TRANSMISSION AND DETECTION OF MAIZE CHLOROTIC MOTTLE VIRUS IN MAIZE SEED

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DECLARATION

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

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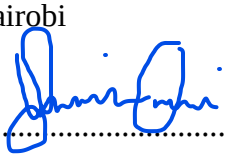

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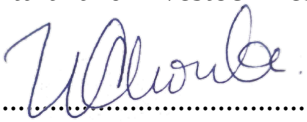

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DEDICATION

To my Heavenly Father

To my parents: Dr. Joseph Kimani Wanjama (RIP) and Esther Wangari

and to my husband John Kageche

I honour you

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I give glory and honour to the Almighty God for everything, His presence was evident throughout the whole study.

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ABBREVIATIONS AND ACRONYMS

A_{405nm}	Absorbance at wavelength 405nm
ANOVA	Analysis of variance
AOSA	Association of Official Seed Analysts
CIMMYT	International Maize and Wheat Improvement Center
CLN	Corn lethal necrosis
Ct	Cycle threshold
DAS-ELISA	Double antibody sandwich ELISA
DNA	Deoxyribonucleic acid
depc	Diethyl pyrocarbonate
ELISA	Enzyme-linked immunosorbent assay
FAOSTAT	Food and Agriculture Organization Corporate Statistical Database
fg	Femtogram
IC-RT-PCR	Immunocapture- RT-PCR
ISTA	International Seed Testing Association
JGMV	Johnson grass mosaic virus
KALRO	Kenya Agricultural and Livestock Research Organization
KEPHIS	Kenya Plant Health Inspectorate Service
KNBS	Kenya National Bureau of Statistics
LAMP	loop-mediated isothermal amplification
MaYMV	Maize yellow mosaic virus
MCMV	Maize chlorotic mottle virus
MDMV	Maize dwarf mosaic virus
MLN	Maize lethal necrosis
µl	Microliter
NCM-ELISA	Nitrocellulose membrane – ELISA
ng	Nanogram
OD	Optical density

PBS-T	Phosphate-buffered saline (with) tween
PCR	Polymerase chain reaction
pg	Picogram
polr	Proportional odds logistic regression
RNA	Ribonucleic acid
RT-LAMP	Reverse transcription-LAMP
RT-PCR	Reverse transcription-PCR
RT-qPCR	Reverse transcription- quantitative real-time PCR
SCMV	Sugarcane mosaic virus
TBE	Tris Borate Ethylenediaminetetraacetic acid buffer
WSMV	Wheat streak mosaic virus

GENERAL ABSTRACT

Maize lethal necrosis (MLN) is an important disease in Kenya and eastern Africa countries causing up to 100% yield losses in farmers field when not controlled. Decrease of production of maize is a threat to food security. The disease is caused by a synergetic infection of two viruses: maize chlorotic mottle virus (MCMV) and a cereal-infecting potyvirus. The frequently identified potyvirus in eastern Africa is sugarcane mosaic virus (SCMV). Maize chlorotic mottle virus is transmitted via insect vectors, mechanically, through plant residues and soil and also by seed. The main objective of this study was to determine the various aspects of seed as a source of transmission of MCMV- Kenya isolate. Serological and nucleic-acid based methods were evaluated for their effectiveness and sensitivity in detecting MCMV using purified virus and seed and leaf tissue. The methods were available and some in progress of development in the country at the time of study. The limit of detection of one infected seed in different sample size and the current sample size in use for seed certification (400-seed) was evaluated for effectiveness in detection of MCMV using ungerminated seed and 10-day grow out seedlings, using double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) and real-time reverse transcription polymerase chain reaction (real-time RT-PCR). The seed transmission rate was determined using MCMV-infected commercial seed lots while seeds from mechanically inoculated plants were used to determine the effect of genotypes on seed transmission of MCMV. The effect of crop stage at time of infection on seed transmission was determined using three genotypes, inoculated at four crop stages. Study of infectivity of the virus was carried out using contaminated seed which were soaked in inoculation buffer and the soak solution as well as seed extract used to inoculate seedlings of a maize genotype PH30G19. Data was analysed using descriptive statistics, analysis of variance (means separated by least significant differences), proportional odds logistic regression, pooled prevalence tests, Kruskal-Wallis test and binomial probabilities using R- software packages and Epitools calculator. The sensitivity levels of detection for MCMV using purified virus were 0.1-0.01 ng/µl, 0.1 pg/µl and 0.001 fg/µl for DAS-ELISA, IC-RT-PCR and lateral flow strips (AGDIA); and up to 0.1 fg/µl, 1 pg/µl and 0.001 fg/µl for RT-PCR, RT-LAMP and real-time RT-PCR, respectively. However, the sensitivity of detection of DAS-ELISA reduced when crude leaf sap and seed extract were used to dilute the purified virus unlike for IC-RT-PCR. The probability of detecting MCMV in a 400-seed sample was higher ungerminated ground seed samples unlike when grow outs from

contaminated seed of similar size were used. Seed transmission rates observed for four seed lots (45,939 seedlings) evaluated ranged from 0-0.57% while that of seed from different genotypes with varying severity scores of MCMV infection ranging from 0 to 1.04%. Higher transmission was recorded from seeds obtained from plants infected at four-leaf stage (0.19%). Infection of 1.64% PH30G19 seedlings was recorded when inoculated with soak solution and seed extract from MCMV contaminated seeds. The methods available for detection of MCMV determine the reliability of the results. While DAS-ELISA is a commonly used method in virus diagnosis, the method is prone to false results when detecting MCMV in seedling grow outs. However, it has been found to be reliable when the samples are ground seeds. Nucleic-acid methods are more reliable as the samples are further purified from the crude state. The use of one 400 seed-sample may lead to a false analysis of seed lot when the seedling grow outs are tested. The study suggests the use of more than one replicate of the sample from a seed lot to determine the presence of transmitted MCMV, or an increase in the number of seeds in a drawn composite sample from a seed lot. Seed transmission of MCMV in all the samples tested was below 1.05%, showing low transmission. The low transmission was despite the level of severity of MCMV in the plant where the seeds were obtained. The results emphasize the need for continuous seed health testing of all the genotypes. The transmission of MCMV was higher at early crop stage, though proper management of MCMV infection should be implemented at all stages, as symptoms were observed on all inoculated plants. Maize chlorotic mottle virus from contaminated seed led to infection to seedlings when used as inoculum. This information is important in ensuring proper detection of MCMV in seed health.

CHAPTER ONE

GENERAL INTRODUCTION

1.1 Background information

Maize (*Zea mays* L.) is the most important cereal crop in Kenya and within the Eastern and Central African region. In 2019, about 3.89 million metric tonnes of maize were produced in Kenya, while 81.89 million metric tonnes were produced in Africa (FAOSTAT, 2021). Maize production, as with other crops, is faced with various production constraints. These include pests and diseases, soil nutrition and other agronomic requirements, quality of seeds and unpredictable weather (Shiferaw *et al*., 2011). The reduced production has led to increase in maize imports into Kenya (KNBS, 2018).

In 2011, maize lethal necrosis (MLN) disease, was reported in Kenya (Wangai *et al*., 2012) and later in other eastern Africa countries (Redinbaugh and Stewart, 2018). Prior to this year, MLN, also referred to as corn lethal necrosis (CLN) disease was reported first in Peru in 1973 and later in Kansas (Niblett and Claflin, 1978). Other reports of maize lethal necrosis disease from other countries in Africa, China and Suth America have been reported after 2011 (Redinbaugh and Stewart, 2018; Xie *et al*., 2011). In Kenya and the surrounding countries, the prevalent potyvirus in MLN infections is sugarcane mosaic virus (SCMV) causing yield losses of up to 100% (Prasanna *et al*., 2020; Wangai *et al*., 2012).

Sugarcane mosaic virus was reported in the East African region as far back as 1930s (Storey, 1936), unlike MCMV that was first reported in 2011 (Wangai *et al*., 2012). Insect vectors that transmit SCMV are corn aphids, while thrips and chrysomelid beetles transmit MCMV (Mahuku *et al*., 2015a). Other modes of transmission are through mechanical transmission via seed (Bockelman *et al*., 1982; Gatunzi, 2018; Jensen *et al*., 1991) and MLN infected residues in the soil (Kinyungu *et al*., 2019; Regassa *et al*., 2022). Maize lethal necrosis disease symptoms severity range from mild leaf chlorosis and mottling to leaf necrosis, “dead heart” syndrome, stunting of plants, small cobs, no grain set and death of the plant (Wangai *et al*., 2012).

Viruses and virions are the smallest pathogens, encompassing of a nucleic acid and a coat protein (Sastry and Zitter, 2014). Management of viral diseases require correct diagnosis of the causative agents. Viruses have the ability to mutate and evolve, thus complicating their identification over periods of time (Rubio *et al*., 2020). Methods of detection of viruses range from time consuming methods such as biological assays and visual observation of symptoms to those requiring specialized equipment and skill such as electron microscopy and nucleic acid methods that have increased sensitivity and specificity while reducing the time of diagnosis (Rubio *et al*., 2020). The goal for choice of a detection method is to get accurate results and at a short time as possible and be cost effective (Albrechtsen, 2006; Sastry and Zitter, 2014).

Research work has been continuing since MLN was reported in Kenya to understand the spread, the causative agents in the region and to screen for sources of resistance to the viruses in the maize genotypes available (Prasanna *et al*., 2020). Management methods to control the spread of MLN include the increasing application of insecticides to control the vectors; scouting of fields and rouging diseased maize plants, crop rotation, having maize-free break seasons to reduce the build-up of virus inoculum, planting early to escape infection, field sanitation, seed dressing and planting of certified seed (Prasanna, 2021). Prior to seed certification, a 400-seed sample is tested for MCMV presence to determine the status of a seed lot (Shango *et al*., 2019).

1.2 Statement of the problem

The reports of the spread of MCMV in the last decade to new areas (countries), many years after it was first reported in the USA raised concern in the maize producing countries. The virus is transmitted by various methods and of interest in this study is seed transmission. Investigation of seed transmission of MCMV in Hawaii resulted in an average transmission rate of 0.33% from 17 seed lots (Jensen *et al*., 1991). Further analysis of seedlings from inbred lines and corn hybrids yielded no seed transmission (Bockelman *et al*., 1982). The seed transmission of MCMV found in the eastern Africa region was studied on few seedlings, yielding a transmission rate of 75% (Mahuku *et al*., 2015a) from 25 seeds, and one pooled sample from 480 seedlings (Gatunzi, 2018). Maize seed infected with MCMV can easily introduce the virus into a clean field, and in the presence of the vectors and a conducive environment, this would cause the spread of MCMV

and MLN where a cereal potyvirus is present. Maize lethal necrosis causes economic yield losses of up to 100% (De Groote *et al*., 2016; Wangai *et al*., 2012).

Despite the reported transmission rate being low, there was need to understand further the factors that contribute to increased seed transmission of MCMV and the infectivity of the virus found on and in the seed. Countries in sub-Saharan Africa included MCMV as a quarantine pest after the emergence, and are routinely testing seed. There have been no prior studies to evaluate the detection methods especially in seed in the region, which play a big part in the seed health assays. Since seed lots have varying levels MCMV infection, there was need to evaluate the effectiveness of testing the 400 seed sample size used in seed health testing in maize (ISTA, 2016) and also to determine the number of seeds required to form a group sample, which reduces the time taken and cost of testing the samples.

1.3 Justification

Maize is the staple food in Kenya with an average consumption supply of 67.3 kg/person in 2019 (KNBS, 2020). A decline in maize production in Kenya would have major impact on food security, not only in Kenya but in sub-Saharan Africa, where maize is the most important cereal (Ranum *et al*., 2014). Maize lethal necrosis disease would further spread and cause loss of more maize in Africa if the problem is not addressed (Isabirye and Rwomushana, 2016). Universally, seed is the main mode of germplasm exchange, thus seeds infected with viruses can be a source of inoculum in the field where it is sown. The virus can then be spread by vectors and disease would develop where the environment is conducive. Infected seed also allows for survival and carryover of viral inoculum from one field to another and to different crop seasons (Sastry, 2013c). Understanding seed transmission and infectivity of MCMV isolate identified in Kenya and the factors that enhance the transmission is important in implementation of management strategies to help curb the spread of MLN which has continued to be also prevalent in seed production fields (Eunice *et al*., 2021).

Seed-borne pathogens are efficiently and effectively detected by ensuring proper sampling of seed lots and utilizing specific, sensitive and reliable methods for correct diagnosis. Comparison of available virus diagnostic methods based on the sensitivity, efficiency and effectiveness of

detection and the cost involved provides important information in guiding the seed health testing for MCMV. Such information will be used by the regulatory institutions and seed industry in ensuring production of MCMV-free seed. Correct detection of MCMV and further understanding of seed transmission of the virus will contribute in management of MLN. This is important especially in sub–Saharan Africa where maize is the highest produced cereal and is consumed by 50% of the population (Ranum *et al*., 2014). The isolate of MCMV found in Kenya has a 4% nucleotide diversity compared to that from Kansas and Nebraska, in USA (Adams *et al*., 2013), and thus it is important to determine the transmission of this MCMV isolate through seed.

1.4 Objectives

1.4.1 General objective

The aim of the study was to reduce losses occasioned by maize lethal necrosis disease in Kenya through determining the various aspects of seed as a source of transmission of maize chlorotic mottle virus.

1.4.2 Specific objectives

The specific objectives of this study were

i. To evaluate the sensitivity and effectiveness of available diagnostic tools in detection of maize chlorotic mottle virus in seed maize.
ii. To determine the limit of detection of sample sizes and evaluate effectiveness of current sample size in detection of maize chlorotic mottle virus.
iii. To determine the seed transmission rates of maize chlorotic mottle virus – eastern Africa isolate and factors influencing seed transmission of MCMV.
iv. To determine the infectivity of maize chlorotic mottle virus detected from contaminated seed.

1.5 Null Hypotheses

i. There are no differences in the sensitivity and effectiveness of virus diagnosis methods used to detect maize chlorotic mottle virus in seed maize.

ii. Sample size of maize seeds does not significantly affect the detection of maize chlorotic mottle virus for ungerminated seed.

iii. There are no differences in levels of seed transmission rates of maize chlorotic mottle virus for seed lots assayed and transmission of the virus by seeds is not dependent on the genotype or crop stage at time of infection.

iv. Maize chlorotic mottle virus contaminant on the maize seed does not cause infection in maize seedlings.

CHAPTER TWO

LITERATURE REVIEW

2.1 Origin and economic importance of maize

Maize, *Zea mays*, of the family Poaceae (Gramineae- grass family), is one of the most common crops in the world. In 2017, the total production of maize globally was 1.148 billion tonnes (FAOSTAT, data accessed June, 2021). Maize was first used as food in Mexico 5000 years ago, as small cobs were found in caves (Ranum *et al*., 2014). It is commonly referred to as 'corn' in the western parts of the world, and highest consumption as food is in Latin America and African countries. It is also used as livestock feed and to generate ethanol for use as vehicle fuel among other industrial uses (Ranum *et al*., 2014). Africa uses 95% of the maize produced as food (Shiferaw *et al*., 2011), and the demand is expected to increase with the predicted rise in population in these regions by year 2050 (Nelson *et al*., 2010). United States of America (USA) is the largest producer of maize (corn) (FAOSTAT, data accessed June, 2021), where it is mainly used is animal feed. It is also consumed as human food and used in production of fuel and alcohol (USDA, 2021; Wallington *et al*., 2012).

There are different types of maize kernel colour in the world, ranging from white, yellow, red, to black. White-coloured maize is the most preferred in Africa (Ranum *et al*., 2014). Maize is adaptable to various areas with ranging rainfall amounts, temperature, altitudes and soils. In east Africa, small holder farmers form 75% of the agricultural producers (Salami *et al*., 2010). The whole, raw, dry white maize grain provides 7.94 g of protein and 4.5 g of fat, and contains 24 mg of calcium, 2.6 mg of iron, 75 mg of magnesium, 367 mg of phosphorus, 226 mg of potassium, 12 mg of sodium, 1.88 mg of zinc, eight microgram of selenium and 20 µg of vitamin A per 100 g (FAO/ Government of Kenya, 2018). Maize kernels also contain oil and phytochemicals that have benefits to human health (Shah *et al*., 2016).

2.2 Maize production and the constraints in Kenya

The average production of maize in Kenya from the year 2015-2019 was 3.65 million tonnes (FAOSTAT, accessed June 2021) as compared to 80.39 million metric tonnes produced in Africa. Kenya is the sixth largest producer of maize in Africa (FAOSTAT, 2021), however, the

production of maize does not meet the requirements of the country, where the average intake of the crop is estimated at 171 g/person/day (Ranum *et al*., 2014). The production of maize in Kenya has been fluctuating over the years (Figure 2.1), with highest production realised in 2018 as a result of the favourable and adequate rainfall (KNBS, 2019). The available maize supply for consumption in 2019 in Kenya was 63.7 kg/person (KNBS, 2020) thus the demand for maize is expected to rise due to population growth.

Maize farming is faced by several biotic and abiotic challenges that negatively affect production. Pests and diseases affect maize pre-harvest and post harvesting. Insect pests that decrease maize production economically include cutworms, ear worms, grain moths, stem borers, beetles, weevils, grain borers, root worms, wire worms, fall army worm, leafhoppers, thrips and aphids (Bosque-Perez, 1995; Day *et al*., 2017). Stem borers are considered to be one of the most damaging pests of maize, the most prominent stem borers in East Africa being the *Chilo partellus* Swinhoe and African stem borer (*Busseola fusca* Fuller) (M'mboyi *et al*., 2010). Fall army worm is a major pest of economic importance in Africa since 2016 (Day *et al*., 2017). Other pests that affect maize production include the chafer grubs, monkeys, termites, red ants, porcupines, rats and wild pigs, depending on the zone where maize is grown (Songa *et al*., 2002).

Disease-causing organisms of maize include bacteria, fungi and viruses. Some of the economically important maize diseases in Kenya include grey leaf spot caused by *Cercospora zeae maydis*, head smut caused by *Sphacelotheca reiliana*, leaf blight caused by *Exserohilum turcicum*, maize streak disease caused by maize streak virus, common rust caused by *Puccinia sorghi*, ear rots caused by different fungi, common smut caused by *Ustilago maydis*, and maize lethal necrosis disease (Charles *et al*., 2019; M'mboyi *et al*., 2010; Wangai *et al*., 2012). Climatic changes have been known to affect the pests and diseases in different regions and also affect crop production. Greenhouse emissions leading to heat and moisture stress have been predicted to reduce the area of land suitable for maize production in the tropics (Ramirez-Cabral *et al*., 2017).

Other production constraints that affect the production of maize are: drought stress, the parasitic weed *Striga hermonthica*, low access to certified seeds for the recommended hybrid varieties due to lack of purchasing power, and soil acidification and low soil fertility (M'mboyi *et al*., 2010).

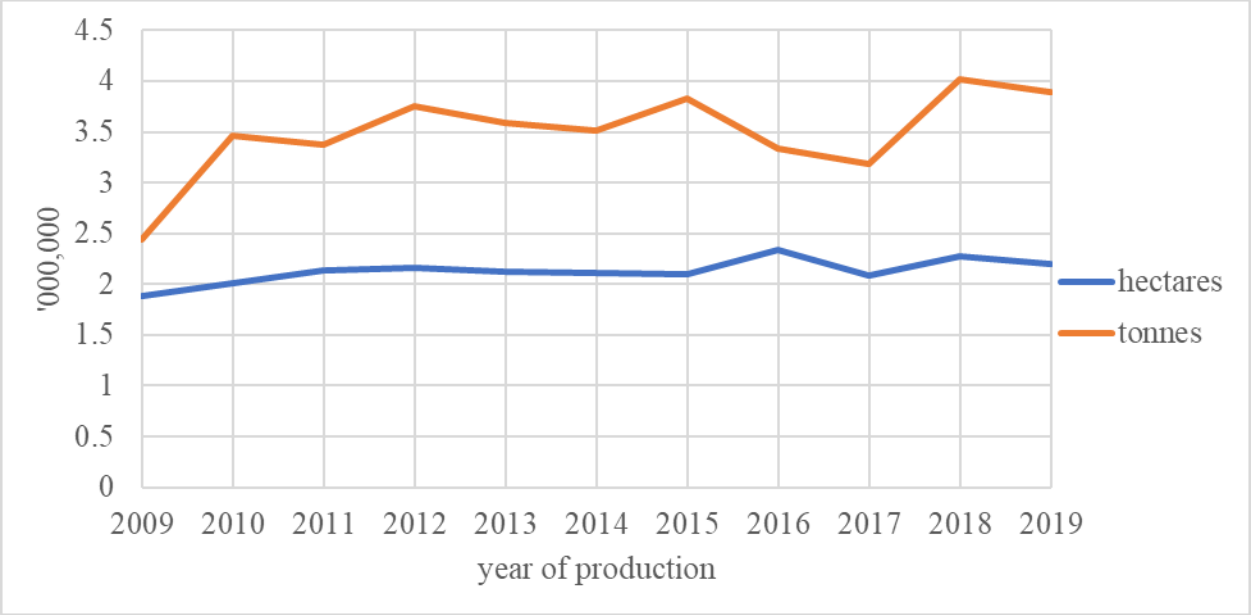

Figure 2.1: Maize production statistics for Kenya for the period 2009-2019
Area harvested in hectares and the production in million tonnes Source: FAOSTAT, accessed June 2021. https://www.fao.org/faostat/en/#data/QCL

2.3 Maize lethal necrosis disease

2.3.1 History and yield losses caused by maize lethal necrosis disease

Maize lethal necrosis (MLN) disease (also referred to as corn lethal necrosis disease, CLN) is caused by a combination of the maize chlorotic mottle virus (MCMV) and any of the cereal-infecting potyviruses including maize dwarf mosaic virus (MDMV), wheat streak mosaic virus (WSMV), sugarcane mosaic virus (SCMV, formerly MDMV-B) and Johnsongrass mosaic virus (JGMV) (Goldberg and Brakke, 1987; Niblett and Claflin, 1978; Scheets, 1998; Stewart *et al*., 2017; Uyemoto *et al*., 1980; Wangai *et al*., 2012). Other viruses have been reported to be present in MLN infected maize plants. They include maize yellow dwarf virus-RMV and maize yellow mosaic virus (MaYMV) (Mwatuni *et al*., 2020a; Wamaitha *et al*., 2018). However, the role of the poleroviruses, in development of MLN is yet to be ascertained.

