EPIDEMIOLOGY AND MANAGEMENT OF MAIZE LETHAL NECROSIS IN ETHIOPIA

PhD DISSERTATION

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DEDICATION

This dissertation is dedicated to my beloved wife Fantaye Guttin, for nursing me with affection and love. It is also dedicated to my children Ebbise Bayissa, Ebba Bayissa and Bontu Abdisa for their endurance and sustained love in the success of my study.

STATEMENT OF THE AUTHOR

By my signature below, I declare and affirm that this Dissertation is my true work and those all sources of materials used for preparation of the Dissertation have been duly acknowledged. This Dissertation is submitted in partial fulfillment of the requirements for PhD degree from the Postgraduate Program Directorate at Haramaya University. The Dissertation is deposited in the Haramaya University Library and is made available to borrowers under the rules of the library. I solemnly declare that this Dissertation has not been submitted to any other institution anywhere for the award of any academic degree, diploma or certificate.

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BIOGRAPHICAL SKETCH

The author was born in Gindeberet, West Shewa Zone, Oromia National Regional State in June 1984. He attended elementary and junior secondary education at Chulute Elementary and Junior Secondary School. He pursued senior secondary education at Gindeberet Senior Secondary School and completed in 2001. After successfully passing the Ethiopian School Leaving Certificate Examination (ESLCE), he joined Jimma University in 2002 and graduated with Degree in Crop Production and Protection in 2006.

After graduation, he was employed by Oromia Regional Bureau of Agriculture and was assigned as Agricultural extensionist in Elfeta district, West Shewa Zone. After having served for four years, he joined the School of Graduate Studies at Ambo University to pursue his Master's Degree in Plant Pathology and graduated in 2012. After graduation, he had been working as Assistant Researcher and Crop Process reperesentative at Ambo Agricultural Research Center (the then Ambo Plant Protection Research Center) until he joined the Postgraduate Program at Haramaya University to pursue doctoral studies in Plant Pathology in October 2015.

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ACRONYMS AND ABBREVIATIONS

ANOVA	Analysis of variance
AUDPC	Area under disease progress curve
CIMMYT	International Maize and Wheat Improvement Center
CSA	Central Statistical Authority
DAP	Diammonium phosphate
DAS-ELISA	Double antibody sandwich Enzyme Linked Immunosorbent Assay
DI	Disease incidence
DNA	Deoxyribonucleic acid
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
ELISA	Enzyme Linked Immunosorbent Assay
GLM	General Linear Model
LSD	Least significant difference
MCMV	Maize chlorotic mottle virus
MDMV	Maize dwarf mosaic virus
MLN	Maize Lethal Necrosis
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
SAS	Statistical Analysis System
SCMV	Sugarcane mosaic virus
SNNP	South Nation, Nationality and Peoples
WSMV	Wheat streak mosaic virus

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I. Regassa, B., Abraham, A., Fininsa, C., Wegary, D., and Wolde-Hawariat, Y. (2020). Distribution of maize lethal necrosis epidemics and its association with cropping systems and cultural practices in Ethiopia. *Crop Protection, 134, 105151.* doi: 10.1016/j.cropro.2020.105151.

II. Regassa, B., Abraham, A., Fininsa, C., and Wegary, D. (2021). Alternate hosts and seed transmission of maize lethal necrosis in Ethiopia. Journal of *Phytopathology,* 169, 303-315.

III. Regassa, B., Abraham, A., Fininsa, C., Wegary, D., and Wolde-Hawariat, Y. (2022). Transmission and persistence of maize lethal necrosis in infested soil and infected maize residue. *Eurropean Journal of Plant Pathology*, 162, 263-273

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V. Regassa, B., Abraham, A., Fininsa, C., Wolde-Hawariat, Y. and Wegary, D. (2021). Evaluation of seed dressing insecticides for the control of maize lethal necrosis vectors. (Submitted to International Journal of Tropical Insect Science)

VI. Regassa B, Wegary D, Fininsa C, Abraham A, (2021). Screening maize genotypes for resistance to maize lethal necrosis disease in Ethiopia. *Tropical Plant Pathology* 46, 583-595.

Epidemiology and Management of Maize Lethal Necrosis in Ethiopia

Bayissa Regassa Feyissa

ABSTRACT

Maize (Zea mays) is one of the most important staple food crops in the Eastern Africa region including Ethiopia. Maize lethal necrosis (MLN) is becoming a threat to maize production and has been challenging food security for the majority of households in East Africa since 2011. MLN is caused by a co-infection of maize chlorotic mottle virus (MCMV) with any one of cereal viruses in the genus Potyvirus, family Potyviridae, such as sugarcane mosaic virus (SCMV) maize dwarf mosaic virus (MDMV), wheat streak mosaic virus (WSMV) or johnson grass mosaic virus (JGMV). The general objective of this research was to study the epidemiology of MLN disease and its management through understanding the survival mechanisms of MLN-causing virures, insect-vector management and identification of resistance/tolerant maize genotypes. A field survey was conducted in major maize producing regions of Ethiopia from 2015-2018 to determine MLN geographical distribution, factors associated with disease intensity, alternate natural hosts, and type and distribution of insect vectors. Simple descriptive statistical analyses were performed to summarize the field survey data. The associations of MLN disease intensity with independent variables were analyzed using logistic regression. To describe and compare different categories of the sample units with respect to the desired characteristics, mean, standard deviation, and percentage were computed using simple descriptive statistics. This study revealed that MLN disease was distributed in major maize production areas of Ethiopia, especially in central, western, southern and southwestern parts of the country. The disease was most prevalent in Southern Nation, Nationality and Peoples (SNNP) region with 66.67% prevalence followed by Oromia region that had a prevalence of 65.62%. Natural MLN alternate host assessment uneder field condition and host range study *by artificially inoculating in the greenhouse were* conducted *to determine potential alternate hosts. Poaceae family had the highest number of grass species that were alternate hosts for MLN causing viruses. Digitaria sanguinalis, Phalaris paradoxa, Oplismenus hirtellus, Echinocloa colona, Cynodon nlemfuensis, Pennisetum purpureum from Poaceae and Cyperus cyperoids from Cyperaceae family were naturally infected by MCMV. Cyperus rotundus, sugarcane (Saccharum officinarum) and sorghum (Sorghum bicolor) were infected by both MCMV and SCMV under natural field conditions. In addition, seed transmission*

study was conducted using growing-on tests to determine the potential of seed transmission and its role in the spread of MLN. The mean overall seed to the seedling transmission rate of MCMV was 0.073% with a range of 0 to 0.17% among 20 different maize genotypes studied. Out of 20 maize genotypes evaluated, 14 genotypes had some levels of seed transmission (0.03-0.017%) for MCMV whereas SCMV seed transmission was observed only in a single plant of one genotype, with an overall average of only 0.003%. The rates of seed transmission of the viruses were influenced by the seed lot and maize varieties used. Field assessment, laboratory, and greenhouse experiments were also conducted using MCMV and SCMV infected maize residue and infested soil to assess the role of MLN infected maize residue and infested soil in the transmission of MLN causing viruses. Serological detection and back-inoculation test result showed that MCMV was detected and confirmed to be transmitted from infested soil to newly raised maize seedlings. However, SCMV was neither detected in soil samples from infected fields nor transmitted to maize seedlings. Under the experimental condition, MCMV remains persistent and transmissible up to 6 months in maize planted on MLN infested soil mixed with MLN infected maize residue. Registered systemic seed dressing insecticides in Ethiopia were evaluated either singly or in combination with fungicide against identified insect vectors of MLN causing viruses. Among seed dressing insecticides evaluated against Franklinella sp and R. maids, thiamethoxam 25% at 2.0g/kg seed and imidalm T 450 at rate 1.5 were showed the superior control efficacy. Vectors were introduced on to the maize seedlings developed from seeds treated with insecticides and the percentage of the reduction of the insect population was determined. Based on morphological and PCR-based vector's identification, transmission and subsequent mechanical inoculation tests, maize thrips (Franklinella sp.) and cereal leaf beetle (Oulema sp.) were identified as potential vectors of MCMV, while corn leaf aphids (Rhopalosiphummaidis) was a potential vector of SCMV. A greenhouse screening study was conducted by artificially inoculating MLN-causing viruses to determine the reactions of various maize genotypes to MLN to identify resistant genotypes that can be either utilized in breeding programs or recommended for commercial production. Nearly 7%, 16.7% and 5.5% of the inbred lines from highland, mid-altitude and lowland maize breeding programs, respectively, showed moderately resistant reaction to MLN. Higher proportion of the inbred lines and varieties showed susceptible to highly susceptible responses. The various weed and cultivated plants identified as alternate hosts, the insect vectors, the transmissibility from infected seed and infested soil to newly raised maize seedlings, and the persistence in soil and maize residue of MLN

causing viruses, are believed to be epidemiologically important and maintain the virus inoculum in the absence of maize crop in the field. These also support the survival of the virus for continuous infection. As part of an integrated management of MLN, farmers and stakeholders involved in maize production should take precautionary measures by using certified and virus-free maize seeds from trusted sources; regular field monitoring, assessment of virus symptoms and rouging-out diseased maize plants; good field sanitation methods including weed control and eliminating alternate host plants within and in the surrounding areas of maize fields together with seed dressing before planting with thiamethoxam 25% WG @ 2.0 g/ kg seed or Imidalm T 450@1.5 g/kg of seeds for early-stage protection.

Keywords: *Disease Intensity; Epidemics; Inoculum source; Insecticide; Level of resistance; Maize chlorotic mottle virus; Sugarcane mosaic virus; Vectors; Virus transmission; Zea mays*

1. INTRODUCTION

1.1 Maize Production and its Constraints in Ethiopia

Maize (*Zea mays* L., 2n = 2x = 20) is a major global commodity that plays a key and increasing role in global agri-food systems including direct food consumption and indirect feed pathways for animal-sourced foods. It is already the leading cereal in terms of production volume and is set to become the most widely grown crop in terms of area in the coming decade (Erenstein et al., 2022). The global maize area (for dry grain) amounts to 197 M ha, including substantive areas in sub-Saharan Africa (SSA), Asia and Latin America (FAO, 2021).

Maize is grown on over 40 M ha of land in SSA. Maize is the primary cereal grown in over half of the countries in SSA, and one of the top two cereals in over three-quarters of these countries (FAO, 2021).

In Ethiopia, maize has a significant share among cereal crops in terms of production, productivity, distribution and adaptation (CSA 2021). Among all cereals, maize is second to teff (*Eragrostis tef*) in area coverage with 2.5 million ha (19.46% area allocated to all cereals) of land planted to maize; but first in productivity (4.6 t ha^{-1}) with total annual production of 11.64 million tons (CSA, 2021).

Maize is grown under diverse agroecologies and socioeconomic conditions, typically under rain-fed production systems by more than 10.5 million smallholder households, more than any other crop in the country (CSA, 2018). Among the 11 administrative regions of Ethiopia, Oromia is the leading region in maize production (54%) followed by Amhara (25%) and South Nation, Nationality and Peoples (SNNP) region account (14%), Tigray and Ben Shangul-Gumuz produce 2% each (Tsedeke *et al.*, 2015).

The maize agro-ecologies in Ethiopia can be broadly divided into six major categories (MOA, 2005); including moist and semi-moist mid-altitudes, moist upper mid-altitudes, dry mid-altitudes, moist lower mid-altitudes, moist lowlands, and dry lowlands. Among these, the moist and semi-moist mid-altitude zones (1700–2000 m above sea level; 1000–1200 mm rainfall) cover the larger proportion of maize production area of Ethiopia. These are mostly located in southwestern and western Oromia, West and Northwestern Amhara and cover about 30% of the national maize

production area. Moist upper mid-altitudes (2000–2400m; >1200 mm rainfall) cover 25%, The dry mid-altitudes (1000–1600 m above sea level; 650–900 mm rainfall) agroecologies cover 20%, which is located in some Parts of SNNP region, southwestern and western Oromia; West and northwestern Amhara; and parts of Benshangul-Gumuz. Moist lower mid-altitudes, moist lowlands and dry lowlands agro-ecologies covers the remaining 25% (Tsedeke *et al.*, 2015).

Despite its importance as a principal food crop, the national average yield of maize in Ethiopia is below the world's average yield (5.67 tons/ha) in 2020/21 (FAO, 2021). A significant portion of this yield gap is attributable to the effect of biotic and abiotic factors (Abate *et al*., 2017; Keno *et al.*, 2018). Environmental conditions such as climate change, drought and soil fertility problems have been the major abiotic factors. The important biotic factors affecting maize production are diseases, weeds, insect pests, especially the maize stalk borer. Diseases such as grey leaf spot (caused by *Cercosporazeae*-maydis), turcicum leaf blight (caused by *Helminthosporium turcicum), common* rusts (caused by *Puccinia sorghi*), smuts and maize streak virus are the most problematic factors on maize production (Tegegne *et al*., 2009; Miano, 2014). Among biotic factors, the recently emerged insect pest fall armyworm (*Spodoptera frugiperda*) in 2017 (Keno *et al*., 2018) and the outbreak of maize lethal necrosis (MLN) in 2014 (Mahuku *et al*., 2015a) were further aggravated the problem and contributed to significant yield losses.

1.2. Plant Virus Disease

Plant viruses are obligate intracellular pathogens composed of a small piece of nucleic acid (5-40%) enclosed within a protein coat known as a capsid (60-95%). The capsid is made up of one or few proteins (capsomeres: coded by viral genome) that form repeating units which assemble around the genome to protect it from enzymatic degradation inside the host cell (Callaway *et al*., 2001). Viral genome codes for only a few structural proteins (besides non-structural regulatory proteins are involved in virus replication). Capsids are formed as single or double protein shells and consist of only one or a few structural protein species and multiple protein copies self-assemble to form the continuous three-dimensional capsid structure. The coat proteins play an important role in almost every step of the viral infection cycle, including virus delivery into the plant cell, disassembly of virus particles, viral RNA translation, viral genome replication, assembly of progeny virus, movement in the plant, activation or suppression of host defense and transmission of the virus to healthy plants (Lal *et al.*, 2015).

The viral nucleic acid surrounded by protein subunits is called a nucleocapsid. A fully assembled infectious virus is called a virion, which may either be a nucleocapsid alone or a nucleocapsid with additional components such as a lipid envelope (located either externally or underneath the capsid) an enzyme or other structural proteins (Lal *et al.*, 2015). Plant viruses are lacking molecular machinery making them unable to replicate without a host and dependent on host cellular machinery for replication.

Plant viruses are among the major factors that affecting food production worldwide and cause vast economic losses. It results in loss by limiting plant produce quality and quantity (Thresh, 2006; Van der Vlugt, 2006) and have an estimated economic impact of more than $30 billion per year (Sastry and Zitter, 2014).

1.3 Plant Virus Epidemics

Plant virus diseases are serious constraints to the productivity and profitability of a wide range of crops. Important agricultural crops are threatened by a wide range of plant viral diseases worldwide, resulting in losses of several billion dollars annually (Mumford *et al*., 2016). Identification of the causal viruses and understanding their epidemiology is the most important pre request to estimating the incidence and economic impact of the diseases they cause on the crop, and to devising significant virus management strategies and tactics. Plant viruse epidemics result from interactions between virus, host plant, vector and environmental factors. Every epidemic may be considered to be a completely unique pathosystem wherein every of the components contributes to the epidemic (Geering and Randles, 2012). Epidemics of existing plant virus diseases and emergence of novel virus diseases have become a serious threat to both subsistence and commercial agriculture. Agroecosystems are less stable, and severe virus epidemics are common, especially where monoculture is practiced. The losses and the resulting financial damage can be limited by controlling epidemics using measures that minimize virus infection sources or suppress virus spread (Jones, 2004).

Factors such as virus virulence, host plant level of resistance or susceptibility, vector, environment, time and anthropogenic factors activities (Agrios, 2005) determine the intensity of epidemics. The ability of a virus to spread and produce a significant epidemic depends on each of these components remaining permissive, that is, none limiting. An epidemic can be considered to be a

pathosystem in which the necessary disease components interact to allow the virus to multiply and spread. Rapidly-expanding global climatic change creates favorable conditions for development and increased spread of plant virus diseases due to direct or indirect impacts on population dynamics of virus-transmitting insect vectors (Pautasso*et et al*., 2012; Geering and Randles, 2012).

Obtaining the critical epidemiological knowledge required for each virus pathosystem involves collecting information on the nature of the primary virus infection source(s), how the virus spreads into and within crops, how it spreads over distance to invade new sites and how it survives outside the main growing period. Then, a clear picture is required of the factors driving epidemics of the virus concerned, which are the key ones favoring spread and which delay its epidemics (Jones, 2001).

Factors favoring epidemics include, sources of infection sources (size, distribution and proximity), monocultures, extended growing periods, successive plantings of short-lived crops all-year-round, planting susceptible cultivars, planting cultivars with long growing periods, lack of crop rotation, poor control of weeds and 'volunteer' plants, old infected plantings left in farm field, temperatures favors virus multiplication, early spread of the virus, polycyclic spread pattern, untimely or absence of control measures (Jones, 2004).

The epidemiology of virus diseases also depends on different pathogen strains which vary in virulence, host range and transmissibility. Viruses continue to change by mutation and selective adaptation by passage through their hosts. Variation may also result from pseudo-recombination since some of them possess divided genomes or by heterologous encapsidation. These phenomena are both examples of direct interaction between virus strains or viruses in mixed infections (Sastry, 2013).

1.4 Mixed Infections of Plant Viruses

Mixed infections of plant viruses are common, and several economically important virus diseases of plants involve interactions between causative agents. The occurrence of any virus changes the environment in which it lives, and viruses interact when they encounter one another in a common host plant (Syller, 2012). Although the process of interaction between viruses occurs in diverse ways, there are three basic effects. One virus may cause, directly or indirectly, an increase in replication or/and transmission of another virus; it may cause a decrease in these processes; or it

may have no effect (Begon and Mortimer, 1986). Some examples of mixed infections of plant viruses are *Tobacco mosaic virus* and *Potato virus* X in tomato, which cause leaf drop streak and often kills plants (Matthews, 1991). A combination of *Maize chlorotic mottle virus* (MCMV) and *Maize Dwarf Mosaic Virus* (MDMV), or *Wheat Streak Mosaic Virus* (WSMV), which cause corn lethal necrosis (Uyemoto, 1983). Similarly, infection with *Sweet potato feathery mottle* virus and *Sweet potato chlorotic stunt* virus results in sweet potato virus disease (Karyeija *et al*., 2000).

Many virus diseases of plants are caused by a synergistic interaction between viruses within the host plant. Such synergism can induce symptoms more severe than would be caused by additive effects. In a synergistic interaction, the virus titre of both, one, or neither virus may be enhanced and, as a consequence, the rate of disease spread may be affected (Zhang *et al*., 2001).

The occurrence of synergism and the associated increase in symptom severity have important practical implications, as some diseases are caused by the direct result of a synergistic interaction. For example, the interactions between MCMV and WSMV results in a significant increase (up to 10-fold) of the MCMV concentration in plants (Scheets, 1998). In addition, WSMV infection is considerably enhanced by the presence of MCMV both in terms of frequency and intensity. Likewise, a strong synergistic interaction was found between *Cucumber mosaic virus* and black-eye *Cowpea mosaic virus* in severely stunted cowpea in fields. In an experimental inoculation study, each virus caused relatively mild disease when inoculated singly and plants showed significantly reduced stunting. In contrast, disease severity and the extent of stunting increased when these two viruses are co-inoculated under the same conditions. Another example is blackberry yellow vein disease (BYVD) complex which is caused by the cooperation between different viral species (Martin *et al*., 2013).

Due to an increase in symptom severity, synergism may cause a larger reduction in total host abundance or host biomass. Concurrently, where an increase in virus concentration occurs, synergism increases the potential of hosts to be sources of inoculum for one or both viruses. A typical increase in virus titre by 3- to 11- folds (Fondong *et al*., 2000) can be expected greatly to increase the transmission rate of the viruses by vectors. Thus, synergism increases the transmission rate by vectors as well as the loss rate of infected hosts. An increased loss rate would be expected to reduce virus fitness, whereas an increased transmission rate would be expected to increase it (Zhang *et al*., 2001).

1.5 Mechanisms of Plant Virus Transmission

1.5.1 Mechanical transmission

Mechanical transmission occurs when an infected plant comes in contact with a healthy plant and leaves rub together or by humans' interferences like tools/hands/clothing. It involves the introduction of infective virus or biologically active virus into a suitable site in the living cells through wounds or abrasions in the plant surface. Spreading viruses by mechanical method is generally used for experimental purposes under laboratory/greenhouse conditions. Despite the ease of mechanical transmission under experimental conditions, transfer of virus from one host to another without the intervention of a vector is not common in nature (Lal *et al.*, 2015). These viruses can contaminate structures, tools, soil debris and wounding of a host plant allowing contact of the tissue with a source of virus can lead to infection (Hu *et al*., 1994).

1.5.2 Seeds

Seeds can sometimes carry virus infection because of external contamination or by an infection of the embryo's living tissues. The location of the virus in seed determines transmissibility of virus through seed. The virus is considered to be externally seed transmitted when it is outside the functional seed and internally seed transmitted when it is within the tissue of the seed. When externally seed transmitted, the virus is confined to the testa as a contaminant (Sastry, 2013).

Host plants show a high degree of protection possessed by embryos of seeds against invasion by viruses that affect the mother plant. Despite this protection, an appreciable number of viruses have been found to pass from one generation to the next through the medium of the seed. This leads to new crops breaking out in disease, which is at first only local in circulation. However, infection can spread to the rest of the crop by mechanical means. About 231 plant viruses have been reported to be seed transmitted in different food, fiber, weed, and ornamental crops (King *et al*., 2011; Sastry, 2013). The most common type of seed transmission of the viruses are found within the tissues of the embryo. The developing embryo can become infected either prior to fertilization by infection of gametes or by direct invasion after fertilization (Sastry, 2013; Lal, *et al.*, 2015).

Seed infection is epidemiologically important since it serves as the primary source of inoculum and forms the starting point for the initiation of the disease. It ensures virus association with the planted crop. As the infected seeds are randomly dispersed in the field, the infected dispersed

seedlings serve as sources of inoculum for secondary spread. When the infected seedlings are the only virtually source of inoculum, seed transmission plays a critical role in virus epidemiology as the seed-transmitted viruses appear to be the sole and or the primary source of inoculum. In considering the inoculum threshold of seed-transmitted viruses (i.e., the maximum amount of inoculum that can be tolerated), this aspect is by far the most significant (Sastry, 2013).

1.5.3 Grafting

Grafting or vegetative propagation is a means of increasing vegetation. Grafting is considered to be a universal method for transmitting viruses because systemic viruses can be transmitted by grafting. Graft transmission of viruses to susceptible host plants is indicated when the virus strain is not readily or not at all mechanically transmissible. Viruses can also develop and multiply from contaminated buds, cuttings and rootstocks. Grafting is particularly useful for transmission of phloem-restricted viruses that cannot be transmitted mechanically and viruses whose vectors remain unknown, and for detecting viruses found in low concentrations (Lal *et al.*, 2015).

1.5.4. Insect vectors

Insect transmission is the most widespread means of virus transmission under field conditions. Due to a strong cell wall boundary and immobility of plants, most plant viruses need vectors for the transmission to new host plants or to a new habitat (Blanc and Drucker, 2011). Approximately 80% of plant viruses depend on insect vectors for transmission, and plant viruses demonstrate a high level of specificity for the group of insects that may transmit them. Vectors of plant viruses are taxonomically very diverse and can be found among arthropods, nematodes, fungi, and plasmodiophorids (Froissart *et al.,* 2002; Hull, 2002).

The important arthropod vectors of plant viruses are: four families of homopterans (aphids, whiteflies, leafhoppers, and delphacid planthoppers), thrips, chrysomellid beetles, and, among the acarines, the eriophyid mites (Bragard*et al*., 2013). Insects in the order homoptera are well adapted to their role as vectors by their capacity to pierce the epidermis and delicately deposit the virus in the cytoplasm without risking the integrity of the plant cell. Hemipteran insects transmit 55%, with aphids, whiteflies and leafhoppers transmitting 28%, 18%, and 4% of plant viruses, respectively (Hogenhout *et al*., 2008). As such, insect vectors are a major driver of plant virus emergence (Fereres, 2015) and are potentially a major factor controlling virus spread (Elena *et al*., 2011).

More than 380 viruses from 27 plant virus genera are transmitted by the homoptera (Lal *et al.*, 2015). More than half of the nearly 550 vector transmitted virus species recorded so far are disseminated by aphids (55%), leafhoppers (11%), 11% beetles (11%) (Emma, 2015). The most common being aphids with more than 200 vector species identified (Ng and Perry, 2004).

Vector-virus transmission consists of several successive steps: acquisition of virions from an infected source, stable retention of acquired virions at specific sites through binding of virions to ligands, release of virions from the retention sites upon salivation or regurgitation, and delivery of virions to a site of infection in a viable plant cell. The vector virus transmission types are generally categorized into non-persistent, semi-persistent and persistent modes, according to the length of the period the vector can harbor infectious particles, which can range from minutes to hours (non-persistent), to days (semi-persistent) and to life-time and even inheritance by the insect progeny (persistent-propagative) (Ng and Falk, 2006; Hogenhout *et al*., 2008).

Non-persistent plant viruses are retained in the insect stylet. The feeding insect loses the virus rapidly when feeding on a non-infected plant (Agrios, 2014). Non-persistent viruses are retained by their vectors for a few hours and transmitted the virus to a new host plant after feeding on an infected plant by the vector lasts from seconds to minutes. Semi-persistent viruses are not thought to be internalized in the insect vector gut, but instead reside in chitin-lined areas (Ng and Falk, 2006).

Persistent viruses are taken up into and retained by insect tissues and are characterized by invading the salivary glands (Hogenhout *et al*., 2008). They require long acquisition times (ranging from hours to days) and long latent periods (ranging from one day to several weeks). Once acquired from infected plants, are associated with the vector for the rest of their lifetime. Successful transmission of persistent viruses requires an internalization of the ingested viruses that are actively transported across several cell membranes. Persistent viruses are also referred to as circulative (Emma, 2015), viruses must escape the insect gut and spread to neighboring organs to reach the salivary glands for transmission (Bragard *et al*., 2013; Hogenhout *et al*., 2008). Persistent viruses can be further divided into propagative, e.g., viruses that replicate in their arthropod vectors in addition to their plant hosts, and nonpropagative viruses, e.g., viruses that replicate only in their plant hosts but not in their vectors (Gray and Banerjee, 1999).

1.6. Plant Virus Disease Management

Disease management is the selection and use of appropriate technologies and techniques (practices) to suppress disease to a tolerable level. The goal of plant disease management is to reduce the economic and aesthetic damage caused by plant diseases. Proper disease management is achieved when the causation and the effect (damage) that the disease could cause are known. The main approach for plant virus management is prevention or delaying virus infection (Rubio *et al*., 2020). Various means have been used to achieve these objectives, including reduction of initial inoculum, reducing the rate of infection, management of insect vectors insecticides or other means and deployment of resistant or tolerant varieties.

1.6.1. Reduction of initial inoculums

1.6.1.1. **Pathogen exclusion**

Pathogen exclusion is the prevention of disease establishment in areas where it does not occur. Planting materials are inspected before entering and going out of countries and within country regions to prevent transmission of the disease especially by seed transmission. This is a major objective of plant quarantine procedures and use of disease-free planting materials throughout the world (Thresh, 2003; Kiruwa*et al*., 2016).

Quarantine: Plant quarantine is a national service and is organized within the framework of Food and Agriculture Organization (Kumar *et al*., 2004). It is considered as one of the best procedures of controlling movement of seed transmitted viruses (Adams *et al*., 2014). In various countries and territories, legislation at this time in force to prevent introduction of important seed-transmitted diseases and pests involves the following quarantine regulations: (i) embargo and import permit, (ii) inspection (field inspection and laboratory testing) in the exporting country before shipment of the consignment, (iii) seed treatment for diseases and pests that can be eliminated by disinfection or fumigation with reasonable certainty, (iv) post-entry growth inspection by the importing country in closed quarantine and (v) certification (Khetarpal and Gupta, 2006; Munkvold, 2009).

Most countries differentiate between the seed imported for scientific purposes and those imported for sowing or commercial purposes. Since more than 231 viruses are seed transmitted (Sastry, 2013), there is a high risk of introducing the virus diseases, which are not known to occur in a

country if proper testing is not carried out. Even minute quantities of soil and plant debris contaminating true seeds can introduce the virus/vector, or both. Thus, man is the direct or indirect cause of most epidemic imbalances that known about, and the high virulence and extreme susceptibility of an epidemic situation is an unnatural imbalance usually brought about by human disturbance (Jones, 2000).

Use of virus free-planting materials: The use of virus-free planting materials for all new plantings is a basic approach to control that is beneficial for several reasons: (i) virus-free material establishes more readily and is more productive than infected; (ii) if virus-free material is adopted there are no initial foci of infection within crops from the outset, during the early most vulnerable stages of crop growth. This delays and curtails the period over which any subsequent spread can occur; (iii) plants not infected until a late stage of crop growth are affected less severely than those infected early; and (iv) infected propagules are particularly dangerous sources of inoculum because they tend to be distributed randomly within crops. This facilitates virus spread from infected to neighbouring healthy plants, whether this is by contact or by vectors. For these reasons, much attention has been given in technologically advanced countries to producing virus-free stocks of seed and of tubers, cuttings or other propagules of crops that are propagated vegetatively (Thresh, 2003).

1.6.1.2. Pathogen eradication

This method reduces pathogen from infected areas before it becomes well established. Pathogen eradication includes sanitation, which involves cleaning of tools such as tractor and clothing used in infected fields, removal of infected maize plant debris that will act as source of inoculum in the next season, rouging of diseased plants (Mawishe and Chacha, 2013), eliminating weeds and other alternative hosts (insect vectors), which serve as reservoir for viruses (Webster *et al*., 2004; Trigiano*et al*., 2008).

Sanitation: Epidemiologically numerous examples of the hazards posed by the debris of previous crops, and by re-growth from the tubers, roots, stems or other plant material left in the ground at harvest. There are also problems due to the growth of ‘self-sown’ seedling ‘volunteers’ of crops such as cereals. This facilitates the survival and perennation of viruses and their vectors, and can provide a ‘green bridge’ between successive growing seasons. Hence the removal and destruction

of these crops deny the virus and its vector a survival opportunity and reduce the disease intensity in the subsequent cropping seasons (Thresh, 2003).

Removal of weeds or wild hosts: Many viruses have weeds or wild hosts that act as foci of infection from which there is spread into or within crops. Weed and volunteer plants being major components of the agroecosystem not only compete with crop plants for water and nutrients but also serve as sources of virus inoculum for both the crop and the vector. In some of the weed hosts, the virus is seed transmitted, and the infected seeds survive in the soil for long periods. In certain cases, the presence of weeds in the field becomes more dangerous as they are symptomless virus carriers and consequently are difficult to assess (Thresh, 1981; Sastry, 1984; Sastry, 2013). Therefore, the removal of weeds and wild hosts will support in the management of virus diseases.

Roguing: Roguing is a well-known phytosanitation measure where virus disease control achieved by eliminating initial sources of infection from which further spread can occur. Roguing is widely applicable and has been used in attempts to control or at least contain diseases of diverse crops in both temperate and tropical regions (Thresh, 1988). The approach is most effective against viruses when the virus incidence is very low, especially in small plantings, help in minimizing the spread of virus (Sastry, 2013).

Crop Rotation: Crop rotation has historically been a major means of disease control in production of annual and biennial crops. Encouraging results are available wherein the soil borne diseases are minimized by crop rotation with non-susceptible hosts of virus/vector (Sastry, 2013; Uyemoto, 1983).

