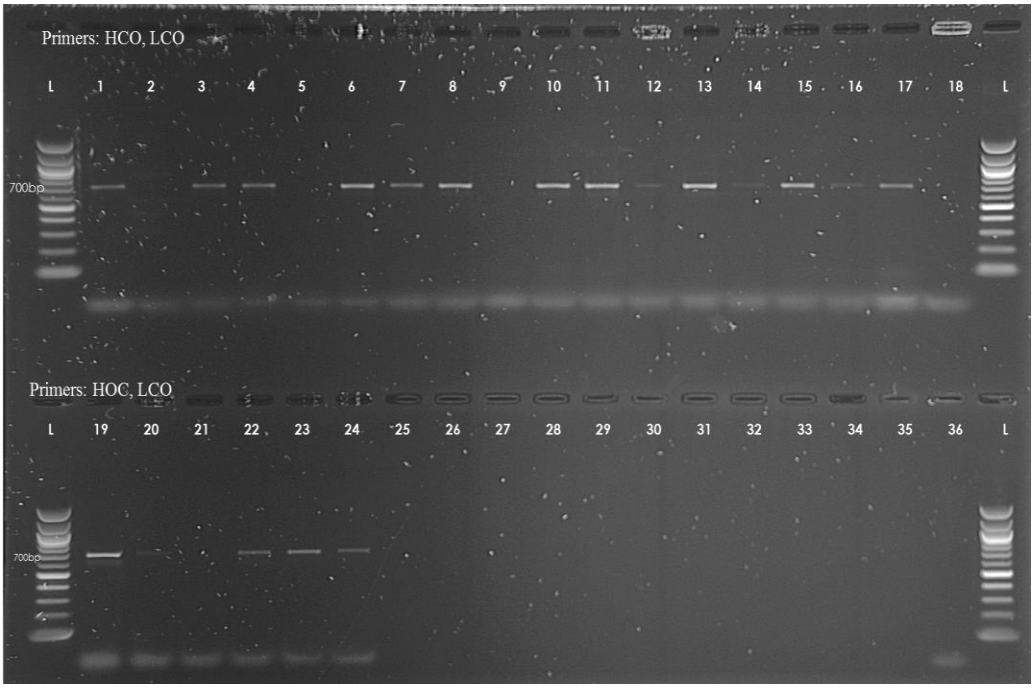


Plate 4.9 Banding patterns detected from PCR products of thrips collected from MLN affected maize.

The universal PCR primers used in this study were useful for confirmation of thrips specimen identity. Application of multiple primers makes PCR more sensitive and thus has a great potential for use species identification. Use of multiple PCR primers can be essential in both adult and larval thrips species discrimination especially those found infesting maize in the MLN endemic zones. Barcode s of about 500bp were recovered from 20 specimens. The barcodes provided at least identities for 15 of the 18 species with only *F. williamsi* and *Chirothrips manicatus* lacking similarity matches. The diversity of thrips infesting maize in the Murang'a region of Kenya was previously poorly understood since no molecular data was unavailable prior to this study. The thrips that were identified morphologically had their barcodes analyzed.

Morphological identification of thrips is difficult and therefore the DNA-based identification method is an important alternative (Mainali *et al.*, 2008). However, an effective use of molecular tools would require an accurate reference library of sequences from previously identified specimens (Virgilio *et al*., 2010). A DNA barcode library has about 263 of the 6,000 known thrips species of Thysanoptera (Kumar *et al*., 2014). The sequence online barcode library would be necessary to accurately identify thrips infesting maize from Kenya. It is important to populate the database with sequences from specimens within the region reference. This molecular data would utilize the gap between maximum intraspecific and minimum interspecific distances for species differentiation (Dellicour and Flot, 2015). Ashfaq *et al*. (2014) utilized barcode gaps to discriminate cryptic species of butterflies. The study has indicated that 70% of the samples matched with *F. occidentalis*. Only one specimen matched with *F. williamsi* and while the rest were identified up to genus level morphologically.