The first reports of CLN were as a result of co-infection of maize chlorotic mottle virus (MCMV) with wheat streak mosaic virus (WSMV) or maize dwarf mosaic virus (MDMV) (Niblett and Claflin, 1978; Uyemoto *et al*., 1980) with yield losses of up to 91%. Maize lethal

necrosis is as a result of combined maize infection with MCMV, and any cereal infecting potyvirus (Goldberg and Brakke, 1987; Scheets, 1998).

Maize lethal necrosis disease was first reported in Peru in 1973, and thereafter in Kansas (Niblett and Claflin, 1978), Nebraska (Doupnik, 1979) and in Hawaii (Jiang *et al*., 1992). In the USA, MCMV acts synergistically with MDMV or with WSMV to produce the corn lethal necrosis disease (Niblett and Claflin, 1978; Uyemoto, 1981), with reported losses as high as 91%. In the second decade of the 21st century, the disease was identified and described in China (Xie *et al*., 2011), Kenya in 2011 (Wangai *et al*., 2012), Rwanda (Adams *et al*., 2014), Democratic Republic of Congo (Lukanda *et al*., 2014), Ethiopia (Mahuku *et al*., 2015b), Uganda and Tanzania (Kagoda *et al*., 2016; Kiruwa *et al*., 2016), Taiwan (Deng *et al*., 2014) and Ecuador (Quito-Avila *et al*., 2016).

In Kenya, MLN was first reported in the areas of Bomet County, Southern Rift Valley region. However, a farmer in highlands recalled observing the symptoms as early as 2009 and others in 2010 (De Groote *et al*., 2016). The disease has been reported in all the maize growing areas of the country, spreading from the high altitudes, moist transitional and moist mid-altitude zones by 2013, with crop losses estimated at USD187 million (De Groote *et al*., 2016). Thereafter the disease has also been reported in the coastal areas of Kenya (Eunice *et al*., 2021). Maize lethal necrosis causes yield losses of 30% - 100% in the farmers' fields, depending on the maturity stage at disease onset and the severity (Wangai *et al*., 2012). The disease has not only affected production but has also increased import restrictions of maize seed. All seed is tested for presence of MCMV and SCMV before packaging for sale or exporting and thus increasing the cost of maize production. The cost of seed is also affected by the need to increase the number of sprayings against vectors while the seed maize crop is in the field (Shango *et al*., 2019).

2.3.2 Symptomatology

Symptoms of MLN infected plants include leaf chlorotic mottling. This usually develops from bright mosaic on the base of the young leaves in the whorl and extends upwards toward the leaf tips. Further, necrosis of the leaves develops from the margins and results in drying up of the whole leaf; or on young leaves leading to plant death. The plants also exhibit severe stunting and

premature aging of plants, while the formation of ears is affected and may be barren or bare cobs with little or no grain set (Niblett and Claflin, 1978; Uyemoto, 1981; Wangai *et al*., 2012). The symptoms of MLN are expressed in all crop stages with varying severity (Eunice *et al*., 2021).

Figure 2.2 Photographs showing symptoms of Maize lethal necrosis (MLN) disease in Bomet county
a- mottling on leaves, b- chlorosis, c- necrosis of leaf tissue d- death of plants in a farmers' field Pictures from year 2018 and 2019. Photo credit: Cyrus Mugambi

The spread of the viruses causing MLN disease is managed by surveillance correct diagnosis of the MLN-causing viruses, use of virus -free certified seed and creation of a phytosanitary community of practice, breeding for resistance to the virus and control of vectors of the viruses by use of spraying (Prasanna *et al*., 2020.

2.4 Maize chlorotic mottle virus

2.4.1 Description and distribution of MCMV

Maize chlorotic mottle virus (MCMV) is the only species in the genus *Machlomovirus* (family *Tombusviridae*). It is a single stranded, positive sense RNA virus that is 4.4 kb (Nutter *et al*., 1989), icosahedral shape, and about 30 nm in diameter and with a single 25 kDa capsid protein

subunit (Lommel *et al*., 1991). The surface of MCMV particles are smooth with no protruding domains (Wang *et al*., 2015), unlike in Melon necrotic spot virus (Wada *et al*., 2007) and cucumber necrosis virus (Li *et al*., 2013), which belong to the same *Tombusviridae* family. Maize chlorotic mottle virus from sap obtained from infected maize had a thermal inactivation point of 80-85^{o}C, making it very stable in high heat. The virus can maintain infectivity at 20^{o}C for 33 days (Uyemoto, 1981). The positive sense MCMV genome has four open reading frames (ORF) of 31.6 kDa, 50 kDa, 8.9 kDa and 25.1 kDa which also overlap and encode for six polypeptides (Nutter *et al*., 1989; Scheets, 2016).

The diversity of MCMV nucleotide sequences globally is very low, with an average of 45.5 nucleotide differences, with highest diversity in the South American and Hawaii isolates, and the least in the African isolates (Braidwood *et al*., 2018). The MCMV isolate identified in Kenya (Adams *et al*., 2013) is 99% identical to four identified in Rwanda (Adams *et al*., 2014) and Ethiopian isolate (Mahuku *et al*., 2015b). It is 98% identical to the China isolate (Adams *et al*., 2013) and 96-97% identical to the genome sequence of the isolates from Kansas and Nebraska in the USA (Mahuku *et al*., 2015a). Phylogenetic tree revealed clades of the MCMV as North American; South American and Hawaii; and Chinese and African isolates (Braidwood *et al*., 2018). Other countries where the virus has been identified are Argentina (Teyssandier *et al*., 1983) and Thailand (Klinkong and Sutabutra, 1983).

2.4.2 Host range, symptoms and mode of infection of MCMV

Crop species that have been identified as natural hosts for MCMV in the field are maize, sugarcane (*Saccharum officinarum*), finger millet (*Eleusine coracana*), sorghum (*Sorghum bicolor*), *Coix* seed (a wild relative of maize), Napier grass (*Pennisetum purpureum*) and Kikuyu grass (*Pennisetum clandestinum*) (Huang *et al*., 2016; Kusia *et al*., 2015; Mahuku *et al*., 2015a; Wang *et al*., 2014). However, some sorghum varieties have been identified as highly tolerant to MCMV in East Africa (Mahuku *et al*., 2015a). There was no detection of MCMV when 230 grasses with mottle or mosaic symptoms from maize growing fields in Kansas, USA were tested. However, several experimental grass hosts, showed infection when mechanically infected in the greenhouse (Bockelman *et al*., 1982). In East Africa, MCMV was detected using ELISA in symptomatic plants of Proso millet (*Panicum miliaceum*), finger millet and Foxtail millet

(*Setaria italica*) (Mahuku *et al*., 2015a). The transmission of MCMV from the alternative hosts identified to maize is not yet known (Redinbaugh and Stewart, 2018). Maize-infected with MCMV have mainly the symptoms of chlorosis, mottling leading to leaf necrosis; stunting of plants, small cobs which have few seeds and a premature husk. Yield losses have been observed and plant death in severe cases (Nelson *et al*., 2011).

There has been effort to understand the mode of infection of MCMV and effects in the host plants. In maize plants, the increase in MCMV infection has been linked to the replication of the virus controlled by the p50 gene and the read through gene p11, and also to the presence of the p32 gene, whose role is yet to be established (Scheets, 2016). Proteomic analysis of maize infected with MCMV using isobaric tags for relative and absolute quantification (iTRAQ)-based comparative approach indicated that 661 proteins were increased and 311 were reduced in the infected maize, with two proteins, ZmPDIL-1 and ZmPrx5, suspected to increase the susceptibility of maize genotypes that have the proteins expressed (Dang *et al*., 2019).

2.4.3. Transmission of MCMV

Maize chlorotic mottle virus is transmitted by insect vectors, mechanically, by seed and through infected soil and infected plant debris.

2.4.3.1 Seed transmission of MCMV

Studies of transmission of MCMV isolate from Hawaii and Nebraska through seed in seedlings of maize inbred lines, hybrids and those obtained from commercial seed lots ranged from 0% to 0.33% (Bockelman *et al*., 1982; Jensen *et al*., 1991). No seed transmission of MCMV was detected from maize seedlings of 14 inbred lines (2,153 plants) and five hybrids (1,898 plants) (Bockelman *et al*., 1982). Transmission of Hawaii MCMV isolate from seeds obtained from 25 seed lots in Hawaii was observed in four seed lots. The number of transmissions were one in 3,000 seedlings for seed lot 5, 10 in 3,000 seedlings from seed lot 7, two out of 3,000 seedlings from lot 8 and four out of 2,000 seedlings from lot 9 (Jensen *et al*., 1991). Seed transmission of the MCMV isolate in Kenya was carried out using 24 inbred lines. Maize chlorotic mottle virus was detected in one pooled sample, giving a transmission rate of 4.17%. The positive sample was from a susceptible line (Gatunzi *et al*., 2022). Similarly, the transmission rate of MCMV

isolates obtained from Kenya and Hawaii was low, at 0.004% from more than 85,000 seedlings tested (Bernardo *et al*., 2023).

Investigations in Kenya revealed MCMV contamination of seeds obtained from maize infected with MCMV ranging from 72-100% (Mahuku *et al*., 2015a; Gatunzi, 2018). However, detection of the virus on whole or ground seeds may indicate virus that is in or on the seed and is not an indication of the transmission to seedlings (Sastry, 2013b).

2.4.3.1.1 Seed transmission of viruses

The transfer of pathogens from seeds to seedlings during plant growth is defined as seed transmission. The seedlings further grow into plants that carry the pathogen and become sources of inoculum, from where the pathogen is stored for duration of time and also spreads (Sastry, 2013b). Various reviews of seed transmitted viruses have been published (Johansen *et al*., 1994; Maule and Wang, 1996; Sastry, 2013c). Of the 231 seed transmitted viruses reported, 68 of the viruses infected leguminous species with varying rates of seed transmission from low to high (Sastry, 2013c). The transmission of viruses is largely through infected embryo. Viruses can be detected from other parts of the seed, however, most of the virus is inactivated during seed maturation period (Dombrovsky and Smith, 2017).

The rate of transmission of viruses through seed to seedling varies depending on the host plant and cultivar; the host range; the maturity of plant at time of infection- with more transmission happening if the plant is infected at early stages; the prevailing environment; the vectors that transmit the virus and their abundance; and finally, the interactions of these factors (Albrechtsen, 2006; Sastry, 2013c).

Seed transmission of maize infecting viruses is at low rates compared to other crops such as legumes and cucurbits. The seed transmission of maize dwarf mosaic virus (MDMV) was in one seed out of 22,189 seeds tested, one in 11,448 and two in 29,735 seeds tested (Williams *et al*., 1968; Hill *et al*., 1974; Mikel *et al*., 1984). Similarly, few seed transmitted seeds were reported for SCMV (previously referred to as MDMV-B), with 17 transmissions from 9,485 seedlings

(Shepherd and Holdeman, 1965). Other seed transmitted viruses in maize are maize leaf spot, maize mosaic virus and high plains virus (Forster *et al*., 2001; Sastry, 2013c).

2.4.3.2 Vector transmission of MCMV

Several insect vectors have been reported to transmit MCMV. These are corn thrips (*Frankliniella williamsi*) (Jiang *et al*., 1992; Cabanas *et al*., 2013), western flower thrips (*F. occidentalis*) (Zhao *et al*., 2014), maize rootworms (*Diabrotica undecimpunctata, Diabrotica longicornis* and *Diabrotica virgifera*), cereal leaf beetles (*Oulema melanopus*), corn flea beetle (*Systena frontalis*) and *Chaetocnema pulicaria* (Jensen, 1985; Nault *et al*., 1978). However, in a study in Kenya, Western flower thrips did not transmit MCMV (Kinyungu *et al*., 2018).

Transmission of MCMV by beetles is reported for the Nebraska and Kansas isolates. The beetles transmit the virus at both the larvae and the adult stage, and have no latent period. The adults would transmit the virus up to six days after acquisition (Nault *et al*., 1978; Jensen, 1985). Corn thrips transmit MCMV in a semi-persistent way in the larvae and adult stages. The adults are able to transmit the virus up to six days post-acquisition (Jiang *et al*., 1992; Cabanas *et al*., 2013).

In Africa, corn thrips have been observed in all the maize fields affected and non-affected with MLN, and are associated with MCMV transmission in the affected countries (Nyasani *et al*., 2012; Wangai *et al*., 2012; Terefe and Gudero, 2019). Corn thrips can survive on other plants such as legumes, other grass species, vegetables and weeds (Frison and Feliu, 1991; Namikoye *et al*., 2017). The infection of maize plants by MCMV causes increase in volatile compounds, such as (E)-4,8-dimethyl-1,3,7-nonatriene (DMNT), methyl salicylate (MeSA), (E,E)-4,8,12-trimethyltrideca-1,3,7,11-tetraene (TMTT) that induced an attraction of corn thrips to maize seedlings (Mwando *et al*., 2018).

2.4.3.3 Soil transmission of MCMV

There are viruses in the *Tombusviridae* family that are harboured in and transmitted via soil. Maize chlorotic mottle virus is detected in soil using laboratory analysis methods. However, sensitive methods are required in situations where the virus titre is low (Gatunzi *et al*., 2022;

Regassa *et al*., 2022). The virus is transmitted to maize seedlings grown in the infested soil with or without infected plant residues (Gatunzi *et al*., 2022; Mahuku, *et al*., 2015a; Nyakundi, 2017; Regassa *et al*., 2022). In the presence of MCMV-infected maize plant residues, the incidence of the virus and the virus titre in the plants reduced with increasing number of days from the incorporation of the residues prior to planting. The highest incidence of MCMV was when the seed was sown in the field 0-15 days after the residues were incorporated in the soil. The least incidence (1-4%) was observed at 90 days after incorporation of the residues (Kinyungu *et al*., 2019). The virus particles in the soil mixed with infected maize residues was found to remain infective after six months (Regassa *et al*., 2022).

2.5 Testing of virus for seed health certification

Seed health testing determines the status of a seed sample that is a representative of a seed lot. A seed lot is a standard number of seed for a particular crop. Testing for seed health requires a representative sample, a suitable method of sample preparation and the detection method that should be effective, sensitive and efficient to ensure correct diagnosis (Albrechtsen, 2006).

2.5.1 Sampling of maize seed for virus seed testing

Seed sampling is the process of obtaining a smaller amount than that of the whole seed lot for purposes of seed health assays. The sample is ideally a representative of the seed lot, and thus care is required in obtaining the sample (Morrison, 1999). The amount obtained is also important as it should be enough to perform the assays without being wasteful. A representative sample of seed is mainly obtained by following the sampling procedures described by seed health testing regulations (ISTA, 2016). Seed lots have unique weight for every plant species, and a procedure for drawing of samples for testing is critical in ensuring representation of the whole lot. The rules have various ways of sampling from large and small seed lots, and also for large and small seeds. The seed lot size for maize is 40 tonnes (ISTA, 2016). The procedure for obtaining a working sample that is small and representative involves drawing a primary sample (PS) from the lot. The portion is taken from a seed lot during one single sampling action, and all the primary samples are combined to form a composite sample (CS). Since the composite sample may be large, further sub sampling is carried out until the quantity is reduced and is referred to as the submitted

sample. This submitted sample is further divided into a working sample and a duplicate sample is stored for future reference (Morrison, 1999; ISTA, 2016).

Seed health testing involves large number of samples and testing of single seeds is costly and would be time consuming. Thus, the working sample is divided into groups of equal seeds, and each group is analysed as one sample (Albrechtsen, 2006). The number of seeds per sample is determined such that one infected seed within a grouped sample can be detected.

Group testing also involves the use of leaf samples from grow-out seedlings, in assays for seed transmission of the virus. The transmission of MCMV through seed has been previously found to be low (Jensen *et al*., 1991), and obtaining a representative sample is paramount especially in seed lots made up of a blend of produce from more than one field. The working sample size that was adopted for MCMV assays in maize is 400 seeds. This number was adopted from that used for determination of germination percentage and purity. The use of this working sample size is evaluated in this study.

2.6 Methods in plant virus diagnosis

Viruses may cause symptoms in plants that are similar to one another or in other cases the plants may not show symptoms especially in cases where the viral titre is low. Various laboratory testing methods have been developed and used in identifying and detecting plant viruses (Boonham *et al*., 2014). The choice of a method depends on the sensitivity and information required, the availability of the reagents, equipment and skill to carry out the tests and also the duration required to obtain the results (Naidu and Hughes, 2003). Identification of viruses by use of biological assays has been in effect for a long time. The assays involve the use of test/ indicator plants to identify virus by using the sap to infect the test plants, and the leaves observed for symptoms of the virus (Albrechtsen, 2006; Sastry and Zitter, 2014). Growing-on tests are employed to test for transmission of viruses from infected seeds to seedlings. Bioassay methods have limitations in the time lapse before the development of symptoms, the space required to grow the seedlings and also where there is low virus titre, there are chances of false negatives, where there are no symptoms (Albrechtsen, 2006). Grow-on tests are coupled with laboratory testing of the specific virus being investigated. The laboratory method is selected based on the

sensitivity, efficiency, effectiveness and cost of running the tests and availability of the test method. One of the challenges in diagnosis of seed pathogens in Africa by the plant protection organizations, is the availability of infrastructure and human capacity to run the tests (Smith *et al*., 2008).

2.6.1 Serological methods for virus detection

Laboratory testing methods include serological methods e.g., enzyme-linked immunosorbent assay (ELISA). ELISA was first used for detection of plum pox virus (genus Potyvirus, family *Potyviridae*) and Arabis mosaic virus (genus Nepovirus, family *Secoviridae*) (Clark and Adams, 1977) and has been the widely used in the detection of viruses (Boonham *et al*., 2014). Other serological methods, which utilise antibodies against the specific virus being tested, are tissue immunoblotting assay (Hsu and Lawson, 1991) and dot immunobinding assay (Nitrocellulose membrane (NCM) – ELISA) (Smith and Banttari, 1987) where the antigen-antibody reaction is carried out on an NCM paper, unlike on a polystyrene plate in the normal ELISA. Both faces of the NCM paper have been used to detect viruses, thereby reducing the cost of the detection method (Abd El-Aziz, 2019; Kawanna and Hosny, 2020). Similar effectiveness was obtained when Canson paper ($300g/m^2$) replaced the nitrocellulose membrane (Kawanna and Hosny, 2020).

Lateral flow strips are immunochromatography tests that make use of antibodies or probe-labelled nucleic acid of the test virus (Rubio *et al*., 2020). The strip is a simple device made of porous material that allows fluid from crude extract being tested to flow by capillary. The device has bio-active particles loaded to it that are conjugated to a first antibody (against the antigen of interest). When a sample fluid with the virus flows over the particles, they bind on the antibodies. The now bound sample- antibody particle keeps moving into the area with two test lines. The first line has another antibody that binds on a different location on the virus. The sample-particle binds to this antibody and a red colour appears due to accumulation of the bio-active particle. The second test line binds the particle conjugated with antibody (without virus/antigen). This second line confirms that the lateral flow test worked well (Koczula and Gallotta, 2016). Lateral flow strips are advantageous as they are simple to use, can be used on location and results are obtained rapidly (Rubio *et al*., 2020).

2.6.2 Nucleic-acid based methods for virus detection

Nucleic-acid based methods increased the sensitivity of detecting plant viruses. The methods include a nucleic acid extraction and purification steps and then amplification of a targeted region of the DNA or RNA using the polymerase chain reaction (PCR) (Boonham *et al*., 2014) with specific or random primers that are for the virus of interest. After PCR, the amplified section of DNA is separated on an agarose or polyacrylamide gel, and then visualized under ultra violet light. Current technology has led to visualization of the presence of the DNA segment of interest in "real time' and also the possibility of quantifying the fragment, in a method referred to as quantitative PCR (qPCR) (Higuchi *et al*., 1993; Wittwer *et al*., 1997). The fragment being considered is detected by fluorescence resonance energy transfer or when a fluorogenic dyes emits a fluorescent signal when it binds to a double-stranded DNA in the reaction. The fluorescence is measured after every cycle and is corresponded to the accumulation of the DNA of interest.

Loop mediated isothermal amplification (LAMP) assay utilises a single- tube for amplification of the DNA at one temperature (Notomi *et al*., 2000). The method is ideal as it does not require specialized equipment thus reduces the cost of operation as compared to RT-PCR and RT-qPCR. It is a highly efficient and rapid method, with reactions as low as 30 minutes and has tolerance to PCR inhibitors and can be used in the field (Tomlinson *et al*., 2013). However, the quantitative LAMP method has lower quantification of DNA than quantitative polymerase chain reaction methods (Nixon *et al*., 2014). The colorimetric LAMP method is ideal where the samples are few and do not require quantification.