1.6.2. Reducing the rate of infection

1.6.2.1. Chemical control

Chemical control method involves protection of the host from invading pathogens (viruses). Many viruses have insect or fungal vectors that spread into, between and within crops. This has led to the use of pesticides or other chemicals to prevent such spread by decreasing vector populations or by impeding transmission (Satapathy, 1998; Perring *et al.*, 1999).

Plant viruses cannot be directly controlled by the use of chemicals, but chemicals can be used to kill vectors that transmit/spread those viruses. Several insecticides, formulated either as granules

or spray applications can be used to manage vectors (e. g. aphids, rootworms, stem borers, mites, thrips) that transmit plant viruses. The introduction of the neonicotinoid class of insecticide seed treatments (imidacloprid, thiamethoxam and clothianidin) are used in a large improvement in insect control. Prior to the commercialization of these neonicotinoids, most insecticide products did not persist long enough to provide effective control on a broad spectrum of insects. Neonicotinoid insecticides possess a number of valuable attributes that have led to their increased adoption by growers (Hahn and Noleppa, 2013).

1.6.2.2. Host resistance

Plants are continuously affected by a number of diseases. Plants have developed resistance mechanisms to prevent and resist attacks from pathogens (Dangl and Jones, 2001). Use of resistant plant cultivars for disease management is an environmentally friendly and cost-effective measure compared to other methods such as chemical control methods (Kumar *et al*., 2004). This is because it is durable, reduces crop losses due to disease and no or little use of chemicals (pesticides) that could affect human and the environment. Resistance to plant viruses can be due to the inability to establish infection, inhibited or delayed viral multiplication, blockage of movement, resistance to the vector, and viral transmission from it (Jones, 1998), and resistance to symptom development, also known as tolerance. Genetically, disease resistance in plants can be either qualitative/complete resistance conditioned by a single gene/major gene or quantitative/incomplete resistance conditioned by one-to-many genes/minor genes (Poland *et al*., 2009).

Qualitative resistance involves resistance genes (R-genes) with gene-for-gene action in which pathogen avilurence genes (Avr-gene) interact directly or indirectly with a plant resistance gene (R-gene) to activate resistance mechanism in the host plant. Flor (1971) found that each avilurence gene in the pathogen has a corresponding resistance gene in the host and the interaction between them initiates a hypersensitive reaction. Lack of compatibility between avirulence and resistance genes results a susceptible reaction (Hammond-Kosack and Jones, 1997). Qualitative resistance is specific for each race and strains of a pathogen. Each species has a large number of R-genes with receptors specific to different strains of pathogens (Ellis *et al*., 2000).

Quantitative disease resistance is conferred by multiple genes (quantitative trait loci) with minor effects. Such kind of resistance is known to be non-specific and controlled by environmental

factors which make it difficult to know the mechanism underlying resistance by multiple genes. Due to high interaction with environment and incomplete gene effects, it is difficult to fine map and clone genes conferring quantitative disease resistance (Ali and Yan, 2012).

1.6.2.3.Quarantine

Quarantine and sanitary certification of virus free seeds and asexual propagative planting materials are the primary procedures to avoid the introduction of new viruses in to previously virus free geographical areas. Plant quarantine is a national service and is organized within the framework of Food and Agriculture Organization (Kumar *et al*., 2004). It is considered as one of the best procedures of controlling movement of seed transmitted viruses. In various countries and territories, legislation at this time in force to prevent introduction of important seed-transmitted diseases and pests involves the following quarantine regulations (i) embargo and import permit (restriction placed on the *import* or export of goods, services by government); (ii) inspection (field check and laboratory testing) in the exporting country before shipment of the consignment; (iii) seed treatment for diseases and pests that can be eliminated by disinfection or fumigation with reasonable certainty; (iv) Post-entry growth inspection by the importing country in closed quarantine; and (v) Certification (Khetarpal and Gupta, 2006; Munkvold, 2009).

Most countries differentiate between the seed imported for scientific purposes and those imported for sowing or commercial purposes. Since more than 231 viruses are seed transmitted (Sastry, 2013), there is a high risk of introducing the virus diseases, which are not known to occur in a country if proper testing is not carried out.

1.7. Maize Lethal Necrotic Disease

1.7.1. History and global distribution of MLN

Maize Lethal Necrosis (MLN), also called Corn Lethal Necrosis, has been known since 1970's in the USA. MLN were first reported from the Americas – Peru in 1973 and followed by Kansas, USA in 1976 (Castillo and Hebert, 1974; Niblett and Claflin, 1978) as a synergistic interaction between *Maize chlorotic mottle virus* (MCMV, family *Tombusviridae*) and *Maize dwarf mosaic virus* (MDMV, *Potyviridae*) (Uyemoto, 1983). The disease was later reported from several countries across the Americas, Asia, Africa and Europe (Table 1).

Table 1. Distribution of maize lethal necrosis (MLN) in different parts of the world and year of first report.

Continent/Country	Year (First report)	Reference
South America		
Peru	1973	Castillo and Hebert, 1974
Argentina	1982	Teyssandier *et al*., 1982
Brazil	1982	Uyemoto, 1983
Ecuador	2015	Quito-Avila *et al*., 2016
North America		
Mexico	1987	Delgadillo and Gaytán, 1987
Hawaii	1990	Jiang *et al*., 1992
USA	1976	Niblett and Claflin, 1978
Europe		
Spain	2015	Achon*et al*., 2017
Asia		
Thailand	1982	Klinkong and Sutabutra, 1982
China	2011	Xie *et al*., 2011
Taiwan	2014	Deng *et al*., 2014
Africa		
Kenya	2011	Wangai *et al*., 2012
Tanzania	2012	Mahuku *et al.*, 2015b
Uganda	2013	Mahuku *et al.*, 2015b
Rwanda	2013	Adams *et al*., 2014
Democratic Republic of Congo	2013	Lukanda *et al*., 2014
Ethiopia	2014	Mahuku *et al.*, 2015a

In Africa, MLN was first reported in Kenya in 2011 (Wangai *et al*. 2012); and has been widespread then and caused significant maize yield reduction in major maize growing countries of East Africa, including Uganda, Tanzania, Rwanda, Democratic Republic of Congo and Ethiopia (Table 1). In East Africa and China, MLN was caused by the co-infection of the SCMV and the MCMV (Adams *et al*., 2014; Lukanda *et al*., 2014, Mahuku *et al.*, 2015b; Xie *et al*., 2011).

1.7.2. Impact and losses associated with MLN

MLN disease causes damage at various levels ranging from low to total failure depending on environmental conditions, varieties grown, crop growth stage during the disease onset and level of infection. Infected maize plants are commonly barren and the ears formed are small, deformed and set very little or no seeds, considerably reducing the yield. In Kansas, USA, crop yield losses due

to MLN (which is referred to as corn lethal necrosis disease) was 91% in 1977 (Nault *et al*., 1978). In China the loss was estimated at more than 2 billion US$ (Rao *et al*., 2010). Recently in Ecuador, in areas where MLN outbreaks caused yield losses of 25–40% in the 2015–2016 production years (Vega and Beillard, 2016).

MLN had a serious impact on maize production and grain yields in eastern Africa (De Groote *et al*., 2016; Marenya*et al*., 2018). The economic impact of the disease on smallholder farmers across Kenya, Tanzania, Rwanda, Uganda and Ethiopia were estimated between 291 and 339 million US$ (Pratt *et al*., 2017). It was reported in Uganda in 2013 with yield loss of 50.5% (Kagoda *et al*., 2016) and in Rwanda in 2013 (Adams *et al*., 2013) with up to 100% crop loss. In Kenya, 23-100% yield loss was reported in severely affected areas, about 0.5 million tons with a value of US$ 180 million (De Groote *et al*., 2016). In Ethiopia, MLN is widespread and has caused from low to complete crop failure in some areas of Oromia, SNNP and Benishangul-Gumuz regional states (Bekele *et al*., 2017; Fentahun *et al*., 2017; Guadie *et al*., 2018). The estimated losses amounting to US$261 million in Ethiopia (Marenya *et al*., 2018).

MLN had a devastating effect not only on the maize production, but also on other key actors in the maize seed/grain value chain, especially small- and medium-enterprise, seed companies and processors. Demand for seed of commercial maize varieties decreased when MLN was a major epidemic in the affected countries, with consequent losses of sales for maize-based seed companies, and carry-over of significant quantities of seed. Thus, in addition to the resource-poor farmers, small and medium-enterprise seed companies were highly affected by the intensity and spread of MLN in eastern Africa (Boddupalli *et al*., 2020).

1.7.3. Viruses causing MLN disease

MLN is caused by a co-infection of *Maize chlorotic mottle virus* (MCMV; genus *Machlomovirus*; family *Tombusviridae*) with any one of several cereal viruses in the family *Potyviridae*, such as *Sugarcane mosaic virus* (SCMV) (Wangai *et al*., 2012; Mahuku *et al*., 2015b), *Maize dwarf mosaic virus* (MDMV) (Niblett and Claflin 1978), *Johnson grass mosaic virus* (JGMV) (Stewart *et al*., 2017) or *Wheat streak mosaic virus* (WSMV) (Scheets 1998). In East Africa including Ethiopia the main cause of the disease is a co- infection with MCMV and SCMV (Wangai *et al*., 2012; Adams *et al*., 2014; Lukanda *et al*., 2014; Mahuku *et al*., 2015a). Both MCMV and SCMV

synergistically interact with one another such that the two comfortably survive in the infected maize plant (Zhang *et al*., 2008; Xie *et al*., 2016). Any of the two viruses can infect the maize plant before the other or both can infect the plant at the same time (Scheets, 1998; Gowda *et al*., 2015; Xie *et al*., 2016). Among the two causal viruses in Ethiopia, the new and the most important component is MCMV while SCMV is known to commonly occur on maize for long time causing mild mosaic symptom (Lencho *et al*., 1997).

The symptoms of MLN are much more severe than the additive symptoms of either MCMV or the potyvirus virus alone. The virus complex causes a severe systemic necrosis which culminates in death of the plant (Niblett and Claflin, 1978; Uyemoto *et al*., 1980; Uyemoto *et al*., 1981)

1.7.3.1. Maize chlorotic mottle virus

Maize chlorotic mottle virus (MCMV) is the only identified member of the genus *Machlomovirus* in the family *Tombusviridae* (King *et al.*, 2011). The virus has a single-stranded RNA genome with virions that are single 30 nm isometric particles with a smooth spherical or hexagonal shape (Goldberg and Brakke, 1987; Lommel *et al*., 1991).

MCMV was first identified in Peru in 1973 (Castillo and Hebert, 1974) and has since then been reported in other areas including Kansas and Nebraska in USA (Nault*et al*., 1978; Philips *et al*., 1982), Argentina (Jiang *et al*., 1992), Mexico and Hawaii (Jensen *et al*., 1991), China in 2011 (Xie*et al*., 2011). In Africa, MCMV was first occurred in Kenya in 2011 (Wangai *et al*., 2012), Tanzania in 2012 (Mahuku*et al*., 2015b), Uganda in 2012 (Kagoda*et al*., 2016), Rwanda in 2013 (Adams *et al*., 2014), Democratic Republic of Congo in 2014 (Lukanda*et al*., 2014) and Ethiopia in 2015 (Mahuku *et al*., 2015a).

Several strains of MCMV have been identified. MCMV-NE is the isolate from Nebraska (Stenger and French, 2008), MCMV-K and MCMV-P are isolates from Kansas and Peru, respectively (Uyemoto, 1983) while MCMV-YN the Chinese isolate from Yunnan (Xie *et al*., 2011). The US isolate (K and NE) share 99.5% Nucleotide sequence identity, a clear indication that the two isolates are related (Stenger and French, 2008). The Yunnan isolates (MCMV-YN) shares nucleotide sequence identity with MCMV-NE and MCMV-K of 97.3% and 97.1%, respectively (Xie *et al*., 2011). MCMV isolates from Thailand were closely related to China strains with 98-99.6% sequence similarity (Wu *et al*., 2013).

The nucleotide sequence similarity of MCMV isolates from East African countries are 99% (Mahuku *et al*., 2015b), indicating that the whole region has similar MCMV viruses interacting mainly with SCMV. Kenyan isolates had 95-98% sequence similarity (Wangai *et al*., 2012). Ethiopia isolate was similar to East Africa isolate with 99% similarity (Mahuku *et al*., 2015b). Rwanda, Kenya, Chaina isolates were identical with 99% and 96-97% with USA isolates (Adams *et al*., 2014).

Under natural condition, MCMV causes 10-15% crop loss and up to 59% loss under inoculated conditions (Castillo and Loayza, 1977). Depending on the host genotype, MCMV infection symptoms range from mild to severe chlorotic mottle, leaf necrosis, stunted growth, a shortened male inflorescence with few spikes, malformed or partially filled ears and premature death of plants (Niblett and Claflin, 1978; Uyemoto*et al*., 1981). Earlier, maize was reported as the only known natural hosts of MCMV (Scheets, 2004). Recent studies, however, have identified MCMV from sugarcane (Wang *et al*., 2014), finger millet (Kusia *et al*. 2015), Napier grass and Kikuyu grass (*P. clandestinium*) (Mahuku *et al*., 2015). Bockelman *et al*. (1982) has also indicated a broad range of MCMV experimental host range that includes at least 19 grass species, but it does not infect dicots.

MCMV is transmitted mechanically by sap, by seed at low rate (0.04%) (Jensen *et al*., 1991) and spread by several insect vectors including maize thrips (*Frankliniella williamsi*) (Jiang *et al*., 1990), maize rootworms (*Diabrotica undecimpunctata, Diabrotica longicornis*and *Diabrotica virgifera*), cereal leaf beetles (*Oulema melanopus*), corn flea beetle (*Systena frontalis*) and *Chaetocnema pulicaria* (Nault*et al*., 1978; Jensen, 1985). The virus may also be spread through soil and through infected plant debris since the virus can survive in plant residues (Nyvall, 1999). Continuous maize production in a field greatly increases the incidence of the viruses and vectors (Miano, 2014).

1.7.3.2. *Potyviridae* (*Sugarecane mosaic virus*, *Maize dwarf mosaic virus* and *Wheat streak mosaic virus*)

Viruses in the *Potyviridae* family are considered the most agronomically destructive. They are distributed worldwide in maize and other crops (Ali and Yan, 2012; Shulka*et al*., 1994). Maize-

infecting viruses in the family *Potyviridae* were first described in the 1960s from Ohio in the United States (Redinbaugh and Zambrano-Mendoza, 2014).

Viruses in the genus *Potyvirus* are single-strand positive sense RNA viruses with flexuous rod-shaped virions of about 12 x 750 nm (Lapierre and Signoret, 2004). MDMV and SCMV are the most important potyviruses causing maize dwarf mosaic around the world. MDMV is prevalent in North America and Europe, and SCMV (formerly known as MDMV-B) is found worldwide (Lapierre and Signoret, 2004; Ali and Yan, 2012). At least thirteen strains of SCMV have been reported throughout the world. Sugarcane, sorghum, maize, *Eteusine* spp., *Panicum* spp. and *Setaria* spp. are hosts of SCMV (Brunt *et al.,* 1990).

Potyviruses are naturally transmitted in a non-persistent manner with acquisition and transmission occurring within minutes by aphids (Shulka *et al*., 1994; Shukla *et al*., 1994), and through seeds at varying rates (<0.5%) (Lapierre and Signoret, 2004). MDMV and SCMV (previously known as MDMV-B) are transmitted mechanically and spread by many aphids such as *Rhopalosiphum maidis*, *Aphis gossipi*, *Myzus persicae, Hysteroneura setariae, Rhopalosiphum padi (*Noone *et al*., 1994; Anon, 1986; Singh *et al*., 2005, Stewart *et al*., 2016). Aphids that transmit these potyviruses have worldwide distribution and appear to be ubiquitous where maize is grown, including East Africa. WSMV is transmitted by the wheat curl mite (*Aceria tosichella*) in a semi-persistent manner (Stenger *et al*., 2005). WSMV and its vector are found in the continental United States, Canada, South America, Eastern Europe, Australia, and the Middle East (Hadi *et al*., 2011), however, it has not been reported in East Africa or Asia (Redinbaugh and Stewart, 2018).

1.7.4. Synergisms between MCMV and potyvirus

MLN is caused by the synergistic infection between MCMV and MDMV, WSMV, SCMV or JGMV, leading to serious yield losses in maize (Goldberg and Brakke, 1987; Scheets, 1998; Mahuku *et al*., 2015b; Xia *et al.,* 2016; Stewart *et al*., 2017). The presence of a potyvirus increases the concentration of MCMV particles up to 5 times in a co-infected plant (Goldberg and Brakke, 1987; Scheets, 1998). For example, the interaction between MCMV and WSMV which cause maize/corn lethal necrosis, results in a significant increase up to 10-fold of the MCMV concentration in plants (Scheets, 1998).

The effect reported for these synergisms is a dramatic increase in MCMV concentrations in mix-infected plants compared with single-infected plants. This increase in concentration is termed unilateral synergism and describes a phenomenon where the presence of one virus increases the concentration of the co-infecting virus resulting to more severe symptoms than when an individual virus infects alone (Goldberg and Brakke, 1987; Scheets, 1998). The observed increase in concentration of MCMV in a co - infected plant compared to the plant infected by MCMV alone is hypothesized to be due to the ability of the potyvirus to suppress regulatory systems that would normally limit MCMV concentrations in a cell allowing easy transmission of the MCMV and increasing the symptom severity (Goldberg and Brakke, 1987).

1.8. MLN Symptom Development

The symptom displayed by MLN disease depends on the stage of the crop affected. The infected plants develop intense chlorosis from the base of young whorl leaves upward to the leaf tips (Niblett and Claflin, 1978; Scheets, 1998; Wangai *et al*., 2012). As the disease progress, the leaves become extremely chlorotic and necrosis sets in starting from the leaf tips and edges progressing inwards (Goldberg and Brakke, 1987; Scheets, 1998). This leads to a dead heart symptom (wangai *et al*., 2012). Early infections lead to complete plant death (Goldberg and Brakke, 1987) while late infections lead to plants aging prematurely, male sterility and malformed or no ear or production of deformed seed (Uyemoto *et al*., 1981), no grain fill and cob rotting (Wangai *et al*., 2012) and general stuntedness (Goldberg and Brakke, 1987). Environmental factors also play a key role in the development of the disease. High temperatures, on average, 27.5 °C (Osunga *et al*., (2017) favor the development of MCMV and hence the development of MLN disease, disease symptoms develop early and disease severity is aggravated (Scheets, 1998).

1.9. Problem Statement and Justification

MLN disease is considered as series threat to maize production in East Africa including Ethiopia, due to its rapid spread and interaction with pre-existed local viruses, transmission by different mechanism, and the absence of resistant commercial maize lines and hybrids currently under production. Hence, MLN represents a significant menace to maize production. Isabirye and Rwomushana (2016) predicted the possibility of increasing the incidence and distribution of the disease to other regions of East and Central Africa with similar climatic conditions to the current

hotspots. This menace of potential spread is one of the reasons to find a solution to MLN. The disease is newly emerged in Eastern African region; and hence, much is not known about its epidemiology and control measures. MLN has a fast-spreading nature, and almost all maize varieties currently under production are susceptible to the disease as these varieties were not originally bred for resistance to the disease. Information is required about the nature and disposition of the primary infection source, how the virus spreads from infected to healthy plants within a crop, how it spreads over distance to invade new sites, and how it survives outside the main growing period. Plant virus diseases including MLN are intrinsically difficult to manage directly by use of chemical pesticides; however, integrated management methods which include cultural practices such as removal of infection sources, field sanitation, removal of alternative hosts, use of healthy seed (virus free seeds); chemical pesticides to control insect vectors indirectly through seed treatment and foliar spray are the most possible management measures of plant viral diseases. For such an approach to succeed, the epidemiology and geographical spread of the disease should have to be studied first.

Good knowledge of factors influencing the occurrence and spread of MLN, its causal agents and their dissemination and survival mechanism is important for developing effective management measures. MLN disease epidemics are multi-component systems resulting from interactions between the viruses, vectors, host plants and environment conditions. Since MLN disease is a new disease to Ethiopia, there is lack of adequate research-based information that can lead to its effective management. These include information on the factors that contribute to MLN disease epidemics including alternative hosts that serve as media for viruses overwintering; the role of insect vectors, soil and plant debris, seed transmission; as well as strategies for the development and use of MLN disease resistant maize varieties were not incorporated in national maize breeding programs.

In Ethiopia, studies conducted on MLN, so far, have focused mostly on the identification and characterization of the causal viruses from maize as well as economic importance and geographical distribution of the disease (Mahuku *et al*., 2015a; Fentahun *et al*., 2017; Demissie *et al*., 2018; Guadie *et al*., 2018). Little attempts were made to relate cropping systems, practices and environmental parameters to MLN disease epidemiology. Understanding the association of disease intensity with different cropping systems and practices will help to identify the most important

variables and focus efforts to develop sustainable management packages (Fininsa and Yuen, 2001). Knowledge of the ways in which a virus maintains itself and spreads in the field is usually essential for the development of satisfactory control measures.

The reports from USA in Hawaii indicated that wild grasses harbour MCMV (Nelson *et al*., 2011). In Ethiopia, only limited number of alternate hosts of MLN causing viruses were reported most of which were not identified to species level (Mahuku *et al*., 2015a; Bekele *et al*., 2017). Moreover, no experimental host ranges studies were conducted by artificially inoculating the viruses onto different weed species and related cereal crops. This information is necessary to establish whether weeds and other cereal crops harbour the MLN causing viruses (MCMV and SCMV) in Ethiopia and how they impact on the disease epidemiology in maize fields.

The knowledge of virus transmission and its survival longevity are also important to understand how the disease transmits from infected to health plants, the virus spreads between and among maize fields and develop management strategies and tactics. However, no experiments have been conducted on MLN soil transmission and longevity of the virus persistence which can be important for effective use of crop rotation as management options. Similarly, in order to better understand the epidemiology of MLN, the insect vector species and their ability to efficiently transmit the disease have to be determined. However, insects observed during the field assessment, such as beetles, thrips and aphids were not experimentally tested for their ability to efficiently transmit MLN causing viruses (Guadie *et al*., 2018; Terefe and Gudero, 2019). Finally, the development of MLN resistant varieties which is an economically feasible and environmentally sustainable approach for disease management requires identification of resistant genotypes, and incorporation of the disease resistance into agronomically desirable varieties. Effective screening of different maize genotypes is vital in identifying genetic resistance for MLN. This research was designed to address the problems indicated above and seek solution for them by means of the different experiments.

1.10. Objectives

The general objective of this research was to study the epidemiology of MLN disease and its management through understanding the survival mechanisms of MLN-causing virures, insect-vector management and identification of resistance/tolerant maize genotypes in Ethiopia.

Specific Objectives:

1. To determine the prevalence, intensity, and distribution of MLN disease and the association of the disease intensity with different cropping systems and cultural practices of maize (**Paper I**);
2. To generate comprehensive information on the identity of alternative hosts, seed transmission and its role in the spread of MLN causing viruses (**Paper II**);
3. To determine the role of MLN virus infected maize plant residue, infested soil in the transmission of MLN causing viruses, and the longevity of the viruses both in the infected plant residue and in the infested soil (**Paper III**);
4. To identify the main insect vectors and their ability to transmit MLN causing viruses ((**Paper IV**);
5. To evaluate the efficacy of selected seed dressing insecticide against MLN causing insect vectors (**Paper V**); and
6. To identify MLN resistant maize genotypes which can be utilized in maize breeding programs for the management of MLN disease by screening the genotypes under artificial inoculation conditions, (**Paper VI**).

2. MATERIALS AND METHODS

2.1. Prevalence and Intensity of Maize Lethal Necrosis in Ethiopia (Paper I)

2.1.1. Survey regions

MLN disease survey was conducted in five main maize growing regions of Ethiopia; namely, Amhara, Oromia, South Nation, Nationality and Peoples (SNNP), Benishangul-Gumuz, and Tigray regions. The regions and zones within the regions were selected based on maize production potential. The selected regions cumulatively account for 97% of maize producing areas of Ethiopia (Abate *et al*., 2015). In addition, the surveyed areas represent three major maize growing agroecologies: (i) moist and semi-moist mid-altitudes located at 1700–2000 m.a.s.l, with an annual rainfall of 1000–1200 mm; (ii) moist upper mid-altitudes, with elevation of 2000–2400 m.a.s.l and an annual rainfall greater than 1200 mm; and (iii) moist lower mid-altitudes located at 900–1500 m.a.s.l, with annual rainfall of 900–1200 mm (Abate *et al*., 2015). Weather data from maize planting to the time of survey were obtained from the Ethiopian National Meteorology Agency for each target locality.

2.1.2. Disease assessments and data collection

MLN disease assessments were conducted during the 2015-2018 main-cropping season (July to August) and off-season (March to April) at the vegetative growth stage of maize. Fields were randomly sampled at 5 to 10 km intervals on the main and accessible rural roads. In each selected field, maize plants were evaluated for the prevalence and disease intensity diagonally in an 'X' pattern in five quadrants (3×3 m) with 10 m distance between two quadrants. MLN disease incidences were rated as a percentage of diseased maize plants with MLN-like symptoms within the quadrant.

$$Percent\ disease\ incidence = \frac{Number\ of\ infected\ plant}{total\ number\ of\ plants\ assessed} \times 100$$

MLN severity was scored on a rating scale of 1 to 5, where 1 = maize with no visible MLN-like symptoms; 2 = symptoms with <25% on leaves; 3 = symptoms with 25 to 50% on leaves; 4 = symptoms with 50 to 75% on leaves and 5 = symptoms with 75 to 100%. The severity scales were converted to percent severity index (PSI) (Osunga *et al*., 2017). Disease prevalence was

determined as the ratio of the number of fields where MLN disease was present to the total number of fields assessed.

$$PSI = \frac{\sum of\ all\ disease\ rating}{total\ numberofrating \times maximumdiseasegrade} \times 100$$

During the survey, data were collected on geographic information (geographic locations, and altitude); cropping system (mono-cropping or crop rotation); crop variables and agronomic management (a maize variety used, planting date and previous crop history); presence/absence of suspected MLN insect vectors (beetles, thrips and aphids), and weed density (low, medium or high) for each field. Information on the type of variety, planting date, and the previous cropping history was obtained from growers through interviews. Representative diseased maize leaves were collected for laboratory diagnosis to confirm the presence of MCMV and SCMV in the samples.

2.1.3. MLN causing virus detection

Serological assays using Double Antibody Sandwich-Enzyme Linked Immuno Sorbent Assays **(**DAS-ELISA) method as described by Clark and Adams (1977) and instructions of the antiserum manufacturer (German Collection of Microorganisms and Cell Culture-DSMZ) was used to test for the presence of both of the MLN causing viruses: SCMV and MCMV.The detection antibodies for MCMV (AS-1087), SCMV (AS-0166) and their respective positives controls were obtained from - DSMZ- Plant Virus Department, Germany.

All samples were tested in duplicates according to a standard protocol described in DSMZ DAS-ELISA kit. Briefly, DAS-ELISA plates were prepared by adding 200 µl coating antibody for each specific MLN causing virus into each well of ELISA plate (dilution 1:1000 v/v of antibody: buffer). The plates were sealed with Para film and incubated at 37˚c for 3 hrs. Plates were thereafter washed three times in PBS-Tween (Phosphate Buffered Saline-Tween 20 pH 7.4) at three min intervals and dried on blotting paper. For each MLN specific virus, 200 µl of the test sample (extracted leaf samples in sample extraction buffer 1: 20 weight/volume) were added into each well in duplicates, negative and positive controls were also loaded, the plate sealed with Para film and incubated in refrigerator at 4˚c overnight. Plates were again washed three times with PBS-Tween, dried on blotting paper and 200µl enzyme conjugate diluted in conjugate buffer at a recommended dilution of 1:1000 (v/v) added to each well. Plates were thereafter incubated at 37ºC

for 3 hours and washed three times with PBS-Tween. Two hundred µl freshly prepared substrate (1 mg/ml p-nitrophenyl-phosphate in substrate buffer) was added to each well, incubated at 37ºC for 30-60 minutes for reaction to take place. Plates were then assessed visually for color change (development of yellow color) and its spectrophotometric absorbance measured at 405 nm wavelength using ELISA plate reader. All samples were assayed in duplicate and the results to be positive if the absorbance was greater than twice the average reading of the negative (healthy) controls.

2.2. Maize Lethal Necrosis Alternative Hosts (Paper II)

2.2.1. Field assessment of MLN alternate hosts

MLN natural alternate hosts were assessed in Oromia and SNNP regions of Ethiopia in 2016 and 2017 during the main rain season (August to September). East Shewa, Arsi and Jimma zones of Oromia and Wolayita zone of SNNP Regional States that have high levels of MLN infestation were selected for the study. Two districts with the history of high MLN incidence were purposively selected from East Shewa (Adama Zuria and Lume), Jimma (Omo Nada and Shebe Sonbo) and Wolayita (Damot Gale and Damot Pulasa) zones, while only one district was picked from Arsi Zone (Jeju district). Weed species with or without virus-like symptoms (chlorosis, mosaic, mottling, stunting, necrosis, yellowing) found in and near MLN infected maize fields were assessed. In addition, cultivated cereals found within or adjacent to the maize fields were included.

Selected maize fields were sampled at the intervals of 5 to 10 km along the main and accessible rural roads using a 1-meter square quadrat. The number of samples collected varied from district to district due to differences in the level of MLN incidence and virus-like symptoms observed in the fields. More samples were collected from fields with higher MLN incidence and virus-like symptoms and fewer samples were collected from fields with lower incidence and symptoms.

Herbarium press was used for preserving plant samples including weeds before the identification to species level. Species identification was made by consulting biosystematics unit herbarium of Ambo Agricultural Research Center and using a weed identification guide book (Stroud and Parker, 1989).

A total of 434 samples belonging to 28 different species and 11 families were collected, out of which 113 samples were displaying virus-like symptoms (29 in East Shewa, 19 in Jimma, 24 in Arsi, 41 in Wolayita Zones), and the remaining 321 samples were non-symptomatic. Among these samples, 399, 24 and 11 were weeds, cereal crops and sugarcane (*Saccharum officinarum*), respectively.

2.2.2 Virus detection and back-inoculation test

The presence of MCMV and SCMV in samples collected from weeds and cultivated crop species were tested using DAS-ELISA following the procedure indicated under section 2.1.3 above. Back-inoculation test was used to determine the ability of MCMV and SCMV that were detected from weeds and cultivated crops to cause MLN disease. For this purpose, plant saps from samples already tested positive for each of the virus using DAS-ELISA were inoculated on maize. An abrasive agent, carborundum dust (SiC), was added to the inoculum solution to cause microscopic injury of the leaves for easy penetration of the virus into the plant cells (Orawu *et al*., 2013). The inoculum was then rubbed with fingers onto 4-6 leaf stage of MLN susceptible maize cultivar (Morka) in an insect proof- greenhouse, and then immediately rinsed with water. The symptom development was monitored after inoculation at least twice a week for 30 days and detection of the viruses was carried out by DAS-ELISA.

2.2.3 MCMV experimental host range

MCMV host range studies were conducted using different weeds and cereal crops species at Ambo Agricultural Research Center in an insect-proof greenhouse at 25-30 °C. Five to eight test plants of each species were planted in 10 cm diameter plastic pots filled with mixed sterilized soil, sand and organic manure at 2:1:1 ratio, respectively, and watered regularly as required. The pots were replicated three times for each plant species. A total of 39 species from 12 weed families, which were naturally associated with maize crop (Hailegiorgis *et al*., 2005) and 10 cereal crops varieties, including wheat (*Triticum aestivum*L.), barley (*Hordeum vulgare* L.), sorghum (*Sorghum bicolor* L.) and finger millet (*Eleusine coracana* L.) were tested to determine potential hosts of MCMV. MLN susceptible maize cultivar (Morka) was used as positive control while non-inoculated plants of all species were included as negative control. MCMV isolates were multiplied on two-week-old susceptible maize cultivar (Morka) seedlings by mechanical inoculation and later used as

inoculum source to infect suspected alternate host plants sampled. MLN symptoms were established fully within two weeks after post-inoculation on the infected susceptible Morka maize variety. The infected maize leaves were collected and ground using sterile mortar and pestle in 10 ml of 100 mM phosphate buffer, pH 7.0 to obtain homogenate solution or extract (1:10; 1g of leaf materials to 10 ml extraction buffer). Carborundum powder was added to the homogenate which was subsequently used to rub on leaves of test plants.