This information could be useful in the analysis of the MLN, virus - vector relationships and disease epidemiology since virus transmission competence varies among species (Wijkamp *et al*., 1995). Several thrips species exist as species complexes (Rebijith *et al*., 2014), yet little information about the role of each complex in MCMV transmission. Western flower thrips, *F. occidentalis*, exists in two species complexes (Rugman-Jones, 2010). Sequence and PCR analyses (Rebijith *et al*., 2014) using species-specific primers discriminates members of a species complex thus identifying target species at any life stage (Asokan *et al*., 2007). A recent study by Dickey *et al*., (2015) found that

S. dorsalis exist in a nine species complex but only one species complex, South Asia 1, transmit viruses. It is therefore important to gain an understanding of *F. occidentalis* and *F. williamsi* species composition and their genetic lineages in order to correctly target develop management strategies of MLN disease within sub Saharan Africa. This study contributes to the need to accurately identify MLN transmitting vectors by employing DNA barcodes that examined the diversity of thrips infesting maize in Kenya.

CHAPTER FIVE: SUMMARY CONCLUSIONS AND RECOMMENDATIONS

5.1. Conclusions

1. *Phaseolus vulgaris* and *Cucurbita pepo* substrates offer suitable substrates for rapid *in vivo* culturing of *F. occidentalis*.
2. Maize stalks with slit leaf sheaths proved the best substrate option for *F. williamsi* colony development, but required timely and regular replacement.
3. *Frankliniella occidentalis* had higher MCMV transmission efficiency than *F. williamsi*. *Frankliniella williamsi* and *F. occidentalis* adults as competent vectors while their larvae have no or limited role in transmission of MCMV.
4. Maize pollen plays a role in MCMV transmission and spread as they provide a pathway for direct horizontal movement within and between maize fields.
5. There was a wide range of insect thrips infesting maize in Murang'a County of Kenya. *Frankliniella occidentalis* is more widely distributed in maize agro ecosystems in Murang'a region of Kenya than *F. williamsi.*

5.2 Recommendations

1. This study recommends *Phaseolus vulgaris* and *Cucurbita pepo* substrates for the rapid *in vivo* culturing of *F. occidentalis.*
2. The young maize stalk - cuttings with slit leaf sheaths should be used in laboratory cage rearing of *F. williamsi* colonies.
3. The *F. occidentalis* and *F. williamsi* vector thrips should be controlled as a strategy for MLN disease management. The control strategies for vectors should target *F. williamsi* and *F. occidentalis* adults since a bigger role in MCMV transmission than larvae.
4. Seed breeders should target maize pollen as pathway for spread of the virus during development of clean seed. Effective vector control will prevent horizontal transmission of the virus through wounds induced during feeding.
5. The COI barcode sequence analysis is recommended for rapid identification of vector thrips.

5.3 Further Research Areas

1. There is need to determine molecular determinants involved in *F. occidentalis* and *F. williamsi* vector specificity for MCMV.
2. The feeding behaviours *F. occidentalis* and *F. williamsi* on maize leaves need to be established to clarify the epidemiology of viruses transmitted at different rates by arthropod vectors within the genus *Frankliniella*.
3. The thrips species complexes infesting maize in Kenya as well as their genetic lineages should be determined towards the development of a comprehensive DNA barcode library for Thysanoptera.
4. There is need for more vector characterization using molecular tools.

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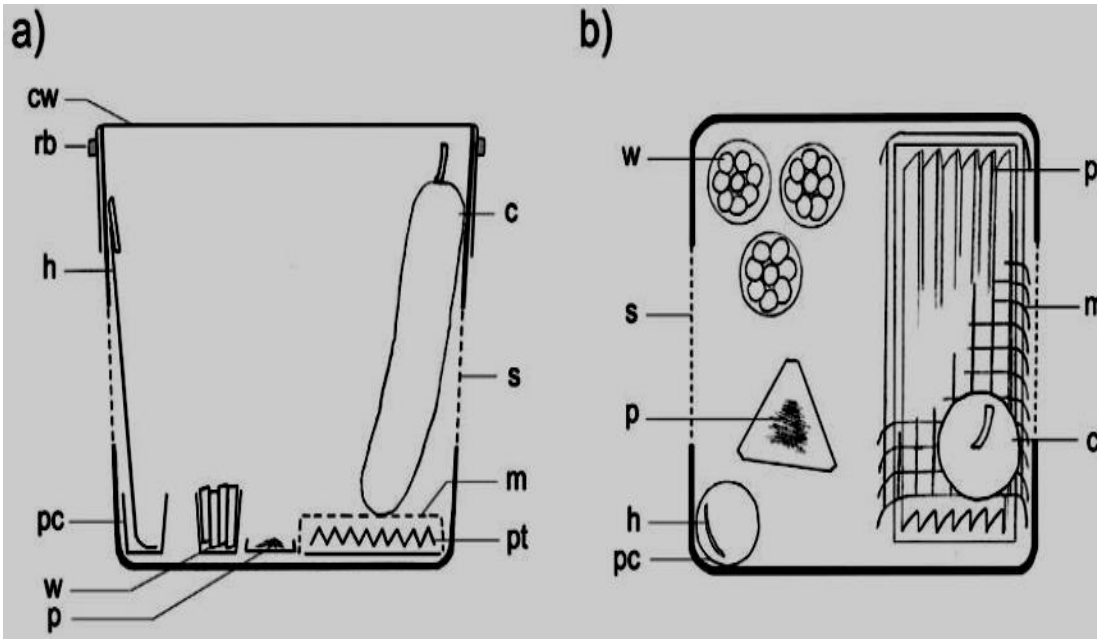
APPENDICES