The different methods summarised above are also used in laboratory assays of viruses in plant seeds. Reverse transcription PCR has been used in detection of cucumber mosaic virus (CMV), an RNA virus, detecting one infected seed in one thousand healthy lupin seeds (Wylie *et al*., 1993). Reverse transcription PCR and ELISA have also been used to study seed borne viruses, alfalfa mosaic alfamovirus, bean yellow mosaic potyvirus, clover yellow vein potyvirus, cucumber mosaic cucumovirus, Subterranean clover mottle sobemovius, in legumes (Bariana, 1994).

2.6.3 Detection methods for maize chlorotic mottle virus

Enzyme-linked immunosorbent assay is the most commonly used virus diagnosis assays (Albrechtsen, 2006; Boonham *et al*., 2014). Commercial antibody kits for MCMV detection using the double antibody sandwich- ELISA are available at Agdia Inc. (Elkhart, USA), BIOREBA (Reinach BL, Switzerland) and DSMZ (Braunschweig, Germany) companies. Other methods that have been used in detection of MCMV in leaves and seeds are: RT-LAMP and RT-PCR on maize leaves infected with MCMV (Liu *et al*., 2016a; Chen *et al*., 2017) real time RT-PCR (Zhang *et al*., 2011; Liu *et al*., 2016b). Maize chlorotic mottle virus like other RNA-viruses is first transcribed to their complementary DNA before PCR, by use of a reverse transcriptase enzyme, and referred to as reverse transcription-polymerase chain reaction (RT-PCR).

Dot immunobinding assay was the least sensitive when compared with triple antibody sandwich ELISA (TAS-ELISA) and immunocapture (IC)-RT-PCR methods in diagnosis of MCMV in maize leaf sap (Wu *et al*., 2013). Real time RT-PCR is the most sensitive method, detecting up to 4fg of total RNA (Zhang *et al*., 2011). The first confirmation reports of MCMV isolates in Kenya and Rwanda were carried out using next generation sequencing (Adams *et al*., 2013; 2014), which is a reliable method, yet requiring highly specialised equipment, reagents and labour (Adams *et al*., 2009).

Employing the methods in seed testing in resource-limited laboratories is limited to the availability of required infrastructure and expertise (Smith *et al*., 2008). However, with MCMV becoming a quarantine pest, testing is necessary. The different methods have been developed mainly using the China and US MCMV isolates and thus the need to evaluate methods in detection of MCMV in seeds and biological assays using the same source of MCMV – Kenya isolate with a goal of right detection while considering the cost of analysis.

CHAPTER THREE

SENSITIVITY AND EFFECTIVENESS OF DIAGNOSTIC METHODS IN DETECTION OF MAIZE CHLOROTIC MOTTLE VIRUS IN SEED HEALTH TESTING

Abstract

Maize is a staple crop in Kenya and in other Sub-Saharan Africa countries, and thus seed health testing for MCMV presence is important to protect the production. There are numerous reports of various methods that are used in detection of the virus. This study sought to compare the sensitivity of Double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA), Immunocapture reverse transcription polymerase chain reaction (IC-RT-PCR), lateral flow strips, RT-PCR, reverse transcription loop mediated isothermal amplification (RT-LAMP) and real-time RT-PCR methods used in detection of MCMV from single source purified virus. The effect of leaf sap and seed extract in detection of MCMV using antibody-based methods was also determined. Maize leaf from plants that were free from MCMV were ground in a sample mesh bag in phosphate buffered saline with tween, while MCMV-free seed was crushed in a seed grinder and PBST buffer added in tube. Nucleic-acid based methods were found to be more sensitive than the serological methods. ImmunoStrips® from Agdia company however could detect very low dilutions of up to 1×10^{-12}. Use of IC-RT-PCR was found to increase the detection limit of MCMV by 1000 times in comparison to DAS ELISA, similar to RT-PCR. The study showed that the use of leaf and seed extract samples did not have any differences as compared to use of purified virus in DAS-ELISA, revealing 10X times lower concentrations. Since MCMV is a quarantine pathogen in many countries in sub-Saharan Africa, the information generated here will be applicable in the selection of seed health tests based on the limit of detection while considering the cost-effectiveness of the methods.

3.1 Introduction

Efforts to curb the spread of maize chlorotic mottle virus (MCMV) and sugarcane mosaic virus (SCMV) in the management of maize lethal necrosis (MLN) disease have included seed health testing prior to seed certification. Maize chlorotic mottle virus is transmitted through seed, though at low rates (Bockelman *et al*., 1982; Gatunzi *et al*., 2022; Jensen *et al*., 1991; Kimani *et al*., 2021). However, infection of maize plants by MCMV led to increase in production of volatile compounds that attracted thrips (*Frankliniella williamsi*), the most common vectors of MCMV (Mwando *et al*., 2018). The vectors thus cause secondary transmission of MCMV and in favourable environment, the low amounts of virus transmitted via seed may become widespread. Infection of maize plants with MCMV before SCMV sets in has also been shown to cause more symptom development and rapid death of the plants as compared to the slow development of MLN when SCMV first infects the plants (Kiambi *et al*., 2019).

Various methods of virus diagnosis have been developed over time (Naidu and Hughes, 2003; Albrechtsen, 2006; Boonham *et al*., 2014). The choice of a method depends on the sensitivity, specificity, cost effectiveness, reproducibility and reliability. Most methods developed for MCMV use leaf tissue as sample. They include next generation sequencing (Adams *et al*., 2013), reverse transcription loop mediated isothermal amplification (RT-LAMP) (Liu *et al*., 2016a; Chen *et al*., 2017; Mwatuni *et al*., 2020b), real time reverse transcription polymerase chain reaction method (real time RT-PCR) (Zhang *et al*., 2011; Liu *et al*., 2016b), monoclonal antibody serological method (Wu *et al*., 2013), lateral flow strips (Eunice *et al*., 2021) and recombinase polymerase amplification (Jiao *et al*., 2019). The next generation sequencing methods, RT-LAMP, recombinase polymerase amplification and real time RT-PCR are specialised methods requiring skilled labour and more costly infrastructure and running costs unlike the DAS-ELISA and lateral flow strips. However, they have increased sensitivity and are amenable to quantification of the virus titre.

Kenya has zero tolerance for MLN in seed farms, rejecting all fields that show any symptoms of the disease by the regulatory agency- Kenya Plant Health Inspectorate Services (KEPHIS). In a previous inspection of maize seed farms, field and laboratory testing of seed for MCMV and SCMV led to rejection of 569 hectares of maize seed, and 1.5 million kilograms of seed after

laboratory testing due to presence of the viruses (Shango *et al*., 2019). Current certification procedures for MCMV and SCMV involve field inspection of seed farms and testing of seed grow outs using real time reverse transcription polymerase chain reaction (real time RT-PCR). Grow out tests detect viruses that are transmitted through seeds to the seedlings (Albrechtsen, 2006). This entails planting the seeds and testing for MCMV in the seedlings. The method in combination with real time RT-PCR has an advantage in that it is sensitive; however, it requires skilled labour, specialized equipment and reagents which are most often not easily accessible to developing countries in the region and is also costly to run. In order to continue the efforts of controlling the spread of MCMV in the region, it is important to evaluate more methods for detection of MCMV that can be utilized in seed health testing.

The study assessed the sensitivity of DAS-ELISA, lateral flow strips, RT-LAMP, RT-PCR and Immunocapture one-step reverse transcription polymerase chain reaction (IC-RT-PCR) against real time RT-PCR. The effectiveness of DAS-ELISA, the most common diagnostic method, and IC-RT-PCR were evaluated in detection of MCMV in seed as compared to leaf as they are cost effective in large-scale testing.

3.2 Materials and Methods

3.2.1 Determination of the sensitivity of different methods in detection of purified MCMV

Purified MCMV virions - Kenya isolate (MCMV-Ke) was obtained from Ohio State University (Bernardo *et al*., 2021). The purified MCMV was serially diluted 10-fold and used in the detection sensitivity tests for the various diagnostic methods. Healthy seed was obtained from previously tested seed (untreated) obtained from commercial seed lots that had been harvested from fields in western Kenya. The clean seeds were planted in a greenhouse at KALRO- Kabete to generate healthy leaf samples for use as negative control in the reactions. The healthy seed and leaves were also used to dilute the purified virions, to simulate samples obtained from the field.

In order to compare the sensitivity of the Double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA), Immunocapture reverse transcription polymerase chain reaction (IC-RT-PCR), lateral flow immunoassays - agristrips (ImmunoStrips®, Agdia Inc, USA), reverse transcription PCR (RT-PCR), reverse transcription loop mediated isothermal

amplification (RT-LAMP) and real time RT-PCR, ten-fold serial dilutions of the purified MCMV were used.

3.2.1.1. Double antibody sandwich enzyme-linked immunosorbent assay

Purified virions of MCMV-Ke (1 µg/µl) were serially diluted in phosphate buffered saline with tween (PBST- 0.8% sodium chloride; 0.115% sodium phosphate, dibasic (anhydrous); 0.02% potassium phosphate, monobasic (anhydrous) - {Sigma-Aldrich, St. Louis, Missouri, USA} 0.02% potassium chloride {Merck KGaA, Darmstadt, Germany} and 0.05% tween 20 at pH 7.4) to concentrations of 100 ng to 0.001 fg/µl and tested using DAS-ELISA (Clark and Adams, 1977; Albrechtsen, 2006). The MCMV antibodies were developed from MCMV-Kenya isolate and sourced from University of Minnesota (UoM).

The reactions were carried out on a high protein-binding capacity polystyrene 96-well flat-bottomed microplate (F96, Maxisorp, Nunc, Thermoscientific). The wells were coated with MCMV primary antibody. The antibodies were diluted in coating buffer (0.349 M sodium bicarbonate and 0.015 M sodium carbonate anhydrous pH 9.6, Sigma-Aldrich, St. Louis, Missouri, USA.) in the antibody buffer ratio of 1:1000. The plates were incubated for one hour at 37°C. After incubation, the plates were washed three times with PBST and left to stand for three minutes in the wash buffer before the next wash step. One hundred microlitres of the 10-fold serial dilutions were loaded on the wells coated with MCMV antibody, and incubated at 37°C for one hour. The serial dilutions were replicated four times. After washing the plates as above, a secondary antibody conjugated with alkaline phosphatase enzyme, in buffer (0.2% bovine serum albumin and 2% polyvinyl pyrollidone molecular weight 40,000 in 1X PBST, pH 7.4) in the ratio of 1:4000 was then added to the wells. The incubation and washing steps were followed as above. In order to visualize the reaction, para-nitrophenyl phosphate (PNPP), a substrate of alkaline phosphatase in substrate buffer (9.7% diethanolamine and 0.01% magnesium sulphate, pH 9.8), was added to the wells after washing, and incubated for one hour at room temperature. The PNPP is hydrolysed by alkaline phosphatase to p-nitrophenol, a chromogenic yellow-coloured product that absorbs light at 405 nm. The absorbance of the yellow-coloured product was detected at 405 nm wavelength, and the optical density (OD) readings (A_{405nm}) obtained using a microplate spectrophotometer reader (Elx808, BioTek Instruments, Vermont, USA).

Positive samples were determined using the absorbance readings of the wells with the buffer. Absorbance readings that were twice that of the buffer were considered a positive detection of MCMV.

3.2.1.2 Lateral flow immunoassays

ImmunoStrips® (Agdia Inc, USA) were purchased and used for detection of MCMV in similar dilutions as those used in the DAS-ELISA according to manufacturers' instructions. Positive detection of MCMV was identified by the presence of two bands on the strip after application of the diluted virus, while negative detection had one band only.

3.2.1.3 Immunocapture reverse transcription polymerase chain reaction

Immunocapture reverse transcription polymerase chain reaction (IC-RT-PCR) was carried out in MicroAmp optical 96-well reaction plate (Applied Biosystems, ThermoFisher Scientific, Massachusetts, USA). The wells were coated with 50 µl of similar antibody coating solution as that used for DAS-ELISA. The plates were incubated at 37°C for one hour, and washed as the DAS-ELISA plate above. The 10-fold serial dilutions of the purified virus in PBST (25 µl), similar to that used for DAS-ELISA were added to the wells and incubated at 37°C for one hour. The dilutions were replicated four times. The plates were then washed three times with PBST as the DAS-ELISA, and after that, rinsed three times with distilled water, prior to tapping them dry on paper towel.

One-step IC- RT-PCR was carried out in a 15 µl reaction mix using the illustra Ready- To-Go RT-PCR beads (GE Healthcare Lifesciences LTD, Buckinghamshire, UK), 0.5 mM of the MCMV forward and reverse primers MCMV-2452F (5'-AGTGGAGGTAGGCAGAGTCA-3') and MCMV-3111R (5'-TCCAACAGCAATGTTTTCCA-3'), that amplify a 660 bp conserved portion of the MCMV replicase open reading frame (Bernardo *et al*., 2021). The PCR amplification program was 42°C for 30 minutes, 95°C for 5 minutes followed by 35 cycles of 95°C for 30 seconds, 55°C for 40 seconds and 72°C for 40 seconds; and a final extension for 10 minutes at 72°C completed the reaction carried out in Applied Biosystems Veriti 96-well thermal cycler (ThermoFischer Scientific, Massachusetts, USA). The PCR products were separated on a 1% agarose (UltraPure, Invitrogen, Thermo Fischer Scientific, Massachusetts, USA) gel in X0.5

Tris Borate EDTA buffer stained with ethidium bromide. The products were viewed on ultraviolet transilluminator. Presence of a 660 bp on the agarose gel indicated a positive detection of MCMV, while no band indicated a negative detection of the virus.

3.2.1.4 Reverse transcription polymerase chain reaction

Reverse transcription polymerase chain reaction (RT-PCR) was carried out using the serially diluted purified virus up to 1 x 10^{-12}. The reaction mix consisted of 1x master mix that included Taq polymerase (MyTaq One-Step RT-PCR kit, Bioline, USA Inc, USA), MCMV forward and reverse primer similar to that used in the IC-RT-PCR, reverse transcriptase, 0.2 µl of inhibitor, one µl of diluted virus and 1.1 µl of depc-treated water. A similar amplification temperature profile and visualization of bands as that of IC-RT-PCR (section 3.2.1.3) were used. Positive detection of MCMV was identified by presence of a 660 bp on the agarose gel, while no band indicated a negative detection of the virus.

3.2.1.5 Reverse transcription loop mediated isothermal amplification

The sensitivity of detecting MCMV in the serial dilutions of the purified virus were also analysed using one-step reverse transcription loop mediated isothermal amplification (RT-LAMP). A negative control from MCMV-free leaf material was included. The reactions were carried out in 8-strip Genie strips. The components of the 15 µl reaction mix included the purified virus (1 µl), 1x RT-isothermal master mix (ISO-001, OptiGene Limited, West Sussex, UK), and 0.5 mM of two outer primers (F3 5'-CGCGGCTGACAAGCAAAT-3' and B3 5'-ACTGGTTGTTCCGGTCTTG-3'), 1 mM of the forward and backward loop primers (LF 5'-CCTACCGCTTGTGTTGCAC-3' and LB 5'-CAGGGCTGGCAAATCATTGA-3') and 2 mM of the forward and backward inner (FIP 5'-CACGGTAGGACACGGAGTACGA-ATTGTGGCTATCCCCAAAGC-3' and BIP 5'-CCTACAACCTGCCCTGGTTCC-TCATGCCGGCTCACCTTA-3') (Mwatuni *et al*., 2020b) and 3.5 µl of nuclease-free water. The RT-LAMP reaction was performed on a Genie III equipment (Gen 3-01, OptiGene Ltd, West Sussex, UK) with the temperatures of 40°C for 2 minutes, followed by 63°C for 30 minutes. An annealing (dissociation) curve analysis step of 98°C to 70°C (ramp of 0.05°C) was included at the end to confirm the specificity of the amplified product. Positive detection of MCMV was

determined by a sigmoid curve with increase in fluorescence combined with detection time lower than that of the negative control.

3.2.1.6 Real time reverse transcription polymerase chain reaction

Real time RT-PCR was carried out in an Applied Biosystems StepOnePlus equipment (ThermoFischer Scientific, Massachusetts, USA). The one-step reverse transcription was performed in a 10 µl reaction mix comprising of 2 µl of the serially diluted purified virus, diluted in DEPC-treated water; 1x SensiFAST probe Hi-Rox One-step buffer (Bioline, USA Inc, USA) 0.5 mM forward and reverse primers (5′-GAGTCCTGCCAATCCAAAGTG-3, reverse primer 5′-TGGGTGGGTCAAGGCTTACTA-3', Liu *et al*., 2016b), 0.3 mM fluorescence-labelled probe (5′-FAM-AGCCGCCGCCCACTCTCCAG-BHQ-3, Liu *et al*., 2016b), 0.2 µl RNase inhibitor and 0.1 µl reverse transcriptase. A no template water control was included in the experiments. The reaction was done by reverse transcription of RNA to the complementary DNA at 50°C for 10 minutes, then denaturation of the double stranded cDNA at 95°C for 1 minute followed by 40 cycles of 94°C for 10 seconds and 60°C for one minute. The data was analysed using the StepOnePlus software and a threshold cycle (Ct) value assigned for all the samples. A positive sample was determined if the mean Ct values was higher than that of the no template control.

3.2.2 Effect of using leaf sap and seed extract as samples in detection of MCMV using antibody-based methods

Antibody-based methods use samples in crude extraction form. This experiment sort to determine the effect of using leaf sap and seed extract in two serological- based methods. The purified virus was serially diluted in MCMV-free leaf sap and seed extract up to a concentration of 0.001 ng/µl (1 $x10^{-6}$). The 1 x 10^{-1} dilution level was not included in the assays due to limitation in the amount of the available purified virus. The methods evaluated were DAS-ELISA and IC-RT-PCR, using the methods described above in section 3.2.1.1 and section 3.2.1.3, but using leaf sap and seed extract for dilution instead of PBST buffer. Healthy leaves were crushed in PBST in a ratio of 1:10, while the seed was ground in the buffer in a ratio of 1:1. Positive detection of MCMV was determined using similar procedure as described for each method in section 3.2.1.1 and section 3.2.1.3. However, healthy controls of leaf and seed extract

were used in place of buffer. Lateral flow strips were not included in the evaluation due to the limitation in purchasing cost.

3.2.3 Data analysis

Data from the agarose gel electrophoresis for RT-PCR, IC-RT-PCR and the immunostrips were described as observed, with presence and absence of bands indicating positive identification of MCMV and a negative sample respectively. Absorbance data from DAS-ELISA was described as positive if the reading was twice higher than that of the average of the negative controls. The absorbance readings were also subjected to analysis of variance (ANOVA) and the means separated using the Fischer's least significant differences using the R software (R Core Team, 2021). Ct values from the real time RT-PCR analysis were also subjected to ANOVA test, and means separated using the Fisher's least significant difference (LSD). Mean Ct values that were above those of the negative controls were assigned as negative detection of MCMV.

3.3 Results

3.3.1 Sensitivity of different methods in detection of purified MCMV

The nucleic acid-based methods exhibited higher sensitivity than the serological methods (Table 3.1). The detection of MCMV purified virus diluted in PBS-T buffer for dilutions 1 x 10^{-5} and lower had absorbance readings that were less than twice that of the negative control (A_{405nm} 0.24). The limit of detection for DAS-ELISA using the antibodies obtained from University of Minnesota was up to 0.1 ng/µl (1 x$10^{-4)}$ dilution) (Table 3.1). Absorbance readings for dilutions above 0.1 ng/µl were not significantly different ($P<0.001$) from those of the negative control. The Real time RT-PCR Ct values were significantly different ($P<0.01$), and were all different from that of the negative control except for that of 0.001 fg/µl concentration (Table 3.1). However, all the mean Ct values were below that of the negative control.

Table 3.1: Sensitivity of detection of maize chlorotic mottle virus (MCMV) from purified virus for different diagnostic methods

Virus concentration	DAS-ELISA (OD)*	IC-RT-PCR (band)	Immunostrips (line)	RT-PCR (band)	RT-LAMP (min)	Real time RT-PCR (Ct)
10 ng/µl	1.16a	√	√	√	-	-
1 ng/µl	0.81b	√	√	√	14.15	17.64e
0.1 ng/µl	0.48c	√	√	√	12.30	19.72de
0.01 ng/µl	0.13d	√	√	√	13.30	20.84de
1 pg/µl	0.11d	√	√	√	14.00	22.99cd
0.1 pg/µl	0.10d	√	√	faint	15.15	25.21c
0.01 pg/µl	0.09d	X	√	faint	17.45	26.16bc
1 fg/µl	0.11d	X	√	faint	18.00	26.40bc
0.1 fg/µl	0.14d	X	√	faint	19.30	25.88bc
0.01 fg/µl	0.07d	X	√	X	22.45	26.74bc
0.001 fg/µl	0.10d	X	√	X	28.45	29.63ab
Negative control	0.12d	X	X	X	None	31.81a

*Double antibody sandwich enzyme-linked immunosorbent assay (DAS- ELISA) (OD) values are the average optical density values at 405 nm obtained after one hour. "√" and "X" under immunocapture reverse transcription polymerase chain reaction (IC-RT-PCR) and RT-PCR represent present and absent bands respectively, and two lines or single line on the ImmunoStrip®. Values under reverse transcription loop mediated isothermal amplification (RT-LAMP) represent average time to amplification, while values of real time RT-PCR represent the average cycle threshold (Ct- number of cycles taken before the fluorescent signal crosses the threshold). Same letter that follows mean values in a column do not differ significantly at $p \leq 0.05$

The lateral flow strips from Agdia detected up to a concentration of 0.001 fg/µl showing high sensitivity (Figure 3.1), while IC-RT-PCR detected up to 0.1 pg/µl (Figure 3.2), thus more sensitive than DAS-ELISA.