MCMV inoculum was then rubbed at 14 days after planting using fingers onto 3-6 leaf stage of seedlings and rinsed immediately with water. The second inoculation was carried out at an interval of one week after the first inoculation to confirm effective viral infection and to avoid possible disease escapes. Dead leaves were removed from the alternate host test plants at the time of inoculation. The development of symptoms was monitored starting from 10 days after the second inoculation and leaf samples were collected for virus detection and back inoculation tests. The viruses were detected using DAS-ELISA as described in section 2.2.

For the back-inoculation tests, the inoculum was prepared from leaf samples (1 g in 10 ml of extraction buffer) and inoculated to a two-week-old susceptible maize cultivar (Morka). The symptom development was monitored after inoculation and disease testing was carried out by DAS-ELISA.

2.3. MLN Seed Transmission (Paper II)

Naturally infected maize plants with MLN in the field and tested ELISA-positive were used as source of seed for seed transmission study (Jensen *et al*., 1991). A naturally MLN infected maize plants, in the experimental fields of three Agricultural Research Centers (Melkassa, Jimma and Wendogenet) were used for seed transmission study. Symptomatic maize plants exhibiting wide chlorotic, mosaic leaf streaks and occasionally stunting and/or general chlorosis were tagged on the stalk for subsequent ear harvest in MLN infected maize fields.

Leaf samples from a representative subset of those tagged maize leaves were collected and DAS-ELISA performed to confirm the presence of MLN disease causing viruses in the samples. The confirmed tagged maize plants that was infected by MLN and its causing viruses were allowed to grow until maturity. At harvest, ears from the maize plant ELISA-positive for MLN disease

causing viruses, individually and in combination (MCMV, SCMV and MCMV + SCMV) were collected, dried to about 12.5-15% moisture, and threshed by hand.

Harvested maize seeds were re-planted separately, which was in three groups according to their virus content (MCMV, SCMV and MCMV + SCMV in combination) in insect proof greenhouse. Seeds were planted in metallic trays (51 x 63 cm) in a greenhouse potting soil mixed with sand and organic manure at 2:1:1 ratio, respectively, and watered regularly. Seed trays had seven rows with 10 seeds per row and were placed on greenhouse benches with no supplemental lighting. Most of the tests were conducted at 25-35°C day temperatures. After emergence, maize seedlings were observed at least twice a week from the two leaf stages until the five to six leaf stages for symptoms appearances, and any symptomatic seedlings were labeled for subsequent testing.

The viruses present were confirmed using DAS-ELISA against the two MLN causing virus (MCMV and SCMV). Non-symptomatic plants were also randomly collected and tested by DAS-ELISA. One month after planting, the numbers of seedlings showing symptoms were expressed as a percentage of germinated seedlings. Transmission percentages were calculated as follows.

$$\mathrm{TP} = \frac{\mathrm{IS}}{\mathrm{TGS}} * 100$$

Where TP= Transmission percentage, IS= Infected seedling which is ELISA Positive, TGS= Total germinated seedlings.

2.4. Role of Soil and Maize Residues on MLN Transmission (Paper III)

2.4.1. Sample collection

One hundred ninety-four soil samples were collected from 68 MLN infected maize fields in Oromia and South Nation, Nationality and Peoples (SNNP) regional states in Ethiopia. These regions are already known to have a high level of MLN infection rate (Regassa *et al*., 2020). Field assessment was conducted in 2017 and 2018 during the main rainy season (August to September) at vegetative stage of maize. Based on MLN incidence and severity, three zones each from Oromia (Jimma, East Shewa and Arsi) and SNNP (Wolayita, Hadiya and Sidama) Regional States, and one to two districts were purposively selected from each zone.

Samples were randomly taken from several points in each field where maize showed severe MLN symptoms (severe leaf chlorotic and mottling, necrosis of leaf margins) at the stage of 10 leaves to near maturity stage. Soil samples suspected to be infested by MLN causing viruses were collected near the roots of symptomatic maize plants. The sampled plants were uprooted and the soil was gently removed from the root by shaking. One hundred ninety-four samples of maize parts (stem and root) from representative subset of maize plant surrounding the soil samples were collected and DAS-ELISA test was performed to confirm the presence of MCMV and SCMV in the samples of maize parts following the method described below. Soil samples collected from the surroundings of ELISA-positive maize plant parts that were infected by MLN causing viruses were used for MLN soil transmission study.

2.4.2. MLN causing viruses detection in infested soil and infected maize samples and mechanical inoculation test

Detection of the viruses (MCMV and SCMV) from soil and maize parts was assayed by using DAS-ELISA following the procedure indicated under section 2.1.3 above. The DAS-ELISA positive soil samples were retested for further confirmation by lateral flow assay for MCMV by dispensing the homogenized sample on the sample spot where the virus reacts with a virus-specific antibody conjugated to colored dye. DAS-ELISA was performed separately on maize parts from individual plants.

To determine disease transmission potential of MCMV and SCMV that were detected from soil and plant part samples, saps from the samples tested positive for each of the virus were used for back-inoculation test. Six DAS-ELISA positive samples were selected from each category (soil, stem and root of plants) for the back-inoculation test. Six seeds MLN susceptible maize variety called Limu (P3812W) (Regassa *et al*., 2020) were planted in 25 cm diameter plastic pots filled with mixed sterilized soil, sand and organic manure at 2:1:1 ratio, respectively. The pots were kept in an insect-proof greenhouse at 25-30 ^{o}C and watered regularly as required. To facilitate mechanical inoculation, carborundum dust was added to the inoculum solution prepared from the leaves. MCMV and SCMV inoculum were then inoculated separately by using fingers onto 4-6 leaf stage of healthy maize seedlings separately in an insect-proof greenhouse and rinsed immediately with water. The symptom development was monitored after inoculation and detection of the virus was carried out by DAS-ELISA.

2.4.3. Soil transmission test

The study was conducted in an insect-proof greenhouse. Seeds of a maize variety called Limu (P3812W) were planted on SCMV, MCMV and MLN infested soil samples collected from the root zones of diseased maize plants. Different soil samples were prepared for transmission tests depending on the type of MLN causing viruses. Accordingly, SCMV infested soil samples were considered as the first group, and MCMV infested samples were considered as the second group. Soil samples from mixed infection of the two viruses (MCMV + SCMV) as the third group.

Seeds were planted in metallic trays (51 × 63 cm) filled with suspected virus-infested soil collected from the root areas of MLN causing viruses infected maize plants. The trays have six rows with 10 seeds per row and were placed on greenhouse benches with no supplemental lighting. Most of the tests were conducted at 25-35 °C day temperatures. After emergence, maize seedlings were observed for symptoms appearances at least twice a week starting from two-leaf stages, and any symptomatic seedlings were labeled for subsequent testing. Several plants with symptoms were recorded at 30 days after planting and the presence of viruses (MCMV and SCMV) were confirmed using DAS-ELISA test as described above under section 2.1.3. Transmission percentages were calculated as follows:

$$\mathrm{TP} = \frac{\mathrm{IS}}{\mathrm{TGS}} \times 100$$

Where TP = Transmission percentage, IS = Infected seedlings which is ELISA Positive, and TGS = Total germinated seedlings.

2.4.4. Persistence of MLN causing viruses in infested soils and plant residues

2.4.4.1. Sources of MLN infected maize residues and infested soils

Infected maize residues and infested soils were obtained from maize which was planted in a greenhouse and inoculated with a mixture of MCMV and SCMV. The MLN causing viruses (MCMV and SCMV) used in this study were collected from the field and maintained and propagated in separate greenhouses periodically by mechanical inoculations. Symptomatic leaves from each virus were weighed and ground using sterile mortar and pestle to obtain homogenate solution or extract (1:10; 1g of leaf materials to 10 ml extraction buffer) separately. The inoculum

extracts were mixed in 1:4 ratios (adding one part of MCMV and four parts of SCMV) in one container to obtain an optimized virus combination known to cause (1:4, MCMV: SCMV) MLN in East Africa (Gowda *et al.* 2015) and inoculated on to healthy maize seedlings in the greenhouse. The inoculated plants were grown until flowering. For the study as a source of MLN infected residue, the infected maize was uprooted, chopped into smaller pieces and used as MLN infected residue. The soil from which the MLN infected maize uprooted were used as MLN infested soil.

2.4.4.2. Treatments and experimental design

The treatments used for the study of MLN causing viruses persistence included maize planted on (1) MLN infested soil mixed with MLN infected maize residue, (2) MLN infested soil only, (3) MLN free soil (uninfested soil) mixed with MLN infected maize residue and (4) sterilized MLN free soil used as control. Plastic pots (30 cm diameter) were used for growing maize. Nine seeds of MLN susceptible maize variety called Limu (P3812W) (Regassa *et al.*, 2020) were sown in each pot and reduced to seven plants after germination. The treatments were arranged in a completely randomized design with four replications. MLN infected maize residue was mixed with MLN infested and MLN free soil at a ratio of 1:4 (residue: soil) by weight. MLN free soil used in the study was collected from Ambo University Guder Campus where maize have never been planted and no MLN disease was found. The treatment categories were stored for one, two, three, four, five and six months before the transmission test. Number of plants with symptoms was recorded at 30 to 40 days after planting and the presence of MCMV and SCMV was confirmed by DAS-ELISA test following the procedure indicated under section 2.1.3 above. Data collected included the number of plants with disease symptoms confirmed by DAS-ELISA and percent disease incidence.

2.5. Identification of Insect Vectorsn of Maize Lethal Necrosis Causing Viruses (Paper IV)

2.5.1. Field assessment, insect specimen and plant sample collection

The assessments and collection of suspected MLN-causing virus insect vectors were undertaken in Oromia and South Nation, Nationality and Peoples (SNNP) regional states of Ethiopia during the main and off-season maize growing periods in 2017-2018. Based on MLN survey results, regions that are identified to have a high prevalence and incidence of MLN infection were selected for the field assessment. Based on MLN incidence and severity, three zones from each of the two

regions and one to three districts were purposively selected from each zone. Maize fields were assessed at 5 to 8 km intervals along the main and accessible rural roads. The altitude of surveyed maize fields ranged from 958 to 1995 m.a.s.l.and the growth stage of maize in most of the fields during the assessment was at a vegetative growth stage. In each field, maize plants were assessed visually for the presence of insects and typical MLN-like symptoms diagonally (in an 'X' way) by counting 25 randomly selected plants at equal distance in each transect. Within selected plants, different types of suspected insect vectors including aphids, thrips and beetles, which are potential vectors of MLN causing viruses (MCMV or SCMV) were assessed and collected. Count of insect was done from 25 randomly selected plants and recorded as the total number of insect species presented in selected plants per field as low (≤30), medium (31 to 60) and high (>60).

Insects were either aspirated directly from plants or swept from plants with a net and then aspirated. For sedentary insects, collection was done by collecting the plant part where they were present and transferred thoroughly using camel brush. Each insect type was collected in sufficient numbers either live in small cage or jar to be used in transmission studies for further rearing and some preserved in 70% alcohol for identification purpose.

2.5.2. Laboratory testing and species identification

2.5.2.1.Virus detection in plant samples and insect specimens

The presence of MLN causing viruses were detected from the collected samples by DAS-ELISA following the procedure indicated under section 2.1.3 above. In addition, field-collected samples of each insect species (aphids, thrips and beetles) were ground in a mortar and pestle and the resulting homogenate was tested for MLN causing viruses (SCMV and MCMV) by DAS- ELISA. To determine whether the virus detected was viable and capable of causing disease, the insect homogenate (maize thrips, *Franklinella* sp. and cereal leaf beetles, *Oulema* sp.) was then mechanically inoculated onto healthy maize seedlings (3-4 growth leaf stage). Controls consisted of inoculations of sap extracted from healthy maize and MCMV were used. Inoculated maize seedlings and control treatments were replicated four times.

2.5.2.2. Insect rearing

The insects (aphids, thrips and beetles) collected frequently from different locations were sorted and reared on healthy maize seedlings for further identification and determination of their capacity to transmit MLN causing viruses (MCMV and SCMV).

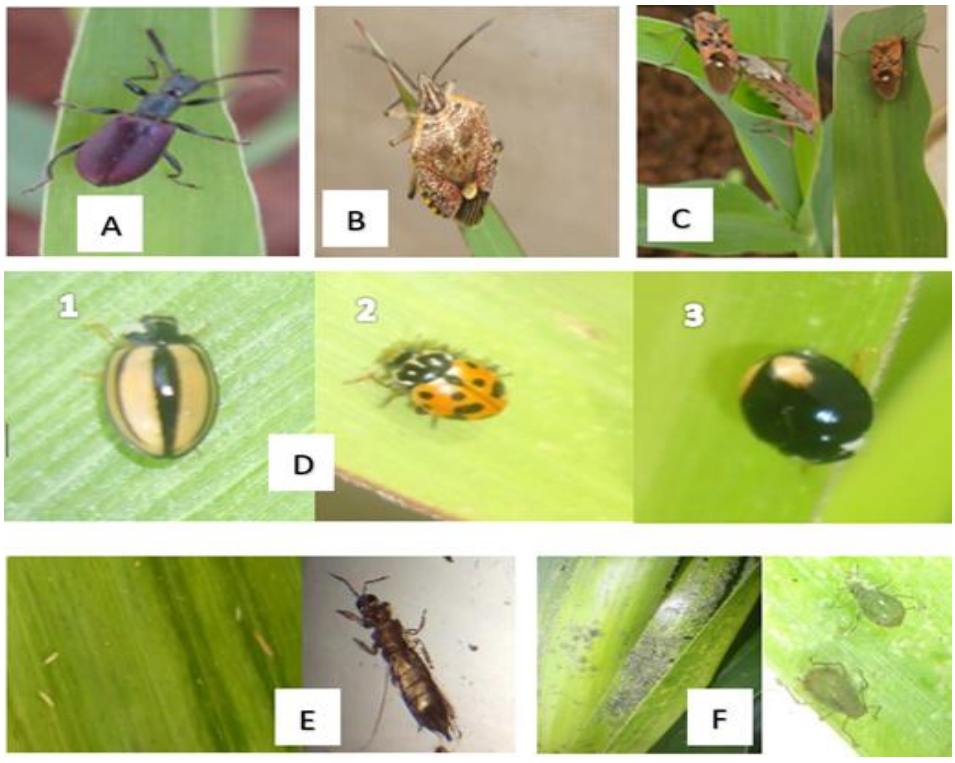

Fig 1. Insect species collected from MLN infect maize field and assessed for the capacity for MLN causing virus transmission: A= Cereal leaf beetle, B= sting bugs, C= Graptostethus spp, D= lead bugs, E= thrips, F= Aphids

MLN susceptible maize variety called Limu (Regassa et al., 2020) obtained from Pioneer Hi-Bred Seeds-Ethiopia was used for insect rearing and transmission assays. Seeds were grown in a 25 cm diameter plastic pots filled with a sterilized soil mixture (soil, sand and yard manure in the ratio of 2:1:1, respectively). The seedlings were grown until 3-4 leaf stage to be used and maintained inside insect-proof cages with a photoperiod of 12 h and a temperature range of 25-30°C. Both potted maize seedlings used for rearing and transmission were kept in a separated section of the cage to avoid uncontrolled insect infestation or MCMV and SCMV contamination.

Colonies of each insect collected were individually reared on healthy maize seedlings grown inside pot and placed in insect proof cages and insect were transferred to potted maize seedlings inside cages. Leaf samples from the maize seedlings used for insect rearing were periodically tested by dDAS-ELISA to confirm the freeness of insects from MCMV or SCMV.

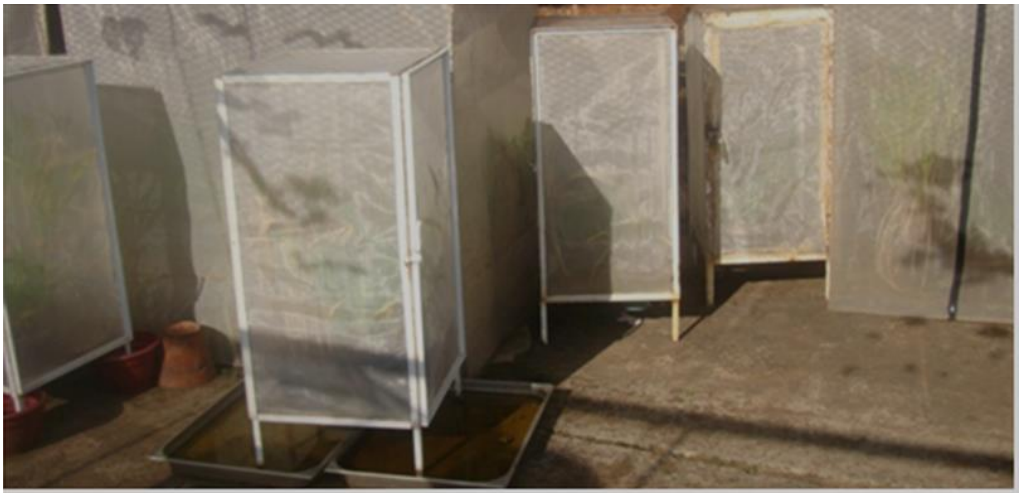

Fig 2. Insect proof cage used for rearing insect-vectors susspeted to transmit MLN

2.5.2.3. Morphological and PCR-based vector species identification

The insects confirmed as vectors of MLN causing viruses (MCMV and SCMV) were identified to genus/species level using morphological charactrization. Morphological identification of the insects to species level was done by known features for aphids using the key by Blackman and Eastop (2000) and Thrips Lucid Key Server by Moritz *et al*. (2017). Further confirmation for some insects (aphids and beetle) was performed by employing a molecular method following DNA extraction and Polymerase Chain Reaction (PCR) analysis at the Molecular Biology Laboratory, Addis Ababa Science and Technology University, Ethiopia. PCR was carried out with Universal Cytochrome Oxidase (COx) primers. Primers used were forward primer: LCO 2518087 5′-GGTCAACAAATCATAAAGATATTG G-3′ and reverse primer: HCO 2518088 5′-TAAACTTCAGGGTGACCAAAAAATCA-3′ (Jalali *et al*., 2015). The extract DNA was subjected to PCR amplification of a 658 bp region near the 5′ terminus of the COX 1 gene following standard protocols. The amplified products were sent to commercial sequencing company, M/s Eurofins Pvt Ltd. India. Each species was bidirectionally sequenced and checked

for quality by Bioedit 7.0.2 software and homology, insertions and deletions, stop codons, and frameshifts by using NCBI BLAST.

2.5.3. MCMV and SCMV maintenance

Leaves from maize plants showing typical symptoms of chlorosis, mottling and mosaic symptoms were collected from MLN disease infected maize fields during the assessment of insect vectors. For the confirmation of the target viruses, the collected samples were assayed for both viruses (MCMV and SCMV) by DAS-ELISA as described above. After the assay, each virus was transferred separately onto 3-4 leaves growth stage of maize seedlings by mechanical inoculation. For the availability of virus isolates, each virus was maintained separately by periodic mechanical inoculation to healthy maize in the insect proof greenhouse.

2.5.4. Vector transmission test

MLN causing viruses' transmission by insect vectors test experiment was conducted in greenhouse in 2018-2019. Healthy seedlings of maize were grown in 25 cm diameter plastic pot filed with sterilized soil mixtures of soil, sand and yard manure in the ratio of 2:1:1, respectively. Seven seeds were sown in each pot and later thinned to five plants. Three weeks after emergence (3-4 leaf stage), the maize seedlings were placed in insect proof cages covered with clear polyester clothing and were used for inoculation experiments.

Adult insects were used for the transmission study. Each insect species was put in a separate Petri-dish containing dry filter paper using camel hairbrush and starved for two to three hours. The starved insects were transferred to Petri dishes containing leaves harvested from MLN infected maize plants. The acquisition and inoculation access periods were adjusted as previously adopted to examine the transmission of MLN causing viruses by the chrysomelid beetles, thrips and aphids (Nault *et al*., 1978; Jensen, 1985; Cabanas *et al*., 2013). The aphids were allowed acquisition access period of 20-35 minutes on the infected maize leaves while thrips and beetles were allowed a period of 2 days. After the acquisition feeding period, varying numbers of insects depending on their abundance (Aphids: 50; thrips: 40; beetles: 25 per pot) were transferred to health maize seedlings in cages using a camel hairbrush and allowed an inoculation access period of 1 hour for aphids, 2 days for beetles and thrips. Maize plants mechanically inoculated with MCMV and

SCMV were used as positive controls, whereas healthy maize (not infested by insect and infected by virus) as negative control. A total of 20 plants (5 plants per replication and repeated four times) were tested for each insect species. After the inoculation access period, the maize plants were sprayed with lamdex® 5% EC (Lambda cyhalothrin 50g/l) to eliminate the insects and were transferred to a greenhouse for symptom development.

2.6. Evaluation of Seed Dressing Insecticides for the Control of MLN Vectors (Paper V)

The experiment was conducted under greenhouse condition. Corn leaf aphid (*Rhopalosiphum maidis*) adults used in this study had been first identified as vector of SCMV and maize thrips (*Frankliniella* sp.) identified as vector for MCMV. *Frankliniella* sp. and *R. maidis* were reared separately on healthy maize seedlings grown inside pot and placed in insect proof cages and insect were transferred to potted maize seedlings inside cages.

2.6.1. Plant materials and seed treatment

The treatments consisted of four systemic insecticides (Apron Star 42 WS, Imidalm T 450 WS, Proseed Plus 63 WS, Evident 25 % WG /Thiamethoxam 25% WG) selected for their ability to be taken up from the treated seed coat and translocate to all parts of the plant during germination and untreated controls. Maize seeds (BH661) were obtained from Bako Agricultural Research Center, Ethiopia. Registered systemic seed dressing insecticides in Ethiopia either singly or with combination of fungicide were used. The seeds were dressed uniformly with lower and above respective dosage of insecticides in three rates of application for each treatment i.e., Thiamethoxam 25% WG and Proseed Plus 63 WS at 1, 2, 3g/kg seed; apron star 42 WS at 2, 2.5 and 3g/kg seed, and imidalm T 450 WS at 0.5, 1 and 1.5 g/kg. The solution volume used (product + water) was 8 ml, 10 ml, 7 ml, and 10 ml per 1 kg of seeds for Apron star 42 WS, Thiamethoxam 25% WG, Imidalm T 450 WS and Proseed Plus 63 WS, respectively. The product slurry was distributed over 1kg of seeds with respective dosage/application rate of insecticide in the bowls and stirred for 5-10 minutes to coat seed uniformly with the insecticide slurry. Then, the treated seeds were allowed to air-dry in the laboratory. The dried seeds treated with respective dosage of insecticides were packed in separate polythene bags and kept under laboratory condition until

sowing. After one- and six-month's storage its effect on seed germination and vector management were evaluated. For comparison, untreated seeds were used as a control.

2.6.2. Germination test of insecticide-treated seeds

The effect of insecticide seed treatment on the germination rate and storage time was evaluated two times after 1- and 6-month storage. The experiment was conducted in completely randomized design with three replications. The test was conducted in a greenhouse with temperature of 25-30 °C during day and 18 °C at night. Randomly selected 20 seeds from each treatment stored at one and six months were placed on water moist double layer of filter paper. Water was added into each plate as required to keep the filter paper moist. Following standard in the germination determination by Gorim and Asch (2012), a seed with visible radicle (longer than 2 mm) was considered as germinated. Number of newly germinating seeds was recorded daily for 8 consecutive days, and the cumulative germination rate was calculated.

2.6.3. Exposing the insects to seedlings derived from treated seeds

The effect of different seed treatments on the vector of MCMV (*Frankliniella* sp.) and SCMV (*R. maidis*) was assessed in an insect-proof cage at room temperature ranging from 25-35 °C. Treated seeds were planted in sterilized soil mixture inside 25 cm diameter plastic pots (five seed per pot). After plants were germinated and reached two leaf stages, a number of vector insects from each of the colony in rearing cage were introduced/transferred (20 *Frankliniella* sp.and 30 *R. maidis* per plant) on to the maize seedlings driven from seeds treated with insecticides. A mixture of adult and immature thrips were exposed to seedlings driven seeds treated withoutdistinction. Three pots (replications) were established for each application rate of each insecticide. Treatments were evaluated by counting the number of live vectors staring from the 3rd day after insects were transferred to maize seedlings at seven days interval for two consecutive weeks (i.e., at 3, 10 and 17 days) after insect vectors were transferred to maize seedlings

The percentage of the reduction (R) of the insect population was calculated according to the following equation (El-Naggar and Zidan, 2013).

$\%\ R = [(NIC - NIT)/ NIC] \times 100$, Where NIC =number of insects in the control and NIT = number of insects in the treatment.

2.7. Screening Maize Genotypes for Resistance to Maize Lethal Necrosis Disease (Paper VI)

2.7.1. Genotypes and experimental design

A total of 306 maize genotypes that included 275 inbred lines and 31 commercial hybrids were used for the study. The inbred lines were collected from major maize breeding programs in Ethiopia. One-hundred inbred lines were obtained from Ambo highland, 66 from Bako mid-altitude and 109 from Melkassa lowland adapted maize breeding programs. Commercial maize varieties were collected from various National Agricultural Research Centers, Dupot Pioneer Hi-Bred Seeds in Ethiopia and Ethiopian Seed Enterprise. Two MLN susceptible maize varieties Limu (P3812W) and Melkassa-2, which were identified by the previous studies (Regassa *et al*., 2020), and six MLN resistant maize cultivars: CKMLN150075, CKMLN150088, CKMLN150076, CKMLN150074, CKMLN15150, and WE5135 were used as checks. The resistant cultivars were developed by CIMMYT in Kenya and are being evaluated across locations in Ethiopia by the National Maize Breeding Program for possible registration and commercialization.

The present experiment was conducted in a greenhouse at 25-35 °C day temperatures at Ambo Agricultural Research Center using artificial inoculation. Two separate sets of experiments that involved 275 inbred lines and 31 commercial varieties were conducted using a completely randomized design with three replications. Each experimental unit consisted of five maize plants grown in a 30 cm diameter plastic pot. Fertilizers were applied at the rates of 100 kg N and 100 kg P_2O_5 in the forms of DAP at planting and UREA 3 weeks after emergence as side dressing, respectively.

2.7.2. MLN causing viruses sources, inoculum preparation and inoculation

Stock isolates of MCMV and SCMV were collected from MLN conducive areas of Oromia region of Ethiopia in hotter Rift Valley areas of East Shewa (Awash Melkassa district) and Arsi (Jeju district) zones. After confirming the presence of SCMV or MCMV by DAS-ELISA, each of the virus isolates were separately propagated on maize and maintained in separate greenhouses transferring periodically by mechanical inoculation.

Symptomatic leaves for each virus isolate were collected separately and cut into small pieces. An extraction buffer of phosphate buffer 0.1 M was made by mixing potassium phosphate dibasic (anhydrous) and potassium dihydrogen orthophosphate (potassium phosphate monobasic) at pH of 7.0 using the following ratios: KH_2PO_4 = 10.8g, K_2HPO_4 = 4.8g and Na_2SO_3 = 1.26 (Sitta *et al*., 2017). Artificial inoculations of MLN causing viruses were done as reported by Gowda *et al*. (2015). Symptomatic leaves for each virus were weighed and ground using sterile mortar and pestle to obtain homogenate solution or extract (1:20; 1g of leaf materials to 20 ml extraction buffer). The inoculum extracts were mixed in 1:4 ratio (adding one part of MCMV and four parts of SCMV) in one container to obtain an optimized virus combination known to cause MLN in East Africa (Gowda *et al*., 2015).

The combination of MCMV and SCMV inoculum was then rubbed onto 4-6 leaf stage of maize seedlings in the greenhouse. Carborundum (SiC) powder, which is an abrasive agent was used to cause microscopic injury of the leaves for easy penetration of the virus into the plant cells (Orawu *et al*., 2013). A second inoculation was done a week from the first inoculation to ensure effective viral dissemination and spread among the genotypes and that there were no diseases escapes. After inoculation, maize inbred lines or varieties that developed minor symptoms and asymptomatic ones were tested serologically for both MCMV and SCMV using DAS-ELISA following the procedure indicated under section 2.1.3 above.

MLN symptoms were assessed for disease severity and incidence to obtain an indication of the disease intensity over time. Weekly MLN disease severity was scored using 1 to 5 scale (Shekha and Kumar, 2012); where: 1 = clean, no MLN symptom on leaves (resistant); 2 = chlorotic mottling on the lower leaves (moderately resistant); 3 = chlorotic mottling and mosaic throughout the whole plant (moderately susceptible); 4 = excessive chlorotic mottling, mosaic, plant necrosis, and/or dead heart (susceptible); and 5 = severe chlorotic mottling, mosaic and necrosis (highly susceptible). Disease severity scoring began one week after the last inoculation and continued for seven consecutive weeks. Delayed scoring of MLN is important to detect late-developing infections as indicated for maize rayado fino virus by Zambrano *et al*. (2013). MLN incidence was measured as the percentage of the number of leaves with MLN infection.

The inbred lines evaluated in this study were classified into five groups (resistant, moderately resistant, moderately susceptible, susceptible and highly susceptible) based on reaction to MLN as

assessed based on final MLN disease severity rating. Resistant inbred lines had final MLN severity score of 1.0, with no disease symptoms. Moderately resistant inbred lines showed mild symptoms with final disease severity score of 2.0 or lower. Moderately susceptible inbred lines had final severity scores of 2-3. Susceptible and highly susceptible inbred lines showed final disease severity scores of 3-4 and higher than 4.0, respectively.

Severity scores were used to generate area under the disease progress curve (AUDPC) values which were analyzed to measure differences among treatments (maize inbred lines and varieties). The disease progress data were summarized into one value by AUDPC, which is suited when host damage and the amount and duration of the disease are proportional (Xu, 2006).

AUDPC was calculated as follows:

$$\text{AUDPC} = \sum_{i=1}^{n-1} [\text{Yi} + \text{Yi} + 1]/2\ x[\big((ti + 1) - ti\big)]$$

Where n = total number of observations, Yi was percent severity index, at the i^{th} observation and ti was the time of observation (days) at the i^{th} observation.

Before calculating AUDPC, the severity scales obtained were converted to percent severity index as indicated under section 2.1.2 above. The AUDPC values for the pooled mean of weekly MLN severity scores, final severity and MLN incidence were subjected to the analysis of variance.

2.8. Data Analysis

Depending on the nature of data collected, different analytical methods were performed. Simple descriptive statistical analyses were performed to summarize the field survey data. The association of MLN disease intensity with independent variables were analyzed using logistic regression (Yuen, 2006; Fininsa and Yuen, 2001) (Paper I), using the Statistical Analysis System procedure of GENMOD (SAS® Version 9.4, SAS Institute, Cary, NC, USA). To describe and compare different categories of the sample units with respect to the desired characteristics, mean, standard deviation, and percentage were computed using simple descriptive statistics and Statistical Package for Social Science (SPSS Version 26). The comparison of treatment means was performed using the least significant difference (LSD) at 5% probability level **(Paper II to VI)**.

3. RESULTS

3.1. Prevalence and Intensity of Maize Lethal Necrosis in Ethiopia

3.1.1. MLN disease symptoms in maize fields

MLN disease infected maize plants at different growth stages starting from early to near maturity stage of the crop. Diverse ranges of symptoms were observed depending on the growth stage of the crop. At the early growth stage, MLN disease symptoms were expressed on the leaves, and the cobs began to show the symptoms at the later growth stage. The typical symptoms included chlorotic mottling of the leaves, usually starting from the base of the young leaves in the whorl and extending upwards toward the leaf tips, mild to severe leaf mottling, the leaves become necrotic at the leaf margins that progress to the mid-rib resulting in drying of the whole leaf. Other symptoms included premature aging of the plants. Severely affected plants formed small cobs with little or no grain.