Appendix 1: Pollen Transmission Experimental Design

	Treatments	
	Plot$_1$	**Plot$_2$**
To: Inoculation by pollination	Vir_1,Vir_3,Vir_2	Vir_2,Vir_1,Vir_3
	Vir_2,Vir_1,Vir_3	Vir_3,Vir_1,Vir_2
	Vir_3,Vir_1,Vir_2	Vir_1,Vir_2,Vir_3
	Vir_1,Vir_3,Vir_2	Vir_2,Vir_1,Vir_3
	Vir_c, Vir_c, Vir_c	Vir_c, Vir_c, Vir_c
To: mechanical inoculation	Vir_1,Vir_3,Var_2	Vir_2,Vir_1,Vir_3
	Vir_2,Vir_1,Vir_3	Vir_3,Vir_1,Vir_2
	Vir_3,Vir_1,Vir_2	Vir_1,Vir_2,Vir_3
	Vir_1,Vir_3,Vir_2	Vir_2,Vir_1,Vir_3
	Vir_c, Vir_c, Vir_c	Vir_c, Vir_c, Vir_c

Key: Vir_1= MCMV, Vir_2 = SCMV, Vir_3 =MCMV+SCMV, Vir_c=control (buffer solution)

Appendix 2: Cross Section of Culture Box For Rearing Thrips



Key: **c**= cucumber, **cw**= clingwrap, **h**=honey wick, **m**= metal mesh, **p**= pollen, **pc** = pill cup, **pt**= paper towel layers, **rb**= rubber band, **s**= silk windows, **w**= wet cotton rolls.

Source: (Degraaf and Wood, 2009)

Appendix 3: Vector Transmission Experimental Design

	Treatments	
	F. williamsi	*F. occidentallis*
Replication 1	Stg_1,Stg_3,Stg_2	Stg_2,Stg_1,Stg_3
Replication 2	Stg_2,Stg_1,St_3	Stg_3,Stg_1,Stg_2
Replication 3	Stg_3,Stg_1,Stg_2	Stg_1,Stg_2,Stg_3
Replication 4	Stg_1,Stg_3,Stg_2	Stg_2,Stg_1,Stg_3

Key: Stg_1-larvae2, Stg_2-larvae3, and Stg_3 -adults

Appendix 4: Research Approval Letter

KENYATTA UNIVERSITY
GRADUATE SCHOOL

E-mail: dean-graduate@ku.ac.ke
Website: www.ku.ac.ke

P.O. Box 43844, 00100
NAIROBI, KENYA
Tel. 8710901 Ext. 4150

Our Ref: A99/28498/2014

DATE: 18th February, 2016

Director General,
National Commission for Science, Technology
and Innovation
P.O. Box 30623-00100
NAIROBI

Dear Sir/Madam,

RE: RESEARCH AUTHORIZATION PAUL ATENGA NYAMWAMU – REG. NO.A99/28498/2014

I write to introduce Mr. Paul Atenga Nyamwamu who is a Postgraduate Student of this University. He is registered for Ph.D Degree programme in the Department of Agricultural Science and Technology.