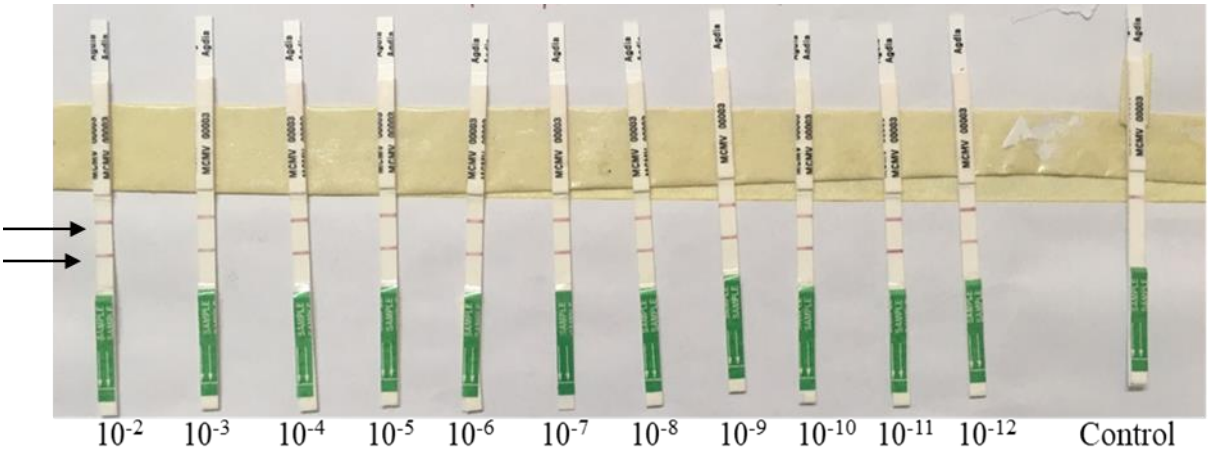

Figure 3.1: Detection of serially diluted maize chlorotic mottle virus (MCMV) using ImmunoStrip®- Agdia
Strip 10^{-2} – 10 ng/µl, strip 10^{-3}- 1 ng/µl, strip 10^{-4}- 0.1 ng/µl, strip 10^{-5}- 0.01 ng/µl, strip 10^{-6}- 1 pg/µl strip, 10^{-7}- 0.1 pg/µl, strip 10^{-8}- 0.01 pg/µl, strip 10^{-9}- 1 fg/µl, strip 10^{-10}- 0.1 fg/µl, strip 10^{-11}- 0.01 fg/µl strip, 10^{-12}- 0.001 fg/µl. Control – buffer. A positive sample is indicated by two red lines on the strip (shown by the arrows)

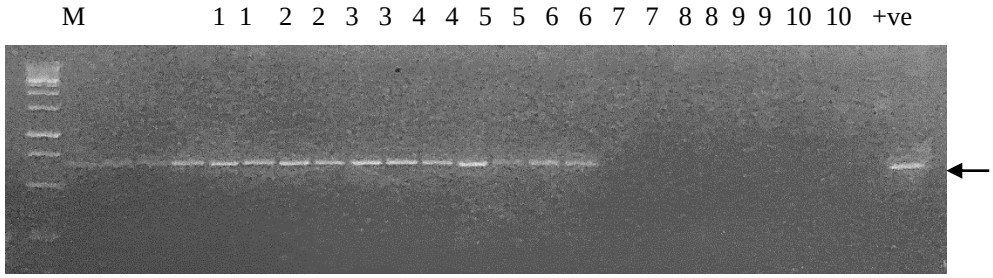

Figure 3.2: Detection of maize chlorotic mottle virus (MCMV) in serially diluted concentrations by immunocapture reverse transcription polymerase chain reaction (IC-RT-PCR)
M- Marker- 1 kb DNA ladder (GeneRuler, ThermoFischer scientific, USA), label 1- 10 ng/µl, label 2- 1 ng/µl, label 3- 0.1 ng/µl, label 4- 0.01 ng/µl, label 5- 1 pg/µl, label 6- 0.1 pg/µl, label 7- 0.01 pg/µl, label 8- 1 fg/µl, label 9- 0.1 fg/µl, label 10- 0.01 fg/µl. Positive samples are indicated by an arrow.

Nucleic-acid based methods detected MCMV at much lower concentrations than DAS-ELISA, the most common diagnosis method. The band of interest (660bp) was present in RT-PCR for concentrations of 10 ng/µl to 0.1 fg/µl (Figure 3.3). MCMV was detected using RT-LAMP for concentrations of up to 0.001 fg/µl, equivalent of dilution of 1 x 10^{-12}. This was similar to the detection limit achieved using real time RT-PCR (Table 3.1). However, the amplification curves were only distinct for up to 1 pg/µl (Figure 3.4).

Figure 3.3: Agarose gel showing band of interest of the serially diluted maize chlorotic mottle virus (MCMV) detected using reverse transcription polymerase chain reaction (RT-PCR)
Concentration on Lane 1-100 ng/µl, lane 2- 10 ng/µl, lane 3- 1 ng/µl, lane 4- 0.1 ng/µl, lane 5- 0.01 ng/µl, lane 6- 1 pg/µl, lane 7- 0.1 pg/µl, lane 8- 0.01 pg/µl, lane 9- 1 fg/µl, lane 10-0.1 fg/µl, lane 11- 0.01 fg/µl, lane 12- 0.001 fg/µl. Marker- 1 kb ladder. Positive bands are indicated with an arrow.

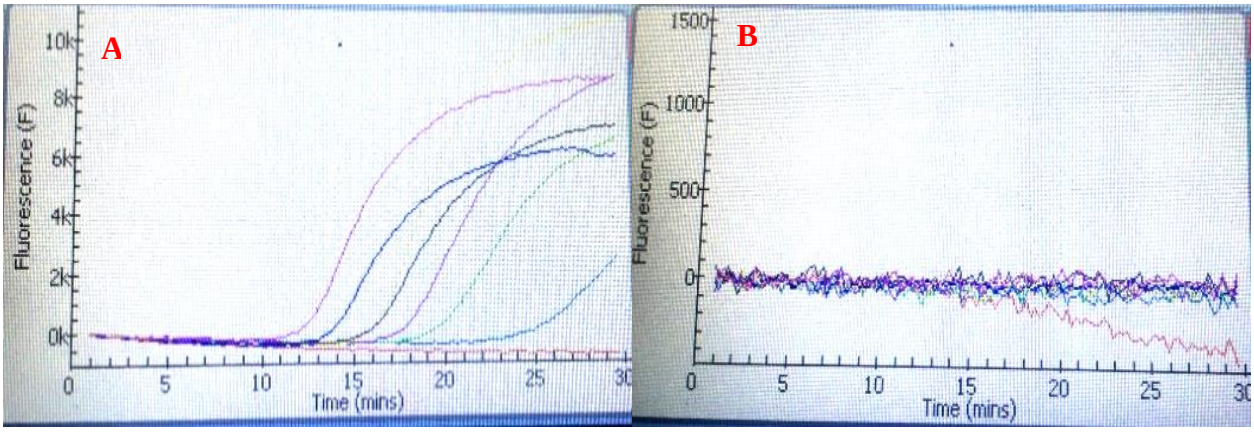

Figure 3.4: Amplification of the purified maize chlorotic mottle virus serial dilutions using reverse transcription loop mediated isothermal amplification (RT-LAMP)
Photo A- curves for virus concentrations of 100 ng/µl to 0.1 pg/µl and photo B- results for concentration to 10 fg/µl to 0.001 fg/µl (10-fold)

3.3.2 Leaf sap and seed extract in detection of purified MCMV using antibody-based methods

3.3.2.1. Sensitivity of detection of MCMV using DAS-ELISA in seed extracts and leaf samples

The sensitivity of DAS-ELISA in detecting the purified virus when diluted in leaf sap and seed tissue was carried out for final virus concentration of 10, 1, 0.1, 0.01 and 0.001 ng/µl (1 x 10^{-6} dilution). The 1 x 10^{-1} dilution level was not included in the assays due to limitation in the amount of purified virus. There was positive detection of MCMV up to the concentration of 0.1

ng/µl with A_{405nm} of 0.18 to 1.22 for the dilutions in healthy leaf sap, and 0.615 to 1.633 in healthy seed extract (Figure 3.5). The A_{405nm} for the virus concentration 0.01 ng/µl and 0.001 ng/µl for the leaf sap and seed extract diluted samples were below twice the average value of those in the healthy controls (0.08 ± 0.002 and 0.11 ± 0.006 respectively) (Figure 3.5).

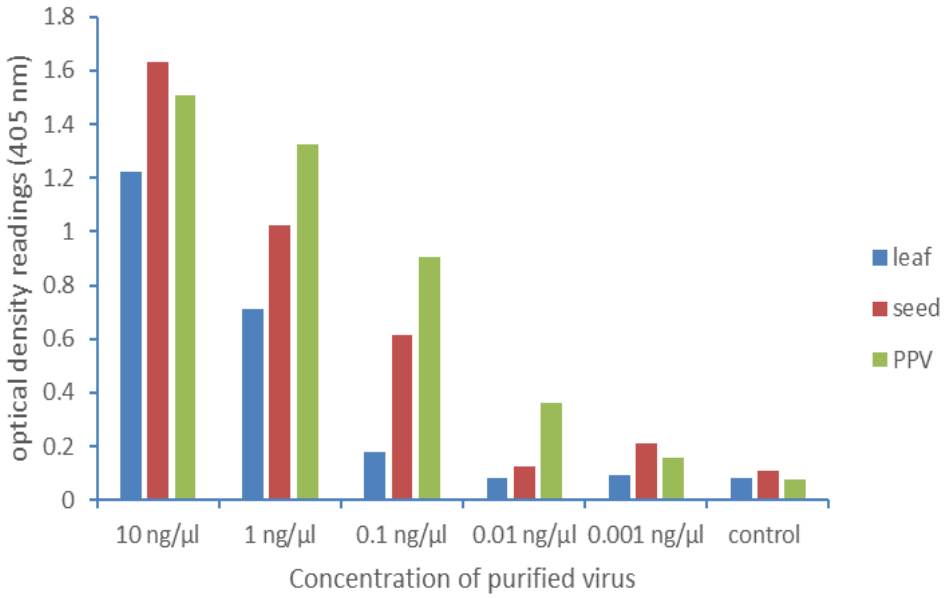

Figure 3.5: Detection of purified maize chlorotic mottle virus (MCMV) serially diluted in healthy leaf sap, healthy seed extract and in phosphate buffer saline with tween (PBST) (labelled as PPV) using DAS-ELISA
Healthy leaves, seeds and buffer were used as negative control

3.3.2.2 Sensitivity of testing purified MCMV using IC-RT-PCR diluted in seed extracts and leaf samples

When the purified virus was diluted in healthy leaf sap and seed extract, the band of interest was visualized on lanes with concentrations up to 0.001 ng/µl (dilution of 1 x 10^{-6}) (Figure 3.6). The lowest dilution where there was detection of MCMV (0.001 ng/µl) was similar to that of the purified virus which was diluted in phosphate buffer.

Figure 3.6: Results of presence of maize chlorotic mottle virus (MCMV) serially diluted in seed extract and leaf sap by IC-RT-PCR and separated on a 1% agarose gel
Figure A- Lanes 1 and 2- buffer; Lanes 3 and 4 healthy leaves; lanes 5 and 6 -healthy seed; Lanes 7 to 30 the purified virus was diluted in healthy seed extract. Lanes 7 to 10 – 0.0001 ng/µl; Lane 11 to 14 - 0.001 ng/µl; lane 15 to 18 - 0.01 ng/µl; lane 19 to 22- 0.1 ng/µl;
Figure A/B- lane 23 to 26- 1 ng/µl and lane 27 to 30 – 10 ng/µl. Lanes 31 to 47 were diluted in healthy leaf sap. Lanes 31 to 34 - 0.0001 ng/µl; lanes 35 to 38 - 0.001 ng/µl; lanes 39 to 42 - 0.01 ng/µl; lanes 43 to 46 - 0.01 ng/µl;
Figure B/C lanes 47 to 50 – 1 ng/µl and lanes 51 to 54 – 10 ng/µl. Marker- 1 kb DNA ladder (Thermoscientific, USA). Positive band indicated with an arrow.

3.4 Discussion

Seed health testing of MCMV was incorporated in countries in sub-Saharan Africa where the virus has been reported. While various methods have been developed for the detection of the virus, the study evaluated the sensitivity of DAS-ELISA, IC-RT-PCR, lateral flow strips, RT-LAMP, RT-PCR and real time RT-PCR using a same source of purified virus; antibodies for serological methods and primer pairs for nucleic acid methods.

ELISA is the most commonly used method in large scale testing of viruses. However, from this and other studies, it has been shown that the method is less sensitive as compared to other methods. In this study DAS-ELISA detected up to 10 pg/µl concentration of purified virus. This was lower sensitivity as compared to the RT-PCR and real-time RT-PCR. The results are comparable to other studies where ELISA was 3000 times lower than RT-PCR and RT-qPCR (Bernardo *et al*., 2021). However, ELISA is prone to false negative results (Albrechtsen, 2006; Bernardo *et al*., 2021). This was also identified in this study where the detection varied with

some reactions having a lower detection of up to100 pg/µl. This may have been attributed to the use of different batch of reagents for preparation of the ELISA buffers, during the separate runs.

Immunocapture one-step RT-PCR method merges the serological and nucleic acid-based method and this increased the sensitivity of detection of the purified virus as compared to DAS-ELISA. The IC-RT-PCR in this study made use of polyclonal antibodies as capture reagents and was able to detect 0.1 pg/µl of MCMV in a reaction. Monoclonal antibodies used in IC-RT-PCR in China detected MCMV in a minimum dilution of 1:3276800 of crude leaf tissue extract (Wu *et al*., 2013), which was 10 times more sensitive than using triple antibody sandwich (TAS)-ELISA. While the monoclonal antibodies have been shown to be more sensitive, the antibodies are more costly. Immunostrips manufactured by Agdia Inc. had higher sensitivity as compared to the other serological methods. However, the method is ideal for smaller samples sizes and field testing and not for large sample numbers characteristic of seed health testing units.

Nucleic acid methods showed to be the most sensitive and amiable to large sample testing. Though more costly and laborious than the serological methods, the RT-PCR and real time RT-PCR method detected lower concentrations of the purified virus of up to 0.1 fg/µl and 0.01 fg/µl. The higher sensitivity of these methods is similar to that used for detection of MCMV of different isolates (Bernardo *et al*., 2021). In this study, the sensitivity of real time RT-PCR was 1 fg/µl to 10 fg/µl depending on the isolate. The study used the SYBR system while the higher sensitivity observed from data presented in this thesis was obtained using Taqman probes. The increased sensitivity realised using nucleic acid methods makes the diagnosis more accurate and are more suitable for adaptation in seed health testing. The methods can be also be automated for large scale testing and reactions can be multiplexed for more than one virus (Sastry and Zitter, 2014).

RT-LAMP in this study detected up to 1 pg/µl of MCMV, as compared to 4.8 pg/µl of MCMV-Agdia 2219 genomic RNA using the colorimetric detection RT-LAMP method (Liu *et al*., 2016a) and 2.5 pg/µl (Chen *et al*., 2017; Mwatuni *et al*., 2020b). The detection of MCMV in higher dilutions, but not showing the curves corresponded to results by Mwatuni *et al*. (2020b). Despite having to use more primers in the RT-LAMP method, the method has advantage in that

the results are obtained immediately after the amplification step by visual observation or real time equipment where fluorescence is used unlike the IC-RT-PCR where electrophoresis is mandatory after amplification. The methods despite requiring sophisticated equipment and being more complicated are reliable and with automation and use of RNA extraction kits, provide results faster (Smith *et al*., 2008; Bernardo *et al*., 2021).

The sample-type condition used during diagnosis was found to have an effect on the results. Use of crude leaf and seed extracts to dilute the purified virus lowered the sensitivity of DAS-ELISA ten times, unlike IC-RT-PCR. Thus IC-RT-PCR was not only more sensitive than DAS-ELISA, but also there was no interference of the leaf and seed tissue in the detection of purified virus. Thus IC-RT-PCR method is useful in increasing the data sensitivity where the crude samples are used. It is an alternative in large-scale testing of the virus to the nucleic acid method as it provided solutions to challenges faced in a laboratory in developing country. Challenges may include power outages that may affect freezers during the storage of RNA; the high cost and availability of required reagents in RNA extraction and purification and the time-consuming step of RNA extraction. However, IC-RT-PCR also requires the use of high-quality antibodies and virus specific primers to ensure reliability of the results. In nucleic acid method, the samples are purified prior to diagnosis, thus increasing ensuring higher detection limit using these methods.

In this study, the sensitivity of detection methods developed for MCMV is detailed. In situations where the use of molecular techniques is challenging, several methods are available for the analysis as described above. Employment of seed health testing for control of spread of MCMV has contributed to the reduced spread of the virus (Eunice *et al*., 2021; Liu *et al*., 2016b). Since maize is a major cereal staple food in sub-Saharan Africa, prevention of the spread of MCMV has been of great importance in the region. The availability of the methods for seed health assays in resource-challenged laboratories will mainly determine the adaptability of any of the protocols. Various expertise, infrastructure and running costs of the methods greatly determine the choices (Smith *et al*., 2008). Despite the need for cost-effective methods, it is important that the methods be sensitive, reliable and specific to ensure no false positives in the detection of the virus and reproducibility of the results (Naidu and Hughes, 2003). However, the study

demonstrated the sensitivity of using the various detection methods for MCMV seed health assay results, and recommends utilizing nucleic-acid based methods for increased accuracy.

CHAPTER FOUR

EVALUATION OF SEED SAMPLE SIZE IN DETECTION OF MAIZE CHLOROTIC MOTTLE VIRUS

Abstract

Sampling for seed health testing is important in determining the infection status of seed lots. Sample sizes obtained for testing are determined using statistical methods guided by a desired probability of detection and the infection level allowable for the pathogen. Despite these methods, application of the statistically determined sample size is influenced by sensitivity of the detection methods and host-pathogen interaction. The adopted sample size for detection of maize chlorotic mottle virus (MCMV) is 400 seeds randomly sampled from a lot. This sample size is determined by the International Seed Testing Association when the threshold for tolerance of a pathogen transmitted through seed is 1%. The purpose of this study was to evaluate the applicability of this sample size for MCMV detection in seed health testing while comparing the effectiveness of using whole seeds and seedling grow outs. Maize chlorotic mottle virus in the grouped samples was tested using double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) and real time reverse transcription polymerase chain reactions (real time RT-PCR). The binomial model was used to calculate the probabilities of detection. The results showed that detection of MCMV in samples with one infected seed in a sample size of up to 1000 whole seeds was possible. Samples that were not soaked, but ground while dry had positive detection of MCMV in 75% of the 100-seed samples, and 62.5% in the 500- and 1000-seed samples. However, when the sample was soaked overnight in general extraction buffer, all the samples had positive detection. The probability of detection of MCMV in 400-seed sample was one (1) when whole seeds were tested using DAS-ELISA, unlike when the seedling grow outs were tested using DAS-ELISA and real time RT-PCR where the probability of detection success was low. The use of seedling grow outs in sample testing for MCMV, despite the low probabilities, is useful in determination of the risk of the transfer of the virus to the field through the seed lot being assessed**.**

4.1 Introduction

Healthy seed is used as a management strategy of seed borne pathogens, as seed is the main media for germplasm exchange worldwide. Despite low transmission of viruses via seed, the risk lies in the secondary spread through vectors (Albrechtsen, 2006). Presence of a pathogen in the seed would lead to its introduction in areas where it has not been present or has been controlled, leading to disease development and spread.

Detection of pathogens in seed lots starts with obtaining the correct working sample that is representative of the seed lot. Standard sampling procedures of seed lots have been developed by international bodies such as International Seed Testing Association (ISTA) and Association of Official Seed Analysts (AOSA). Over the years, despite these procedures being first developed for evaluating purity and germination of seeds, the procedures have been found to be sufficient in obtaining samples for plant health from seeds (Morrison, 1999). A part of seed from a lot that is required for tests to be carried on, is obtained by undertaking sampling procedures steps. It is referred to as a working sample. The size of this working sample is determined by statistical probabilities of detecting the pathogen guided by the threshold that may cause an epidemic infection and the choice/availability of the testing method (Morrison, 1999). The choice of detection method is also informed by the virus-host interaction.

Maize chlorotic mottle virus (MCMV) is a quarantine pest in Kenya, with a zero-tolerance level during seed certification. The standard procedures for detection and sample sizes have not been well documented, However, 400 seeds have been adopted for detection (Hodson *et al*., 2021) from the statistical probabilities at 95% confidence interval, while allowing a threshold infection of 1%. Each seed in the sample can be tested individually, however, this is time consuming as well as expensive. Testing of more than one seed together is referred as group testing, and it is adopted to reduce the time for analysis as well as the cost of testing the samples (Albrechtsen, 2006). Testing of seedling grow-outs is time consuming and laborious, however, it gives the true picture of transmission of virus from seed to seedlings, as compared to testing of whole seeds. Seed health testing for MCMV was introduced in Kenya after the outbreak of maize lethal necrosis (MLN) disease in 2011 (Wangai *et al*., 2012), and there was need to evaluate all the maize seed locally produced and those imported, hence the need to evaluate the sample sizes for

detection of the MCMV isolate found in the region. The study was carried out to determine the sensitivity of detection of one MCMV-infected seed in maize composite samples and evaluating probability of successful detection of MCMV from 400 seed sample size using whole seeds and seedling grow outs currently being used during testing.