3.1.2. MLN Disease prevalence and distribution

MLN disease was most widespread in SNNP regional state, with 66.7% prevalence followed by Oromia regional state with 65.6% prevalence, whereas only 47.4% of the surveyed maize field in BG, 18.2% in Tigray and 8.8% in Amhara were infected by MLN and its causative viruses (**Paper I**).

Among 11 Zones surveyed in Oromia regional state, the disease was highly prevalent in Jimma (100%), Ilu Ababor (100%), West Wellega (100%), East Arsi (87.5%), West Arsi (87.5%), East Shewa (75%), West Shewa (62.5%) and East Wellega (40.7%) (Table 4). Among the six zones surveyed in SNNP regional state, Hadiya (100%), Sidama (100%), Wolayita (87.1%) and Alaba (50%) had high MLN prevalence. In BG regional state, Asosa and Metekel showed MLN prevalence of 50 and 44.4%, respectively. Relatively higher MLN prevalence was observed in Awi (22.2%) and East Gojjam (21.4%) zones among the areas survey in Amhara regional state while North West (Alamata) zone of Tigray region had higher MLN prevalence of 33.3%. MLN and its causative viruses were not observed and detected in East Guji and Borana zones of Oromia regional state, West Gojjam zone of Amhara regional state and North Western zone of Tigray regional state.

3.1.3. MLN disease intensity with different cropping systems and cultural practices

The maximum mean incidence of 29.98% and severity of 30% was recorded in SNNP regional state whereas the least incidence of 2.05% and severity of 3.11% were recorded in Amhara regional state. Among maize fields surveyed in zones of Oromia regional state, the maximum MLN incidence of 39.41% was recorded in Jimma Zone. Other Oromia zones such as East Arsi (35%), East Shewa (32.31%), West Arsi (23.18%), West Wellega (21.13%), IlluAbabor (20.34%) and East Wellega (16.77 %%) also showed considerable levels of MLN incidence. On the other hand, the least disease incidence of 4.37% was recorded from West Guji, followed by West Shewa (10.72%) zone. Among SNNP regional state, the maximum MLN incidence was recorded in Wolayita zone (56%) followed by Sidama (40.62%) Hadiya (28%), Alaba (14.17%) zones, wheras the minimum incidence was observed in Segen (3%) followed by GamoGofa (6.46%) zone (**Paper I**).

A total of nine maize varieties and local cultivars were observed during the survey. MLN disease intensity was greater under mono cropping system with mean incidence of 23.62% and severity of 27.41% than the field where crop rotation was used (fields planted with other crops in previous year) that had 12.13% incidence and 15.48% severity.

A higher mean MLN incidence (42.92%) and severity (51.65%) was observed in fields with higher weed density (41-100 weeds m^{-2}) as compared to the field with lower weed density (0-20 weeds m^{-2}) that showed MLN incidence of 10.77% and severity of 13.23%.

Maize fields cultivated at altitudes between 1700 and 2000 m.a.s.l had higher MLN incidence (27.55%) and severity (31.74%) as compared to the areas that lie at altitudes between 900 and 1600 m.a.s.l (with incidence of 16.91% and severity of 31.74%). Areas with altitude greater than 2000 m.a.s.l had mean incidence and severity of 3.76% and 4.51%, respectively.

In terms of cropping seasons, higher incidence and severity of 27.11 and 31.05% were observed on the maize crop grown during the off-season as compared to the main season cropping that showed relatively low incidence and severity of 17.90 and 21.47%. Different maize varieties grown by farmers had different levels of reaction to MLN. The highest MLN incidence (59.29%) and severity (54.86%) was recorded on Jabi, an old hybrid marketed by Dupont-Pioneer, followed by Melkassa-2, BH540 and BH660.

MLN incidence and severity is associated with the presence at least one or more of the suspected MLN insect vectors (thrips, beetles or aphids). Higher mean incidence (29.20%) and severity (33.41%) was recorded for the maize fields where the suspected insect vectors were present as compared to the field without suspected vectors (7.80% incidence and 10.76% severity) (**Paper I**).

3.1.4. Distribution of MLN causing viruses

The two MLN causing viruses were found in almost all major maize growing regional states. The viruses MCMV and SCMV were identified to cause MLN disease in 20 (83.3%) out of 24 zones, 54 (71.0 %) out of 76 districts and 231 (55.93%) out of 413 fields surveyed in the study.

From over all MLN causing viruses in the country, MCMV was the most prevalent virus that was detected on 543 (29.6%) of the 1833 samples assayed, whereas SCMV and MCMV combined with SCMV were detected on 474 (25.9 %) and 421 (23%) of the samples assayed, respectively. The maximum MCMV (34.5%), SCMV (52%) and combination of the two (MCMV+SCMV) (34.4%) virus were detected in samples collected from Oromia, BG and SNNP regional states, respectively.

3.2. Maize Lethal Necrosis Alternative Hosts

3.2.1. Natural alternative hosts of MLNcausing viruses

Overall, a total of 434 samples (113 symptomatic and 321 non-symptomatic) were tested for MCMV and SCMV by DAS-ELISA. Out of these samples of symptomatic samples tested, MCMV was detected in 23 (20.35%), SCMV in 4 (3.53%) and mixed infection of both in 11 (9.73%) samples. However, no MLN causing viruses (MCMV and SCMV) were detected from asymptomatic samples collected (**Paper II**).

MCMV was detected and identified in *Amaranthus hybridus* L. from *Amaranthaceae*, in seven species of *Poaceae* family in *Cyperus cyperoids* L. from *Cyperaceae* family whereas SCMV was detected only in *Sorghum bicolor* (L.) and mixed infection of MCMV and SCMV was detected in three species from *Poaceae*and *Cyperaceae* families.

The back-inoculation from detected DAS-ELISA positive to susceptible maize cultivar was successful for *Cyperus rutundus, Cyperus cyperoides, Snowdenia polystachya, Cynodon*

nlemfuensis, Digitaria sanguinalis, Echinocloa colona, Oplismenushirtellus, Pennisetum purpureum and *Phalaris paradoxa*which were capable of producing symptoms and positive ELISA readings for MCMV. *Sorghum bicolor* and *Saccharum officinarum* developed symptoms and positive result for SCMV while *Amaranthus hybridus*, which tested positive for MCMV by ELISA, did not produce symptoms and also tested negative by DAS-ELISA.

3.2.2. Experimental host ranges of MCMV

In experimental/artificial host range studies in the green house, 39 different species of broad and grass weeds from 12 families, and 10 cereal crop varieties from barley (*Hordeum vulgare*), wheat (*Triticum aestivum*), sorghum (*Sorghum bicolor*) and finger millet (*Eleusine coracana* were tested for MCMV by mechanical inoculation. Among these *Cyperus assimilis* from *Cyperaceae* family; *Andropogon abyssinicus, Cyndon dactylon, Denebraretroflexa, Digitaria abyssinica, D. ternate, Echinocloa colona, Eleusine indica, Eragrostiscilianesis, Pennisetum polystachion, Phalaris paradoxa, Setaria pumila, S. verticillata, S. verticillata, Snowdeniapolystachyaand Sorghum arundinaceum*from family *Poaceae*were identified experimentally as hosts of MCMV. Among cereal crops varieties HB-1307 from barley, Kilinto/DZ 918 and Alidoro/HK-14-R251 from wheat and Geremew/ 87BK-4122 from sorghum were infected by MCMV. Back inoculations of extracted sap from symptomatic of all species and inoculated to susceptible maize variety (Morka) were also successful. Furthermore, inoculum prepared from symptomatic plants was able to reproduce symptoms in healthy seedlings of the respective plants species. No symptom was developed from asymptomatic plants of any plant species, which confirmed the absence of virus detection in these plants (**Paper II**).

3.3. MLN Seed Transmission

A total of 37,140 seeds from 20 maize genotypes were evaluated by grow-out test in the greenhouse. Out of which 32, 856 seedlings were germinated with mean germination of all genotypes was 88.37% with a range of 62.14 to 97.09%. Out of these seedlings only 24 (0.073%) seedling from 14 maize genotypes (1 of 1551 seedlings in BH546, 3 of 3366 in Melkassa-2, 2 of 1,844 in 124-b (109) DUSL, 2 of 1,408 in142-1-e DUSL, 2 of 1,603 in CUBA DUSL, 2 of in 2,005 in CML 124-b (113), 2 of 1,192 in CML 144 DUSL, 1 of 1,542 in CZH131009, 1 of 3,476 in CZH131010, 1 of 1,128 in CZH141029, 2 of 2,118 in CZH141022, 2 of 2,004 in EHYB, 1 of

1,741 in BH540 and 2 of 2,187 in QPM-A seedlings) showed were found infected with MCMV. Only one (0.003%) 1of 3,366 seedlings in Melkassa-2 were infected with SCMV based on symptoms and DAS-ELISA testing. The result indicates low level of transmission of MLN causing viruses in Ethiopian maize genotypes in general with MCMV showing relatively higher transmission rates compared to extremely low transmission rate of SCMV. The infected seedlings were showed symptoms on leaves above the second leaf (**Paper II**).

3.4. Role of Soil and Maize Residue on MLN Transmission

3.4.1. Detection of MLN causing viruses from maize plant parts and soil

Out of 194 maize stem samples, 94 (48.4%), and 24 (12.4%) were tested positive for MCMV and SCMV, respectively, as single infections and 76 (39.2%) had mixed infection of both viruses (MCMV + SCMV). Similarly, from the same size of root samples, single infection of MCMV was detected in 96 (49.5%) and SCMV was detected in 26 (13.4%) of the samples, whereas mixed infection of both viruses was detected in 77 (39.7%) of the samples using DAS-ELISA test. Out of 194 soil samples collected and assessed, 13 (6.7%) of the samples were positive for MCMV but all were negative for SCMVwhen tested using DAS-ELISA. Serological detection and back-inoculation test results showed that MCMV was detected and confirmed to be transmitted from infested soil to newly germinated maize seedlings. However, SCMV was neither detected in soil samples from infected fields nor transmitted to maize seedlings (**Paper III**).

3.4.2. MLN persistence in the soil and plant residues

Maize planted in MLN infested soil mixed with MLN infected maize residue and MLN free soil mixed with MLN infected maize residue that was stored for one month before planting had a higher MLN incidence of 50% than MLN infested soil, which had MLN incidence of 21.4%. Whereas, maize planted in MLN infested soil that was stored for three months had the lowest MLN incidence of 3.57% and no MLN symptoms were observed in maize planted on the soil stored for more than three months. The maize planted on MLN infested soil mixed with MLN infected maize residue generally had more infected plants than those planted on MLN infested soil alone across the storage duration. No symptom was observed on maize planted on sterilized MLN free soil that was used as control.

Out of 119 symptomatic samples tested, 113 of them were positive for MCMV, 3 for SCMV and the other 3 for mixed infection (MCMV+SCMV). MCMV was detected from maize planted in all treatments, while SCMV was detected from samples of maize planted on both MLN infested soil and MLN free soil mixed with MLN infected residue stored for 1 month. The mixed infection of both MCMV and SCMV was detected only from maize planted on MLN infested soil with MLN infected residue stored for 1 month. MCMV did not survive in MLN infested soil (no MLN infected residue) that was stored for more than three months but up to 6 months to maize planted on both MLN infested soil mixed with MLN infected maize residues and MLN free soil mixed with MLN infected maize residue.

3.5. Insect Vector Transmission of MLN Causing Viruses

Morphological identification of potential insect vectors collected indicated that the most predominant species in MLN infected maize fields were maize leaf aphid (*Rhaphalosiphum maydis)*, beetles (cereal leaf beetle and ladybug beetles), and thrips (*Franklinella* sp*.)*. Clear amplicons of expected size (658bp) were obtained from aphid DNA whereas weaker bands from beetle. Sequence analysis showed that the sequences obtained were indeed of those *Rhaphalosiphum maidis* with 100% identity, confirming the results of morphological identification method. However, cereal leaf beetles (*Oulema* sp.) did not show any resemblance with available sequences in the Genebank indicating that this was a new record of vector for the Genebank. Among the insects collected from MLN-infected maize fields and directly caged on plants without an acquisition feeding period, *Oulema* sp. and *Franklinella* sp. transmitted MCMV to healthy plants (**Paper IV**).

Healthy maize plants developed symptoms of MCMV infection were ELISA positive after being caged with thrips (*Franklinella* sp*.)* and cereal leaf beetles (*Oulema* sp.) that had previously fed on MCMV infected maize. True bugs (Stink bugs sp.) and ladybug beetles (*Hippodamia quindecimmaculata, Hyperaspis bigeminata* and *Paranaemia vittigera)* after feeding on MCMV and SCMV-infected plants, did not transmit the virus to healthy maize seedlings, while *Rhapolosiphum maidis* transmit SCMV to healthy maize seedlings. Plants inoculated with MCMV and SCMV homogenates developed similar symptoms and tested positive by DAS-ELISA. Plant sap extracted from uninfected (healthy maize) did not produce symptoms and also tested negative for both viruses by DAS-ELISA.

Ten out of twenty (50%) plants inoculated mechanically from the homogenized field-collected *Oulema* sp. and 11 of 20 (55%) of *Franklinella* sp. were produced typical symptoms of MCMV and positive DAS-ELISA result. MCMV was recovered both by transmission feeding and by mechanical inoculation from *Oulema* sp. and *Franklinella* sp. Plants inoculated with MCMV homogenate developed similar symptoms and tested positive for MCMV by DAS-ELISA. Plant sap extracted from healthy maize did not produce symptoms of MCMV infection in healthy maize seedlings when mechanically inoculated into the seedlings and these seedlings also tested negative for MCMV by DAS-ELISA.

3.6. Evaluation of Seed Dressing Insecticides for the Control of MLN Vector

3.6.1. Effect of seed treatments on maize seed germination

Irrespective of insecticides used in seed treatment, the germination percentage did not differ significantly over different periods of storage. It slightly decreased from 100 - 96.67% in treated seed stored for 1 month and 98.33 – 95% for treated seed stored for six months before planting. These levels of reduced germination rates were comparatively low, never exceeded 4% of the controls in the final evaluation. The germination percentage was higher (100%) in Apron star 42 WS @2g/kg, Imidalm T 450 WS @ 1g/kg, Proseed Plus 63 WS @2g/kg, Apron star 42 WS @ 2.5g/kg and untreated seed, and lower in Thiamethoxam 25% WG (95-96.67%). Comparing all tests, the germination percentage ranged from 95 to 100% (**Paper V**).

3.6.2. Effect of seed treatments on control efficacy against maize thrips (*Frankliniella* sp.) and corn aphids (*R. maidis*)

Significant reduction of both vector species population was observed in all treatments over control (untreated). All the treatments reduced *Frankliniella* sp. population over untreated and the reduction varied from 90.67 to 99.67% for treated seeds stored for one months before planting and 60 to 95.33% for six months stored. All the tested dosages of thiamethoxam 25 WG and Proseed Plus 63 WS @1g/kg (at low rate) were found more effective in reducing *Frankliniella* sp. than other treatments and untreated control. While Imidalm T 450 WS@1.5g/kg (at high dosage) showed lower *Frankliniella*s preduction percentage on both maize seedlings derived from treated seeds stored for one and six months before planting.

The effectiveness of the seed treatments showed that numbers of aphids decreased from 99.48 - 96.15% on maize seedlings derived from treated seeds stored for one month before planting and 95.85-80.59% in treated seeds stored for 6 months before planting. As that of *Frankliniella* sp., thiamethoxam 25% WG at all dosage and storage time were effective and showed insecticidal activity against *R. maidis*. Imidalm T 450 WS at high rate (1.5g/kg) also effective against *R. maidis*, while Proseed Plus 63 WS at 1.0 and 2.0 g/kg (lower and medium dosage) showed lower reduction percentage on both maize seedlings derived from treated seeds stored for one and six months before planting.

Overall, the results revealed that treatments thiamethoxam 25% at 2.0g/kg seed and imidalm T 450 at rate of 1.55g/kg were significantly superior in reducing of both species population when compared with others. While Proseed Plus 63 WS @1g/kg (at low rate) is the lowest in reduction of *Frankliniella* sp. and *R. maidis* population.

3.7. Screening Maize Genotypes for Resistance to Maize Lethal Necrosis Disease

3.7.1. Reaction of maize genotypes to MLN

3.7.1.1. Inbred lines

Based on final MLN severity rating, only 2% of the highland maize inbred lines showed resistant reaction whereas none of the inbred lines from mid-altitude and lowland maize breeding programs had resistant reaction to the disease. Nearly, 7% of the highland, 17% of the mid-altitude and 6% of the lowland inbred lines were moderately resistant to the disease. Larger proportion of the inbred lines from all breeding programs showed susceptible and highly susceptible reactions to MLN. Among the 275 inbred lines about 1%, 9%, 20%, 44 % and 27% showed resistant, moderately resistant, moderately susceptible, susceptible and highly susceptible reactions to the disease, respectively. Except two highland inbred lines (ABL-91 and AMB17KN20-1), all the inbred lines evaluated showed MLN symptoms.

Among the top twenty MLN resistant highland inbred lines, two inbred lines showed resistant and another seven moderately resistant reaction to MLN, with lower values of mean weekly and final disease severity scores (1.0 -2.0), pooled mean of weekly disease severity (1.0 - 2.1), AUDPC

(45.5 - 99.7), and diseaseincidence (0.0 - 54.3%) (Table 4). Inbred lines ABL-91 didn't show MLN disease symptom and no virus was detected when tested with DAS-ELISA. Whereas, AMB17KN20-1 was symptomless but tested positive for MCMV. Some inbred lines showed MLN disease symptoms in the later weeks. For example, inbred lines AMB12N33-84, AMB14N17-2-6, ABL-35 and ABL-64 showed MLN symptoms in the third week, Breeder-k, AMB17N17-8 and ABL-77 in the fourth week, ABL-44 in fifth week and Breeder-w in the sixth week after inoculation. Inbred lines ABL-11, ABL-19, ABL-67, ABL-10, and ABL-42 were severely infected by MLN.

Among the top 20 most resistant inbred lines collected from mid-altitude maize breeding program, CML204, CML464, TZMI754, TZMI751 and TZMI717 showed moderately resistant reactions to MLN with 1.0 – 2.0 weekly disease severity score and lower AUDPC (79.9-86.8), pooled mean of weekly disease severity (1.7-1.9), final disease severity (2.0) and disease incidence (25.0-66.7%). Delayed appearances of MLN symptoms were observed in CZLQ2 that showed MLN symptoms in the thirdweek, and TZMI730 and CML159 inthe fourth week after inoculation (Table 5). Among the inbred lines from lowland maize breeding program, MKL17K0422, MKL17K0597, MKL17K0343, MKL17K0343 and MKL1K0716 showed relatively lower values of weekly severity scores, AUDPC and final MLN severity. These inbred lines also showed delayedMLN symptoms (**Paper VI**).

7.3.1.2.Varieties

Maize varieties evaluated in this study showed significantly variable levels of resistance to MLN disease. Among the disease parameters, AUDPC ranged from 45.5 to 173.8 with a mean of 116.4. Final severity score ranged from 1.0 to 4.4 with a mean of 3.0. Pooled mean of weekly MLN severity ranged from 1.0 to 3.7 with a mean of 2.5, while disease incidence ranged from 0.0 to 95.1% with a mean of 69.5. As expected, the susceptible check varieties had higher AUDPC values of 135.0 (Melkassa-2) and 154.6 (Limu, P3812W), whilst the resistant check varieties had low AUDPC values ranging from 45.5 to 78.9. The six varieties from CIMMYT-Kenya CKMLN150075, CKMLN150088, CKMLN150076, CKMLN150074, CKMLN15150, and WE5135) that were used as resistant checks did not show MLN disease symptom (except WE5135 which had weekly severity rating ranging between 1.0 and 2.0 and AUDPC of 78.9. Serological test using DAS-ELISA, however, showed that these varieties were positive for MCMV, which is

the main cause of MLN disease. All the maize varieties evaluated in this study were infected by MLN but had different levels of reaction to the disease. Most varieties showed moderately susceptible to highly susceptible reactions. Two varieties, which were collected from Ambo highland maize breeding program (Wenchi and Kolba) showed moderately resistant reaction to the disease (**Paper VI**).

8. DISCUSSION

A field survey was conducted in major maize producing regions of Ethiopia to determine MLN geographical distribution and factors associated with disease intensity, MLN and its causative viruses were widely distributed throughout major maize growing areas of Ethiopia, especially in Oromia and SNNP regions. Most farmers in these regions practice continuous maize production throughout the year due to the availability of residual moisture and irrigation water. From results of the study, it was evident that rotating maize with other crop types decreased MLN disease incidence as compared to maize mono-cropping. The presence of maize crop in the field throughout the year provided a favorable environment for the preservation of insect vectors and MLN causative viruses, whereby infected plants used as a bridge between cropping seasons. This finding is in agreement with the results reported from USA by Uyemoto (1983) who reported that fields planted with crops other than maize in the previous year have mostly lower the intensity of MLN disease.

High prevalence and intensity of MLN disease were observed at altitudinal ranges of 900-1600 m.a.s.l and 1700-2000 m.a.s.l as compared to higher altitude of >2000 m.a.s.l, indicating that mid and low-altitude maize growing environments of Ethiopia were more favorable for MLN disease than the high-altitude environments. A similar report by Guadie *et al*. (2018) showed high prevalence of viral diseases of maize in low to mid-altitude areas. Higher altitude areas of Ethiopia are characterized by high rainfall (Abate *et al*., 2015) and cool temperature, which could hinder insect vector reproduction and ease of mobility to spread the viruses. On the other hand, maize grown at an altitude range of 1700 to 2000 m.a.s.l receives moderate rainfall (Abate *et al*., 2015). Such environments are characterized by warm and semi-humid weather conditions, which could be favorable for insect vectors development and spread that result in increased prevalence and incidence of MLN disease. Insect populations of most virus vectors build up faster in areas with

high temperature and high relative humidity, and decline at low temperature and high rainfalls (Islam *et al*., 2017).

The findings of this study showed that the seasonal variability of MLN disease incidence as higher incidence was observed during the off-season than the main rainy season. Recently, Guadie *et al*. (2018) also reported a relatively higher incidence of MLN disease during the off-season than the main rainy season. A plausible explanation for such variation might be that in the off-season, maize grown under irrigation was the only green vegetation in the area that can attract the insect vectors, and also dry and hot conditions during the off-seasons would be favorable environment for reproduction and movement of vectors to transmit MLN causing viruses.

Plant viruses have weeds or other alternate natural hosts that act as the source of inoculum from which the economically important crop plants may become infected (Neeraj and Zaidi, 2008; Mathews and Dodds, 2008). In this study, the prevalence and incidence of MLN disease were found to be higher in maize fields with high weed density than weed-free maize fields. This suggested that weeds play a significant role as the source of infection for the spread of viruses. Similar findings were previously reported by Thresh (1982). Seven species of *Poaceae*, two species of *Cyperaceae*, and two species of cultivated crops from *Poaceae* were infected by MCMV and SCMV in the field either individually or in a mixed infection. Even though some of the species were previously identified as alternate hosts of MLN causing viruses (Mahuku *et al*., 2015a; Bekele *et al*., 2017), several other species were identified by this study as new records. Symptomatic samples of *Cyperus cyperoides* and *Snowdenia polystachya* collected from the fields adjacent to MLN infected maize fields were positive for MCMV and showed a clear yellow colored symptom. *Phalaris paradoxa*, *Oplismenus hirtellus*, *Echinochloa colona*, *Cynodon nlemfuensis* and *Pennisetum purpureum* from *Poaceae* family and *Cyperus cyperoids* from *Cyperaceae* family were identified as natural hosts of MCMV by this study for the first time in Ethiopia and possibly globally. The natural hosts identified newly in this study indicated the availability of several previously unknown favorable hosts that can act as the source of infection for the spread of MLN causing viruses. This study indicated that MCMV is fairly commonly found on grass weeds within or nearby maize fields. In a similar study however, Bockelman (1982) in Kansas, USA, didn't observe MCMV on grass weed samples nearby the maize field indicating tha epidemiological factors there may be different from that currently exists in Ethiopia.

Among the cultivated plants, sugarcane (*Saccharum officinarum*) and sorghum (*Sorghum bicolor*) were naturally infected by both MCMV and SCMV in combination and individually in the field and thus, acting as possible virus reservoirs. Since these two crops are economically important, MLN would also be a potential threat to the production of these crops.

Results of the host range identification experiments for MCMV indicated that three species from the *Cyperaceae* family and 17 species of *Poaceae* family were identified as hosts of MCMV. Among cultivated crops, a barley variety (HB-1307) and wheat varieties (DZ 918 and HK-14-R251) were infected by MCMV and showed clear disease symptoms. These indicate the presence of new MCMV host plants that are favorable for multiplication and distribution of MLN causing viruses. The highest numbers of alternate host plant species identified in this study belonged to the family *Poaceae* followed by *Cyperaceae*, indicating that these families contain larger numbers of plants that are susceptible to MLN causing viruses. Some of the weed species that had previously been identified in Ethiopia as hosts of MCMV also belong to the *Poaceae* family (Mahuku*et al*., 2015a).

The present study showed that 6.7% of soil samples collected from MLN infected maize fields were tested positive for MCMV using DAS-ELISA. However, a large proportion (93.3%) of suspected MLN infested soil was tested negativefor MLN causing viruses, which might be due to the low concentration of the virus inoculum in the samples which were not in detectable amounts by DAS-ELISA and requires further investigation using molecular methods such as RT-PCR. SCMV was not detected in all samples, indicating that the virus inoculum either existed in the soil at a very low concentration that may not be in detectable amounts by DAS-ELISA test or do not survive in the soil environment. Similar to MCMV, other plant viruses for example *Pepper mild mottle virus* (Ikegashira *et al*., 2004) and *Tobacco mosaic virus* (Gülser *et al*., 2008) were detected from the soil by DAS-ELISA, however, future research may require for the development of optimized protocol for more efficient detection of MCMV from soil sample by DAS-ELISA. Both MCMV and SCMV were detected in maize parts (stem and root), indicating that maize plant parts might serve as a reservoir to maintain MLN causing viruses after harvesting. A similar result was reported by Uyemoto (1980), Jiang *et al*. (1992), and Sheets (2004) who reported that both viruses can be found in any parts of maize plants so long as the plant was infected.

The study also confirmed that MCMV can be transmitted from infested soil to newly raised maize seedlings. Abiotic transmission of viruses from soil or plant residues to plant hosts has been reported for several crop-virus combinations including tomato mosaic virus in tomato (Pares *et al*. 1996), tobamoviruses (*Tomato mosaic tobamovirus* and *Tobacco mosaic virus*) in forest soils (Fillhart *et al*., 1998), *Tobacco bushy stunt virus* in tomato (Kleinhampel and Kegler, 1982) and *Southern bean mosaic virus* in beans (Teakle *et al*., 1986). Since naturally plant viruses require wound or vectors for entrance to the plant cell, the possibility of soil transmission increased by the activities of microorganisms in the soil or during cultural practices such as weeding and through cutting implements that may create a wound and generating virus entry sites.

In addition, this study showed that virus persistence and transmission were observed in maize planted on MLN free soil mixed with MLN infected maize residue, suggesting that the transmission sources of MLN causing virus within the soil were not only the infected roots exuded during the cropping season but also from infected maize plant residue left within the soil after harvest. Similar findings were previously reported on MCMV transmission through soil (Nyvall, 1999). The persistence of MLN causing viruses in the soil and maize residue had a significant effect on the incidence of the disease. The highest percentage of disease incidence was observed on maize planted in infested soil stored for one-month and mixed with MLN infected residue, and in virus free soil mixed with MLN infected residue. Freshly incorporated infected maize residue had higher concentration of MCMV and SCMV, and resulted in higher disease incidence.

MLN causing viruses did not survive in MLN infested soil that was stored for more than three months. However, in MLN infested soil that was mixed with maize residue the virus (MCMV) survived up to six months. This indicates that continuous presence of MLN infected maize residue in the soil/field provides a virus reservoir and bridges the virus between seasons.

The seed transmission study revealed that both MCMV and SCMV can be introduced into maize production areas through seed transmission. Results indicated that out of 32,856 seedlings, 24 (0.073%) seedlings from 14 maize genotypes were infected with MCMV, whereas only one (0.003%) maize variety (Melkassa-2) was infected with SCMV. This verylow-rate seed transmission of both MLN causing viruses and suggests the minor role of seed-borne inoculum in MLN epidemiology. The rate of MCMV seed transmission indicated in this study was comparable

to the reports of Jensen *et al*. (1991) who evaluated 42,000 seedlings and found 0.04% transmission rate in Hawaii, USA. In contrast to this finding Quito-Avila *et al*. (2016) from Ecuador reported more than 8% seed transmission of MCMV although they did not present specific data on the methodology and genotypes yused. However, for vector-borne viral diseases like MLN, even a low rate of seed transmission can be epidemiologically important particularly where there is high vector pressure, because it is the primary source of inoculum that forms the starting point for the disease onset. As the infected seeds are randomly dispersed in the field, the seedlings germinating from these seeds serve as sources of inoculum for secondary spread by insect vectors (Sastry, 2013). Moreover, infected seed, even in very low proportion, can serve as a means for long-distance dissemination of viruses. Further investigation needs to be carried out to determine the localization of the viruses in maize seed.

The spread of MLN causing viruses are linked to the free movement of insect vector and contributes for its widespread from plant to plant, field to field and to different new geographical areas. In the current investigation, *Rhopalosiphum maidis* was identified as vector of SCMV, while maize thrips (*Frankliniella* sp.) and cereal leaf beetle (*Oulema sp.)* were identified as vectors of MCMV in Ethiopia. Many authors including Mansoor-ul-Hasan *et al*. (2003) reported that *R. maidis* is the most efficient vectors of SCMV in maize and other crops like sugarcane and sorghum (Singh *et al*., 2005; Perera *et al*., 2012). Reports arevailable that indicate that MCMV is also transmitted in a semi persistent manner by adult maize thrips, *Frankliniella williamsi* (Jiang *et al*., 1992; Cabanas *et al*., 2013) and flower thrips, *Frankliniella occidentalis* (Zhao *et al*., 2014). It has been also reported that MCMV is transmitted in a semi persistent manner by six different species of chrysomelid beetles including cereal leaf beetle (*Oulema melanopa*), maize flea beetle (*Chaetocnema pulicaria*), flea beetle (*Systena frontalis*), southern maize rootworm beetle (*Diabrotica undecimpunctata*), Northern maize rootworm (*D. longicornis*) and western maize rootworm (*D. virgifera*) (Nault*et al*., 1978; Jensen, 1985). Beetle transmitted viruses enter plant tissues through the wound created by beetle chewing and rapidly translocate far from the wounded sites through the plant xylem (Cabanas *et al*., 2013).

For the control of the selected insects identified as vectors, seed dressing insecticides were evaluated against these vectors at green house condition. The study is a first attempt to investigate the effects of insecticide seed treated on the germination revealed that it did not significantly

influence the germination of maize seed even up to six months storage before planting. The slight decline in germination percentage may be due to ageing effect leading to depletion of food reserves (Laxman *et al*., 2017). Thiamethoxam and imidacloprid singly or with mixed other pesticides are commonly used as a systemic seed treatment to protect seeds and seedlings against injury by early season insects (Tharp *et al*., 2000; Wilde *et al*., 2001). Both imidacloprid and thiamethoxam have the potential to provide long-term residual control of a broad spectrum of insect pests (Maienfisch*et al*., 2001; Wilde *et al*., 2001).