Mr. Nyamwamu intends to conduct research for a Ph.D Proposal entitled, "Transmission Mechanisms of Maize Lethal Necrosis Disease in Kenya."

Any assistance given will be highly appreciated.

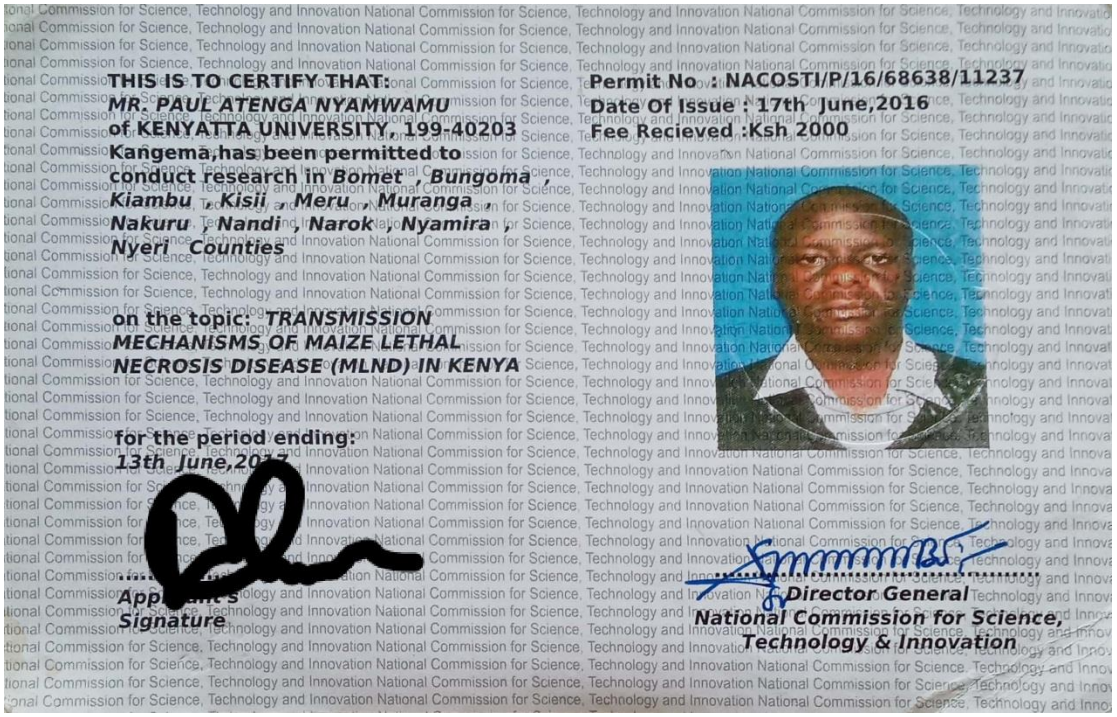
KENYATTA UNIVERSITY * OFFICE OF DEAN * 18 FEB 2016 GRADUATE SCHOOL P. O. Box 43844-00100 NAIROBI

Yours faithfully,

MRS. LUCY N. MBAABU
FOR: DEAN, GRADUATE SCHOOL

EO/rwm

Appendix 5: Research Authorization Permit



THIS IS TO CERTIFY THAT:
MR. PAUL ATENGA NYAMWAMU
of KENYATTA UNIVERSITY, 199-40203
Kangema,has been permitted to
conduct research in Bomet , Bungoma ,
Kiambu , Kisii , Meru , Muranga ,
Nakuru , Nandi , Narok , Nyamira ,
Nyeri Counties

on the topic: TRANSMISSION
MECHANISMS OF MAIZE LETHAL
NECROSIS DISEASE (MLND) IN KENYA

for the period ending:
13th June,2017

Applicant's
Signature

Permit No : NACOSTI/P/16/68638/11237
Date Of Issue : 17th June,2016
Fee Recieved :Ksh 2000

Director General
National Commission for Science,
Technology & Innovation

Appendix 6: RUFORUM Biennial Conference Certificate

CERTIFICATE OF ATTENDANCE

This certifies that

PAUL NYAMWAMU

participated in the 6th African Higher Education Week and RUFORUM Biennial Conference in Nairobi, Kenya

22-26 October 2018

RUFORUM

Professor Adipala Ekwamu

Appendix 7: Certificate of Participation in the Phytosanitary Conference