4.2 Materials and Methods

4.2.1 Sampling of the seed lots

Maize chlorotic mottle virus contaminated seed was obtained from infected plants from MLN screenhouse at the Kenya Agricultural and Livestock Research Organization (KALRO) Biotechnology Research institute, Kabete centre, while healthy seed was obtained from Seed Science Centre at Iowa State University, USA for experiment on the group testing. Two maize seed lots that were identified to be infected with MCMV were availed for the other experiments by a seed company through Kenya Plant Health Inspectorate Service (KEPHIS). Each seed lot was made up of one maize hybrid variety weighing 40 tonnes. The seed lots were labelled seed lot A and seed lot B.

Seed samples were obtained from the two seed lots following the international sampling guidelines (ISTA, 2016). Seed lot A was a blend of produce from different farmers, while seed lot B was obtained from a single source. A primary sample was drawn from all the bags of seed lot A, while for seed lot B, selected bags were identified using the sampling procedures and a primary sample drawn from these bags. The type of sampling tool used was the thief-trier, which collected approximately 80 – 100 gm of maize seed from each bag.

The bags were arranged into subunits for ease of sampling. Seed in lot A was packaged in bags of 50 kg, and were arranged into subunits of 30 bags each. Lot B had bags of 90 kg, and was arranged in seven subunits. The primary samples obtained from the bags were collected in a clean bucket to make the composite samples. The composite samples were thoroughly mixed and subdivided using a Gourmet sample divider or the quarter method depending on the accessibility to the seed lot. The composite sample (at least one kilogram) was labelled and submitted for laboratory analysis.

4.2.2 Determination of sensitivity of detecting MCMV in different group sizes

4.2.2.1 Group test using whole seed

The sensitivity of detecting MCMV was determined using three sample amounts. Group testing reduces the cost of testing individual seeds. These were:

i) One seed from MCMV infected plant in 99 healthy seed,

ii) One seed from MCMV infected plant in 499 healthy seed and

iii) One seed from MCMV infected plant in 999 healthy seed.

The MCMV contaminated seed was obtained from infected plants at the maize lethal necrosis (MLN) disease screenhouse at KALRO Biotechnology Research institute, Kabete while the healthy seed was obtained from Seed Science Centre at Iowa State University, USA. The healthy seed was from seed lots that had tested negative for MCMV and were grown in MCMV- free areas of Iowa.

The healthy seed was counted out and placed in one-litre conical flasks, and one infected seed was added and thoroughly mixed together. The experiment was laid out in a completely randomised design in the shaker. The mixtures were soaked overnight in ELISA general extraction buffer and ground in a Waring 7011HS blender (Waring Commercial, Stamford, CT, USA) the next day. However, the healthy controls were ground first, then the samples with one infected seed in 1000 seed-sample, followed by 500-seed samples and 100-seed samples to avoid chances of contamination (Figure 4.1). The experiments were repeated five times and the extracts tested for presence of MCMV using DAS-ELISA as described in 3.2.1.1 with Agdia MCMV antibodies according to manufacturer's instruction (Agdia, Indiana, USA).

Further, similar samples with the same ratios of healthy and infected seed were tested using real time reverse transcription polymerase chain reaction (real time RT-PCR). Real time RT-PCR was carried out on similar sample sizes used for ELISA tests. Dry seed of one infected seed in 1000, 500 and 100 were ground in a Cyclone sample mill (Seedburo Equipment Co. Chicago, IL, USA). Optimization of a protocol for extracting quality RNA from dry maize seed (50mg) was carried out using five methods. These were using RNA kit (EZNA Total Plant RNA kit, Omega Bio-Tek 2012, GA 30071, United States); sodium dodecyl sulphate (SDS) method (Wang *et al*., 2012); a CTAB method adapted from extraction of RNA for MCMV testing

(Adams *et al*., 2009), Kingfisher Total RNA Kit (Thermo Fischer Scientific Inc., Vantaa, Finland, 2012) and another SDS method (Li and Trick, 2005). The RNA extraction method that produced the best results was that by Li and Trick, (2005) and this was used in the subsequent tests with modifications. The method yielded good quality and quantity of RNA. To the remaining ground seed, extraction buffer was added for testing using DAS-ELISA according to manufacturer's instruction (Agdia kit) for comparison.

Figure 4.1: Photograph of soaked seed samples in ELISA general extraction buffer containing one infected seed in varying sample sizes, and healthy controls
The samples had one infected seed in total sample sizes of either 100, 500 and 1000. Control seed samples having no infected sample were also soaked in the ELISA general extraction buffer, and were ground first

Three different primer pairs (Table 4.1) were optimized for detection of MCMV. The 25 µl reactions consisted of 1x qScript XLT One-step RT-qPCR with ROX buffer, 300 nm of each forward and reverse primers for MCMV specific gene and *nad*5 reference gene, 200 nm each of the MCMV specific and reference probe (Table 4.1), six microlitres of RNA and depc-treated water. Real time PCR profile and data collection stages were similar to that described in section 3.2.1.6. Samples with Ct values that were lower than that of the negative healthy control were designated as positive for MCMV.

Table 4.1: Primer sets used for optimization of the real time RT-PCR assay

Primer set	Primer Name	Sequence	Reference
1	MCMV-FP	5′-GAGTCCTGCCAATCCAAAGTG-3,	(Liu *et al*., 2016b)
	MFMV-RP	5′-TGGGTGGGTCAAGGCTTACTA-3’	
	MCMV probe	5′-FAM-AGCCGCCGCCCACTCTCCAG-BHQ-3’	
2	MCMV-F	5’-CCGGTCTACCCGAGGTAGAAA-3’	(Adams *et al*., 2013)
	MCMV-R	5’- TGGCTCGAATAGCTCTGGATTT-3’	
	MCMV Probe	5’FAM-CAGCGCGGACGTAGCGTGGA-BHQ-3’	
3	MCMVfp ZHA	5’-CGTATCACTTGGGAAACA-3’	(Zhang *et al*., 2011)
	MCMVrp ZHA	5’-CAGAGAGGAATGCCATGGA-3’	
	MCMV probe ZHA	5’ FAM-TCACAGCAGACACCACTAGCGGATACA-BHQ 3’	
Reference	*nad5*-F	5’-GATGCTTCTTGGGGCTTCTTGTT-3’	(Menzel *et al*., 2002)
	nad5- R	5’-CTCCAGTCACCAACATTGGCATAA-3’	
	nad5- probe	5’ VIC-AGGATCCGCATAGCCCTCGATTTATGTG-BHQ-3’	(Botermans *et al*., 2013)

4.2.2.2. Group test of varying number of grow-out samples seedlings

Healthy seed was sowed in trays with sterile soil mixed with vermiculite at a ratio of 2:1 in an insect-free laboratory at KALRO Kabete. Each tray was planted with fifty seeds. About one centimetre of the seedling leaf was sampled 10 days after sowing (Shango *et al*., 2019) and grouped in 50s and 100s. Leaf of seedlings infected with MCMV was sampled and chopped into similar size, and a piece added to the healthy leaf samples. The test was repeated 10 times since getting a positive detection was more difficult than when whole seeds were used. Healthy seedlings were also included in the experiments as negative control. The grouped samples were then ground in DAS-ELISA extraction buffer and tested as described in section 3.2.1.1.

Data collected from DAS-ELISA consisted of the absorbance (optical density-OD) readings at 405 nm. Group samples with absorbance readings that were twice that of the healthy control were reported as a positive detection of MCMV.

4.2.3 Determination of the effectiveness of detecting MCMV in working sample sizes of 400 seed

Working sample size of 400 seeds was evaluated for effectiveness in detection of MCMV. The seeds were tested in subsamples of 100 for whole seed and 50 for the grow outs. The lower amount used in grow outs was due to the infrequency of MCMV detection in this type of sample materials.

Four hundred seeds were obtained from the samples of commercial seed lots A and B and repeated three times. The samples were sub divided into 100-seed sub samples and ground in an IKA tube mill control grinder (Tube mill CS000, IKA, Staufen, Germany). The 100-seed subsamples allowed for all the seed to be ground well and also mixed together evenly. Each of the subsample was tested separately by DAS-ELISA. A working sample was recorded as positive if one of the sub samples tested positive for MCMV.

Similarly, 400 seeds working samples were sowed and tested as grow outs described in section 4.2.2.2. After 10 days, each of the germinated seedlings was sampled and grouped into sub sample of 50-growouts. The grouped samples were tested for MCMV using DAS-ELISA and real time RT-PCR as described in section 4.2.2.1. Positive subsamples were identified if the absorbance at A405nm was more than twice that of the negative control in DAS-ELISA, and if the Ct value in the real time RT-PCR was below that of the negative control. A working sample was labelled positive if one of the subsamples tested positive for MCMV.

A binomial distribution model was used to calculate the probability of successfully detecting MCMV infection/contamination in the seed and grow out samples from seed lots designated code A and B using R software, binGroup2 package (R Core Team, 2021). Each seed lot had three 400-seed sample. The assumption applied was that all the seeds had an equal probability to be tested and be positive if infected. The 400-samples were divided into four 100-seed subsamples for ungerminated seed and eight 50-seed subsamples for grow outs to increase the efficiency of detection of MCMV. If any of the subsample tested positive, the original 400-sample was recorded as positive.

4.3 Results

4.3.1 Seed sub-sample group size for detection of MCMV using ungerminated seeds

In the experiments where ground ungerminated (whole) seeds were used as materials, MCMV could be detected from samples with 100, 500 and 1000 seeds of which one seed was infected, using the DAS-ELISA and real time RT-PCR methods (Table 4.2). All the 12 replicates for the 100, 500 and 1000 seed samples with one infected seed soaked in buffer prior to grinding, tested positive for MCMV, with the A_{405nm} values being twice higher than those of the corresponding healthy seed amounts. However, when the extraction buffer was added after dry seed was ground, the 100-seed sample had the highest rate of detection of 0.75%. The method assumed that all the seeds from the infected plants were infected with MCMV.

The absorbance (A_{405nm}) values did not show any pattern or trend with increasing number of seeds. A qualitative DAS-ELISA method was used in this experiment, as the starting amount of virus in the sample was not quantified. Nevertheless, the $A405_{nm}$ values of the samples assigned as positive were more than twice that of the healthy seed control with similar number of seeds (Table 4.2).

Maize chlorotic mottle virus was detected in all four seed samples having one infected seed in 100, 500 and 1000, using real time RT-PCR. The cycle threshold (Ct) values increased as the sample group size increased (Table 4.2). The Ct values for the samples were all lower than those of the healthy seed negative control.

Table 4.2: DAS-ELISA absorbance readings and real-time RT-PCR cycle threshold values of maize chlorotic mottle virus presence in various sample sizes with one infected seed

		DAS-ELISA		Real-Time RT-PCR		
1 infected seed in no. of healthy seeds	No. of replicates*	Average# A405nm	No. of '+' samples	No. of reps	Average* Ct values	MCMV presence/absence
1000sg	12	1.17 ± 0.39	12			
500sg	12	1.43 ± 0.50	12			
100sg	12	1.30 ± 0.59	12			
1000sg healthy	3	0.1 ± 0.05	0			
500sg healthy	3	0.09 ± 0.02	0			
100sg healthy	3	0.11 ± 0.04	0	4	33.6 ± 2.66	Negative
1000d	8	1.31 ± 0.58	5	4	24.3 ± 0.67	Positive
500d	8	1.7 ± 0.86	5	4	22.1 ± 1.51	Positive
100d	8	1.34 ± 0.62	6	4	19.6 ± 1.31	positive
100d healthy	4	0.14 ± 0.01	4	4	33.6 ± 2.66	

'DAS-ELISA'- Double antibody sandwich enzyme linked immunosorbent assay; 'Real-Time RT-PCR'- Real time reverse transcription polymerase chain reaction; 'sg' - seeds soaked prior to grinding; 'd'- seeds ground while dry (without a soaking step). *Average of duplicates. # Average of the replicates ± standard deviation. '+'- MCMV positive

4.3.2 Seed sub-sample group size for detection of MCMV using seedling grow outs

Healthy seedlings were raised from seed and were all negative for MCMV when tested using DAS-ELISA. However, efforts to obtain an MCMV infected grow out seedlings, from seeds of MCMV infected plants obtained from the greenhouse, yielded no positive sample. However, when an infected piece of leaf from MCMV infected plant of similar size to the grow outs was added to make a group size of 50 and 100, MCMV was detected on all the samples. MCMV was detected in serial dilutions of the infected leaves sap up to 1 X 10^{-4} level (Table 4.3).

4.3.3 Probability of successful detection of MCMV in 400-seed working sample size using whole seeds and seedling grow outs

The whole seeds were ground in sub samples of 100-seeds, to allow all seed to be ground well, and mixed together properly. MCMV was positively detected using DAS-ELISA from the three replicates of the two seed lots used in the experiment (Table 4.4). The DAS-ELISA absorbance values were twice higher than those of the negative controls. The subsamples of Lot A sample I had high variance of the absorbance readings which may be attributed to the higher virus titre in the seeds contained in some of the sub samples.

Table 4.3: Absorbance readings of serially diluted maize chlorotic mottle virus (MCMV) leaf sap by DAS - ELISA

Dilution level	Average absorbance
10^{-7}	0.105
10^{-6}	0.131
10^{-5}	0.200
10^{-4}	0.425
10^{-3}	0.446
10^{-2}	0.474
10^{-1}	0.363
Undiluted sample	0.613
Healthy leaf	0.150

Table 4.4: Detection of maize chlorotic mottle virus (MCMV) by DAS-ELISA on ground seed subsamples for 400-seed sample size Lot A and B

	DAS ELISA readings of 100-seeds subsample			
	subsample 1	subsample 2	subsample 3	subsample 4
Lot A				
Sample I	0.597	0.720	1.106	0.816
Sample II	0.427	0.280	0.305	0.620
Sample III	0.411	0.559	0.637	0.345
Lot B				
Sample I	0.750	1.258	0.878	1.021
Sample II	0.721	1.390	0.78	1.054
Sample III	0.491	0.467	0.454	0.496
negative control	0.071	0.081	0.077	0.095
2 x negative control	0.142	0.162	0.154	0.190
positive control	0.650	0.527	0.639	0.590

Absorbance values that were higher than twice the negative control absorbance values were considered as a positive detection

The calculated probability of successfully detecting MCMV in 400-samples of ungerminated seeds using the detection data from DAS-ELISA for the two seed lots is 100%. However, when three 400-seed samples were sowed and grow outs tested for MCMV using DAS-ELISA in subsample sizes of 50, two samples from lot A had sub samples that were positive (Table 4.5). No sub sample was positive for MCMV in lot B. Further, when three 400-seed samples were tested for MCMV using real time RT-PCR, one samples each of lot A and B were positive for MCMV (Table 4.6).

Table 4.5: Detection of maize chlorotic mottle virus (MCMV) from seedling grow outs subsamples for 400-seed sample size for Lot A and B using DAS-ELISA

	50-seedlings subsample							
	1	2	3	4	5	6	7	8
Lot A								
Sample I	0.108	0.093	0.099	**0.486**	0.099	0.095	0.112	0.098
Sample II	0.137	0.096	0.095	0.16	0.095	0.092	0.154	0.098
Sample III	0.115	0.102	0.092	**0.311**	0.094	0.092	**0.260**	0.101
Lot B								
Sample I	0.103	0.094	0.097	0.121	0.092	0.096	0.108	0.096
Sample II	0.110	0.101	0.094	0.096	0.090	0.102	0.111	0.091
Sample III	0.104	0.0945	0.095	0.087	0.093	0.093	0.101	0.094
negative control	0.076	0.093	0.096	0.102	0.103	0.094	0.097	0.098
2 x negative control	0.152	0.186	0.192	0.204	0.206	0.188	0.194	0.196
positive control	0.250	1.236	0.294	1.259	0.2965	0.323	0.390	1.245

Values displayed are the average absorbance values of a duplicate sample. A positive detection of MCMV was identified if the absorbance values were higher than twice that of the negative controls. Values of subsamples in bold font indicate a positive detection of MCMV.

Table 4.6: Ct values from Real time RT-PCR detection of maize chlorotic mottle virus (MCMV) from seedlings grow outs for 400-seed sample size for Lot A and B

	50-seedlings subsample							
	1	2	3	4	5	6	7	8
Lot A								
Sample I	nd	nd	nd	nd	nd	nd	nd	Nd
Sample II	nd	nd	nd	nd	nd	nd	nd	Nd
Sample III	nd	36.33	nd	nd	**29.59**	38.55	**27.24**	nd
Lot B								
Sample I	32.11	**29.09**	32.18	31.44	33.65	32.80	**27.10**	nd
Sample II	33.09	32.98	37.05	33.01	32.35	32.64	31.58	36.29
Sample III	nd	nd	nd	34.28	nd	nd	nd	nd
average negative control	32.09							
average positive control	17.40							

nd- no detectable value. Values of subsamples in bold font indicate a positive detection of MCMV.

The probabilities of successfully detecting MCMV from 400-seed sample of grow out seedlings from the two seed lots was 0.101%, when testing was achieved using DAS-ELISA and real time RT-PCR.

4.4 Discussion

An adequate working sample is critical in the final determination of the status of seed lot, while ensuring a cost effectiveness of the seed health test. The statistical working sample size used in seed health testing of MCMV is 400 seeds (Shango *et al*., 2019). This study set to evaluate the assay sensitivity of one infected seed in a sample size and to determine the adequacy of the 400-seed working sample in detection of MCMV in seed lots. The study has shown that use of DAS-ELISA and real time RT-PCR detected one MCMV infected seed in up to 1000-seeds sample of ungerminated whole seed samples. The assays are thus sensitive enough for testing for MCMV contamination in ungerminated samples from seed lots. The limitation of detection of the 1000-seed sample was the availability of equipment to grind such large seed samples, and also the samples required an overnight soaking in extraction buffer while using DAS-ELISA. These are some of the factors that influence the determination of subsample size to ensure assay sensitivity (Albrechtsen, 2006). However, when detecting MCMV in seedling grow outs, positive outcomes were rare. All seedlings grown out from seeds that were obtained from MCMV infected maize plants, yielded no MCMV positive samples. Due to the rarity of obtaining a seed transmitted seedling from the available contaminated seed, the limit of detection of MCMV in seedlings was determined as the lowest concentration of virus detected in the samples analysed earlier study objectives (section 3.3).

Sampling procedures in seed health testing are borrowed from those of seed lot features such as germination and physical purity (Morrison, 1999). In maize seed lots a representative working sample has 400 seeds. The use of whole seeds in detection of MCMV in maize samples of 400-seeds each from two seed lots using DAS-ELISA generated a probability for successful detection of 98%, revealing the adequacy of the sample size. The samples were grouped into smaller subsamples of 100-seed each, which ensured all the seeds were well ground, and also managed the cost of detection by having only four subsamples for each 400-seed sample. When 400-seed samples from two seed lots were evaluated for the probability of MCMV detection using grow

outs, the success of detection of was low (77%) as compared to the use of whole seeds. The low detection in grow outs was similar to that reported in other studies (Jensen *et al*., 1991; Gatunzi *et al*., 2022). The use of real-time RT-PCR is thus of importance when detecting MCMV in seed grow-outs due to the low viral load in the seedlings. Real-time RT-PCR was more sensitive as compared to the other methods of detection (section 3.4). The low probability of detection would require that more seed per sample obtained from a seed lot to be increased, in order to improve the detection probability. However, this would increase the testing cost. In seed health testing there is a caveat that the results of a test are confirmed as the true status of the sample tested and not the whole seed lot (Morrison, 1999), thus for a pathogen that has low detection in seedling grow-outs, a negative status of a seed lot can be achieved due to this. Transmission of viruses from seed to seedlings is affected by a number of factors, including the host-virus interaction, level of seed infection and environmental conditions (Sastry, 2013c). Both viable and inactivated viruses are found on whole seeds samples and these increase the detection limit when whole seeds are used. This reflects the contamination level of the seed lot (Albrechtsen, 2006). However, more seed lots should be tested to confirm the probabilities that are presented in this study.

The results in this study show that the type of sample i.e., whole seeds and seedling grow outs, were found to influence the detection of MCMV. Since not all virus is viable, use of whole seeds is recommended only when the results obtained correspond closely to the actual seed transmission of the virus (Albrechtsen, 2006). From the data, the variance of detection in whole seed as compared to grow outs shows that this is not the case for MCMV. Similar results were obtained with the barley stripe mosaic virus (BSMV; Malgorzata, 2001), tomato leaf curl New Delhi virus (ToLCNDV; Kil *et al*., 2016) and tomato brown rugose fruit virus (ToBRFV; Davino *et al*., 2020), with high detection in whole (ungerminated) seed samples unlike in seedlings. Seed borne virus is detected from the various seed parts, while transmitted virus in the seedlings is mainly found in the embryo (Sastry, 2013c). Not all the detected virus in whole-seed assays is transmitted to the next progeny. This depends on the virus-host interactions, with whole seed assays giving an overestimation of the transmission of the virus (Albrechtsen, 2006).