Results of the study conducted evaluate seed dressing insecticides against vectors of MCMV and SCMV indicated differences in the efficacy among seed dressing insecticides with different doses. More satisfactory reduction levels of maize thrips *(Frankliniella* sp) and corn aphids *(Rhopalosiphum maidis)* were achieved using thiamethoxam 25% WG @ 2.0 g/ kg seed than other insecticides used in this study at the same dosage. In a related study conducted under field condition, Ding *et al*. (2018) reported that treating maize seeds with thiamethoxam (1.0 and 2.0 g/kg of seeds) reduced thrips infestations on maize However, compared with other tested seed dressing insecticides, Imidacloprid + Thiram + Carboxin (2.0 and 1.0 g/kg of seeds) had a lower control efficiency for both vectors.

The differences in efficacy may be associated to the toxicity of the different insecticides to thrips. As reported by Byrne *et al*. (2007) other than maize plant, thiamethoxam and imidacloprid provide good control of avocado thrips in bioassays. The toxicities of thiamethoxam to larvae and adult females of western flower thrips (*Frankliniella occidentalis*) were higher than those of other tested neonicotinoids (nitenpyram, imidacloprid, and thiacloprid) (Shan *et al*., 2012).

Farmers in most maize growing areas were planting maize in different months, which made maize crops to exist at different growth stages in the field simultaneously. In such instances, the disease can easily be transmitted from the older to the younger maize plants by the insect vectors and leads to continuous MLN disease infection.

During the survey, nine known improved maize varieties and local varieties were observed to be frequently grown by farmers. All varieties were infected by one or a combination of MLN causing viruses. The presence of MLN disease on commonly grown maize varieties in Ethiopia showed that none of the improved and local varieties grown by farmers were resistant to MLN and this at

least partly accounted for the high prevalence and incidence of the disease in the surveyed areas. Findings of this study confirmed the reports of Gowda *et al*. (2015), who highlighted that a large number of pre-commercial and commercial maize germplasm in East Africa are susceptible to MLN disease. The susceptibility of all the assessed maize varieties in Ethiopia to MLN disease justifies the need to develop resistant/tolerant varieties to minimize the negative effects of MLN disease on maize production in the country.

The results of maize genotypes screening against MLN under greenhouse condition showed that most genotypes were susceptible to MLN. None of the inbred lines and varieties evaluated were immune from MLN based on DAS-ELISA tests, except one highland maize inbred line (ABL-93). Genotypes that showed resistant reaction and restricted development of the disease symptoms might carry desirable genes for MLN resistance. Ingvardsen *et al*. (2010) indicated that plant defense mechanism against viruses could be mediated by resistance genes which are observed as complete resistance or extreme resistance and that the virus replication could be hindered or gone undetectable among the infected cells. In some cases, viruses may not be detected in infected plant cells due to low titer of the virus that are not at the concentration of detectable by a less-sensitive DAS-ELISA test and requires further investigation by a molecular method such as real-time quantitative-PCR. A resistant highland maize inbred line (AMB17KN20-1) and CIMMYT-Kenyan resistant check varieties (CKMLN150075, CKMLN150088, CKMLN150076, CKMLN150074, and CKMLN15150) did not show MLN symptoms but MCMV was detected when tested with DAS-ELISA, indicating that these genotypes may carry desirable genes for MLN tolerance. The absence of one or more of the virus factors results in lack of infection, reduced virus replication, or retarded virus infection by inhibiting the movement of the virus inside the host cells, causing low virus accumulation and mild symptoms in infected plants (Gowda *et al.*, 2015; Garcia-Ruiz *et al*., 2018).

9. CONCLUSIONS

Understanding the spatial distribution and disease epidemiology as it is influenced by different variables is useful to design sustainable virus management strategies. This study revealed that MLN disease was distributed in major maize production areas of Ethiopia, especially in central, western, southern and southwestern parts of the country. Region, cropping system, cropping

season, altitude, weed density, insect vector and maize variety were distinguished as significant factors that impact the MLN disease epidemic. SNNP, Oromia and Benishangul-Gumuz regions, maize cultivated at an elevation of 900-1600 and 1700-2000 m.a.s.l, mono-cropping, presence of insect vectors, medium and high weed density had a high probability of association with MLN disease intensity.

Various weed and cultivated plants identified as alternate hosts, insect vectors, the transmissibility from infected seed and infested soil to newly raised maize seedlings, and the persistence in soil and maize residue of MLN causing viruses (MCMV and SCMV), are epidemiologically important and maintain the virus inoculum in the absence of maize crop in the field, and support the survival of the virus for continuous infection. *Poaceae* family had the highest number of species that were identified as alternate host for MLN causing viruses. These alternate hosts have a potential to serve as sources of inoculum for the virus that can be further spread by insect vectors.

Genotypes that showed resistant reaction and restricted development of disease symptoms might carry desirable genes for MLN resistance. Almost none of the inbred lines, except one highland maize inbred line (ABL-93), and the commonly grown improved maize varieties evaluated in this study were immune from MLN, however, different levels of reactions that ranged from moderately resistance to highly susceptible were observed. The use of genetic resistance is considered as the most economically and environmentally sustainable approach for plant virus disease management. Resistant inbred lines identified in this study might serve as sources of MLN disease resistant gene (s) in maize breeding programs.

The knowledge generated by this study on the incidence and geographical distribution of MLN and its causal viruses has substantially improved our understanding of the extent and economic importance of the disease in the country. The knowledge of the ways and extent of virus spread via seeds, insect vectors and soil and alternate hosts that can act as virus reservoir can help in devising the use of cultural practices such as roguing, crop rotation and sanitation together with the set of specific management options developed that include the sources of resistance and seed treatment chemicals will be important input as component of integrated MLN management depending on the specific local context. Hybrids and breeding lines with resistance/tolerance to MLN identified in this study can be utilized either in breeding systems (inbred lines) for inclusion

in national performance trials for fast-track release of resistant hybrids or for scaling up in MLN-affected areas (commercial hybrids) while the effective seed treatment insecticides can be incorporated into integrated MLN management. The national agricultural research system can use the outputs of this research for integrated management of this newly emerged disease that has rapidly become a serious challenge to maize production in the country.

10. RECOMMENDATIONS

As part of integrated management of MLN, farmers and stakeholders involved in maize production should take precautionary measures by using certified and virus-free maize seeds from trusted sources and all seed coming into a country from other country should be tested and quarantined for MLN; regular field monitoring, assessment of virus symptoms and rouging-out diseased maize plants; apply good field sanitation methods, including weed control and eliminating alternate host plants within and in the surrounding areas of maize fields. Seed dressing before planting with thiamethoxam 25% WG @ 2.0 g/ kg seed or Imidalm T 450@1.5 g/kg of seeds for early-stage protection against potential vectors of the MLN causing viruses can be effective, as this prevents secondary transmission of the viruses. Removal of all infected maize materials/residues and alternate hosts from and around the field, avoiding any activity that moves the soil from MLN infected fields or infected maize residue from one place to another play an important role in MLN management. Rotating maize crops with non-cereals or non-grass crop will likely reduce the virus inoculum of MLN causing viruses. The use of tolerant or resistant varieties is possibly the most effective means of managing MLN. Eventually, resistant varieties need to be developed for all major maize agro-ecological zones (highland, mid-altitude and lowland). Inbred lines that showed good level of resistance reaction to the disease can be used as sources of desirable genes in maize breeding programs.

11. FUTURE RESEARCH DIRECTION

MLN disease is still widespread and prevalent in various areas of the country. Thus, additionally adequate field and laboratory research-based information will be required including more assessments of alternative hosts in which the virus overwinters and insect vectors that are transmitting the virus from plant to plant. Since both MLN causing viruses (MCMV and SCMV)

are also vector transmitted, monitoring insect vector pressure and establishing the tolerance level might help in disease management as done for Lentil mosaic virus in California (Grogan, 1980).

The susceptibility of almost all the field assessed and greenhouse evaluated maize genotypes in Ethiopia to MLN disease suggests the need to develop resistant/tolerant varieties to minimize the negative effects of MLN disease on maize production in the country. Screening/evaluation of untested maize genotypes and further evaluation of these inbred lines and varieties for responses to individual MLN causing viruses would help to establish information on the genetics of MLN resistance for effective utilization of the materials in the breeding programs.

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APPENDICES

PAPER I

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Distribution of maize lethal necrosis epidemics and its association with cropping systems and cultural practices in Ethiopia

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Zea mays

ABSTRACT

Four hundred thirteen fields in five major maize growing regions of Ethiopia were surveyed to determine the prevalence, intensity and distribution of maize lethal necrosis (MLN) disease, and its associations with different maize cropping systems and cultural practices. The disease was most prevalent in South Nation, Nationality and Peoples (SNNP) region with 66.67% prevalence followed by Oromia region that had a prevalence of 65.62%. Similarly, MLN disease intensity and distribution varied among the regions surveyed, cropping systems and cultural practices used by the farmers. The associations of MLN disease intensity with independent variables were analyzed using logistic regression. Region, altitude, cropping season, cropping system, variety, insect vector, planting month and cropping year were significantly associated with high disease intensity of MLN in a multiple variable model. SNNP, Oromia and Benishangul-Gumuz regions, maize cultivated at altitudes of 900–1600 and 1700–2000 m.a.s.l, mono-cropping, presence of insect vectors, medium and high weed density and the 2015, 2016 and 2017 cropping years had a significant association with MLN disease epidemics. This study indicated that MLN disease is a major maize production constraint in Ethiopia. The findings suggested that planting maize at the beginning of the main rainy season, proper weed management and crop rotation practices can minimize the negative impact of the MLN disease until resistant maize genotypes are developed and distributed to the farmers.

Distribution of Maize Lethal Necrosis Epidemics and its Association with Cropping Systems and Cultural Practices in Ethiopia

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ABSTRACT

Four hundred thirteen fields in five major maize growing regions of Ethiopia were surveyed to determine the prevalence, intensity and distribution of maize lethal necrosis (MLN) disease, and its associations with different maize cropping systems and cultural practices. The disease was most prevalent in South Nation, Nationality and Peoples (SNNP) region with 66.67% prevalence followed by Oromia region that had a prevalence of 65.62%. Similarly, MLN disease intensity and distribution varied among the regions surveyed, cropping systems and cultural practices used by the farmers. The associations of MLN disease intensity with independent variables were analyzed using logistic regression. Region, altitude, cropping season, cropping system, variety, insect vector, planting month and cropping year were significantly associated with high disease intensity of MLN in a multiple variable model. SNNP, Oromia and Benishangul-Gumuz regions, maize cultivated at altitudes of 900-1600 and 1700-2000 m.a.s.l, mono-cropping, presence of insect vectors, medium and high weed density and the 2015, 2016 and 2017 cropping years had a significant association with MLN disease epidemics. This study indicated that MLN disease is a major maize production constraint in Ethiopia. The findings suggested that planting maize at the beginning of the main rainy season, proper weed management and crop rotation practices can minimize the negative impact of the MLN disease until resistant maize genotypes are developed and distributed to the farmers.

***Keywords**:* Disease Intensity; Logistic regression; Prevalence; *Zea mays*

1. Introduction

Maize (*Zea mays* L.) is the most important crop in terms of production and distribution in Ethiopia. Among cereals, maize ranks second to teff (*Eragrostis tef*) in area coverage with 2.13 million hectares, but first in productivity (3.94 t ha^{-1}) and grown by more than 10.5 million smallholder households, more commonly than any other crop in the country (CSA, 2018). Despite its importance, the average yield of maize in Ethiopia (3.9 t ha^{-1}) is below the world average (5.6 t ha^{-1}) (FAO, 2017). A significant portion of the yield gap is attributed to the effects of abiotic and biotic factors, and insufficient use of varieties tolerant or resistant to these factors (Abate et al., 2017; Keno et al., 2018).

The recently emerged maize lethal necrosis (MLN) disease further threatens maize production in the country. MLN disease that is caused by double infection of *Maize chlorotic mottle virus* (MCMV; genus *Machlomovirus*; family *Tombusviridae*) along with *Sugarcane mosaic virus* (SCMV; genus *Potyvirus*, family *Potyviridae)* is known to commonly occur on maize in Ethiopia (Mahuku et al., 2015; Fentahun et al., 2017). Even though SCMV is the most common virus in Ethiopia and other countries in East Africa, different members of the *Potyviridae* family, including *Maize dwarf mosaic virus* (MDMV) (Niblett and Claflin, 1978) or *Wheat streak mosaic virus* (WSMV) (Scheets, 1998) are also known to cause MLN disease by forming synergy with MCMV. In Africa, MLN was first reported in 2011 in Kenya, causing extensive to complete yield losses (Wangai et al., 2012). It was first reported in Ethiopia in 2014 (Mahuku et al*.,* 2015). Since its occurrence in Ethiopia, MLN disease has become widespread and, in some cases, has lead to total crop failure (Fentahun et al., 2017; Guadie et al., 2018).

In Ethiopia, few survey activities were carried out in different maize growing areas to assess MLN disease distribution and establish its economic importance (Fentahun et al., 2017; Guadieet al*.,* 2018). However, no attempts were made to relate cropping systems, cultural practices and environmental parameters to MLN disease epidemic. Understanding the association of disease intensity with different cropping systems and cultural practices will help to identify the most important variables and focus efforts to develop sustainable management strategies (Fininsa and Yuen, 2001). Therefore, the objectives of this study were to determine (1) the prevalence, intensity, and distribution of MLN disease in Ethiopia and (2) the association of the disease intensity with different cropping systems and cultural practices of maize in the country.

2. Materials and methods

2.1.Survey Regions

The MLN disease survey was conducted in five maize growing regions of Ethiopia; namely, Amhara, Oromia, South Nation, Nationality and Peoples (SNNP), Benishangul-Gumuz, and Tigray regions. The regions and zones within the regions were selected based on maize production potential. The selected regions cumulatively account for 97% of maize produced in the country (Abate et al., 2015). In addition, the surveyed areas represent three major maize growing agro-ecologies: (i) moist and semi-moist mid-altitudes located at 1700–2000 m.a.s.l, with an annual rainfall of 1000–1200 mm; (ii) moist upper mid-altitudes, with elevation of 2000–2400 m.a.s.l and an annual rainfall greater than 1200 mm (iii) moist lower mid-altitudes located at 900–1500 m.a.s.l, with annual rainfall of 900–1200 mm (Abate et al., 2015). Weather data from maize planting to the time of survey were obtained from the Ethiopian National Meteorology Agency (Table 1).

Table 1. Temperature and rainfall ranges from planting to the survey period during the main cropping season (May-August).

Region	Altitude (m)	Rainfall (mm)	Minimum temperature (°C)	Maximum temperature (°C)
Oromia	918-2765	66.2-322.2	10.05-17.18	17.6-28.95
Amhara	1607-2354	248.40-308.03	12.43-12.50	23.58-25.20
SNNP[a]	979- 1995	61.38-110.29	14.98-20.08	27.75-28.83
BG[b]	1022-1590	178.35-257.40	16.02-18.75	26.10-26.97
Tigray	1472-1977	17.6-98.9	17.7-18.7	31.5- 33.7

[a] SNNP = South Nation, Nationality and Peoples, [b] BG = Benishangul-Gumuz.

2.2.Diseases assessments and data collection

MLN disease assessments were conducted during the 2015-2018 main-cropping season (July to August) and off-season (March to April) at the vegetative growth stage of maize. Fields were randomly sampled at 5 to 10 km intervals on the main and accessible rural roads. In each selected field, maize plants were evaluated for the prevalence and disease intensity diagonally in an 'X' pattern in five quadrants (3×3 m) with 10 m distance between two quadrants. MLN disease incidences were rated as a percentage of diseased maize plants with MLN-like symptoms within

the quadrant. MLN severity was scored on a rating scale of 1 to 5, where 1 = maize with no visible MLN-like symptoms; 2 = symptoms with <25% on leaves; 3 = symptoms with 25 to 50% on leaves; 4 = symptoms with 50 to 75% on leaves and 5 = symptoms with 75 to 100%. The severity scales were converted to percent severity index (PSI) (Osunga et al., 2017). Disease prevalence was determined as the ratio of the number of fields where MLN disease was present to the total number of fields assessed.

$$PSI = \frac{\sum of\ all\ disease\ rating}{totalnumberofrating \times maximumdiseasegrade} \times 100$$

During the survey, data were collected on geographic information (geographic locations, and altitude); cropping system (mono-cropping or crop rotation); crop variables and agronomic management (a maize variety used, planting date and previous crop history); presence/absence of suspected MLN insect vectors (beetles, thrips and aphids), and weed density (low, medium or high) for each field. Information on the type of variety, planting date, and the previous cropping history was obtained from growers through interviews. Representative diseased maize leaves were collected for laboratory diagnosis to confirm the presence of MCMV and SCMV in the samples. Samples were tested serologically by double-antibody sandwich enzyme-linked immunosorbent assays (DAS-ELISA) following the method described by Clark and Adams (1977).

2.3.Data analysis

Simple descriptive statistical analyses were performed to summarize the field survey data. Disease intensity was categorized into distinct groups of binomial qualitative data as described by Woldeab et al. (2007). Thus, class boundaries were chosen as ≤ 20 and > 20 for incidence and ≤ 25 and > 25 for PSI data yielding a binary dependent variable. Contingency tables of disease intensity and the independent variables were constructed to represent the bivariate distribution of the fields (Table 2). The associations of MLN disease intensity with cropping systems and cultural practices were analyzed using logistic regression (Fininsa and Yuen, 2001, Yuen, 2006), using the SAS procedure of GENMOD (SAS Institute, Cary, NC, USA). The logistic regression model permits to evaluate the importance of multiple independent variables that have an effect on the response variable (Fininsa and Yuen, 2001). The response variable was the chance that MLN incidence exceeds 20% and severity exceeds 25% in a given maize field.

Table 2. Independent variable by disease contingency table for logistic regression analysis of MLN incidence and severity during the 2015 to 2018 cropping seasons in Ethiopia.

Variable	Variable Class	No. of Fields with MLN (%)				Variable	Variable Class	No. of Fields with MLN (%)			
		Incidence		Severity				Incidence		Severity	
		≤20	> 20	≤25	> 25			≤ 20	> 20	≤ 25	> 25
Region[a]	Oromia	117	107	115	109	Cropping System	Mono-cropping	128	130	128	130
	Amhara	54	3	54	3		Crop Rotation	119	36	114	41
	SNNP	52	50	51	51	Insect[b]	Absent	154	37	154	37
	BG	14	5	12	7		Present	93	129	88	134
	Tigray	10	1	10	1	Weed density[c]	Low	118	120	119	119
Altitude	1700–2000 m	74	82	70	86		Medium	95	26	90	31
	>2000 m	48	3	48	3		High	34	20	32	22
	900-1600m	125	81	124	82	Season[d]	Main-season	223	127	179	171
Variety	Shone	41	38	45	34		off-season	24	39	25	38
	BH661	14	10	12	12	Planting month	February	10	20	10	20
	BH660	18	18	17	19		March	8	12	9	11
	Limu	53	32	50	35		April	5	7	5	7
	BH540	33	26	31	28		May	182	67	176	73
	BH543	12	3	13	2		June	38	55	38	55
	Local	61	28	59	30		July	4	5	4	5
	Melkasa – 2	2	5	2	5	Year	2015	28	36	29	35
	BH140	11	1	11	1		2016	22	42	23	41
	Jabi	2	5	1	6		2017	128	60	124	64
							2018	69	28	66	31

[a] SNNP = South Nation, Nationality and Peoples, BG = Benishangul-Gumuz; [b] Present = observed suspected vectors of MLN causing viruses as indicated in literatures (thrips, beetles or aphids) in MLN disease infected fields whereas, absent = these insects were not observed in surveyed maize field; [c] Low = 0-20 weeds m^{-2}, medium = 21-40 weeds m^{-2}, and high = 41-100 weeds m^{-2}, [d] main-season = a season with long-rain that extends from May to September, off-season = a cropping season with short or no rain but irrigation or residual moisture used for maize production and it extends from November to April.

The importance of the independent variables was examined in two ways (Woldeab et al., 2007; Belete et al., 2013). First, the association of all the independent variables with MLN disease intensity was tested in a single-variable model. This consisted of testing the deviance reduction attributed to a variable when it was first entered into the model. Second, the association of an independent variable with MLN disease incidence or PSI was evaluated when entered last into the

model with all other independent variables. Lastly, selected independent variables that have a significant association with MLN disease incidence or PSI when entered first and last into a model were added to a reduced multiple variable model. The parameter estimates and standard errors were analyzed in both single and multiple models using the GENMOD procedure in SAS (SAS Institute, Cary, NC, USA) for the single and multiple models. The deviance table was constructed for the final reduced multiple variable model as described by Fininsa and Yuen (2001). Deviance reduction was calculated for variables as it was added to the reduced model and the likelihood ratio test was used to evaluate the significance of the variables and was examined against Chi-square value (McCullagh and Nelder, 1989). The odds ratio that was obtained by exponentiation of the parameter estimate used for comparing variable classes based on the reference point.

3. Results

3.1.MLN disease prevalence and distribution

Out of the 413 maize fields evaluated in five regions, 231 (55.9%) fields had plants that were infected with MLN disease. The disease was more prevalent in the SNNP region followed by Oromia whereas the lowest prevalence was noted in Amhara region (Fig. 1). In this study, regions that showed higher MLN disease prevalence and distribution had moderate level of rainfall and higher maximum temperature relative to the other regions (Table 1). The distribution of MLN disease in different zones of the five surveyed regions is presented in Table 3.

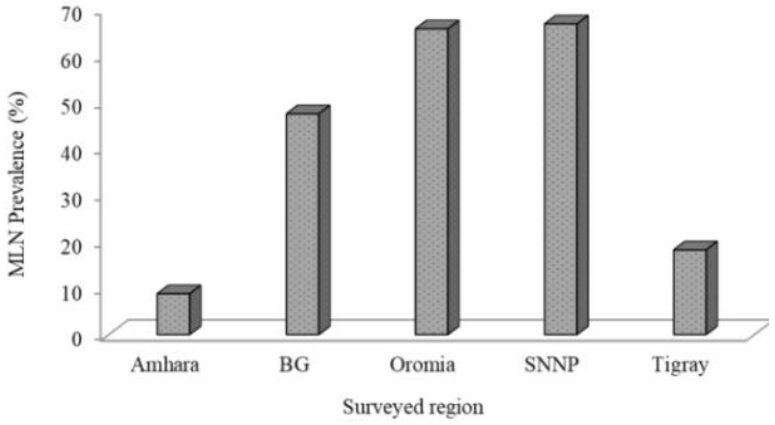

Fig.1 Prevalence of MLN disease in five regions of Ethiopia surveyed during the 2015-2018 cropping seasons. BG=Benshangul-Gumuz, SNNP=South Nations, Nationality and People.

Table 3. Distribution of MLN disease in different Zones of the five surveyed regions of Ethiopia.

Region	Zone	Prevalence	Incidence		Severity (PSI[a])	
			Mean	SD[b]	Mean	SD
Amhara	Awi	22.22	4.63	9.42	6.30	15.02
	East Gojjam	21.43	5.36	10.82	8.21	17.06
	West Gojjam	0.00	0.00	0.00	0.00	0.00
BG[c]	Asosa	50.00	13.00	9.77	20.00	8.50
	Metekel	44.44	11.67	14.14	18.89	23.15
Oromia	Arsi	87.5	35.00	22.99	44.87	24.47
	East Guji	0.00	0.00	0.00	0.00	0.00
	East Shewa	75.00	32.31	27.40	34.32	26.60
	East Wellega	40.68	16.77	18.76	21.61	22.71
	Ilu Ababor	100.00	20.34	11.19	26.85	20.34
	Jimma	100.00	39.41	14.52	47.96	16.37
	West Arsi	87.50	23.18	13.33	39.67	20.22
	West Guji	18.75	4.37	9.46	5.62	12.09
	West Shewa	62.50	10.72	10.71	17.15	16.64
	West Wellega	100.00	21.13	10.79	29.38	14.93
	Borana	0.00	0.00	0.00	0.00	0.00
SNNP[d]	GamoGofa	25.00	6.46	13.80	7.25	13.85
	Hadiya	100.00	28.00	18.08	37.86	22.65
	Segan	20.00	3.00	6.75	4.80	10.29
	Sidama	100.00	40.62	22.97	48.46	23.49
	Wolayita	87.10	56.00	73.00	48.88	29.57
	Alaba	50.00	14.17	16.13	19.75	22.08
Tigray	South Tigray	33.33	10.83	18.55	8.33	13.29
	North Western Tigray	0.00	0.00	0.00	0.00	0.00

[a] PSI = Percentage severity index; [b] SD = Standard deviation; [c] BG= Benishangul-Gumuz;

[d] SNNP = South Nation, Nationality and People.

The prevalence slightly decreased in zones of Amhara, Benishangul-Gumuz and Tigray regions. MLN disease was not observed and detected in East Guji and Borana zones of Oromia, West Gojjam zone of Amhara and North-Western zone Tigray regions.

DAS-ELISA test showed that the two MLN causing viruses (MCMV and SCMV) were found in most surveyed regions. Among MLN causing viruses, MCMV was the most prevalent virus that was detected on 543 (29.6%) out of 1833 samples tested, while SCMV was detected in 474 (25.9%) of the samples. Combined infection of both viruses (MCMV + SCMV) was found in 421 (23.0%) of the tested samples. The maximum proportions of MCMV, SCMV and MCMV + SCMV infections were detected in samples collected from Oromia, Benishangul-Gumuz and SNNP regions, respectively (Fig. 2).

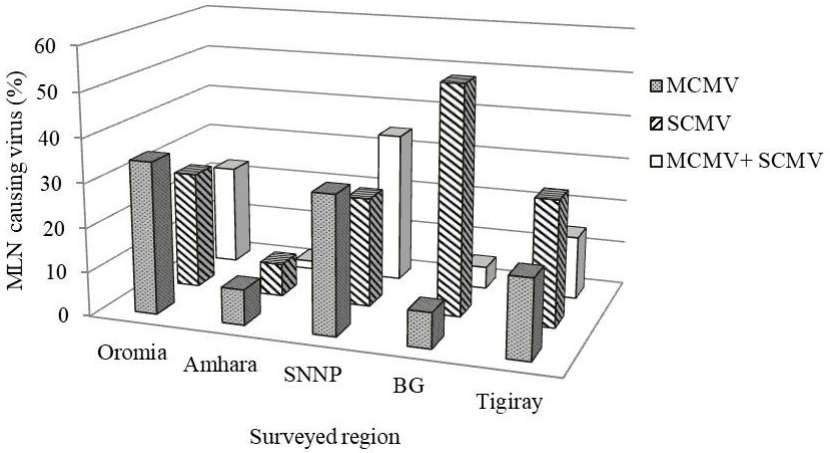

Fig.2. MLN disease causing viruses detected by DAS-ELISA in five regions of Ethiopia surveyed. BG=Benshangul-Gumuz, SNNP = South Nation, Nationality and Peoples.

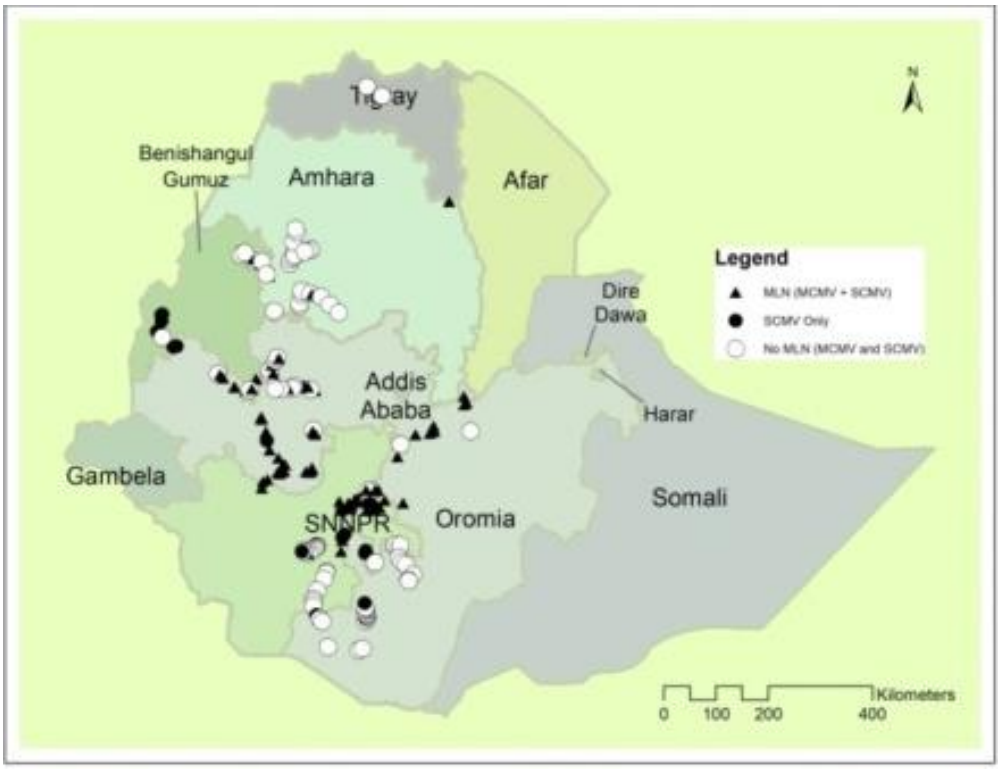

Fig. 3. Distribution of MLN disease causing viruses mapped based on 413 maize fields surveyed during the 2015-2018 cropping seasons in Ethiopia.

3.2. MLN disease intensity

The intensity of MLN disease varied among the regions assessed (Table 3). The maximum mean incidence and PSI were recorded in SNNP region followed by Oromia region, whereas the lowest

levels of incidence and PSI were recorded in Amhara region. In SNNP region, MLN disease incidence ranged from 3.0 to 56.0% while PSI ranged from 4.80 to 48.88%; and the highest incidence and PSI were recorded in Wolayita zone. In Oromia region, incidence ranged from 0.0 to 39.41% while PSI ranged from 0 to 47.96%; and the highest incidence and PSI were recorded in Jimma zone.

The effects of different maize production variables on MLN disease intensity was shown in Table 4. Under the mono-cropping system, the MLN disease intensity was higher than the field under crop rotation (fields planted with other crops in the previous year). Higher mean incidence and PSI was observed in fields with higher weed density as compared to fields with lower weed density. Maize fields situated ataltitudes ranging between 1700 and 2000 m.a.s.l had higher MLN incidence and PSI as compared to areas with lower altitude of 900 to1600 m.a.s.l and higher altitude of >2000 m.a.s.l. Among the cropping years, MLN incidence and PSI decreased from 2015 to 2018. In terms of cropping seasons, higher incidence and PSI were observed during the off-season as compared to the main cropping season that showed relatively lower incidence and PSI. Different maize varieties grown by farmers had different levels of reaction to MLN disease. The highest MLN incidence and PSI was exhibited Jabi, an old hybrid marketed by Dupont-Pioneer, followed by Melkassa-2, BH540 and BH660, in order listed. MLN incidence is associated with the presence of at least one or more of the suspected MLN insect vectors (thrips, beetles or aphids). Higher mean incidence was recorded for the maize fields where the suspected insect vectors were present as compared to the field without suspected vectors incidence.

Table 4. Mean incidence and PSI of maize lethal necrosis (MLN) disease assessed for different independent variables during the 2015-2018 cropping seasons in Ethiopia.

Variable	Variable Class	Incidence		Severity (PSI[a])	
		Mean	SD[b]	Mean	SD
Cropping system	Mono-cropping	23.62	25.17	27.41	25.36
	Crop rotation	12.13	19.82	15.48	22.78
Insect	Present	29.20	26.46	33.41	25.63
	Absent	7.80	13.43	10.76	17.94
Weed density	Low	10.77	17.78	13.23	19.76

Variable	Variable Class	Incidence		Severity (PSI[a])	
		Mean	SD[b]	Mean	SD
	Medium	25.55	23.39	29.21	24.48
	High	42.92	28.60	51.65	23.23
Altitude	900-1600	16.91	20.40	20.83	23.09
	1700-2000	27.55	28.06	31.74	27.05
	>2000	3.76	9.55	4.51	10.90
Planting month	April	27.50	27.27	32.92	27.91
	May	12.32	19.09	16.02	22.99
	February	25.51	16.16	30.30	17.33
	July	20.78	18.45	28.22	22.46
	June	32.36	28.15	35.19	25.53
	March	30.65	28.55	31.42	28.79
Variety	Shone	20.47	32.16	24.68	23.83
	BH540	25.83	27.59	28.51	26.10
	BH543	6.97	12.40	7.24	14.53
	BH660	22.89	21.90	27.18	22.94
	BH661	19.37	19.10	27.78	24.08
	Jabi	59.29	36.79	54.86	28.75
	Limu	16.75	22.57	21.77	26.20
	Local	14.71	21.65	17.49	23.52
	Melkessa- 2	37.86	36.15	37.14	28.85
	BH140	2.14	7.41	2.94	10.10
Season	Off-season	27.11	24.25	31.05	20.12
	Main rain season	17.90	23.64	21.47	25.10
Cropping year	2015	30.52	28.87	34.09	28.16
	2016	27.13	16.10	34.95	18.24
	2017	16.56	24.92	19.57	25.94

Variable	Variable Class	Incidence		Severity (PSI[a])	
		Mean	SD[b]	Mean	SD
	2018	12.07	18.45	14.18	19.22

[a] PSI = Percentage severity index; [b] SD = Standard deviation

3.3. Association of MLN with cropping systems and environmental variables

The independent variables varied in their association with MLN disease incidence and PSI (Table 5). All variables were significantly associated with MLN incidence and PSI when entered first into a logistic regression model. However, cropping season and planting month lost significance for both incidence and PSI, and variety lost significance for PSI when entered last into the model with the addition of other variables. The region, altitude, cropping system, weed density, suspected insect vectors and cropping year were significantly associated with MLN incidence and PSI when entered first and last into the model as evident from higher deviance reductions and χ^2 values.

Table 5

Independent variables used in logistic regression modeling of maize lethal necrosis incidence and PSI and likelihood ratio test (LRT) for nine variables entered first and last into a model[*]

Independent variable	Df	MLN Incidence LRT				MLN Severity LRT			
		VEF		VEL		VEF		VEL	
		DR	Pr>χ^2	DR	Pr>χ^2	DR	Pr>Pr>χ^2	DR	Pr>Pr>χ^2
Region	4	53.00	<.0001	22.30	0.0002	53.29	<.0001	12.82	0.0122
Altitude	2	41.77	<.0001	19.00	<.0001	45.88	<.0001	16.76	0.0002
Season	1	14.29	0.0002	1.06	0.3034	10.79	0.0010	1.43	0.2315
Cropping system	1	30.88	<.0001	8.30	0.0040	23.53	<.0001	4.32	0.0376
Variety	9	21.59	0.0103	17.67	0.0392	30.31	0.0004	11.11	0.2684
Insect	1	66.88	<.0001	9.69	0.0019	74.34	<.0001	14.97	0.0001
Weed density	2	78.36	<.0001	28.01	<.0001	93.04	<.0001	41.16	<.0001
Planting month	5	46.97	<.0001	8.26	0.1425	38.82	<.0001	5.69	0.3378
Year	3	34.42	<.0001	31.02	<.0001	25.83	<.0001	11.79	0.0081

[*] DR = Deviance reduction; Pr = Probability of a χ^2 value exceeding the deviance reduction; VEF = Variable entered first; VEL = Variable entered last; Df=degrees of freedom.

A group of seven variables namely region, altitude, cropping system, variety, weed density, suspected insect vectors, and year significance were tested in a reduced multiple variable model. Analysis of deviance, odd ratio and standard error of added variables in a reduced model showing the importance of variable and variable classes of MLN incidence is presented in Table 6. Low MLN disease incidence (≤20%) had a high probability of association with Amhara region and all

maize varieties, except BH543. High MLN disease incidence (>20%) had a high probability of association with Benishangul-Gumuz, Oromia, and SNNP regions; altitudinal ranges of 900-1600 and 1700-2000 m.a.s.l, mono-cropping, presence of suspected insect vectors, medium to high weed density and cropping years of 2015, 2016 and 2017.

Table 6. Analysis of deviance, natural logarithms of odds ratio and standard error of added variables in a reduced model of maize lethal necrosis incidence.

Added variable[a]	Residual deviance	df[b]	LRT[c]		Variable class	Estimate[d]	SE[e]	Odds ratio
			DR	Pr>χ^2				
Intercept	521.8655					-19.44	1.91	0.00
Region	423.3239	4	53.00	<.0001	Amhara	-1.13	1.71	0.32
					BG	0.39	1.41	1.48
					Oromia	1.57	1.20	4.81
					SNNP	2.55	1.21	12.83
					Tigray	0*	0*	1
Altitude	397.0022	2	26.32	<.0001	900-1600	3.03	0.82	20.78
					1700-2000	2.97	0.82	19.40
					>2000	0*	0*	1
Cropping System	376.4436	1	19.50	<.0001	Mono-cropping	0.89	0.32	2.44
					Crop Rotation	0*	0*	1
Variety	347.0261	9	29.42	0.0006	Shone	-3.58	1.41	0.03
					BH540	-4.14	1.44	0.02
					BH140	-5.79	2.12	0.00
					BH660	-3.35	1.41	0.04
					BH661	-4.54	1.47	0.01
					Jabi	-3.88	1.79	0.02
					Limu	-3.51	1.39	0.03
					local	-4.21	1.41	0.01
					Melkessa 2	-2.58	1.75	0.08
					BH543	0*	0*	1
Insect	317.9203	1	29.11	<.0001	Present	0.97	0.31	2.64
					Absent	0.00	0.00	1.00
Weed density	281.1302	2	36.79	<.0001	Medium	1.18	0.32	3.25
					High	2.10	0.49	8.14
					Low	0*	0*	1
Year	252.0156	3	29.11	<.0001	2015	2.41	0.63	11.14
					2016	3.23	0.67	25.39
					2017	1.54	0.52	4.64
					2018	0*	0*	1

* = Reference group; [a]Added Variable = Variables added into the model in order of presentation in the Table; [b]df = Degrees of freedom; [c] LRT = likelihood ratio test, DR = deviance reduction, Pr =

probability of a χ^2 value exceeding the deviance reduction; [d] Estimates from the model with all independent variables added; [e] SE = Standard Error.

Analysis of deviance, parameter estimates and their standard error of added variables in a reduced model that analyze MLN disease PSI are shown in Table 7. Low MLN disease severity (≤25%) had a high probability of association with maize varieties shone, BH540, BH140, BH660, BH661, Limu, and local. On the other hand, high MLN severity (>25%) had a high probability of association with Amhara, Benishangul-Gumuz, Oromia, and SNNP regions; altitudes of 900-1600 and 1700-2000 m.a.s.l, mono-cropping, Jabi and Melkassa-2 varieties, presence of suspected insect vectors, medium to high weed density and cropping years of 2015, 2016 and 2017.

Table 7. Analysis of deviance, natural logarithms of odds ratio and standard error of added variables in a reduced model analyzing maize lethal necrosis Percentage severity index (PSI).

Added variable[a]	Residual deviance	df[b]	LRT[c]		Variable class	Estimate[d]	SE[e]	Odds ratio
			DR	**Pr>χ^2**				
Intercept	504.6507					-16.87	2.02	0.00
Region	423.3239	4	53.29	<.0001	Amhara	0.04	1.40	1.04
					BG	2.02	1.34	7.51
					Oromia	1.90	1.19	6.67
					SNNP	2.39	1.20	10.87
					Tigray	0*	0*	1
Altitude	385.0198	2	31.89	<.0001	900-1600	2.68	0.82	14.61
					1700-2000	2.88	0.82	17.84
					>2000	0*	0*	1
Cropping System	371.5824	1	12.65	0.0004	Mono- cropping	0.62	0.30	1.86
					Crop Rotation	0*	0*	1
Variety	377.4963	9	25.27	0.0027	Shone	-1.34	1.38	0.26
					BH540	-1.15	1.41	0.32
					BH140	-3.20	1.98	0.04
					BH660	-0.41	1.39	0.66
					BH661	-1.35	1.45	0.26
					Jabi	0.26	1.89	1.30
					Limu	-0.69	1.35	0.50
					local	-1.44	1.39	0.24
					Melkessa - 2	0.36	1.72	1.43
					BH543	0*	0*	1

Added variable[a]	Residual deviance	df[b]	LRT[c]		Variable class	Estimate[d]	SE[e]	Odds ratio
			DR	**Pr>χ^2**				
Insect	314.4093	1	31.64	<.0001	present	1.17	0.31	3.21
					Absent	0*	0*	1
Weed density	266.2350	2	48.17	<.0001	Medium	1.24	0.31	3.47
					High	2.69	0.54	14.68
					Low	0*	0*	1
Year	257.1869	3	9.05	0.0287	2015	1.48	0.58	4.38
					2016	1.94	0.61	6.93
					2017	1.02	0.47	2.78
					2018	0*	0*	1

* = Reference group; [a]Added Variable = Variables added into the model in order of presentation in the Table; [b]df = Degrees of freedom; [c] LRT = likelihood ratio test, DR = deviance reduction, Pr = probability of a χ^2 value exceeding the deviance reduction; [d] Estimates from the model with all independent variables added; [e] SE = Standard Error.

4. **Discussion**

MLN and its causative viruses were widely distributed throughout major maize growing areas of Ethiopia, especially in Oromia and SNNP regions (Table 3 and Fig. 2). Most farmers in these regions practice continuous maize production throughout the year due to the availability of residual moisture and irrigation water. From the cropping history, it was evident that rotating maize with other crop types decreased MLN disease incidence as compared to maize mono-cropping. The presence of maize crop in the field throughout the year provided a favorable environment for the preservation of insect vectors and MLN causative viruses, whereby infected plants used as a bridge between cropping seasons. Similarly, Uyemoto (1983) reported that fields planted with crops other than maize in the previous year have mostly lower disease intensity of MLN disease.

Farmers in most maize growing areas were planting maize in different months, which makes maize crops existed at different growth stages in the field simultaneously. In such instances, the disease can easily be transmitted from the older to the younger maize plants by the insect vectors and leads to continuous MLN disease infection. These areas can also serve as sources of inoculum for the spread of the disease into new areas.

High prevalence and intensity of MLN disease were observed at altitudinal ranges of 900-1600 m.a.s.l and 1700-2000 m.a.s.l as compared to higher altitude of >2000 m.a.s.l (Table 4), indicating that mid and low-altitude maize growing environments of Ethiopia were more favorable for MLN

disease than the high-altitude environments. A similar report by Guadie et al. (2018), showed a high prevalence of viral diseases of maize in low to mid-altitude areas.

Higher altitude areas of Ethiopia are characterized by high rainfall (Abate *et al*., 2015) and cool temperature, which could hinder insect vector reproduction and ease of mobility to spread the viruses. On the other hand, maize grown at an altitude range of 1700 to 1200 m.a.s.l receives moderate rainfall (Abate et al., 2015). Such environments are characterized by warm and semi-humid weather conditions, which could be favorable for insect vectors development and spread that result in increased prevalence and incidence of MLN disease. Insect populations of most virus vectors build up faster in areas with high temperature and high relative humidity, and decline at low temperature and high rainfalls (Islam et al., 2017).

SNNP and Oromia regions that had higher MLN disease prevalence were characterized by moderate rainfall amounts and higher maximum temperatures (Table 1), depicting that these weather conditions were favorable for MLN development. Osunga et al. (2017) also reported that, on average, 27.5°C temperature, 85.6 mm rainfall and a1888.5 m.a.s.l altitude are the most favorable conditions for MLN development.

During the survey, nine known improved maize varieties and local variety (field collected seed; variety and source provenance unknown) were observed to be frequently grown by farmers. All varieties were infected by one or a combination of MLN causing viruses (Table 4). The presence of MLN disease on commonly grown maize varieties in Ethiopia showed that none of the improved and local varieties grown by farmers were resistant to MLN and this at least partly accounted for the high prevalence and incidence of the disease in the surveyed areas. Findings of this study confirmed the reports of Gowda et al. (2015), who highlighted that a large number of pre-commercial and commercial maize germplasm in East Africa are susceptible to MLN disease. The susceptibility of all the assessed maize varieties in Ethiopia to MLN disease suggests the need to develop resistant/tolerant varieties to minimize the negative effects of MLN disease on maize production in the country. However, since the current surveys were conducted on farmers' fields with different inoculum levels, further testing of maize varieties under known inoculum pressures and its temporal development would be required to identify the severity of MLN disease reaction levels.

The findings of this study depicted the seasonal variability of MLN disease incidence as higher incidence was observed during the off-season than the main rainy season. Similarly, Guadie et al. (2018) reported a relatively higher incidence of MLN disease during the off-season than the main rainy season. The plausible reasons might be maize grown under irrigation was the only green vegetation in the area that can attract the insect vectors, and also dry and hot conditions during the off-seasons would be favorable environment for reproduction and movement of vectors to transmit MLN causing viruses. Higher temperature that is usually experienced during the dry/off-season could increase the susceptibility of host plants to virus infection and rather accelerates the fitness of viruses to cause infection (Mitchell et al., 2005).

The spread of MLN causing viruses are linked to the free movement of insect vector and continuous availability of the host plants. However, insects observed during the field assessment, such as beetles, thrips and aphids were not identified to species level nor tested for their ability to efficiently transmit MLN causing viruses, although reports are available that indicate aphids are vectors of SCMV (Xia et al., 1999; Brault et al., 2010), and chrysomelid beetles (Jiang et al., 1992) and thrips (*Frankliniellawilliamsi*) (Cabanas et al., 2013) are vectors of MCMV. Different levels of weeds were observed in maize fields during the survey. The prevalence and incidence of MLN disease were higher in maize fields with high weed density than weed free maize fields. This suggested that weeds play a significant role as the source of infection for the spread of viruses. Similar findings were previously reported by Thresh (1982).

Assessment of MLN incidence during four consecutive years showed that the disease incidence generally decreased from 2015 to 2018. MLN awareness campaigns implemented by different governmental institutions and maize stakeholders over the years had increased maize growers' level of understanding about the challenges of MLN and its management. In addition, continuous spraying of insecticides to control fall army worm that has become an important maize production challenge since 2017 (Keno et al., 2018) in most maize growing areas might have also indirectly controlled insect vectors of MLN causing viruses, and thus considerably reduced the disease spread.

This study revealed that MLN disease was distributed in major maize production areas of Ethiopia, especially in central, western, southern and southwestern parts of the country (Fig. 3). Logistic regression analysis recognized factors that are associated with MLN epidemics. Understanding

disease epidemiology as it is influenced by different variables is useful to design sustainable management strategies. In the present study, region, cropping system, cropping season, altitude, weed density, insect vector, variety and year of assessment were distinguished as significant factors that impact the MLN disease epidemic. SNNP, Oromia and Benishangul-Gumuz regions, maize cultivated at an elevation of 900-1600 and 1700-2000 m.a.s.l, mono-cropping, presence of suspected insect vectors, medium and high weed density and cropping years of 2015, 2016 and 2017 had a high probability of association with MLN intensity. This study suggested that planting maize at the beginning of the main rainy season, removing weeds and other alternative host plants that can serve as a reservoir for viruses and insect vectors, crop rotation with alternative non-cereal crops, use of tolerant/resistant varieties should be considered as important components in designing MLN disease management strategies.

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ORIGINAL ARTICLE

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Alternate hosts and seed transmission of maize lethal necrosis in Ethiopia

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Abstract

Maize lethal necrosis (MLN) disease caused by double infection of *Maize chlorotic mottle virus* (MCMV) and *Sugarcane mosaic virus* (SCMV) has become a major maize production constraint in Ethiopia. A field survey was conducted in areas where MLN infection was reported to identify naturally infected alternate hosts. MCMV host range was also studied by artificially inoculating various weed species in the greenhouse to determine potential alternate hosts. Using the serological method of double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) test, MCMV was detected in 23 (20.35%), and SCMV was detected in 4 (3.53%) of 113 MLN symptomatic samples collected from the field, while combined infection of both viruses was detected in 11 (9.73%) of the samples. *Poaceae* family had the highest number of grass species that were alternate hosts for MLN causing viruses. *Digitaria sanguinalis*, *Phalaris paradoxa*, *Oplismenus hirtellus*, *Echinocloa colona*, *Cynodon nlemfuensis*, *Pennisetum purpureum* from *Poaceae* and *Cyperus cyperoids* from *Cyperaceae* family were naturally infected by MCMV. *Cyperus rotundus*, sugarcane (*Saccharum officinarum*) and sorghum (*Sorghum bicolor*) were infected by both MCMV and SCMV under natural field conditions. In addition, seed transmission study was conducted using growing-on tests to determine the potential of seed transmission and its role in the spread of MLN. Overall mean seed to the seedling transmission rate was 0.073% with a range of 0 to 0.17% among 20 different maize varieties studied. Fourteen maize genotypes had some levels of seed transmission (0.03%–0.017%) for MCMV, whereas, SCMV seed transmission was observed only in a single plant of one genotype, with an overall mean of only 0.003%. Seed transmission rates of the viruses were influenced by the seed lot and maize varieties used. The wide ranges of natural and experimental alternate hosts and seed transmissibility of MLN causing viruses suggest that the viruses play a greater role in MLN epidemiology.

KEYWORDS

disease epidemiology, inoculum source, *maize chlorotic mottle virus*, *Sugarcane mosaic virus*

Alternate Hosts and Seed Transmission of Maize Lethal Necrosis in Ethiopia

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Abstract

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Key words: Disease epidemiology; Inoculum source; *Maize chlorotic mottle virus*; *Sugarcane mosaic virus*

1. INTRODUCTION

Maize (*Zea mays* L.) is an important staple cereal in terms of production, acreage, and number of households producing the crop in Ethiopia (CSA, 2018). Despite its importance, maize production is threatened by maize lethal necrosis (MLN), a devastating viral disease that is widely spreading in the country (Regassa et al., 2020). The disease was initially reported in the Rift Valley area of Ethiopia in 2014 (Mahuku et al., 2015). Subsequent assessments showed that the disease is widespread across various geographical locations, and causing significant maize yield losses. In some areas of Oromia and South Nation, Nationality and Peoples (SNNP) regions of Ethiopia, a complete crop failure was observed and forced the farmers to replace maize with other crops (Bekele et al., 2017; Fentahun et al., 2017; Guadie et al., 2018).

In Eastern African countries, including Ethiopia, MLN disease is caused by double infection of *Maize chlorotic mottle virus* (MCMV, genus *Machlomovirus*, family *Tombusviridae*) and *Sugarcane mosaic virus* (SCMV, genus *Potyvirus*, family *Potyviridae*) (Adams et al., 2014; Mahuku et al., 2015; Wangai et al., 2012). In addition, other viruses of the *Potyviridae* family including *Maize dwarf mosaic virus* (Niblett and Claflin, 1978) or *Wheat streak mosaic virus* in the genus *Tritimovirus* (Scheets, 1998) can cause MLN disease by forming synergy with MCMV. In Ethiopia, SCMV is known to commonly occur on maize for a long time (Lencho et al., 1997); however, MCMV is a newly introduced virus, which is the most important component of MLN (Mahuku et al., 2015). MCMV is transmitted by vectors, mainly thrips (Jiang et al., 1992) and beetles including the cereal leaf beetle (*Oulema melanopa*), corn flea beetle (*Chaetocnema pulicaria*), flea beetle (*Systena frontalis*), southern corn rootworm (*Diabrotica undecimpunctata*), western corn rootworm (*Diabrotica virgifera*), and northern corn rootworm (*Diabrotica longicornis*) (Nault et al., 1978; Jiang et al., 1992). SCMV is transmitted by several species of aphids (Zhang et al., 2008).

Means of field spread of MCMV as a component of MLN is poorly understood and a very low percentage (0.04%) of seed transmissionof MCMV was reported (Jensen et al., 1991). On the other hand, earlier studies that involved several thousands of maize seeds concluded that MCMV is not

seed transmitted (Uyemoto, 1983). However, it is known that in vector-borne virus diseases like MLN, a very low level of seed transmission can be economically important (Sastry, 2013). For example, a very low-level seed transmission rate (0.001%) of the *Lettuce mosaic virus* has been shown to be sufficient for the spread of the virus by aphid vectors (Dinant and Lot, 1992; Johansen et al., 1994).

A study conducted in the USA indicated that MLN causing viruses are harbored by a number of alternate non-maize grass hosts that contribute to the epidemiology of the disease by serving as overwintering hosts (Uyemoto, 1983). According to Scheets (2004), the host range for both SCMV and MCMV is limited to members of the *Poaceae* family, maize and sugarcane being the natural hosts of MCMV and SCMV, respectively. In contrast, Bockelman et al. (1982) reported that MCMV was not found in 230 grass samples of 14 species collected near fields of infected maize and suggested that MCMV does not overwinter on grass weeds.

In Ethiopia, studies on MLN so far have focused mostly on the identification and characterization of the causal viruses from maize, its economic importance and geographical distribution (Mahuku et al., 2015; Fentahun et al., 2017; Demissie et al., 2018; Guadie et al., 2018). Limited alternate hosts of MLN causing viruses were reported from grass weeds, but most of them were not identified to species level (Mahuku et al., 2015; Bekele et al., 2017). Moreover, an experimental host range study of MCMV was not conducted in Ethiopia as it is a newly emerging virus. To our knowledge, there are also no documented research findings on the rate of seed transmission of MLN causing viruses to different maize genotypes. Plant virus disease management has to be knowledge-based, and thus, it is important to understand the role of alternate hosts in the emergence and development of disease epidemics. In addition, in order to develop an effective MLN management strategy, empirical data on the role of alternate hosts and the rate of seed-transmission should be determined. Therefore, there is a critical need to generate information on natural modes of transmission and survival of MLN causing viruses in Ethiopia. The objectives of the study were to (1) provide comprehensive information on the identity of alternative hosts of MLN causing viruses, and (2) determine the potential of seed transmission and its role in the spread of MLNcausing viruses in Ethiopia.

2 MATERIALS AND METHODS

2.1. Field assessment of MLN alternate hosts

MLN natural alternate hosts were assessed in Oromia and SNNP regions of Ethiopia in 2016 and 2017 during the main rain season (August to September). East Shewa, Arsi and Jimma zones of Oromia and Wolayita zone of SNNP Regional States that have high levels of MLN infestation (Regassa et al., 2020) were selected for the study. Two districts with the history of high MLN incidence were purposively selected from East Shewa (AdameZuria and Lume), Jimma (Omo Nada and ShebeSonbo) and Wolayita (Damot Gale and DamotPulasa) zones, while only one district was picked from Arsi Zone (Jeju district). Weed species with or without virus-like symptoms (chlorosis, mosaic, mottling, stunting, necrosis, yellowing) found in and near MLN infected maize fields were assessed. In addition, cultivated cereals found within or adjacent to the maize fields were included.

Selected maize fields were sampled at the intervals of 5 to 10 km along the main and accessible rural roads using a 1-meter square quadrate. The number of samples collected varied from district to district due to differences in the level of MLN incidence and virus-like symptoms observed in each field. More samples were collected from fields with higher MLN incidence and virus-like symptoms and fewer samples were collected from fields with lower incidence and symptoms. This procedure was used in order to generate more information on the occurrence of MLN causing viruses.

Herbarium press for the identification of the weeds to species level was used, and species identification was made by consulting biosystematics unit herbarium of Ambo Agricultural Research Center and using a weed identification guide book (Stroud and Parker, 1989).

A total of 434 samples belonging to 28 different species and 11 families were collected, out of which 113 samples were displaying virus-like symptoms (29 in East Shewa, 19 in Jimma, 24 in Arsi, 41 in Wolayita Zones), and the remaining 321 samples were non-symptomatic. Among these samples, 399, 24 and 11 were collected from weeds, cereal crops and sugarcane (*Saccharum officinarum*), respectively.

2.2. Virus detection and back-inoculation test

Samples collected from weeds, wild grasses and cultivated crop species were analyzed using DAS-ELISA to detect the presence of SCMV and MCMV. The detection of antibodies for MCMV (AS-1087), SCMV (AS-0166) and their respective positive controls were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) Plant Virus Collection, Germany. DAS-ELISA test was conducted following the procedures described by Clark and Adams (1977) and the standard protocols at the DSMZ- Plant Virus Collection. The extracts used as sources of the antigen/virus were obtained by grinding the leaf samples (1 g in 10 ml of extraction buffer). Back-inoculation test was used to determine the ability of MCMV and SCMV that were detected from weeds and cultivated crops to cause MLN disease. For this purpose, plant saps from samples already tested positive for each of the virus using DAS-ELISA were inoculated on maize. An abrasive agent, carborundum dust (SiC), was added to the inoculum solution to cause microscopic injury of the leaves for easy penetration of the virus into the plant cells (Orawu et al., 2013). The inoculum was then rubbed with fingers onto 4-6 leaf stage of MLN susceptible maize cultivar (Morka) in an insect proof- greenhouse, and then immediately rinsed with water. The symptom development was monitored after inoculation at least twice a week for 30 days and detection of the viruses was carried out by DAS-ELISA.

2.3. MCMV experimental host range

MCMV host range studies were conducted using different weeds and cereal crops species at Ambo Agricultural Research Center in an insect-proof greenhouse at 25-30 °C. Five to eight test plants of each species were planted in 10 cm diameter plastic pots filled with mixed sterilized soil, sand and organic manure at 2:1:1 ratio, respectively, and watered regularly as required. The pots were replicated three times for each plant species. A total of 39 species from 12 weed families, which were naturally associated with maize crop (Hailegiorgis et al., 2005) and 10 cereal crops varieties, including wheat (*Triticum aestivum*L.), barley (*Hordeum vulgare* L.), sorghum (*Sorghum bicolor* L.) and finger millet (*Eleusine coracana* L.) were tested to determine potential hosts of MCMV. MLN susceptible maize cultivar (Morka) was used as positive control while non-inoculated plants

of all species were included as negative control. MCMV isolates were multiplied on two-week-old susceptible maize cultivar (Morka) seedlings by mechanical inoculation and later used as inoculum source to infect suspected alternate host plants sampled. MLN symptoms were established fully within two weeks after post-inoculation on the infected susceptible Morka maize variety. The infected maize leaves were collected and ground using sterile mortar and pestle in 10 ml of 100 mM phosphate buffer, pH 7.0 to obtain homogenate solution or extract (1:10; 1g of leaf materials to 10 ml extraction buffer). Carborundum powder was added to the homogenate which was subsequently used to rub on leaves of test plants.

MCMV inoculum was then rubbed at 14 days after planting using fingers onto 3-6 leaf stage of seedlings and rinsed immediately with water. The second inoculation was carried out at an interval of one week after the first inoculation to confirm effective viral infection and to avoid possible disease escapes. Dead leaves were removed from the alternate host test plants at the time of inoculation. The development of symptoms was monitored starting from 10 days after the second inoculation and leaf samples were collected for virus detection and back inoculation tests. The viruses were detected using DAS-ELISA as described in section 2.2.

For the back-inoculation tests, the inoculum was prepared from leaf samples (1 g in 10 ml of extraction buffer) and inoculated to a two-week-old susceptible maize cultivar (Morka). The symptom development was monitored after inoculation and disease testing was carried out by DAS-ELISA.

2.4. Seed transmission

2.4.1. Seed source

Seed samples of naturally infected 20 representative maize varieties were collected from experimental fields of three Agricultural Research Centers; namely Melkassa, Jimma and Wendogenet. Maize plants that showed typical symptoms of MLN disease, such as chlorotic, mosaic leaf streaks and occasionally stunting and/or general chlorosis were tagged for subsequent ear harvest to obtain seeds that are used for seed transmission study.

Leaf samples from a representative subset of those tagged maize plants were collected and a DAS-ELISA was performed to confirm the presence of MCMV and SCMV. ELISA-positive tagged

maize plants that were infected by MLN causing viruses were allowed to grow until maturity. At harvest, ears from plants infected by individual or combination of MLN causing viruses (MCMV, SCMV and MCMV + SCMV) were harvested, shelled and dried to 12.5% moisture level.

2.4.2. Greenhouse grow-out tests

Harvested maize seeds were sown separately in an insect-proof greenhouse in three categories according to the type of virus detected (MCMV, SCMV or mixed infection of MCMV and SCMV). In order to avoid accidental infection, stringent measures of isolation, confinement, handling and insect control were taken. The grow-out tests were conducted during the off-season to avoid the possibility of infection by insect vectors from the surrounding areas. During the off-season, there was no MLN causing viruses maintained in the greenhouse and also there was no maize and other cereal crops in the fields of surrounding area.

Seeds were planted in metallic trays (51×63cm) in a greenhouse potting filled with a sterilized mix of soil, sand and organic manure in 2:1:1 ratio, respectively, and watered regularly as required. Seed trays had seven rows with 10 seeds per row and were placed on greenhouse benches with no supplemental lighting. Most of the tests were conducted at 25-35°C day temperatures. After emergence, maize seedlings were observed at least twice a week from the two-leaf stages until five to six- leaf stages for symptoms appearances, and any symptomatic seedlings were labeled for subsequent testing.

The plants were tested for the two MLN causing viruses (MCMV and SCMV) using DAS-ELISA. One month after planting, the seedlings showing symptoms were counted and expressed as a percentage of germinated seedlings. Transmission percentages were calculated as follows.

$$\mathrm{TP} = \frac{\mathrm{IS}}{\mathrm{TGS}}\ X\ 100$$

Where TP = Transmission percentage, IS = Infected seedling which is ELISA-Positive, TGS = Total germinated seedlings.

3. RESULTS

2.2.MLN disease symptoms in maize fields

MLN disease infected maize plants at different growth stages starting from early to near maturity stage of the crop. Diverse ranges of symptoms were observed depending on the growth stage of the crop. At the early growth stage, MLN disease symptoms were expressed on the leaves, and the cobs began to show the symptoms at the later growth stage. The typical symptoms included chlorotic mottling of the leaves, usually starting from the base of the young leaves in the whorl and extending upwards toward the leaf tips, mild to severe leaf mottling, the leaves become necrotic at the leaf margins that progress to the mid-rib resulting in drying of the whole leaf. Other symptoms included premature aging of the plants. Severely affected plants formed small cobs with little or no grain as shown in Fig 1.

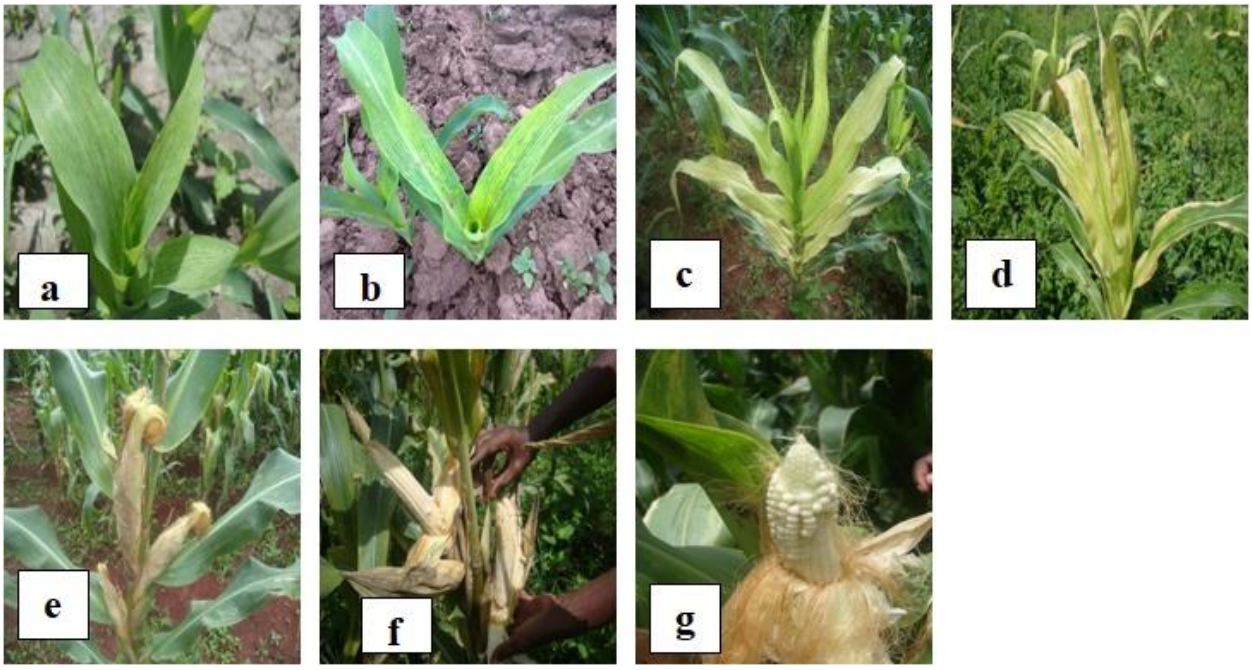

Fig. 1. Maize lethal necrosis (MLN) disease symptoms commonly observed in the field: (a) chlorotic, (b and c) mild to severe leaf mottling, (d) necrosis of leaf margins, (e) drying cob, (f and g) poor or no grain filling.