This study has shown that testing of whole seeds gives a higher chance of detecting MCMV unlike in seedlings generated from the seeds that are being tested. Since the probability of detecting the virus in the seedlings is low, the effect of different levels of virus contamination/infection in seeds, on the transmission of the virus, is required to determine the threshold allowable when the seed health tests use whole seed. Tolerance is the allowable limit of contamination/infection on seeds by a pathogen (Morrison, 1999). The tolerance levels of a pathogen are useful in management during production. This study has presented the actual probabilities of detection of MCMV using the standard 400 seed-samples.

CHAPTER FIVE

ANALYSIS OF SEED TRANSMISSION RATE OF MAIZE CHLOROTIC MOTTLE VIRUS OF CONTAMINATED COMMERCIAL SEED LOTS AND THE EFFECT DUE TO GENOTYPES AND TIME OF INFECTION

Abstract

The first report of Maize chlorotic mottle virus (MCMV) in eastern Africa region was in 2011, in Bomet county in Kenya. The virus isolate was found to have 96-99% similarity to those reported globally. The virus causes maize lethal necrosis (MLN) disease when in combination with a cereal potyvirus. The study was set to determine the seed transmission rate of MCMV isolate found in Kenya. Commercial seed lots that had previously tested positive for MCMV were obtained and used in the study. Further, several genotypes were mechanically inoculated with MCMV and assessed for their potential to transmit MCMV from the seed obtained. Similarly, three varieties were mechanically inoculated with MCMV at four crop stages and the seed obtained here also evaluated for differences in transmission of MCMV to seedlings. Overall, low seed transmission of less than 1% was reported for all the experiments carried out. Genotypes with tolerance to MCMV were found to transmit the virus, as well as the susceptible ones. Transmission of MCMV from seeds obtained from plants inoculated at four-leaf stage was 0.1% and that at eight-leaf stage was 0.04%. The level of severity of MCMV infection for plants inoculated at fifteen-leaf and tasselling stages was not significantly different. Seed transmission of MCMV Kenyan isolate compares to that reported from the Hawaii isolate. The low transmission rates may however be rendered complex by the secondary transmission of MCMV by vectors in the field. The results of this study emphasize on the importance of continual testing for MCMV for seed certification to avoid the introduction of the virus into fields, and management of the seed production fields to protect the crop from the viruses causing maize lethal necrosis disease.

5.1 Introduction

In 2011, MCMV was reported in Kenya. The spread of the virus was identified to be by vectors, especially corn thrips, and also mechanically and via seed (Wangai *et al*., 2012; Mahuku *et al*., 2015a). Despite the low seed to seedling transmission of MCMV, the problem is compounded by the secondary transmission by corn thrips in the field that would spread the low amounts presented through the seedlings. Transmission of MCMV through seed has been reported from seed lots in Hawaii at an average transmission rate of 0.33%. Further low transmission has been reported from hybrids and inbred lines inoculated with the virus (Uyemoto *et al*., 1980; Bockelman *et al*., 1982; Gatunzi *et al*., 2022). However, there are few studies on the transmission of MCMV from commercial seed lots infected naturally, and none has been reported for the Kenyan isolate. The studies carried out on the Kenyan isolate have also utilized small amounts of seed, thus a lower confidence level. Merchants produce maize seed from fields that are rain-fed or irrigated and are exposed to various factors that can affect transmission of the virus. These factors include environmental conditions, cultivars and virus isolate-host and the interactions (Albrechtsen, 2006). The aim of this study was to estimate the rate of seed to seedling transmission of MCMV Kenyan isolate in commercial seed lots in Kenya that were naturally infected with the virus, and to determine the effect of genotype and crop stage at the time of infection on the transmission of the virus from seed. This information will contribute to the knowledge of the spread of MCMV and further guide management of MLN.

5.2 Materials and Methods

5.2.1 Seed to seedling transmission from commercial seed lots

5.2.1.1 Source of seed used in the study

Maize chlorotic mottle virus isolates contaminated seed from maize seed lots were obtained from a seed merchant, under the supervision of KEPHIS. This were used to study the rate of seed transmission. Further, experiments were laid out to generated seeds from maize infected with MCMV as explained below and were used to determine the role of the genotypes and the time of infection to seed transmission of the virus.

Twenty-nine commercial seed lots (numbered as lots K1-K27, plus lots A and B) were sampled and used for testing for the presence of MCMV. One seed lot from the same seed merchant was

used as a negative control, having been tested for MCMV by Kenya Plant Health Inspectorate Service (KEPHIS). The MCMV-positive seed lots had been rejected for seed processing by KEPHIS, the national seed regulatory agency, due to non-compliance to seed certification standards, while some had been rejected due to detection of MCMV. The seed was naturally infected with MCMV in the fields. Sampling of the seed lots was carried out following the guidelines described by the International seed testing association (ISTA, 2016) in year 2017 for K1-K27 lots, and year 2018 for lots A and B. Seeds for K1-K27 were harvested in 2015 and were used in the study from year 2017, while those from lots A and B were harvested in 2017 and used from 2018. During the period of the study, the seed was stored at moisture content of below 13% at room temperature in gunny bags. Seed lots K4, K27, A and B were further used to determine the seed transmission rate of MCMV Kenyan isolate, since they were each obtained from one source and not blended from various farms. Since the storage period from time of harvesting was shorter for Lots A and B, more of these seeds was used in the assays.

5.2.1.2 Detection of MCMV in seedling grow outs of commercial seed lots

Four hundred seeds from the representative sample of the seed lots were sowed in trays filled with sterile forest soil vermiculite mixture (ratio 2:1). Each tray had fifty seeds, and the trays were well watered and placed in insect-free laboratory. The laboratory was sprayed weekly with pesticides to control any MCMV vectors. After 10 days, the germinated seedling grow outs were counted and recorded per tray before obtaining a leaf sample from all the seedlings. Leaf samples from seedlings of the same tray were tested as a grouped sample.

Real time RT-PCR, as described in chapter four, was used to detect MCMV from the seedling grow out samples of the 29 seed lots. Positive detection of MCMV in at least one of the subsamples of the seed lots, when the Ct value was below that of the negative control, was interpreted as MCMV presence in the 400-seed sample.

5.2.1.3 Determination of seed transmission rates in commercial seed lots in Kenya

Seeds from lots K4, K27, A and B were sowed and the seedling grow outs counted, recorded and sampled. The grouped samples per tray were tested using DAS-ELISA with antibodies raised against MCMV prepared at the University of Minnesota. A total of 400, 4,800, 15,319, and

17,098 seedlings were used for determination of the seed transmission rates for seed lots K4, K27, B and A respectively. These number of seedlings were determined by the quantity of seed available release for research from the seed merchant per lot at time of the study. However, the acceptable sample per sampling is one (ISTA, 2016), thus 400 seeds were an adequate amount from a maize seed lot as it represents one sample. A positive detection of MCMV was determined when the absorbance value obtained by DAS-ELISA was twice that of the negative control. The number of positive grouped samples were recorded against the number of individual samples in each of the groups.

Further, samples from 4,837 and 3,485 grow out seedlings of Lot A and B were tested for MCMV transmission from seed using real time RT-PCR. The data collected was the threshold cycle (Ct) values from the StepOnePlus software (Applied Biosystems, Thermo Fisher Scientific). Ct values that were lower than the negative controls were considered as positive samples.

5.2.1.4 Seed transmission determination using visual symptoms on plants raised from MCMV contaminated seed

Testing for seed to seedling transmission of MCMV was also carried out using visible symptoms on the leaves of 720 plants of each of commercial seed lots A and B. Six seeds were sown in a pot in the MLN screenhouse at the KALRO Kabete Centre. The screenhouse was sprayed using pesticides every week to control any MCMV vectors using either 50g/litre lufenuron (Match 050EC, Syngenta), 480g/litre flubendiamide (Belt 480SC, Bayer Crop Science) and 19g/litre emamectin benzoate (Escort 19EC, Greenlife Crop Protection).

Observation of MCMV symptoms on the plants was carried out for seven weeks. Maize chlorotic mottle virus symptoms were scored on a 1 to 5 scale, where 1 was no visible MCMV symptoms, 2 = fine chlorotic streaks on leaves, 3 = chlorotic mottling throughout plant, 4 = excessive chlorotic mottling, necrosis on leaves and in some cases dead heart symptom and 5 = complete plant necrosis (Karanja *et al*., 2018).

5.2.2 Development of seed from various genotypes infected with maize chlorotic mottle virus

5.2.2.1 Source of planting material

Certified seed for commercial maize hybrids were obtained from the local agrovet and breeder's hybrids and two inbred lines (MLN009 and MLN 012) from KALRO for use in the experiment (Table 5.1). The commercial hybrids are all susceptible to MLN except DK777, which is tolerant to MLN. The breeders' hybrids and inbred lines have varying tolerance to MLN. The seeds for the genotypes labelled as missing were not available in the specified year.

Table 5.1: List of the maize genotypes included in the experiments evaluating the effect of genotype on seed transmission of maize chlorotic mottle virus (MCMV)

Genotype	Source	2017	2018
DK777	Agrovet	√	√
DK8031	Agrovet	√	√
DH02	Agrovet	-	√
DUMA 43	Agrovet	√	√
H614	Agrovet	√	√
H6213	Agrovet	√	√
H625	Agrovet	√	√
KH500-33A	Agrovet	√	√
PHB3253	Agrovet	√	√
PH30G19	Agrovet	√	√
WE1101	CIMMYT/KALRO	√	-
WE5138	CIMMYT/KALRO	√	√
WE5139	CIMMYT/KALRO	√	√
KATEH 16-01	KALRO	√	√
KATEH16-02	KALRO	√	√
WE5140	CIMMYT/KALRO	√	√
WE5135	CIMMYT/KALRO	√	-
KATEH 17-01	KALRO	√	-
KATEH17-08	KALRO	√	-
KATEH 17-03	KALRO	√	-
KATEH 17-10	KALRO	√	-
KATEH 17-13	KALRO	√	-
MLN009	KALRO	√	-
MLN012	KALRO	√	-

"-" missing seeds, not available. "√"- present in the year for evaluation

5.2.2.2 Planting and field management

The seeds from the genotypes were planted at a quarantine field at KALRO Njoro centre in 2017 and 2018. The centre is situated in Nakuru county in Kenya at an altitude of 2172 m above sea level, and at Latitude 0° 20°S; Longitude 35°. 56E'. Each plot had five rows and 11 plants per row at a spacing of 0.25 m between holes and 0.75 m between rows. The experiment was laid out in a split-plot design, with the genotypes as the main plots and the concentration of virus inoculum assigned to each plot. Three guard rows of a commercial variety PH30G19 were planted around the experimental area. Di-ammonium phosphate (DAP) fertilizer was applied at planting at a rate of 60 kg/ha of phosphate (P_2O_5). Calcium ammonium nitrate (CAN) fertilizer was applied eight weeks after planting at a rate of 60 kg/ha of nitrogen. Weeds were removed from the plots throughout the experiment period. Maize chlorotic mottle virus and SCMV vectors and fall army worm, were controlled using different insecticides which included 50 g/litre lufenuron (Match 050EC, Syngenta), 45 g/litre Chlorantraniliprole and 18 g/litre Abamectin (Voliam targo® 063SC, Syngenta) and 19 g/litre emamectin benzoate (Escort 19EC, Greenlife Crop Protection).

5.2.2.3 Preparation of MCMV inoculum and inoculation of the maize seedlings

Maize chlorotic mottle virus collected from Bomet County, Kenya and maintained in H614 maize hybrids were sourced from the KALRO Biosafety level II green house. Young leaves from the plants were harvested, chopped into smaller pieces and ground in 0.1 phosphate buffer (ratio 1:10) using a commercial blender. The mixture was passed through a muslin cloth and then stored at 4°C during transportation and prior to inoculation. Further, the inoculum was serial diluted to 1:100, 1:1000 and 1:10000 with the phosphate buffer. Prior to inoculation, carborundum (0.6% of 22 µm grit) was added to the inocula.

At four leaf stage, the seedlings were inoculated using the four concentration levels of inoculum using a mechanized sprayer. The process was repeated after one week. The leaves were sprayed and covered with the diluted MCMV inoculum (1:10, 1:100, 1:1000 and 1:10000). The severity of MCMV symptoms were scored every week using a scale of 1 to 5 (Karanja *et al*., 2018) as details outlined in section 5.2.1.4. At maturity, the maize seed was harvested from the middle three rows threshed and winnowed by hand to avoid cross contamination between the different

plots. The seeds were then sun-dried to a moisture content of 13%, before storage at room temperature.

5.2.2.4 Detection of MCMV transmitted from seeds to seedlings in grow outs

The seeds obtained from the genotypes inoculated with 1:10 virus inocula concentration were sowed in trays in the insect-free laboratory as explained in section 5.2.1.2. The seeds were used within one year from time they were harvested. Ten-day old leaves were obtained from the seedlings in each tray and tested in groups using DAS-ELISA and real time RT-PCR. The data collected was presence or absence of MCMV as explained in section 3.2.3.

5.2.3 Development of seed from maize crop infected with MCMV at different growth stage

5.2.3.1 Planting materials

Certified seeds of three commercial maize varieties were purchased from the agrovet stores. The varieties were Duma 43 and H614 which are susceptible to MCMV and DK777 which is tolerant to the virus.

5.2.3.2 Planting, inoculation and field management

Maize chlorotic mottle virus infected plants were obtained through an experiment laid out at the quarantine field at KALRO Njoro. The experiment was laid out in plot sizes, and field management practices similar to what is described in section 5.2.2.2, with three replications in a split plot design. The main plots were crop stages at time of mechanical inoculation which were: four-leaf (V4), eight leaf (V8), 15-leaf (V15), tasselling (VT) stages and a control that was not inoculated. The three varieties were included in all the replications in the main plots. Inoculation was carried out at each crop stage using 1:10 diluted inocula described above, using a mechanized sprayer.

Guard rows of variety PH30G19 were planted in between the main plots to separate the crops inoculated at different crop stages. Maize chlorotic mottle virus symptoms severity was scored using the scale described above (section 5.2.2.3) once a week. Harvesting, threshing and storage was similar to the previous experiment (section 5.2.2.3).

5.2.3.3 Detection of MCMV in seedlings from the seeds obtained from plants infected at different crop stage

The seeds obtained from the cobs developed from the maize plants infected with MCMV at different crop stages were sowed in trays in an insect-free laboratory. The seedlings were allowed to grow for ten days as previously described, before sampling the leaves and testing them in groups represented by the tray where they were obtained from. The samples were tested for presence of MCMV using DAS-ELISA and real-time RT-PCR. The data obtained was recorded as MCMV present or absent.

5.3 Data analysis

For the determination of seed transmission rates, data on the count of samples that were positive for MCMV was recorded as well as the total number of seedlings per grouped sample. The seed transmission rates were estimated at 95% confidence interval using the pooled prevalence tests in Epitools epidemiological calculators (Sergeant, 2018) applying a maximum-likelihood estimate of prevalence of the virus for the grouped samples. Since the germination varied per tray, the variable pool sizes option was applied. Seed transmission was calculated using each data obtained from DAS-ELISA, real time RT-PCR and visual symptoms separately. The percentage seed transmission rates for experiment using visual symptoms were calculated using the number of seedlings showing MCMV symptoms at seven weeks divided by the total number of plants that germinated multiplied by 100.

The severity of MCMV in the experiment where different genotypes were inoculated with the four levels of inoculum concentrations were analysed using the proportional odds logistic regression (polr) function (R Core Team, 2021). The seed transmission rate of MCMV for the genotype evaluated was determined by subjecting the data of the number of MCMV positive grouped samples and total number of samples assayed to the maximum likelihood method. The pooled prevalence tests on Epitools epidemiological calculators using varying pools were used (Sergeant, 2018).

The severity of MCMV in the plants infected at different crop growth stages was also compared using ordinal logistic regression model using the proportional odds logistic regression (polr)

function from the MASS package on the R software (R Core Team, 2021). The severity scores were grouped into three: group one had scores that were 1.5 and below, group two had scores that were above 1.5 up to 3, and group three had scores that were between 3 and 5. The association of crop stage at time of infection and to the disease severity was first determined independently, then the effect of the varieties factored in. Seed transmission rates were determined as described in previous paragraph above.

5.4 Results

5.4.1 Maize chlorotic mottle virus in commercial seed lots in Kenya

When samples from the 29 maize seed lots were tested for MCMV, 17 of them had at least one sub sample that had a positive detection of MCMV (Table 5.2).

Table 5.2: Detection of maize chlorotic mottle virus (MCMV) in commercial seed lots in Kenya using real time RT-PCR

Seed Lot label	MCMV Presence/absence	Seed Lot label	MCMV Presence/absence
K01	+	K15	+
K02	+	K16	-
K03	+	K17	+
K04	+	K18	+
K05	+	K19	-
K06	-	K20	-
K07	+	K21	-
K08	-	K22	-
K09	+	K23	+
K10	+	K24	+
K11	+	K25	-
K12	-	K26	-
K13	-	K27	+
K14	-	A	+
		B	+

'+'- MCMV present, '-' MCMV absent

5.4.2 Seed transmission rates of MCMV from commercial seed lots in Kenya

The transmission rate of MCMV was estimated using samples from seed lots K4, K27, A and B. The transmission rate ranged from 0-0.57% when testing was done using DAS-ELISA, with seed lot K4 having the highest seed transmission rate despite fewer numbers of seeds tested (Table 5.3). Of the 820 grouped samples with varying sample sizes tested, 21 grouped samples tested positive.

Table 5.3: Number of seedlings grow-outs from commercial seed-lots, groups tested and number of positive samples observed in detection of maize chlorotic mottle virus (MCMV) using DAS-ELISA, and estimated seed transmission rate

Seed Lots	No. of seedlings tested	No. of positive groups/ Total no. of groups tested	Estimated seed transmission rate
Lot A	17,098	15 /360	0.09%
Lot B	15,319	4/356	0.03%
Lot K27	4,800	0 /96	0
Lot K4	400	2/8	0.57%

When real time RT-PCR was used to assess samples from seed lot A and B, the transmission rates were 0.02 and 0.03% for Lot A and B, respectively. The number of seedlings tested were 4,837 and 3,485 for Lot A and B, respectively, with each lot having one sample positive for MCMV.

5.4.3 Visual diagnosis of MCMV infection in seedlings from contaminated seeds

Out of the 1,228 seedlings that germinated from a possible 1,440, only two plants, from seeds from Lot B, developed visible MCMV symptoms (0.16% transmission rate). The symptoms were observed at 35 days after emergence of the plants. Majority of the plants had an average score of one, and showed no significant difference ($P>0.05$) in the severity of MCMV symptoms that were visible.

5.4.4 Seed transmission rates from various genotypes infected with MCMV

The mean severity of MCMV decreased with increased dilution of the inoculum, with more symptoms observed where the inoulum dilution was lowest. Plants inoculated with the lowest dilution of 1:10, had the highest mean MCMV severity score of 2.78 for the two years. At 63 days after inoculation, the mean severity scores for all the genotypes were significantly higher

for plants inoculated with 1:10 inoculum (mean severity 2.55) and least for 1:10000 (mean severity 1.99) (Table 5.4). The mean severity was significantly different for the 15 genotypes that were evaluated, with DH02 having the highest mean severity scores for the genotypes available in year 2017 and 2018.

Only seeds from plants inoculated with 1:10 concentration were used for determination of seed transmission rates of MCMV. Transmission from seeds to seedlings ranged from 0 to 0.48%. Testing of seeds obtained from 2017, four grouped samples were positive for MCMV, while only one grouped sample was positive for the seedlings tested from the seeds obtained in 2018 (Table 5.5).

Table 5.4: Regression coefficients, standard error and odds ratio of maize chlorotic mottle virus (MCMV) severity scores for 15 genotypes and virus inoculum dilutions

	Coefficient	**Std.Error**	**p.value**	**Odds ratio**
Genotype				
DK777	-5.64	1.12	0.000	0.00
DK8031	-0.11	0.95	0.911	0.90
DUMA 43	-1.60	0.95	0.094	0.20
H614	-1.67	0.93	0.071	0.19
H6213	-2.63	0.93	0.005	0.07
H625	-1.15	0.95	0.223	0.32
KATEH 16-01	-2.33	0.91	0.011	0.10
KATEH 16-02	-3.25	0.94	0.001	0.04
KH500-33A	-2.31	0.92	0.012	0.10
PH30G19	-0.92	0.93	0.324	0.40
PHB3253	-0.52	0.93	0.578	0.60
WE5138	-6.27	1.21	0.000	0.00
WE5139	-21.74	0.00	0.000	0.00
WE5140	-7.18	1.41	0.000	0.00
DH02	*			
Virus Inoculum concentration				
A (1:10)	2.99	0.56	0.000	19.91
B (1:100)	1.28	0.52	0.014	3.61
C (1:1000)	0.32	0.51	0.531	1.37
D (1:10000)	*			

Std.error- standard error, * reference group

Table 5.5: Seed transmission rates of maize chlorotic mottle virus (MCMV), number of seedlings grow outs for the various genotypes and the number of grouped samples that tested positive using DAS-ELISA

Genotype	No. of seedlings tested	No. of positive grouped samples	seed transmission (%)	Genotype	No. of seedlings tested	No. of positive grouped samples	seed transmission (%)
2017				**2018**			
DH02	-	-	-	DH02	1205	1	0.08
DK777	1283	0	0	DK777	1463	0	0
DK8031	929	4	0.48	DK8031	1371	0	0
DUMA 43	995	2	0.21	DUMA 43	1116	0	0
H614	1345	0	0	H614	1272	0	0
H6213	998	0	0	H6213	1332	0	0
H625	974	0	0	H625	1119	0	0
KATEH 16-01	875	0	0	KATEH 16-01	1361	0	0
KATEH 16-02	864	0	0	KATEH16-02	1417	0	0
KATEH 17-01	859	0	0	KATEH 17-01	-	-	-
KATEH 17-03	1019	0	0	KATEH 17-03	-	-	-
KATEH 17-08	948	0	0	KATEH 17-08	-	-	-
KATEH 17-10	952	0	0	KATEH 17-10	-	-	-
KATEH 17-13	862	0	0	KATEH 17-13	-	-	-
KH500-33A	1000	4	0.45	KH500-33A	1399	0	0
PH30G19	-	-	-	PH30G19	1211	0	0
PHB3253	987	0	0	PHB3253	1258	0	0
WE1101	990	0	0	WE1101	-	-	-
WE5135	928	0	0	WE5135	-	-	-
WE5138	963	4	0.46	WE5138	1315	0	0
WE5139	1021	0	0	WE5139	1379	0	0
WE5140	864	0	0	WE5140	1261	0	0

When real time RT-PCR was used to detect MCMV in 1,642 seedlings harvested in 2018 from plants inoculated with 1:10 concentration of virus inoculum, nine grouped samples were positive for MCMV (Table 5.6). Transmission rates of below 1.04% were obtained.

Table 5.6: Seed transmission rates of maize chlorotic mottle virus (MCMV) of genotypes where samples tested positive using real time RT-PCR

Genotype	Seed transmission rate (%)
DK8031	1.00
H6213	0.29
H625	0.64
KATEH 16-01	0.30
KATEH 16-02	0.67
PH30G19	1.04
PHB3253	0.38
WE5138	0.32
WE5140	0.57

5.4.5 Maize chlorotic mottle virus in seedlings grow outs for seed obtained from plants inoculated at different crop stages

The single variable models showed that the effect of crop stage at inoculation, variety and year to severity of MCMV were significantly different at $p<0.001$, $p<0.001$ and $p<0.05$ respectively. The model with the lowest residual deviance and Akaike information criteria (AIC) was selected for ordinal logistic regression analysis. This model comprised the interaction of the treatment (crop stage at time of infection), the varieties and the year (Table 5.7).

The effect of time of inoculation in development of MCMV severity in maize was significantly higher ($P<0.0001$) for crops inoculated at V4 and V8 stages, while the severity due to MCMV was higher for Duma 43 and H614 as compared to DK777. MCMV severity was significantly higher in year 2017 as compared to year 2018 (Table 5.8).

Table 5.7: Comparison of ordinal logistic regression models prior to selection of analysis

Variables	Number of parameters	Residual deviance	AIC
Crop stage	5	272.32	290.32
Variety	3	280.26	294.26
Year	2	299.44	311.44
Crop stage x Variety	15	227.45	249.45
Crop stage x Year	10	265.37	285.37
Variety x Year	6	269.58	285.58
Crop stage x Variety x Year	30	213.68	237.68

AIC- Akaike information criteria

Table 5.8: Analysis of the coefficient's estimates, odds ratio and standard error of maize chlorotic mottle virus (MCMV) severity scores during the 2017 and 2018

Variable	Variable category	Estimate	S.E	p-value	odds ratio
Crop stage	Control				1.00
	four-leaf	24.71	0.48	<0.0001	5.38×10^{10}
	eight-leaf	23.06	0.48	<0.0001	1.04×10^{10}
	fifteen-leaf	0.0002	1.04	0.1	1.00
	Tasselling	0.0002	1.04	0.1	1.00
Variety	DK777				1.00
	Duma43	4.67	1.3	<0.001	1.06×10^{2}
	H614	1.64	0.7	0.018	5.17
Year	2017				
	2018	1.71	0.68	0.011	0.18

S.E. standard error

Seed transmission testing of grow out seedlings from the seed harvested in 2017 and 2018 from the plants infected at V4 and V8 stages were analysed using DAS-ELISA, as the severity scores of MCMV were significantly different from those of plants from the control plots. A total of four samples were positive for MCMV from the 2017 seeds, three from the plants infected at four-leaf stage and one from the eight-leaf stage, with overall seed transmission rate of 0.1% and 0.04%, respectively. Seed transmission was highest from seeds of H614 inoculated at V4 stage (Table 5.9). Analysis of the 2018 seedlings using real time RT-PCR yielded one positive grouped sample. The seedlings were from seeds of which the plants were inoculated at eight leaf stage (Table 5.10).

Table 5.9: Maize seedlings grow outs tested by DAS-ELISA and seed transmission rates of maize chlorotic mottle virus (MCMV) from seed obtained from plants of three varieties mechanically inoculated at four leaf and eight leaf stage

		Four-leaf			Eight-Leaf		
Year seed harvested	Variety	No. seedlings tested	positive samples	seed transmission %	No. seedlings tested	positive samples	seed transmission %
2017	DK777	1,031	1	0.1	1142	1	0.1
	DUMA 43	796	0	0	798	0	0
	H614	1,117	2	0.19	741	0	0
2018	DK777	1,640	0	0	1602	0	0
	DUMA 43	1,630	0	0	1528	0	0
	H614	1,632	0	0	1596	0	0

Table 5.10: Real-time RT-PCR of seedling grow outs to determine effect of crop stage at time of infection for plants inoculated at four- and eight-leaf stages on transmission of maize chlorotic mottle virus (MCMV) via seed

	Four-leaf			Eight-Leaf		
Variety	No. seedlings tested	positive samples	seed transmission %	No. seedlings tested	positive samples	seed transmission %
DK777	668	0	0	636	1	0.17
DUMA 43	639	0	0	652	0	0
H614	637	0	0	579	0	0

5.5 Discussion

Viruses that are transmitted through seed are important as they enhance the spread of disease in different areas (Bhat and Rao, 2020). This study set to ascertain seed transmission rate of MCMV isolate found in Kenya and to determine the effect of genotypes and crop stage at time of infection on the rate of transmission of the virus from seeds to seedlings.

Overall, the results from this study present the transmission of MCMV isolate found in Kenya from seed to seedlings. The rates of seed transmission were all below one percent, similar to that of isolates identified in Hawaii (Jensen *et al*., 1991), Kansas and Peru (Bockelman *et al*., 1982) and China (Zhang *et al*., 2011). Despite the study employing a large number of seeds for detection of MCMV in seedlings from the contaminated seed lots, the highest transmission recorded was 0.57%. The commercial seed lots that were assessed all had seed that had no visual

degradation of the quality of seed. Variation of the seed transmission rates maybe as a result of some of the seeds in the lots being obtained from farms with varying levels of MCMV infection.

Low transmission of MCMV from seed to seedlings was observed from the results from different genotypes assessed in this study. Despite there being significant differences in the severity of MCMV symptoms in the field, the seed transmission rates ranged from 0 - 0.48%. Transmission through seed was also detected for hybrid WE5140, KATEH 16-01 and KATEH 16-02 which are tolerant to MLN (Prasanna *et al*., 2020) as well as in genotypes that are susceptible to MCMV. Transmission of MCMV in seeds from genotypes that are tolerant to MCMV has also been reported (Mwatuni, 2021). All the genotypes used in the mentioned study had potential of transmitting MCMV, confirming the results in our study of no preferential genotype in the transmission of MCMV through seed. All the genotypes used in this study were the white, flint type, which is preferred in Kenya as human food.

The severity of MCMV in plants that were infected at four and eight-leaf stage were found to be significantly different from what was observed when the plants were infected at 15-leaf and tasselling stages, which were not significantly different ($P>0.05$) from the control. Despite the higher severity scores at four- and eight-leaf stages, the seed transmission rates detected were low at 0.1% and 0.04%, respectively. Early infection by viruses, earlier than flowering, leads to more severe symptoms, and allows for more seed transmission of viruses (Sastry, 2013b) infected. Seed transmission of viruses is influenced by different factors which include the virus-host interaction, age of seed and time of infection of the crop (Sastry, 2013b). The speed of movement of viruses in the host and ability to multiply in the plant inflorescence have been reported as the best estimators for seed transmission (Cobos *et al*., 2019).

The low transmission of MCMV from seed to seedlings in the above experiments is similar to that reported for other maize-infecting viruses. This includes maize dwarf mosaic virus (MDMV) with one detected seedling with seed transmitted virus out of 22,189 seedlings (Mikel *et al*., 1984) and three seedlings out of 38,473 tested seedlings for High plains virus in sweet corn seed (Forster *et al*., 2001). In the *Tombusviridae* family, seven other viruses alongside MCMV are transmitted by seed. These are cowpea mottle virus (CMeV), melon necrotic spot virus (MNSV),

pea stem necrosis (PSNV) and soybean yellow mottle mosaic (SYMMV) viruses all from the Gammacarmovirus genus, olive latent virus 1 (OLV-1) from genus Alphanecrovirus, cucumber leaf spot virus (CLSV) from genus Aureusvirus and tomato bushy stunt virus (TBSV) of the Tombusvirus genus (Sastry, 2013a; Sandra *et al*., 2020). As high as 65% - 73% seed transmission rates of some of the viruses have been reported (Nawaz *et al*., 2014; Sandra *et al*., 2020), in comparison to the low transmission rates revealed in this study of MCMV, the only member in the genus, Machlomovirus.

High transmission of viruses has been reported in legumes. In bean cultivars, bean common mosaic virus transmission was at a rate of 25.3% (Mandour *et al*., 2013), while in soybean mosaic virus was transmitted at a rate of 30% in soybean (Domier *et al*., 2007). Further in lablab beans, dolichos yellow mosaic virus was transmitted at a rate of 46.6% (Suruthi *et al*., 2018). Genetic material and plant structures have been shown as some of the pathways associated with transmission of viruses via seed (Wang and Maule, 1994). Legumes have more cytoplasmic connections that allow for more passage of virus to embryo, leading to more seed transmission of viruses (Sastry, 2013b). The transmission of viruses through seed is also controlled by either single or multiple genes in host plants (Wang and Maule, 1994; Sastry, 2013b). Other factors that may affect seed transmission are the ability of the virus to multiply in the seed, as well as the speed the virus moves in the host (Cobos *et al*., 2019). Transmission of MCMV through seed has also been reported to be higher (up to 3.1%), where seeds were from plants with synergistic infected with maize lethal necrosis causing viruses (Mwatuni, 2021).

Transmission of viruses through seeds is made complex by secondary transmission of the viruses by vectors in the field. Introduction of viruses in the field, even in low amounts might lead to spread where the vectors of the virus are present. Corn thrips have been identified in most areas where maize lethal necrosis disease has been reported (Mahuku *et al*., 2015a; Wangai *et al*., 2012). Maize chlorotic mottle virus is transmitted by corn thrips (*Frankliniella williamsi*) in a semipersistent manner by larvae and adults (Cabanas *et al*., 2013), with the adult thrips causing more damage due to the ability to move from the source of initial virus acquisition (Redinbaugh and Stewart, 2018). The thrips are attracted by volatile compounds in plants that are infected with MCMV (Mwando *et al*., 2018), thus presence of MCMV transmitted from seeds leads to

early spread of the virus. It is also reported that the development of maize lethal necrosis disease is more severe when MCMV infects a maize plant before the potyvirus, unlike when the potyvirus infects first (Kiambi *et al*., 2019).

Considering the economic damage due to MLN, introduction of MCMV in maize fields through seeds, even in low amounts, and the spread by vectors need to be managed. In seed health testing, detection of MCMV in a seed lot leads to the rejection of the batch for use as seed (Shango *et al*., 2019), thereby leading to huge losses in the seed production sector. This study provides information that contributes to the complex implementation of management approaches of MCMV (Prasanna *et al*., 2020), in the field especially at early growth stages to prevent the infection of seeds.

CHAPTER SIX

INFECTIVITY OF MAIZE CHLOROTIC MOTTLE VIRUS FROM CONTAMINATED SEED

Abstract

Maize chlorotic mottle virus (MCMV), one of the viruses causing maize lethal necrosis, leads to high yield losses. Limited information of MCMV infection or contamination of seeds and further transmission to the next progeny, is available. Seeds are exchanged globally, and thus the use of MCMV-free seeds will prevent the spread and prevalence of the virus in non-endemic areas. This study aimed at determining the infectivity of MCMV from contaminated seeds. The contamination levels of the commercial seed lots were determined by using ground seed extract. The presence of MCMV was confirmed using double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA). Infectivity assays were done by mechanically inoculating maize test plants with the seed soak solution and extracts from the contaminated seed. The range of seed contamination of the four seed lots tested was 4.9-15.9%, while visible MCMV symptoms were observed on less than 2% of the 547 seedlings inoculated with the seed soak and seed extract from contaminated seed. There were few infected seedlings observed from plants inoculated with contaminated seed extract. The results show evidence of low infectivity of MCMV from contaminated seed. However, the low infectivity is important since this can lead to introduction into the fields, and lead to secondary spread of the virus by vectors from seedlings developing from such seed. Seed testing of maize is important in order to confirm MCMV-free certified seed, thus controlling the introduction of the virus.

6.1 Introduction

Seed has been identified as a major source of introducing and spreading pathogens in crop fields (Buddenhagen *et al*., 2021). Seed infection is important as it is associated with reduced seed quality and seed coat damage, as well as impact on seed germination and spread of pathogens (Jeżewska and Trzmiel, 2009; Li *et al*., 2011). Viruses and other pathogens, have been found

located inside the seeds as well as on seed coats and endosperm with viable virus particles occurring outside the embryos (Dombrovsky and Smith, 2017). Such virus on the seed coat is transmissible to the seedlings, when the seed is sowed and through abrasion by soil particles. The virus then invades the embryo and moves to the seedlings during germination (Sastry, 2013b). The stability of viruses in seed depends on the type of virus, the location on the seed and the host (Sastry, 2013b). However, most viruses are viable in the embryo unlike other seed parts and are inactivated during seed maturation (Konate *et al*., 2001). However, the inactivated virus is detectable using serological and nucleic acid-based methods isolated from whole seed (Albrechtsen, 2006). Positive detection of MCMV has been reported from seed samples obtained from infected maize plants (Mahuku *et al*., 2015a; Zhang *et al*., 2011). Detection of the virus has also been confirmed from seeds obtained from infected maize genotypes which are resistant, tolerant and susceptible to MCMV (Gatunzi, 2018). Maize chlorotic mottle virus, unlike other maize-infecting viruses, is readily transmitted mechanically to maize seedlings (Redinbaugh and Stewart, 2018). Thus, it is important to determine the infectivity of the virus and ultimately the role of the detectable virus from the seed parts in epidemiology of MCMV. The objective of this study was to determine the infectivity of MCMV from contaminated/ infected seeds. The information generated in this chapter will further guide the management opportunities against the spread of MCMV.

6.2 Materials and Methods

6.2.1 Detection of MCMV in the seed lots samples using DAS-ELISA

Maize seed contaminated with MCMV was obtained from two sources; commercial seed lots and from plants mechanically inoculated with MCMV. Four commercial seed lots that were contaminated with MCMV, coded Lot A, B, K27, K4, were sampled and are described in chapters four and five. While seed from experimental plots were obtained from plants mechanically inoculated with the virus at four-leaf stage for varieties H614, DK777 and Duma 43 (described in section 5.2.3). Seeds from a commercial seed lot that was free from MCMV was used as the healthy control. Seedlings were raised from variety PH30G19, which is susceptible to MCMV.

Maize seeds from the four commercial seed lots were tested for presence of MCMV. The whole seeds were ground to a coarse texture as described in section 4.23 and 4.2.4.

Double antibody sandwich enzyme-linked immunosorbent assay (DAS ELISA) was used to detect MCMV. Antibodies developed against MCMV were coated on a high protein-binding capacity polystyrene 96-well flat-bottomed microplate (F96 Maxisorp, Nunc, Thermoscientific). The samples from seed lots K27 and K4 were tested using ELISA reagent set for MCMV (Agdia Inc, Elkhart, IN, USA) as per manufacturer's instructions. A yellow colour developed where the primary antibody-antigen-secondary antibody sandwich was present, as the p-nitrophenyl phosphate is hydrolysed by alkaline phosphatase to p-nitrophenol, a chromogenic product that absorbs light at 405 nm. The colour development was quantified by measuring the optical density (OD) absorbance values using a microplate spectrophotometer (PowerWave HT, BioTek Instruments, Vermont, USA) at wavelength of 405 nm one hour after incubation at room temperature. Three controls were included in all the microplates: two negative controls- general extraction buffer and MCMV-free maize seed obtained from the Seed Science Center, Iowa State University, USA; a positive control used in the experiment was maize seed infected with MCMV obtained from the MLN screenhouse at KALRO, Kabete, Kenya.

DAS-ELISA was carried out on the samples from the seed lots A and B, using MCMV antibodies obtained from Plant Pathology Department, University of Minnesota, USA and the details are presented in section 3.2.1.1. The buffers were prepared in the laboratory with similar components as those used with the Agdia MCMV antibodies kit. Three controls were also included in all the microplates which included two negative controls- ELISA general extraction buffer and an MCMV-free maize seed sample; and a positive control from maize seed harvested from maize infected with MCMV in the MLN screenhouse at KALRO Kabete, Kenya. The optical density (OD) absorbance values of the reaction were measured using a microplate reader (Elx808 BioTek Instruments, Vermont, USA) at wavelength of 405 nm, one hour after incubation at room temperature.

All samples that had average optical reading higher than twice that of the negative maize seed control were considered positive for MCMV. The sum of the samples that displayed a positive

result for MCMV were recorded. The most probable percentage of infected seeds was calculated using a binomial statistical method formula (Albrechtsen, 2006).

$$P = [1 - (Y/N)^{1/n}] \times 100$$

where: n is the number of seeds per group sample; N is the number of groups; and Y is the assay-negative groups. The total number of seeds tested for the four seed lots was 4,560, 3,860, 1,850 and 3,100 for lots A, Lot B, K27 and K4 respectively, due to the available seed amounts at time of assays.

6.2.2 Preparation of virus inoculum from the MCMV contaminated seeds

The seeds were counted out and soaked in tubes at the laboratories at Kenya Agricultural and Livestock Research Organization (KALRO) - Food Research Institute, Kabete Center. Twenty seeds from the MCMV contaminated sources (K27, K4, A, B, DK777, H614 and Duma43) and the MCMV free source (healthy control) were drawn for each sample. In 50 ml centrifuge tubes, 0.1 M phosphate inoculation buffer was added to the seeds at a ratio of 1:1 (amount of seeds/volume of buffer) and allowed to stand overnight at 4°C. The inocula was then allowed to stand acclimatize to room temperature for 30 minutes prior to application. The solution used to soak the seeds (referred in hereafter as soak solution) was used as one of the inoculum sources, while the solution from the ground seed in buffer was used as the second inoculum (referred to as seed extract). The seed extract was left to stand for 20 minutes at room temperature before using the clear solution to inoculate the maize seedlings using the procedure detailed below.

An MCMV-infected leaf was used to prepare the positive control inoculum by grinding the leaves in similar phosphate buffer as that used for the seed, at a ratio of 1:10. The MCMV-infected inoculum was left overnight at 4°C in similar conditions as the seed soak and the seed extract solution prior to inoculation. Freshly ground seed from MCMV contaminated H614 seeds mixed with the inoculation buffer above were used to evaluate the effect of soaking and incubation of inoculum overnight on infectivity. Maize chlorotic mottle virus detection in all the inoculum used was confirmed by testing an aliquot of the soak solutions, seed extracts and the positive control using DAS-ELISA method.

6.2.3 Inoculation of maize seedlings with seed soak solution from contaminated seeds

Seeds of cultivar PH30G19, a variety susceptible to MCMV, were planted in 20cm-diameter pots half filled with sterile soil mixed with diammonium phosphate fertilizer in the MLN screenhouse located at Biotechnology Research Institute, Kabete Center. The experiments were laid out in randomized complete block design with four replications per treatment (seed source for virus inoculum). The experiments were repeated six different times in the period of 2018-2020.

Two weeks after planting the seedlings were inoculated using the i) seed soak solutions and ii) seed extract obtained from the seven MCMV contaminated seed and the controls. Each tube of inoculum and controls was used to inoculate six seedlings. Carborundum powder was lightly dusted on the leaves of the seedlings and the leaves rubbed with a muslin cloth soaked with the inoculum. Gloves were changed after using each tube of inoculum.

The controls included in the experiment were; i) seedlings inoculated using seed soak and seed extract solution from MCMV-free seed which was confirmed in the laboratory by DAS-ELISA. ii) Maize seedlings not inoculated and the third control were seedlings inoculated using inoculum from MCMV-infected leaf (positive control). All the seedlings were watered daily and the screenhouse sprayed weekly against possible MCMV vectors using either 50 g/litre lufenuron (Match 050EC, Syngenta), 480 g/litre flubendiamide (Belt 480SC, Bayer Crop Science) or 19 g/litre emamectin benzoate (Escort 19EC, Greenlife Crop Protection).

The severity of MCMV symptoms was scored once every week for four weeks after inoculation using a scale of 1 to 5 as described previously in section 5.2.2.3.