3.2. Alternate hosts of MLN causing viruses

Different types of MLN symptoms were observed on different species of assessed plants. The symptoms observed included mosaics, mottling, yellowing, necrosis that develop from leaf margins to the mid-rib, and purple discoloration of leaves. For instance, MCMV symptoms on *Cyperus cyperoids was* expressed as yellowing, while it showed mosaic and chlorotic symptoms on *Oplismenushirtellus* (Fig.2). Combined infection of MCMV and SCMV on *Cyperus rotundus* showed yellowing and necrotic symptoms. Mixed infection of the two viruses on sorghum (*Sorghum bicolor*) showed yellowing and purple discoloration in the form of strips on the leaves. On sugarcane (*Saccharum officinarum)* the symptoms of combined infections of both viruses were expressed as yellow coloration and necrosis on the whole leaf (Fig.3). The infection of SCMV on sorghum was mild mosaic which could not clearly be observed from a distance (Fig. 4).

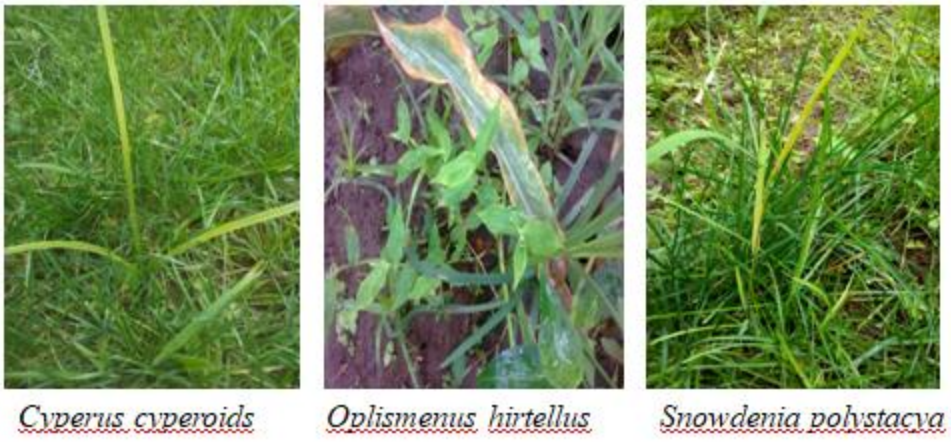

Fig. 2. *Maize chlorotic mottle virus* (MCMV) on naturally infected different alternate hosts shows mosaic and yellowing symptoms.

Fig. 3. Mixed infection of MLN causing viruses (MCMV and SCMV) on naturally infected different alternate hosts show symptoms of yellowing and necrosis, yellowing and purple discoloration in the form of strips.

Fig. 4. Maize lethal necrosis (MLN) causing *Sugarcane mosaic virus* (SCMV) on naturally infected sorghum shoes mild mosaic symptoms.

Among the 113 symptomatic samples tested, 23 (20.35%) samples were tested positive for MCMV, 4 (3.53%) for SCMV and 11 (9.73%) for the combination of both viruses (Table 1). However, no MLN causing viruses (MCMV or SCMV) were detected in all the 321 asymptomatic samples. MCMV was the most prevalent virus that was detected in the samples collected from all the surveyed zones and districts except in samples from Omo Nada district, whereas SCMV was

detected in samples collected from Adama, ShebeSonbo and Jeju districts of East Shewa, Jimma and Arsi zones, respectively.

Table 1. Total number of maize lethal necrosis (MLN) symptomatic and asymptomatic samples collected from seven selected districts in Ethiopia during the 2016 and 2017 growing seasons (August to September) and types of viruses detected

Region	Zone	District	Number of samples collected		Virus detected from symptomatic samples		
			Symptomatic	Asymptomatic	MCMV	SCMV	MCMV + SCMV
Oromia	East Shewa	Lume	11	52	2	0	0
		AdamaZuria	18	40	4	2	2
	Jimma	Omo Nada	11	40	0	0	2
		ShebeSonbo	8	28	3	1	2
	Arsi	Jeju	24	55	5	1	5
SNNP	Wolayita	Damot Gale	24	56	4	0	0
		DamotPulasa	17	50	5	0	0
Total			**113**	**321**	**23**	**4**	**11**

SNNP = South Nation, Nationality and Peoples, MCMV = *Maize chlorotic mottle virus*, SCMV = *Sugarcane chlorotic mottle virus.*

MCMV was detected and identified in *Amaranthus hybridus* L. spp. of *Amaranthaceae*, family, seven species of *Poaceae* family, *Cyperus cyperoids* L. spp. of *Cyperaceae* family whereas SCMV was detected only in *Sorghum bicolor* (L.) spp. of *Poaceae* family and mixed infection of both MCMV and SCMV was detected in three species from *Poaceae*and *Cyperaceae* families (Table 2).

Back-inoculation using plant sap samples obtained from MLN virus-positive alternate hosts (Table 2) to susceptible maize cultivar successfully produced MLN symptoms and subsequent DAS-ELISA test showed positive readings for some of the sampled plant spp. Accordingly, *Cyperus rotundus, Cyperus cyperoides, Snowdeniapolystachya,Cynodonnlemfuensis, Digitariasanguinalis, Echinochloacolona, Oplismenushirtellus, Pennisetum purpureum*

and *Phalaris paradoxa*were identified as alternate hosts for MCMV. *Sorghum bicolor* and *Saccharum officinarum* were identified as alternate hosts for SCMV. Back-inoculation using extracts from *Amaranthus hybridus* did not produce disease symptoms on the susceptible maize variety and also showed negative when tested using DAS-ELISA.

Table 2. Natural alternate hosts of maize lethal necrosis (MLN) viruses identified in Ethiopia using DAS-ELISA test

Family	**Species**	**Life cycle**	**Type**	**Virus detected**
Amaranthaceae	*Amaranthus hybridus* L.	Annual	Broad leaves	MCMV
Asteraceae	*Bidens pachyloma*olve and Hiern.	Annual	Broad leaves	-
Asteraceae	*Bidens pilosa* Linn.	Annual	Broad leaves	-
Asteraceae	*Galinsoga parviflora* Cav.	Annual	Broad leaves	-
Asteraceae	*Guizotiascabra* (Vis.) chiov	perennial	Broad leaves	-
Brassicaceae	*Sinapis arvensis* L.	Annual	Broad leaves	-
Brassicaceae	*Erucastrumarabicum*Fisch and Mey.	Annual	Broad leaves	-
Commelinaceae	*Commelina bengalensis* L.	Annual	Broad leaves	-
Cyperaceae	*Cyperus rotundus*L.	Perennial	Sedges	MCMV + SCMV
Cyperaceae	*Cyperus cyperoides* L.	Perennial	Sedges	MCMV
Nyctaginaceae	*Boerhaaviaerecta*L.	Annual	Broad leaves	-
Oxalidaceae	*Oxalis triangularis* L.	perennial	Broad leaves	-
Plantaginaceae	*Plantago lanceolata* L.	Perennial	Broad leaves	-
Poaceae	*Cynodondactlon*L.	Perennial	Grasses	-
Poaceae	*Snowdenia polystachya* (Fresen.) plig.	Annual	Grasses	MCMV
Poaceae	*Cynodon nlemfuensis*Vanderyst.	Perennial	Grasses	MCMV
Poaceae	*Digitariasanguinalis* (L.) Scop.	Annual	Grasses	MCMV
Poaceae	*Echinochloacolona*L.	Annual	Grasses	MCMV
Poaceae	*Oplismenushirtellus*L.	Perennial	Grasses	MCMV
Poaceae	*Pennisetum purpureum* Schumach.	Perennial	Grasses	MCMV
Poaceae	*Phalaris paradoxa*L.	Annual	Grasses	MCMV
Poaceae	*Sorghum arundianaceun* (Desv.) Stapf	Annual	Grasses	-
Poaceae	*Sorghum bicolor* L.	Annual	Grasses	MCMV + SCMV
Poaceae	*Sorghum bicolor* L.	Annual	Grasses	SCMV
Poaceae	*Triticum aestivum* L.	Annual	Grasses	-
Poaceae	*Eleusine coracana* L	Annual	Grasses	-

Family	Species	Life cycle	Type	Virus detected
Poaceae	*Saccharum officinarum* L.	Perennial	Grasses	MCMV + SCMV
Polygonaceae	*Polygoniumnepalense*-Meissn	Annual	Broad leaves	-
Solanaceae	*Datura stramonium* L.	Annual	Broad leaves	-

MCMV = *Maize chlorotic mottle virus*, SCMV = *Sugarcane chlorotic mottle virus*

- = negative (both MCMV and SCMV was not detected by DAS-ELISA test).

3.3. MCMV experimental host ranges

Various types of MCMV symptoms (mild chlorotic, yellowing, necrosis starting from leaf merges to mid-rib) were observed on different grass weeds and cereal crops (wheat and sorghum) (Fig. 5) under the experimental conditions in the greenhouse. The susceptible maize control also showed typical symptoms of MCMV while no symptoms were observed on non-inoculated control weed and cereal crop species studied.

Among the 39 weed species that were tested for reaction to MCMV using artificial inoculation in the greenhouse, 20 species and the susceptible control maize variety (Morka) were susceptible to MCMV infection (Table 3). This study identified *Cyperus assimilis, C. esculentus and C. rotundus*from*Cyperaceae*family*; Andropogon abyssinicus,Cenchurusciliaris, Cyndonnlemfuencis,C. dactylon,Denebraretroflexa, Digitariaabyssinica, D. ternate, D. ischaemum, Echinochloacolona, Eleusine indica, Eragrostiscilianesis, Pennisetum ramosum, Phalaris paradoxa, Setaria pumila, S.verticillata, Snowdeniapolystachya* and *Sorghum arundinaceum*from*Poaceae*family as alternate hosts of MCMV.Among the 10 cereal crop varieties, a barley variety HB-1307, wheat varieties Kilinto/DZ 918 and Alidoro/HK-14-R251 and a sorghum variety Geremew/87BK-4122 were infected by MCMV (Table 4).

Table 3. Weed species artificially inoculated with *Maize chlorotic mottle virus* (MCMV) to identify its alternate hosts and reaction of the weeds to the MCMV as detected by DAS-ELISA test

Family name	Species name	Life cycle	Type of weed	ELISA result*
Amaranthaceae	*Amaranthus graecizans* L.	Annual	Broad leaves	-
Astraceae	*Bidens pachyloma* (Oliv. And Hiern) Cufod.	Annual	Broad leaves	-

Family name	Species name	Life cycle	Type of weed	ELISA result*
Astraceae	*Bidens pilosa* L.	Annual	Broad leaves	-
Astraceae	*Flaveriatrinervea*Spreng.	Annual	Broad leaves	-
Astraceae	*Galinsoga parviflora* Cav.	Annual	Broad leaves	-
Astraceae	*Guizotiascabra* vis. Chiov.	Perennial	Broad leaves	-
Astraceae	*Xanthium strumarium* L.	Annual	Broad leaves	-
Brassicaceae	*Erucastrumarabicum*. Fisch. And Mey	Annual	Broad leaves	-
Commelinaceae	*Commelinabenghalensis* L.	Annual	Broad leaves	-
Commelinaceae	*Commelinalatifolia*A. Rich.	Perennial	Broad leaves	-
Convolvulaceae	*Convolvulus arvensis* L.	perennial	Broad leaves	-
Cyperaceae	*Cyperus assimilis*Steud.	Annual	sedges	+
Cyperaceae	*Cyperus esculentus* L.	perennial	sedges	+
Cyperaceae	*Cyperus rotundus* L.	Perennial	Sedges	+
Nyctaginaceae	*Boerhaaviaerecta* L.	Annual	Broad leaves	-
Plantaginaceae	*Plantago lanceolata* L.	Biennial	Grasses	-
Poacceae	*Cenchurusciliaris* L.	perennial	Grasses	+
Poacceae	*Cyndonnlemfuencis*Vanderyst.	Perennial	Grasses	+
Poaceae	*Andropogon abyssinicus* (Fresen.) R. Br.	Annual	Grasses	+
Poaceae	*Cyndondactylon* (L.) Pers.	Perennial	Grasses	+
Poaceae	*Denebraretroflexa* (Vahl.) panzer	Annual	Grasses	+
Poaceae	*Digitaria abyssinica* (A. Rich) Stapf	Perennial	Grasses	+
Poaceae	*Digitaria ischaemum* (Schreb.) muhl.	Annual	Grasses	+
Poaceae	*Digitaria ternate* (A. Rich.) Stapf	Annual	Grasses	+
Poaceae	*Echinocloa colona* (L.) Link	Annual	Grasses	+
Poaceae	*Eleusine indica* L. Gaertn.	Annual	Grasses	+
Poaceae	*Eragrostiscilianesis* (All.) Lut.	Annual	Grasses	+
Poaceae	*Pennisetum ramosum* (Hochst.) Schweinf.	Annual	Grasses	+
Poaceae	*Phalaris paradoxa* L.	Annual	Grasses	+
Poaceae	*Setaria pumila* (poir.) Roem. &schult.)	Annual	Grasses	+
Poaceae	*Setariaverticillata* (L.) P.Beauv.	Annual	Grasses	+
Poaceae	*Snowdeniapolystachya* (Fresen.) pilg	Annual	Grasses	+
Poaceae	*Sorghum arundinaceum* (Desv.) Stapf	Annual	Grasses	+
Polygonaceae	*Polygonum aviculare* L.	Annual	Broad leaves	-

Family name	Species name	Life cycle	Type of weed	ELISA result*
Polygonaceae	*Polygonum nepalense* Meisn.	Annual	Broad leaves	-
Polygonaceae	*Rumex bequartii* De Wild.	Annual	Broad leaves	-
Primulaceae	*Anagallis arvensis* L.	Annual	Broad leaves	-
Solanaceae	*Datura stramonium* L.	Annual	Brood leaves	-
Solanaceae	*Solanum nigrum* L.	Annual	Broad leaves	-
Poaceae	*Zea maize.* L. (Morka: susceptible maize control)	Annual	Grasses	+

* - = negative (both MCMV and SCMV were not detected by DAS-ELISA).

Table 4. Cereal crops artificially inoculated with Maize chlorotic mottle virus (MCMV) to identify hosts and reaction of tested crops to the MCMV as detected by DAS-ELISA

Common name	Species	Variety	ELISA result*
Barley	*Hordeum vulgare* L.	HB-1307	+
Barley	*Hordeum vulgare* L.	Shege	-
Wheat	*Triticum aestivum* L.	Kilinto/ DZ 918	+
Wheat	*Triticum aestivum* L.	Alidoro/HK-14-R251	+
Wheat	*Triticum aestivum* L.	Hidase /ETBW5795	-
Sorghum	*Sorghum bicolor* L.	Geremew/87BK-4122	+
Sorghum	*Sorghum bicolor* L.	Chiro/COLL#4	-
Sorghum	*Sorghum bicolor* L.	Baji/85 MW 5334	+
Sorghum	*Sorghum bicolor* L.	Tashale/3443-0P	-
Finger Millet	*Eleusine coracana* L.	-	+

* - = negative (both MCMV and SCMV were not detected by DAS-ELISA).

Inoculum prepared from symptomatic alternate host plant species and back inoculated to a susceptible maize variety (Morka) showed clear symptoms of MLN causing viruses. Furthermore, inoculum prepared from symptomatic plants reproduced symptoms in healthy seedlings of the respective plant species. Whereas, no symptom was developed when the inoculum extracted from

asymptomatic plants of any species was used, this indicates the absence of viruses in these plant species.

3.4.MLN seed transmission

For the grow-out test in the greenhouse, a total of 37,140 seeds from 20 maize genotypes were evaluated. Out of these 32,894 seedlings were germinated with a mean germination rate of 88.37% and a range of 62.14 to 97.09%, out of which only 24 (0.073%) seedlings representing 13 maize genotypes were infected with MCMV. Only one (0.003%) out of 3,366 seedlings of Melkassa-2 was infected with SCMV based on symptoms and also as confirmed by ELISA test (Table 5). The infected seedlings showed symptoms on leaves above the second leaf.

Table 5. Seed transmission of maize lethal necrosis (MLN) causing viruses (MCMV and SCMV) in 20 maize genotypes in Ethiopia

Genotype	Harvested seed and sown	Total seedling emerged	Seedling with symptoms and virus detected by ELISA				
			Virus type			Seedling number	Transmission %
			MCMV only	SCMV only	MCMV + SCMV		
BH546	1853	1551	1	0	0	1	0.06
CZ132080	1030	993	0	0	0	0	0.00
BH547	877	545	0	0	0	0	0.00
CZH141027	2969	2139	0	0	0	0	0.00
Melkassa -2	3793	3366	3	1	0	4	0.09 (MCMV), 0.03 (SCMV)
SC-22	688	553	0	0	0	0	0.00
A-7033	620	449	0	0	0	0	0.00
124-b (109)	2000	1844	2	0	0	2	0.11
142-1-e DUSL	1562	1408	2	0	0	2	0.14
CUBA DUSL	1851	1603	2	0	0	2	0.12
CML 124-b (113)	2180	2005	0	0	0	2	0.10
CML 144 DUSL	1300	1192	0	0	0	2	0.17
CZH131009	1867	1542	1	0	0	1	0.06
CZH131010	3580	3476	0	0	0	1	0.03
CZH141029	1166	1128	1	0	0	1	0.09
CZH131013	1092	1012	0	0	0	0	0.00
CZH141022	2352	2118	1	0	0	2	0.09

Genotype	Harvested seed and sown	Total seedling emerged	Seedling with symptoms and virus detected by ELISA				
			Virus type			Seedling number	Transmission %
			MCMV only	SCMV only	MCMV + SCMV		
EHYB	2113	2004	2	0	0	2	0.10
BH540	1890	1741	0	0	0	1	0.06
QPM-A	2395	2187	2	0	0	2	0.09
Total	**37,140**	**32,894**					

MCMV = *Maize chlorotic mottle virus,* SCMV = *Sugarcane mosaic virus*

4. DISCUSSION

Vector- and seed-borne virus diseases of crops such as MLN are inherently difficult to manage directly. Hence, integrated management options such as resistant crop varieties, cultural practices like removal of alternate hosts, use of virus-free seeds and application of chemical pesticides against insect vectors are often used as management options. For successful management of viral diseases, the role of alternate hosts and the extent and significance of seed transmission in wider geographical areas have to be clearly understood. The results of this study present the first comprehensive report on potential and known alternate hosts and the level of seed transmission of the viruses causing MLN in larger maize growing geographic areas of Ethiopia.

The study indicated that over a third (33.63%) of symptomatic samples collected from weed and cultivated plant species grown under field conditions were tested positive for MLN causing viruses (MCMV and SCMV), either for an individual virus or the combination of both viruses (Table 1). A larger proportion (66.37%) of the symptomatic samples were tested negative for MLN causing viruses, which suggested that it may be due to the low concentration/titer of the virus inoculum in the samples which were not in detectable amounts by DAS-ELISA and also the symptoms might be due to other types of viruses that infect the sampled plant species and producing MLN-like symptoms. It is known that several other viruses are developing mosaic symptoms that resemble the symptoms of MCMV and SCMV, especially at earlier stages of infection (Lencho et al. 1997, Guadie et al., 2018).

Seven species of *Poaceae*, two species of *Cyperaceae*, and two species of cultivated crops from *Poaceae*were infected by MCMV and SCMV in the field either individually or in a mixed infection (Table 2). Even though some of the species were previously identified as alternate hosts of MLN

causing viruses (Mahuku et al., 2015; Bekele et al., 2017), several other species were identified by this study as new records. *Phalaris paradoxa*, *Oplismenushirtellus*, *Echinochloacolona*, *Cynodonnlemfuensis* and *Pennisetum purpureum* from *Poaceae* family and *Cyperus cyperoids* from *Cyperaceae* family were identified as natural alternate hosts of MCMV by this study for the first time in Ethiopia and possibly globally. Both MCMV and SCMV were transmitted from plants with the detectable virus to maize plants in the back-inoculation assay, indicating that these plants species can play an important role as sources of the viruses' inoculums to maize. Most of these weed species are frequently and abundantly associated with the maize crop and they may play an important epidemiological role as a viral reservoir and alternate hosts, where the virus can persist in the absence of the maize crop. The natural alternate hosts identified newly in this study indicated the availability of several unknown favorable hosts that can act as the source of infection for the spread of MLN causing viruses. Symptomatic samples of *Cyperus cyperoides* and *Snowdenia polystachya* collected from the fields adjacent to MLN infected maize fields were positive for MCMV and showed a clear yellow colored symptom (Fig. 2). This study indicated that MCMV is fairly commonly found on grass weeds within or nearby maize fields. Contrary to the current finding, Bockelman (1982) in Kansas, USA, didn't observe MCMV on grass weed samples nearby the maize field.

Most of the natural alternate hosts identified in this study were annual and perennial grasses in nature (Table 2) and commonly occur in the maize growing areas. This indicates that maize fields contaminated with these weeds are under threat of MLN infections. Plant viruses have weeds or other alternate natural hosts that act as the source of inoculum from which the economically important crop plants may become infected (Neeraj and Zaidi, 2008; Mathews and Dodds, 2008). In cultivated plants, sugarcane (*Saccharum officinarum*) and sorghum (*Sorghum bicolor*) were naturally infected by both MCMV and SCMV in combination and individually in the field (Table 3); and thus, acting as virus reservoirs. Since these two crops are economically important, MLN would also be a potential threat to the production of these crops.

Results of the experimental host range test (Table 3) for MCMV indicated that three species from the *Cyperaceae* family and 17 species of *Poaceae* family were identified as hosts of MCMV. Among cultivated crops (Table 4), a barley variety (HB-1307) and wheat varieties (DZ 918 and HK-14-R251) were infected by MCMV and showed clear disease symptoms. These indicate the

presence of new MCMV host plants that are favorable for multiplication and distribution of MLN causing viruses. The highest numbers of alternate host plant species identified in this study belonged to the family *Poaceae* followed by *Cyperaceae*, indicating that these families contain larger numbers of plants that are susceptible to MLN causing viruses. Some of the weed species that had previously been identified in Ethiopia as hosts of MCMV also belong to the *Poaceae* family (Mahuku et al., 2015).

The presences of weeds that serve as alternate hosts for MLN causing viruses indicate their epidemiological significance and the importance of their management. Weeds tolerate drought and continue to grow within the field at the absence of crop plants. These weeds support the survival of the viruses at the absence of desired hosts and serve as an important initial source of virus inoculum, which can be spread not only to the main host crops but also to other weed plants after harvesting periods (Asala et al., 2014). Moreover, weeds may also harbor insect vectors that are responsible for viral transmission, resulting in rapid vector population growth (Wisler and Norris, 2005). More studies are required on the relationships between the weeds as vector hosts and virus sources to clarify their impact on the survival and spread of MLN causing viruses.

The results of the seed transmission test indicated that the infected seedlings had symptoms on all levels above the second leaf, which is similar to the findings of Jensen et al. (1991). The transmission percentages among 20 maize genotypes ranged from 0 - 0.17% for MCMV and 0 - 0.003% for SCMV. The highest transmission rates were exhibited by Melkassa-2 maize variety. This indicated that virus seed transmission varies considerably by seed lot and might be influenced by genetic variation among the genotypes.

Results of the seed transmission study indicated that out of 32,856 seedlings, 24 (0.073%) seedlings from 14 maize genotypes were infected with MCMV, whereas only one (0.003%) maize variety (Melkassa-2) was infected with SCMV (Table 5). This indicated low-rate seed transmission of both MLN causing viruses and their importance in MLN epidemiology. The rate of MCMV seed transmission observed in this study was comparable to the reports of Jensen et al. (1991) who evaluated 42,000 seedlings and found 0.04% transmission rate in Hawaii, USA. In contrast to this finding Quito-Avila et al. (2016) from Ecuador reported more than 8% seed transmission of MCMV. However, for vector-borne viral diseases like MLN, even a low rate of seed transmission can be epidemiologically important, because it is the primary source of inoculum that forms the

starting point for the disease onset, and serves as a means for long-distance dissemination. As the infected seeds are randomly dispersed in the field, the seedlings germinating from these seeds serve as sources of inoculum for secondary spread by insect vectors (Sastry, 2013). Seed transmission of plant viruses happens in either embryonic or non-embryonic (Sastry, 2013), hence, further investigation needs to be carried out to determine the localization of the viruses in maize seed.

This study revealed that both MCMV and SCMV can be introduced into maize production areas through seed transmission. Among the two MLN causal viruses, SCMV has been known to commonly occur on maize in Ethiopia for a long time (Lencho et al., 1997), and its transmission rate is very low (Abraham, 2019). Whereas, MCMV, which is the most important component of MLN, is new to Ethiopia and many other East African countries (Mahuku et al., 2015; Fentahun et al., 2017; Demissie et al., 2018; Guadie et al., 2018). This virus is also transmitted with a low rate, but relatively with higher rate as compared to SCMV. It might be possible that MCMV was introduced to Ethiopia through infected seed from countries where MCMV was reported previously through imported maize seed for different purposes, including research and trade. Abraham (2019) suggested that MCMV was probably introduced with maize seeds, due to the fact that the Ethiopian quarantine system is unable to effectively detect viruses, that is the inspection is not capable of using reliable and sensitive virus detection techniques like ELISA and polymerase chain reaction (PCR). The increase in the worldwide exchange of maize seeds between countries has been pointed out to sustain a high risk of MCMV transmission by seed (Liu et al., 2016).

Since the first report of MCMV and SCMV as causative viruses of MLN in Eastern Africa (Wangai et al, 2012; Mahuku et al. 2015; Kagoda et al., 2016), there has been conflicting information on the rate of seed transmission of MCMV. Maize researchers, seed companies, and regulatory authorities working in the maize seed industry have been looking for clear information on the rate of MLN seed transmission. Earlier research conducted in Kenya (Mahuku et al., 2015) indicated 72% (18 out of 25) detection of MCMV in maize seeds harvested from infected plants. It is, however, important to note that the presence of the virus in seed does not necessarily imply its transmission to the seedling. A good example for this suggestion is the fact that the *Rice yellow mottle virus* which is consistently present in the seed (ranging from 65-100% in most cultivars) is not transmitted to the seedlings at all (Konte et al., 2001). Seed transmission of viruses differs

among plant species/varieties, time of plant infection and the virus isolate. Recently, Zhang et al. (2011) reported MCMV seed transmission of 2 seeds in 600 (0.33%) in a Chinese maize sample which is also not too far from to the highest seed transmission rate (0.17%) we observed with one of the maize genotypes (CML 144 DUSL) used in the current study. Quito-Avila et al. (2016) however reported a considerably higher level of MCMV seed transmission (8% and 12%) compared to the current finding and also compared to the finding of other authors (Jensen et al., 1991; Zhang et al., 2011). Such discrepancy might be due to differences in host genotype, virus strain or experimental condition. In general, however, the findings of this study are highly convincing since the results were generated under strict green-house and laboratory test conditions, which reflect the true level of MCMV transmission by seeds for the tested varieties. Epidemiologically, this apparently low level of transmission can be important under environmental conditions with significant vector pressure. Since both MCMV and SCMV are also vector transmitted, monitoring insect vector pressure and establishing the tolerance level might help in disease management as done for *Lentil mosaic virus* in California (Grogan, 1980) where seeds will be rejected from being certified if one seed is infected out of 30,000 seeds (0.003% infection rate). Future work should, therefore, focus on the infection threshold level of seed and vector transmission to use them in an integrated MLN management package.

Based on the findings of this study, it can be concluded that various weed and cultivated plants identified as alternate hosts, and the seed transmissibility of MLN causing viruses (MCMV and SCMV) are epidemiologically important and maintain the virus inoculum in the absence of maize crop in the field, and support the survival of the virus for continuous infection. Farmers and stakeholders involved in maize production should take precautionary measures by using certified and virus-free maize seeds from trusted sources, and eliminating alternate host plants within and in the surrounding areas of maize fields. Regular field monitoring, assessment of virus symptoms and rouging-out diseased maize plants are recommended to prevent further spread by insect vectors.

In conclusion, this study showed occurrence of a wide range of weed and crop plants representing various families as actual and potential hosts of MLN causing viruses in Ethiopia. *Poaceae* family had the highest number of species that were identified as alternate host for MLN causing viruses. These alternate hosts have a potential to serve as sources of inoculum for the virus that can be

further spread by insect vectors. The study also provided clear information on the level of maize seed transmission of these viruses in Ethiopia. The information provided by the study represents a valuable input for the development of integrated MLN management strategies, which may include the use of resistant varieties and pesticides to control the insect vectors, removal/management of alternate hosts and virus- free seeds.

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Paper III

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Transmission and Persistence of Maize Lethal Necrosis in Infested Soil and Infected Maize Residue

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Abstract Maize lethal necrosis (MLN) is a viral disease caused by a co-infection of maize chlorotic mottle virus (MCMV) and any one of cereal viruses including sugarcane mosaic virus (SCMV), maize dwarf mosaic virus, wheat streak mosaic virus or Johnsongrass mosaic virus. MLN has been identified as the most devastating maize disease that causes the highest yield loss in major maize growing areas in Eastern Africa including Ethiopia. In this study, field assessment, laboratory, and greenhouse experiments were conducted using MCMV and SCMV infected maize residue and infested soil to: (1) assess the role of MLN infected maize residue and infested soil in the transmission of MLN causing viruses, and (2) determine the longevity of the viruses both in the infected maize residue and in the infested soils. Serological detection and back-inoculation test results showed that MCMV was detected and confirmed to be transmitted from infested soil to newly germinated maize seedlings. However, SCMV was neither detected in soil samples from infected fields nor transmitted to maize seedlings. The present study confirmed that MLN infested soil is an essential medium for the survival and spread of MCMV under natural conditions. Under experimental condition, MCMV remained persistent and transmissible up to 6 months to maize planted on MLN infested soil mixed with MLN infected maize residues. Proper management or clearing of crop residues in the field after harvest is necessary to minimize adverse effects of MLN on maize production. Crop rotation is also one of the ways of freeing the soil from MLN disease.