6.2.4 Detection of viruses

6.2.4.1. Using double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA)

Leaf samples from the seedlings in each pot were collected 28 days after inoculation, and tested for MCMV by DAS-ELISA as described in section 3.2.1.1. Each sample was made up of a composite of leaf samples from three seedlings from the same pot which were inoculated with the inoculum from the same tube. In each microplate, three controls were included in DAS-

ELISA, two negative controls i.e., general extraction buffer and sap from MCMV-free leaf from plants maintained at Plant pathology greenhouses at KALRO Kabete, and one positive control i.e., sap from MCMV infected leaf from plants maintained at the KALRO Biosafety level II greenhouse. A sample was referred as positive for MCMV infection when the mean absorbance optical density (OD) reading of duplicate samples at 405 nm were above twice the mean A_{405nm} of the negative controls in the same microplate.

6.2.4.2. Using real time reverse transcription polymerase chain reaction (Real time RT-PCR)

Two seed sources of MCMV-contaminated samples of the H614 variety were tested using the real time Taqman RT-PCR as described in section 3.2.1.6, due to limitation with the PCR reagents. RNA was isolated from leaves of seedlings obtained from each pot using the Purelink RNA minikit (Ambion 1283018A, ThermoFischer Scientific, USA) as grouped samples.

The controls included in the real time RT-PCR plates were the master mix (without template) RNA obtained from MCMV-free leaf and RNA obtained from RNA from MCMV-positive leaf (similar to that used in DAS ELISA). The cycle threshold (Ct) values obtained from the StepOnePlus software were used to determine the presence of MCMV. The Ct values were interpreted qualitatively and samples that had mean Ct value higher than that of negative controls were MCMV-free, while those that were lower than that of the negative controls were MCMV positive.

6.2.5 Data analysis

MCMV severity ordinal data was analysed for differences using the Kruskal-Wallis test using the base package in R (R Core Team, 2021), and the scores of the various entries compared against each other using the Dunn's test in the rstatix package in R software. The sum of the plants that showed MCMV symptoms are presented and the mean severity of the symptoms from plants inoculated with the inoculum from same seed source was reported. The absorbance data from DAS-ELISA is presented and the number of positive samples identified were recorded.

Analysis of variance (ANOVA) was used to analyse the Ct values data from real time RT-PCR, and the means separated using the Tukey's honestly significant differences (HSD) test in R software (R Core Team, 2021) since the sample sizes were unbalanced. A positive sample was determined if the mean Ct values of the samples were lower than that of the negative control and also significantly different at $P<0.05$.

6.3 Results

6.3.1 Extent of contamination of seed by MCMV

All the four seed lots tested (A, B, K4 and K27) were found to be contaminated with MCMV at varying levels at a confidence level of 95%. The average contamination rate for the four seed lots was 8.75%, with a range of 4.9% (K27) to 15.93% (Lot B) (Table 6.1).

6.3.2 Maize chlorotic mottle virus severity on maize seedlings inoculated with soak solution from contaminated seeds

The seed soak solutions from all the contaminated seed sources were confirmed to test positive using DAS-ELISA for presence of MCMV prior to inoculation. Soak solutions from Lot B seed source had more inoculum that were positive for MCMV. Thus, more seedlings were inoculated with this seed source (Table 6.2). There were significant differences of the inoculum entries on the severity of MCMV on seedlings ($\chi^2(9) = 297.39$, $p<0.0001$).

Out of the seedlings inoculated with the virus from the different inoculum sources, eight seedlings showed fine MCMV chlorotic streaks. Symptoms were first observed at 21 days post inoculation. No symptoms were visible on the leaf of the seedlings inoculated with the healthy controls. The significant differences were observed to have been caused by the seedlings that were inoculated with sap from MCMV-infected leaf, by Dunn's test. All the seedlings in this category developed symptoms. When samples from the seedlings were tested for MCMV using DAS-ELISA, there was no sample with positive result (Table 6.2). The negative results were due to the low sensitivity of DAS-ELISA.

Table 6.1: ELISA-based estimation of percentage of maize chlorotic mottle virus (MCMV) contamination in four maize seed lots

Seed Lot	Total number of seeds tested	Number of MCMV+ seed pools[a]/total number of seed pools tested	Proportion of positive samples	% MCMV contamination rate[b]
A	4,560	165/228	0.72	6.23
B	3,860	187/193	0.96	15.93
K27	1,850	34/37	0.92	4.90
K4	3,100	61/62	0.98	7.92

[a] Samples were tested in pools, with a pool size of 20 seeds for Lot A and B; and 50 seeds for Lot K27 and K4. [b] Contamination rate was calculated using a binomial statistical method

Table 6.2: Number of seedlings inoculated using seed soak solutions and seedlings with maize chlorotic mottle virus (MCMV) symptoms; the mean MCMV severity at 28 days post inoculation and DAS-ELISA absorbance results of the inoculum and seedlings

	Seed soak (SS)				
Seed Source	Mean absorbance (OD) of Inoculum	No. of tested seedlings	Plants with symptoms	Mean severity	[†] Mean absorbance (OD) of seedlings
K4	-	44	1	1.04	-
K27	1.77	73	2	1.02	0.09 ± 0.01
Lot A	2.12	78	2	1.01	0.09±0.00
Lot B	1.84	100	3	1.03	0.09±0.00
DK777	1.56	12	0	1	0.11 ± 0.03
Duma43	2.39	12	0	1	0.09±0.00
H614	3.24	12	0	1	0.09±0.00
Plants not inoculated	-	37	0	1	0.09±0.00
MCMV-free seed	0.10	20	0	1	0.09±0.00
Positive control	1.18	18	18	3****	0.56± 0.12

"-" samples were not tested using DAS-ELISA; the positive control was obtained from MCMV infected plant. ****= significant at $P<0.0001$. [†]= absorbance readings ± standard deviation at 60 minutes, each sample was duplicated. OD- optical density

6.3.3 Maize chlorotic mottle virus severity on maize seedlings inoculated with seed extract from contaminated seed

When seed extract from the contaminated seed was used to inoculate the seedlings, most of the seedlings did not exhibit MCMV symptoms. However, there was significant differences in the mean severity of MCMV symptoms ($\chi^2(8) = 273.23$, $P<0.0001$). Fine chlorotic streaks were

observed on one seedling at 21 days post inoculation, inoculated with seeds from Lot A. The other seedlings did not show any symptoms except those inoculated with sap from MCMV infected leaf (Table 6.3). DAS ELISA did not detect MCMV in the samples from the seedlings that had no symptoms of MCMV, with lower absorbance readings that were twice that of the negative controls. The seedlings inoculated with MCMV-infected leaf sap showed progressive MCMV symptoms development from seven days post inoculation and was confirmed as positive using DAS-ELISA.

Table 6.3: Number of seedlings inoculated with maize chlorotic mottle virus (MCMV) contaminated seed extract; seedlings that developed MCMV symptoms, the mean severity at 28 days post inoculation and DAS-ELISA absorbance readings results of MCMV for the inoculum and seedlings

Seed Source	Mean absorbance (OD) for inoculum	No. of tested seedlings	No. of plants with symptoms	Mean severity	† Mean absorbance (OD) for seedlings
K4	-	-		-	
K27	1.53	12	0	1	0.09 ± 0.00
Lot A	1.79	36	1	1.02	0.09 ± 0.00
Lot B	1.57	60	0	1	0.09 ± 0.01
DK777	3.33	12	0	1	0.09 ± 0.00
Duma43	3.29	12	0	1	0.08 ± 0.01
H614	3.46	84	0	1	0.09 ± 0.01
Plants not inoculated	-	24	0	1	0.09 ± 0.01
MCMV-free seed	0.11	36	0	1	0.10 ± 0.01
Positive control	4.01	15	15	3****	0.42 ±0.01

'-' Seeds not available, ****= significant at $P<0.0001$. †DAS-ELISA absorbance readings ±standard deviation at 60 minutes, each sample was duplicated; OD- optical density.

6.3.4 Maize chlorotic mottle virus detection using real time RT-PCR on maize seedlings inoculated with seed extract from contaminated seed

Seventy-two seedlings inoculated with seed extract from MCMV contaminated seed of variety H614 were tested using real time RT-PCR. Despite the seedlings not showing visible MCMV symptoms in the screenhouse, seven samples tested positive for MCMV. The Ct values were higher than those of the negative and healthy controls, ranging from 27.04 to 30.22 and were

significantly different (P<0.001) (Table 6.4). The Ct values however were much lower than those obtained from samples that had been inoculated with MCMV-infected leaf sap.

Table 6.4: Seedlings inoculated with MCMV seed extract prepared in different ways and tested for MCMV using real time reverse transcription polymerase chain reaction

Seed source	Number of seedlings tested	No. samples with Ct values lower than negative control	Ct Mean	MCMV status
H614-1	12	0	34.83 a	Negative
H614-1B	12	0	34.48a	Negative
H614-1A	12	0	34.14a	Negative
not inoculated	12	0	35.22a	Negative
healthy_B	12	0	33.00a	Negative
healthy_A	12	0	32.77a	Negative
H614-2	12	2	29.40b	Positive
H614-2A	12	2	28.73b	Positive
H614-2B	12	3	28.21b	Positive
MCMV_leaf sap-A	12	12	13.70c	Positive
MCMV_leaf sap	12	12	12.36cd	Positive
MCMV_leaf- sap B	12	12	11.53cd	Positive
PCR Negative control			33.53a	
PCR Positive control			9.79d	
Tukey's HSD			2.44	

Ct- mean cycle threshold obtained. Seed sources with a letter A or B were inoculated with inoculum of freshly ground seed extract while those without the letters were ground and soaked overnight in buffer at 4°C before the inoculation process the following day. Healthy seed source was from MCMV-free seed. 'MCMV-leaf' labelled samples were seedlings inoculated with sap from MCMV-infected leaf, while the 'PCR positive' was obtained from RNA of fresh MCMV-infected leaves. Negative control represents non template control included in the real-time RT-PCR. Same letter after the Ct meanvalues indicate no significant difference. *P*<0.001

6.4 Discussion

Due to the importance of MCMV infection in maize production, this study determined the contamination levels of selected seed lots and the infectivity of these detectable virus in the seed. This was achieved by infecting maize seedlings with inoculum obtained from soaking solution and extract from the MCMV contaminated seeds.

The contamination level of the seed lots tested varied from 4.9 to 15.93%, detected from whole seed using DAS-ELISA. The method was found to be sensitive enough to detect one

contaminated seed in a sample of 1000 seeds (section 4.3.1.1). Varying contamination rates of seed lots is similar to previous study of 26 lots assayed for MCMV using 10-seed samples. Twelve of the 26 seed lots were positive for MCMV (Mahuku *et al*., 2015a). Similar variability in contamination levels were reported in tomato and capsicum seed lots tested for pospiviroids (Dall *et al*., 2019). Other variability has been reported for cucumber green mottle mosaic virus (CGMMV) from watermelon and melon seeds that had a high contamination of 100% in one study (Wu *et al*., 2010) and ranged from 2.7% to 15.8% in another study (Constable *et al*., 2018) in the same crops. Factors that cause the variability in contamination rates include the extent of infection in the field; whether the seeds were obtained from one field or samples from different fields were blended together and the ease of detection of the virus from the seeds by the methods applied. The detected virus in seeds does not give accurate confirmatory indication of transmission of the virus to next progeny (Johansen *et al*., 1994), as inactivated and viable virus are all detected (Albrechtsen, 2006).

There are few studies that have described the infectivity of viruses found especially on seed coats. In this study, MCMV infection was observed in less than 2% of the seedlings inoculated with the soaking solution and seed extract. The visible symptoms were mild, with fine chlorotic streaks. The rarity of MCMV infection was also evident in the negative results obtained by the less sensitive method of DAS-ELISA (Bernardo *et al*., 2021). Similarly, real time RT-PCR detected low viral amounts in some of the seedlings that were tested when this method was applied.

The low infectivity (2%) observed on the seedlings may be as a result of the lack of viability of the virus in the samples. Viruses that are seed- borne have been shown to lose viability as the seed matures and dries up, altering the environment for the virus survival (Konate *et al*., 2001; Li *et al*., 2007). Viable virus is mainly found in the embryo, having penetrated the seed early in the development cycle of the plant (Wang and Maule, 1994; Sastry, 2013b). Infectivity of the virus may also be inhibited by compounds found in the maize seed components.

Viable viruses on the surface have been reported for tobamoviruses (Dombrovsky and Smith, 2017). High infectivity of 100% was reported for cucumber green mottle mosaic virus from

seeds of cucumber and melon (Reingold *et al*., 2015) and 33% from watermelon seeds (Sui *et al*., 2018). Seed treatment procedures are available to reduce the infectivity, but they could not totally eliminate the virus (Reingold *et al*., 2015). This was attributed to the stable particles that are characteristic for tobamoviruses, and that the treatment could not remove the virus that is inside the seed. Tomato brown rugose fruit virus (ToBRFV) has also been reported in seed coats of tomato, with low infectivity on seedlings (Davino *et al*., 2020). Infectious pepino mosaic virus (PepMV) has also been reported from tomato seed extract (Ling, 2008), causing 100% infection on *Nicotiana benthamiana* seedlings and 5% of *Solanum lycopersicum* seedlings. *Nicotiana benthamiana* was more easily infected with the virus as compared to the tomato seedlings. The infectivity of the virus was attributed the hairy structure of the tomato seed coat, since the same virus from *Nicotiana benthamiana* seeds was not infectious (Ling, 2008).

Maize chlorotic mottle virus is easily transmitted mechanically and the detection of virus from seeds, though low, is of concern as that may be a route of transmission into fields. Though the infectivity of MCMV is low, the extent of contamination of the virus on seeds could also contribute to the infectivity of the virus and transmission to seedlings (Néya *et al*., 2007). Thus, there is need to determine the amount of virus on the maize seed that is viable and the location of the virus, and the percentage prevalence of contamination that may be of economic importance, leading to disease development. The information is necessary to advise management of the virus on/in seed through treatment methods including those with chemicals or requiring heat application. The study has been instrumental in additional knowledge on the spread of MCMV and is important in the management of spread of the virus.

CHAPTER SEVEN

GENERAL DISCUSSION, CONCLUSION AND RECOMMENDATIONS

7.1 General discussion

Transmission of viruses through seed confounds trade of crops as the seed can be a source of inoculum in new areas where the virus has not been reported before or introduce new isolates to an area. This study presents several aspects related to the transmission via seed and diagnosis of maize chlorotic mottle virus. Overall, it is evident that the transmission of MCMV through seed is low (1% and below), and detection of the virus is most convenient and probable from ungerminated seeds.

The use of DAS-ELISA is most common in detection of viruses. However, in this study the method was the least sensitive. Similar sensitivity results have been reported for detection of MCMV and other viruses (Bernardo *et al*., 2021; Torre *et al*., 2020). The use of crude (not purified) plant tissue as samples i.e., leaf sap and seed extracts, led to further decrease in the sensitivity of DAS-ELISA. DAS-ELISA was also found to be less sensitive than the other serological methods using lateral flow strips and IC-RT-PCR, though it is less costly and requires less specialised skill to implement. The use of nucleic-acid based methods provide higher sensitivity of MCMV detection, using RNA samples that are to be purified. Sensitive and specific detection of MCMV for seed certification is important to avoid false negative or positive results (Albrechtsen, 2006).

The choice of a method of detection is also determined by the sample type. MCMV was easily detected using DAS-ELISA in samples with 100, 500 and 1000 seeds with one infected seed. However, the samples needed to have been soaked overnight, to increase chances of detection. In seedlings obtained from the MCMV-contaminated seeds tested, the detection of the virus was challenging as no positive samples were identified. This was despite grouping the samples into smaller subunits of 50 seedlings. Further use of groups of 20 seedlings did not yield any different results. Group testing of seeds is commonly used for the estimation of infection of a seed lot by pathogens (Albrechtsen, 2006), as it saves on time and costs. However, when the use of 400-seed

sample size was used for detection of MCMV was evaluated, the probability of detecting MCMV in whole ungerminated seeds was 98%, while in grow-outs the probability was 77%. The lower probability of detection in grow-outs may require an increase in the number of seeds in a sample to be tested, to achieve a desired probability of 95% and above.

This study aimed at determining the seed transmission rate of MCMV -Kenya isolate from commercial seed lots as well as from mechanically inoculated maize. The virus transmitted to seedlings were found to be uncommon with all transmission rates below one percent. The transmission of viruses via seed is influenced by a number of factors. Various genotypes studied here had varying MCMV severity score in the field. Both those with high and low MCMV symptoms exhibited transmission of MCMV via the seed obtained from the inoculated plants. This confirmed the results that all the maize genotypes had a potential to transmit MCMV via seed (Mwatuni, 2021). However, this study and previous ones involved the use of white flint maize (Gatunzi, 2018; Mwatuni, 2021). Other maize genotypes could be studied to examine for possible resistance to seed transmission of MCMV. Higher symptoms severity was observed for maize inoculated at early growth stages, with higher severity for MCMV susceptible varieties as compared to DK777 that is tolerant to maize lethal necrosis disease. The transmission of MCMV from the seeds obtained from the plants inoculated early showed seed transmission rate of less than 0.2%. This low seed transmission rates of MCMV are comparable to other maize-infecting viruses (Mikel *et al*., 1984; Li *et al*., 2011; Gatunzi, 2018).

Maize chlorotic mottle virus has been reported in maize seed production areas in Kenya between 2014 and 2019. Five agroecological zones for incidence of MLN yielded no significant difference (Eunice *et al*., 2021), and different varieties had varying severity levels of the disease, with highest incidence for DK8031. The now endemic nature of MLN in the country predisposes the produced seed to transmission of the virus. There is need to increase the management of the disease in the fields in order to minimise further loss in seed production. Rejection of seed maize crop may lead to shortage of certified seed in the country thereby affecting the next planting season.

Unlike other maize infecting viruses, MCMV is readily transmitted mechanically (Redinbaugh and Stewart, 2018; Prasanna, 2021). This study assessed the infectivity of MCMV found on contaminated seeds. Despite the positive detection of MCMV on the contaminated seed, less than 2% of the seedlings that were inoculated with seed extract or soak solution from these sources showed MCMV symptoms. The symptoms were mild. The infectivity observed is considered as low when compared to that observed by tobamoviruses where up to 100% infectivity has been reported (Reingold *et al*., 2015). Mechanical infection from the virus found on the seed testa and in the endosperm to seedling occurs when the roots are bruised and the virus is transmitted mechanically (Dombrovsky and Smith, 2017). The introduction of this low amount may lead to an epidemic if the vectors for MCMV are present and under favourable environment. Inactivation of virus in seed is due to dehydration during the drying process as well as by inhibitors found in seeds (Konate *et al*., 2001; Li *et al*., 2007: Sastry, 2013b). There is need to determine the location of viable MCMV in the maize seeds.

7.2 Conclusion

The first objective demonstrated the sensitivity of the various methods in detection of purified MCMV, as well as using MCMV in leaf sap and seed extract for the serological methods. Despite the higher cost and longer time required to carry out the nucleic-acid methods, the methods detected lower concentration of the virus and eliminate the problem of false negatives, due to their sensitive nature. Lateral flow strips (ImmunoStrips®, Agdia Inc, USA), were also found to be sensitive and they are easier to use, since the assays can be carried out in the field, however, they are not easily adaptable to high throughput testing as those required during seed certification, due to the cost implication. The second objective determined the limit of detection of MCMV in seed samples and evaluated the 400-seeds as sample size in seed certification. The study exhibited that testing of MCMV in seed extract was possible in samples with up to one infected seed in 1000 using DAS-ELISA and real time RT-PCR, and the 400-seed sample was adequate when using ungerminated seed. However, the probability of detection greatly reduced when the seeds were grown out and the germinated seedlings tested for MCMV, and there is need to test more seedlings in a sample obtained from a seed lot to have a probability of at least 95%.

The low detection of MCMV in seedlings from grown out contaminated seed was confirmed by the low seed transmission rate determined in the third objective of this study. The seed transmission rate was not influenced by the level of response to MCMV (resistance/susceptibility) of the genotypes assessed. The transmission of MCMV via seed was also low when seed was obtained from plants that showed higher infection at earlier growth stages. Maize chlorotic mottle virus is transmitted horizontally to other plants in the field by vectors. This makes the low seed transmission of the virus important as it could introduce the virus in the field and lead to further spread by the vectors. Corn thrips, vectors that have been identified in maize lethal necrosis infected fields in Kenya, are attracted to MCMV-infected plants by volatile compounds that are produced. Thus, when the virus is introduced in the field early through seed, would lead to earlier transmission by vectors, thereby affecting plants at earlier growth stages.

Management of contaminated seed is also important as the study also showed a small percentage of viable virus in the seed. The contaminated seed may spread the virus to progeny through mechanical means especially through the developing roots. Maize chlorotic mottle virus is a stable virus and may persist in seed for a while. The virus can also be spread through equipment used in the processing of seed, if not well handled and machines not well disinfected.

7.3 Recommendations

Based on the research results detailed in the study, these recommendations are made:

i. The detection of MCMV using ungerminated seed samples and employing double antibody sandwich enzyme linked immunosorbent assay (DAS-ELISA) can be incorporated in seed health assays, while other lateral flow strips from different companies should also be evaluated for sensitivity.
ii. Sample size for detection of MCMV in seedling grow outs should be increased as a result of low transmission rate of the virus to avoid false negative assessment of seed lot.
iii. Seed certification testing of MCMV should continue despite the low seed transmission rate due to the possibility of horizontal transmission by vectors attracted by the infected plant.

iv. Seed certification should be done in all maize genotypes, whether exhibiting susceptibility or varying resistance levels to the MCMV, as all are prone to transmission of MCMV via seed.

v. Management of MCMV vectors should be done at all stages of crop growth to control infection

vi. More studies should be done to determine the location of the virus in the maize seed to identify the areas where viable virus is found in order to manage transmission where the virus is infectious.

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