Keywords Crop rotation · maize chlorotic mottle virus · Virus survival · sugarcane mosaic virus · *Zea mays*

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Introduction

Maize (*Zea mays* L.) is the most widely cultivated cereal crop in the Sub-Saharan Africa (SSA), and it is greatly important for food security and livelihoods

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Abstract

Maize lethal necrosis (MLN) is a viral disease caused by a co-infection of *Maize chlorotic mottle virus* (MCMV) and any one of cereal viruses including *Sugarcane mosaic virus* (SCMV), *Maize dwarf mosaic virus*, *Wheat streak mosaic virus* or *Johnson mosaic virus*. MLN has been identified as the most devastating maize disease that causes the highest yield loss in major maize growing areas in Eastern Africa including Ethiopia. In this study, field assessment, laboratory, and greenhouse experiments were conducted using MCMV and SCMV infected maize residue and infested soil to (1) assess the role of MLN infected maize residue and infested soil in the transmission of MLN causing viruses, and (2) determine the longevity of the viruses both in the infected maize residue and in the infested soils. Serological detection and back-inoculation test results showed that MCMV was detected and confirmed to be transmitted from infested soil to newly germinated maize seedlings. However, SCMV was neither detected in soil samples from infected fields nor transmitted to maize seedlings. The present study confirmed that MLN infested soil is an essential medium for the survival and spread of MCMV under natural conditions. Under experimental condition, MCMV remained persistent and transmissible up to 6 months to maize planted on MLN infested soil mixed with MLN infected maize residues. Proper management or clearing of crop residues in the field after harvest is necessary to minimize adverse effects of MLN on maize production. Crop rotation is also one of the ways of freeing the soil from MLN disease.

Key words: Crop rotation; *Maize chlorotic mottle virus*; Virus survival; *Sugarcane mosaic virus*; *Zea mays*

1. Introduction

Maize (*Zea mays* L.) is the most widely cultivated cereal crop in the Sub-Saharan Africa (SSA), and it is greatly important for food security and livelihoods of the people (Tesfaye et al. 2015). In SSA it covers over 35 million hectares, largely grown by smallholder farming communities that produce over 70 million metric tons of grain (FAO, 2018). However, insect pests and diseases are key constraints to maize production and productivity (Mahuku et al. 2015b). In the SSA region, especially in East Africa, MLN epidemics that occurred in 2011 in Kenya caused 30-100% maize yield loss (Wangai et al. 2012). Since its outbreak, MLN has become widespread and caused significant maize yield reduction in major maize growing countries of East Africa, including Uganda, Tanzania, Rwanda, Democratic Republic of Congo and Ethiopia (Adams et al. 2014; Lukanda et al. 2014, Mahuku et al. 2015a; Mahukuet al. 2015b; Kogoda et al. 2016; Regassa et al. 2020) and continues to constrain maize production in the region (Boddupalli et al. 2020).

MLN is caused by a double infection of *Maize chlorotic mottle virus* (MCMV; genus *Machlomovirus*; family *Tombusviridae*) with any one of several cereal viruses in the family *Potyviridae*, such as *Sugarcane mosaic virus* (SCMV) (Wangai et al. 2012; Mahuku et al. 2015), *Maize dwarf mosaic virus* (MDMV) (Niblett and Claflin 1978), *Johnson grass mosaic virus* (Stewart et al. 2017) or *Wheat streak mosaic virus* (WSMV) (Scheets 1998). While SCMV has a worldwide distribution, the outbreak of MLN in SSA including in Ethiopia was driven by the emergence and spread of MCMV (Boddupalliet al. 2020). The causative viruses are transmitted from plant to plant and field to field by insect vectors. MCMV is transmitted mainly by chrysomelid beetle species (Naultet al. 1978; Jiang et al. 1992) and thrips (Jiang et al. 1992), and SCMV by aphids (Zhang et al. 2008). Both viruses also transmitted through infected maize seeds at low rates, which ranged from 0% to 0.17% for MCMV and 0 to 0.003% for SCMV (Jensen et al. 1991; Regassa et al. 2021).

MLN is widespread and has caused from low to total crop failure in major maize growing areas since its first outbreak in 2014 in Ethiopia (Fentahunet al. 2017; Guadieet al. 2018; Regassaet al. 2020). In Ethiopia, investigations on MLN distribution, identification and characterization of the causal viruses from maize (Mahuku et al. 2015a; Fentahun et al. 2017; Guadie et al. 2018), its association with cropping systems and cultural practices (Regassa et al. 2020), alternative hosts and seed transmission (Regassa et al. 2021) have been conducted. In Kenya, preliminary work

done by Mahuku et al. (2015b) showed a high incidence of MCMV in seedlings planted in soil taken from around MLN-infected maize. Regassa et al. (2020) reported that continuous maize cultivation in the same field is associated with increased incidence of MLN in Ethiopia and suggested crop rotation as a management option. To manage a disease successfully using crop rotation, disease transmission mechanisms and longevity of the pathogen survival in the soil and in plant residues need to be determined.

Understanding plant viruses' transmission and persistence play a significant role in designing appropriate management options. Plant viruses being obligate pathogens must be spread from one susceptible host plant to another and need to be introduced into the living cells for their survival and continuity. Knowledge of the ways in which a virus maintains itself in the absence of a living host and spreads in the field is essential for the development of effective management measures.

Knowledge of virus transmission and its survival longevity are important to understand how the disease transmits from infected plant debris to healthy plants, and how the virus spreads between and among maize fields. Such information is very important to develop appropriate disease management strategies and tactics. To the best of our knowledge, no experimentally supported studies have been conducted so far on longevity of the virus persistence/survival for effective use of crop rotation as management options in Africa. Thus, the objectives of this study were to (1) assess the role of MLN infected maize plant residues and infested soil in the transmission of MLN causing viruses, and (2) determine the longevity of the viruses in infected plant residues and in infested soil.

2. Materials and methods

2.1.Sample collection

One hundred ninety-four soil samples were collected from 68 MLN infected maize fields in Oromia and South Nation, Nationality and Peoples (SNNP) regional states in Ethiopia. These regional states are known to have a high level of MLN infection rate (Regassaet al. 2020). Field assessment was conducted in 2017 and 2018 during the main rainy season (August to September) at vegetative todough stages of the crop. Based on MLN incidence and severity, three zones each from Oromia (Jimma, East Shewa and Arsi) and SNNP (Wolayita, Hadiya and Sidama) Regional States, and one to two districts were purposively selected from each zone.

Samples were randomly taken from several points in each field where maize showed severe MLN symptoms (severe leaf chlorosis, mottling and necrosis) at the stage of 10 leaves to late dough stage. Soil samples suspected to be infested by MLN causing viruses were collected near the roots of symptomatic maize plants. The sampled plants were uprooted and the soil was gently removed from the root by shaking. One-hundred ninety-four samples of maize parts (stem and root) from representative subset of maize plant surrounding the soil samples were collected and double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) test was performed to confirm the presence of MCMV and SCMV in the samples of maize parts following the method described below. Soil samples collected from the surroundings of ELISA-positive maize plant parts that were infected by MLN causing viruses were used for MLN soil transmission study.

2.2. Detection of MCMV and SCMV from infested soil and infected maize samples, and back inoculation of extracted MLN causing viruses to susceptible hosts

Detection of the viruses from soil and maize parts was assayed serologically using DAS-ELISA for the presence of MCMV and SCMV. Extraction of the virus from soil samples was conducted as described by Gülser et al. (2008) as used for *Tobacco mosaic virus*. One gram of air-dried MLN-infested soil samples were suspended in 1 ml extraction buffer (0.01 M phosphate buffer of PH 7.0) in Eppendorf tubes. The suspensions were agitated on a vortex mixer for 1 min, shaken on a rotary shaker for 30 min at room temperature, and then incubated at 4°C overnight. The supernatants were used for DAS-ELISA test. DAS-ELISA was performed separately on soil samples and maize parts from individual plants. The detection antibodies for MCMV (AS-1087), SCMV (AS-0166) and their respective positive controls were obtained from DSMZ- plant virus collection, Germany. Tests were conducted following the method described by Clark and Adams (1977) and the standard protocols at the DSMZ- Plant Virus Collection.

To determine disease transmission potential of MCMV and SCMV that were detected from soil and plant part samples, saps from the samples that tested positive for each of the virus were used for back-inoculation test. Six DAS-ELISA positive samples were selected from each category (soil, stem and root of plants) for the back-inoculation test. Six seeds from MLN susceptible maize variety called Limu (Regassa et al. 2020) were planted in 25 cm diameter plastic pots filled with mixed sterilized soil, sand and organic manure at 2:1:1 ratio, respectively. The pots were kept in

an insect-proof greenhouse at 25-30ºC and watered regularly as required. To facilitate mechanical inoculation, carborundum dust was added to the inoculum solution prepared from the leaves. MCMV and SCMV inoculum were then inoculated separately by using fingers onto 4-6 leaf stage of healthy maize seedlings separately in an insect-proof greenhouse and rinsed immediately with water. The symptom development was monitored after inoculation and detection of the virus was carried out by DAS-ELISA.

2.3. Soil transmission test

This study was also conducted in an insect-proof greenhouse five days after soil samples were collected and maintained at 25-35°C in greenhouse. Seeds of a maize variety (Limu) were planted on SCMV, MCMV and MLN infested soil samples collected from the root zones of diseased maize plants. Different soil samples were prepared for transmission tests depending on the type of MLN causing viruses. Accordingly, SCMV infested soil samples were considered as the first group, and MCMV infested samples were considered as the second group. Soil samples from mixed infection of the two viruses (MCMV + SCMV) as the third group.

Seeds were planted in metallic trays (51 × 63cm) filled with suspected virus-infested soil collected from the root areas of MLN causing viruses infected maize plants. The trays had six rows with 10 seeds per row and were placed on greenhouse benches with no supplemental lighting. Most of the tests were conducted at 25-35°C day temperatures. After emergence, maize seedlings were observed for symptoms development at least twice a week starting from two-leaf stages, and any symptomatic seedlings were labeled for subsequent testing. Several plants with symptoms were recorded at 30 days after planting and the presence of viruses (MCMV and SCMV) were confirmed using DAS-ELISA test as described above under section 2.2. Transmission percentages were calculated as follows:

$$\mathrm{TP} = \frac{\mathrm{IS}}{\mathrm{TGS}} \times 100$$

Where TP = Transmission percentage, IS = Infected seedlings which is ELISA Positive, and TGS = Total germinated seedlings.

2.4. Persistence of MLN causing viruses in infested soils and plant residues

2.4.1. Sources of MLN infected maize residues and infested soils

Infected maize residues and infested soils were obtained from maize plants which were established in a greenhouse and inoculated with a mixture of MCMV and SCMV. The MLN causing viruses (MCMV and SCMV) used in this study were collected from the field and maintained and propagated in separate greenhouses periodically by mechanical inoculations. Symptomatic leaves from each virus were weighed and ground using sterile mortar and pestle to obtain homogenate solution or extract (1:10; 1g of leaf materials to 10 ml extraction buffer) separately. The inoculum extracts were mixed in 1:4 ratios (adding one part of MCMV and four parts of SCMV) in one container to obtain an optimized virus combination known to cause MLN in East Africa (Gowda et al. 2015) and inoculated on to healthy maize seedlings in the greenhouse. The inoculated plants were grown until flowering. The infected maize was uprooted, chopped into smaller pieces and used as MLN infected residue. The soil from which the MLN infected maize uprooted were used as MLN infested soil.

2.4.2. Treatments and experimental design

The treatments used in this experiment included maize planted on (1) MLN infested soil mixed with MLN infected maize residue, (2) MLN infested soil, (3) MLN free soil (uninfected soil) mixed with MLN infected maize residue and (4) sterilized MLN free soil used as control. Nine seeds of MLN susceptible maize variety known as Limu (Regassaet al. 2020) were sown in each plastic pot (30 cm diameter) and reduced to seven plants after germination. The treatments were arranged in a completely randomized design with four replications. MLN infected maize residue was mixed with MLN infested and MLN free soil at a ratio of 1:4 (residue: soil) by weight. MLN free soil used in the study was collected from Ambo University Guder Campus where maize have never been planted and no MLN disease was found. The treatment categories had a one-month storage period differences between one to seven months in greenhouse (25-30°C day temperature) before the transmission test. Number of plants with symptoms was recorded at 30 to 40 days after planting and the presence of MCMV and SCMV was confirmed by DAS-ELISA test following the procedure indicated under section 2.2 above.

Data collected included the number of plants with disease symptoms confirmed by DAS-ELISA and percent disease incidence (DI) as follows:

$$\mathrm{DI} = \frac{\text{Total number of plants with disease symptoms}}{\text{total number of plants per treatment}} \times 100$$

Disease incidence data recorded was subjected to analysis of variance (ANOVA) to determine effects of different treatments and differences among the means were separated using Fischer's protected least significant differences (LSD) test at 5% probability level.

3. Results

3.1.Detection of MLN causing viruses from soil and maize plant parts

Out of 194 soil samples collected and assessed, 13 (6.7%) of the samples were positive for MCMV but all were negative for SCMVwhen tested using DAS-ELISA. MCMV was detected in samples collected from Shebe Sombo, Jeju, Boricha, Misirak Badawacho and Damot Gale districts (Table 1). Out of 194 maize stem samples, 94 (48.4%), and 24 (12.4%) were tested positive for MCMV and SCMV, respectively, as single infections and 76 (39.2%) had mixed infection of both viruses (MCMV + SCMV). Similarly, from the same size of root samples, single infection of MCMV was detected in 96 (49.5%) and SCMV was detected in 26 (13.4%) of the samples, whereas mixed infection of both viruses was detected in 77 (39.7%) of the samples using DAS-ELISA test (Table 1).

Table 1. DAS-ELISA test results for MCMV and SCMV from 194 soil, maize stem and root samples collected from maize lethal necrosis (MLN) infected maize fields in 2017-2018 main cropping season.

Region	Zone	District	DAS-ELIS tested positive samples for								
			MCMV			SCMV			MCMV + SCMV		
			Soil	Stem	Root	Soil	Stem	Root	Soil	Stem	Root
Oromia	Jimma	Omo nada	0	15	15	0	3	3	0	5	5
		Shibe Sombo	2	13	13	0	2	2	0	9	9
	East Shewa	Fantale	0	10	11	0	4	3	0	7	7
		Lume	0	9	9	0	3	3	0	8	8
	Arsi	Jeju	5	5	5	0	4	4	0	7	7

SNNP	Sidama	Boricha	3	9	9	0	1	1	0	13	13
	Hadiya	MisirakBadawacho	1	10	10	0	2	2	0	8	8
	Wolayita	DamotPulassa	0	14	15	0	3	2	0	6	6
		Damot Gale	2	9	9	0	2	1	0	13	14
	Total		**13**	**94**	**96**	**0**	**24**	**21**	**0**	**76**	**77**

MCMV = *Maize chlorotic mottle virus*; SCMV = *Sugarcane mosaic virus;* SNNP = South Nation, Nationality and Peoples

Soil sample sap that was tested positive for MCMV using DAS-ELISA could produce symptoms of MCMV infection and positive ELISA readings when mechanically inoculated onto healthy maize plants. Plant saps from stem and root of the positive maize plants also developed symptoms and tested positive for MCMV and SCMV by DAS- ELISA.

3.2. Soil transmission test

Maize seedlings raised on MLN infested soil were tested positive for MCMV. However, all the seedlings were tested negative for SCMV when detected by DAS-ELISA (Table 2). MCMV symptoms started to appear two weeks after planting and the virus was detected in 4.24-13.5% of the seedlings emerged. The seedlings did not show SCMV symptoms and the virus was not detected when tested for using DAS-ELISA.

Table 2. Transmission of maize lethal necrosis (MLN) causing viruses from infested soils to maize seedlings.

District	Sample type	Number of plants tested	Number of DAS-ELISA tested positive for[a]		Percent infected (%)
			MCMV	SCMV	
Omo nada	Infested soil	237	32	0	13.50
	Control	118	0	0	0.00
Shibe sombo	Infested soil	236	23	0	9.75
	Control	120	0	0	0.00
Lume	Infested soil	179	19	0	10.61
	Control	119	0	0	0.00
Fantale	Infested soil	178	21	0	11.80
	Control	120	0	0	0.00

District	Sample type	Number of plants tested	Number of DAS-ELISA tested positive for[a]		Percent infected (%)
			MCMV	SCMV	
Jeju	Infested soil	118	5	0	4.24
	Control	120	0	0	0.00
DamotPulassa	Infested soil	238	24	0	10.08
	Control	120	0	0	0.00
Damot Gale	Infested soil	235	25	0	10.64
	Control	119	0	0	0.00
MisirakBadawacho	Infested soil	179	17	0	9.5
	Control	117	0	0	0.00
Boricha	Infested soil	237	27	0	11.39
	Control	118	0	0	

[a] 0 = negative (both MCMV and SCMV was not detected by DAS-ELISA); MCMV = *Maize chlorotic mottle virus*; SCMV = *Sugarcane mosaic virus*

3.3.MLN persistence in the soil and plant residues

The incidence of MLN causing viruses in different treatments and their survival duration (longevity) is presented in Fig. 1. There was a statistically significant difference ($P < 0.001$) between treatments. Maize planted in MLN infested soil mixed with MLN infected maize residue and MLN free soil mixed with MLN infected maize residue that was stored for one month before planting had a higher MLN incidence of 50% than MLN infested soil, which had MLN incidence of 21.4%. Whereas, maize planted in MLN infested soil that was stored for three months had the lowest MLN incidence of 3.57% and no MLN symptoms were observed in maize planted on the soil stored for more than three months. The maize planted on MLN infested soil mixed with MLN infected maize residue generally had more infected plants than those planted on MLN infested soil alone across the storage duration. No symptom was observed on maize planted on sterilized MLN free soil that was used as a control.

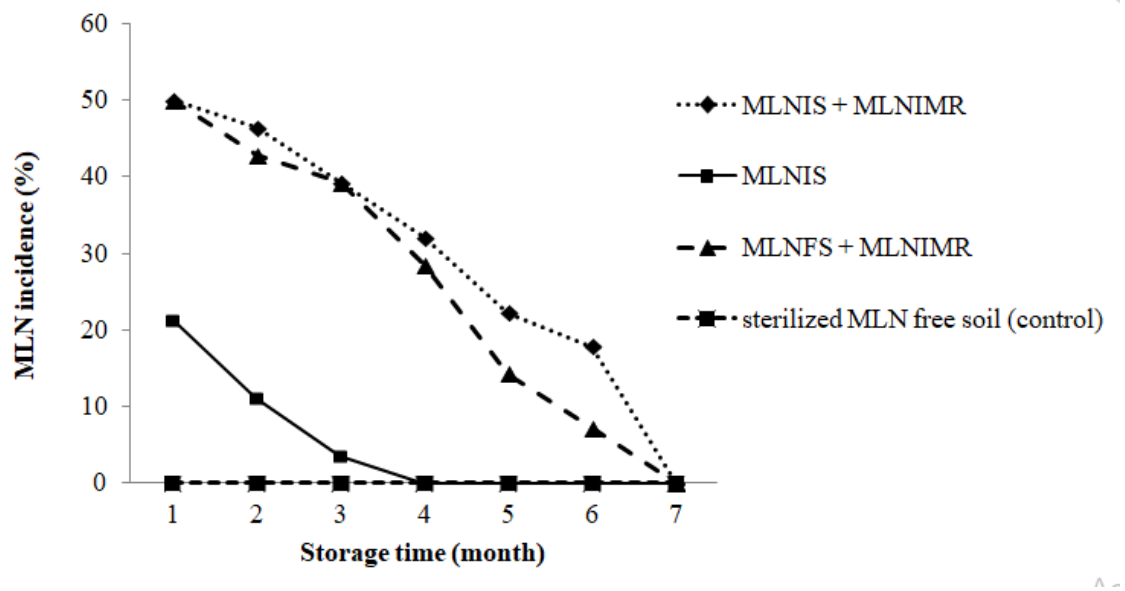

Fig.1. Incidence and survival of maize lethal necrosis (MLN) in the soil and maize plant residues. MLNIS + MLNIMR = maize planted on MLN infested soil mixed with MLN infected maize residue; MLNIS = maize planted in MLN infested soil; MLNFS + MLNIMR = maize planted in MLN free soil mixed with MLN infected maize residue.

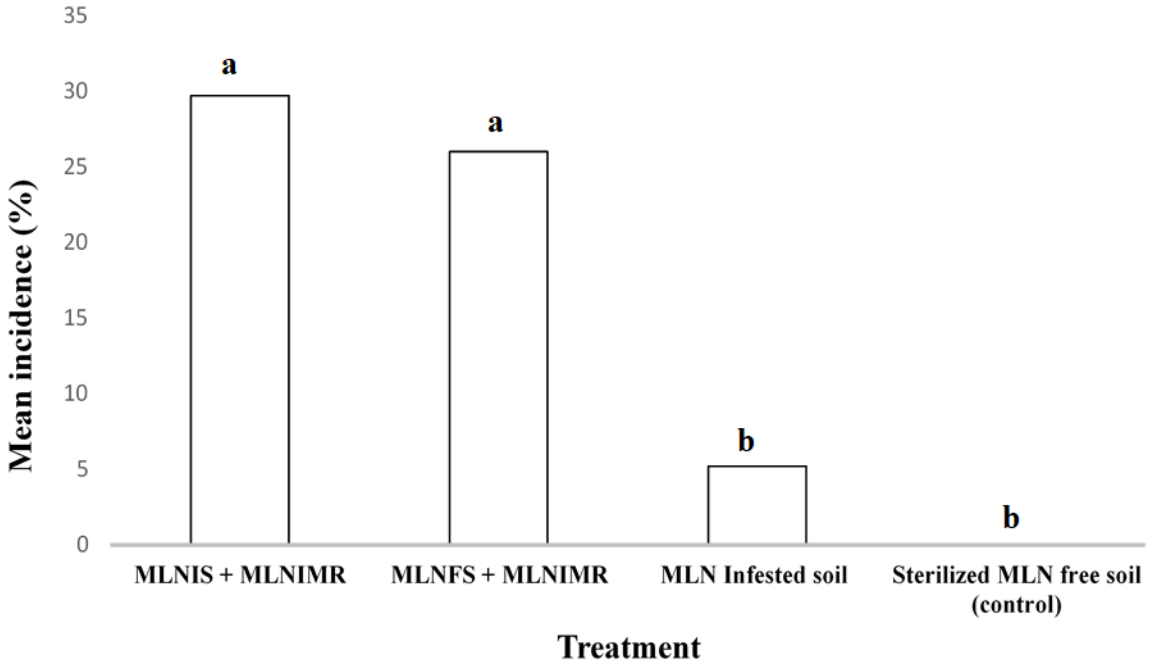

Fig. 2. Mean of all time incidences of maize lethal necrosis (MLN) in the soil and maize plant residues. Means with the same letter are not significantly different ($p \leq 0.05$). MLNIS + MLNIMR

= maize planted on MLN infested soil mixed with MLN infected maize residue; MLNIS = maize planted in MLN infested soil; MLNFS + MLNIMR = maize planted in MLN free soil mixed with MLN infected maize residue.

The plant started to show symptoms (chlorosis, mottling and mosaic) two weeks after emergence. From each treatment, all symptomatic samples were collected for ELISA analysis. Number of virus-positive and symptomatic plants during one to seven months of storage duration of MLN from different treatment is presented in Table 3. MCMV was detected from maize planted in all treatments, while SCMV was detected from samples of maize planted on both MLN infested soil and MLN free soil mixed with MLN infected residue stored for 1 month. The mixed infection of both MCMV and SCMV was detected only from maize planted on MLN infested soil with MLN infected residue stored for 1 month. MCMV did not survive in MLN infested soil (no MLN infected residue) that was stored for more than three months but up to 6 months to maize planted on both MLN infested soil mixed with MLN infected maize residues and MLN free soil mixed with MLN infected maize residue.

Table 3. Number of virus-positive and symptomatic plants during one to seven months of storage duration of maize lethal necrosis (MLN) infested soil, and infected maize residue mixed with infected and uninfected soil.

Treatment [a]	MLN symptomatic (number and %) after storage (month) [b]							DAS-ELISA (positive frequency) [c]		
	1	2	3	4	5	6	7	MCMV	SCMV	MCMV +SCMV
MLNIS + MLNIMR	14/28 (50.0%±8.2)	13/28 (46.4%±7.1)	11/28 (39.3%±7.1)	9/28 (32.1%±7.1)	6/27 (22.6%±9.8)	5/28 (17.9%±7.1)	0/28 (0.0%±0.0)	52	3	3
MLNIS	6/28 (21.4%±8.2)	3/27 (11.3%±7.6)	1/28 (3.6%±7.1)	0/28 (0.0%±0.0)	0/28 (0.0%±0.0)	0/27 (0.0%±0.0)	0/28 (0.0%±0.0)	10	-	-
MLNFS + MLNIMR	14/28 (50.0%±8.2)	12/28 (42.9%±0.0)	11/28 (39.3%±7.1)	8/28 (28.6%±0.0)	4/28 (14.3%±0.0)	2/28 (7.1%±8.2)	0/28 (0.0%±0.0)	49	2	-

[a]MLNIS + MLNIMR = maize planted on MLN infested soil mixed with MLN infected maize residue; MLNIS = maize planted on MLN infested soil; MLNFS + MLNIMR = maize planted on MLN free soil mixed with MLN infected maize Residue

[b]Symptomatic plant/total plant

[c] - = negative (both MCMV and SCMV were not detected by DAS-ELISA).

4. Discussion

Previous studies indicated that MLN is transmitted by seed and insect vectors. For MCMV, soil transmission was also suggested by the reduced incidence of MCMV in maize after rotation with sorghum (Phillips et al. 1982). Although there are some preliminary reports suggesting a high incidence of MCMV in seedlings planted in soil taken from around MLN-infected maize (Mahuku et al. 2015b), more detailed investigation was needed to be conducted using well designed and controlled experiments to define the importance of soil transmission and the role of host residues on MLN development. It is therefore important to clearly understand the role of MLN infested soil and infected maize plant residue in MLN causing viruses' transmission and persistence/survival to use appropriate management options like crop rotation and field cleaning. The present study showed 6.7% of soil samples collected from MLN infected maize fields were tested positive for MCMV using DAS-ELISA. However, a large proportion (93.3%) of suspected MLN infested soil was tested negativefor MLN causing viruses, which might be due to the low concentration of the virus inoculum in the samples which were not in detectable amounts by DAS-ELISA and requires further investigation using molecular analysis (RT-PCR) methods. SCMV was not detected in all samples, indicating that the virus inoculum either existed in the soil at a very low concentration that may not be in detectable amounts by DAS-ELISA test or do not survive in the soil environment. Like MCMV, other plant viruses for example *Pepper mild mottle virus* (Ikegashira et al., 2004) and *Tobacco mosaic virus* (Gülser et al., 2008) were detected from the soil by DAS-ELISA, however, future research may require for the development of optimized protocol for detection of MCMV from soil sample by DAS-ELISA. Both MCMV and SCMV were detected in maize parts (stem and root), indicating that maize plant parts might serve as a reservoir to maintain MLN causing viruses after harvesting. A similar result was reported by Uyemoto (1980), Jiang et al. (1992), and Scheets (2004) who reported that both viruses can be found in any parts of maize plants so long as the plant was infected.

Our study also confirmed that MCMV can be transmitted from infested soil to newly raised maize seedlings. The plants showed symptoms (chlorosis and mottling) and detected positive by DAS-ELISA, which agrees the report of Mahuku et al. (2015b) that stated maize planted in infected soil

with MCMV had characteristic symptoms of the virus and gave positive results by serological detection.

Abiotic transmission of viruses from soil or plant residues to plant hosts has been reported for several crop-virus combinations including tomato mosaic virus in tomato (Pares et al. 1996), tobamoviruses (*Tomato mosaic tobamovirus* and *Tobacco mosaic virus*) in forest soils (Fillhartet al. 1998) *Tobacco bushy stunt virus* in tomato (Kleinhampel and Kegler 1982) *Southern bean mosaic virus* in beans (Teakle et al. 1986). Since naturally plant viruses require wound or vectors for entrance to the plant cell, the possibility of soil transmission increased by the activities of microorganisms in the soil, during cultural practices such as weeding and through cutting implements that may create a wound and generating virus entry sites.

In this study, low soil transmission of 4.24-13.5% is recorded (Table 3). Such a level can, however, play a major role in the epidemiology of the disease, since MLN causing viruses are also vector transmitted. Hence, soil transmission can be important under environmental conditions with significant vector pressure.

In addition, this study showed that virus persistence and transmission were observed in maize planted on MLN free soil mixed with MLN infected maize residue, indicating that the transmission sources of MLN causing virus within the soil were not only the infected roots exuded during the cropping season but also from infected maize plant residue left within the soil after harvest. Similar findings were previously reported on MCMV transmission through soil (Nyvall 1999). Previous investigators also documented that MCMV can be transmitted through infected plant residues that play important roles for the survival of the virus especially when maize is planted during the off-season (Uyemto 1983; Montenegro and Castillo 1996). In sorghum plant, SCMV was found to be transmitted by soil as non-inoculated sorghum plants become infected with SCMV when grown in containers with infected plants (Bond and Pirone 1970).

The persistence of MLN causing viruses in the soil and maize residue had a significant effect on the incidence of the disease. The highest percentage of disease incidence was observed on maize planted in infested soil stored for one-month and mixed with MLN infected residue, and in virus free soil mixed with MLN infected residue. Freshly incorporated infected maize residue had higher concentration of MCMV and SCMV, and resulted in higher disease incidence. Shorter survival duration of SCMV might be influenced by its amount/concentration of the inoculum in the residue.

The higher survival rate of MCMV suggested that the virus was more stable in soil or plant debris, and hence had higher transmission rate with greater opportunity of persistence and incidence compared to SCMV. This study clearly showed that MLN incidence decreased as the storage period was prolonged, suggesting viability of the virus in the soil and maize residue was reduced. It is expected that decomposition of the maize residue reduces the virus inoculum and therefore resulted in reduced incidence and disease development.

MLN causing viruses did not survive in MLN infested soil that was stored for more than three months. However, in MLN infested soil that was mixed with maize residue the virus (MCMV) survived up to six months (Fig. 1). This indicates that continuous presence of MLN infected maize residue in the soil/field provides a virus reservoir and bridges the virus between seasons. This signifies the importance of maize residue management in the field, use of crop rotation and maize-free period in the field for the management of MLN disease. A previous study (Regassa et al. 2020) showed that MLN incidence is higher in fields continuously planted with maize than in the field where maize was rotated with other crop types. Crop rotation is an important practice that is widely emphasized around the world to avoid the inoculum buildup of pathogens. In Kansas and Nebraska (USA), MCMV infections re-occur in the same locations within maize fields year after year indicating that the virus is maintained in the soil from season to season and overwinter in maize residues (Uyemoto 1983; Montenegro and Castillo 1996). Commercial seed producers in Hawaii control MCMV with maize-free periods of 60 days each year, as well as regular insecticide treatments for vector control (Jiang et al. 1992).

The present study showed that MLN infested soil and infected maize residue play an important role in the survival, inoculum source and spread of MCMV, the major component of MLN. This suggests that apart from being inoculum sources within the same field, any activity that moves infested soil or infected maize residue from one place to another can spread the viruses. MCMV remains persistent and transmissible even up to 6 months in maize planted on MLN infested soil mixed with MLN infected maize residue. Nearly, 50% of maize plants became infected when grown experimentally in soil containing plant residue infected with MLN that was stored for one month before planting andreduction with time (Table 3), which seems a high risk. The inoculum level in this work was almost certainly much higher than what would occur in crop conditions in the field, therefore, further research is required to determine how long would the viruses persists in the soil under field conditions.

Thus, proper management of crop residues in the field after harvest is necessary to minimize the adverse effects of MLN on maize production. Crop rotation is one of the ways of freeing the soil from MLN disease. Therefore, as part of integrated management of MLN, maize growers should remove all infected maize residues from the field, ignore any activity that moves the infected soil and maize residues from one place to another, and practice sustainable crop rotation with non-host crops coupled with efficient weed management practices.

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Paper IV

Identification of insect vectors of maize lethal necrosis viruses in Ethiopia

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Abstract

Maize lethal necrosis (MLN) is a viral disease caused by co-infection of *Maize chlorotic mottle virus* (MCMV) and a potyvirus mostly *Sugarcane mosaic virus* (SCMV). MLN is widely distributed across sub-Saharan Africa, and it is one of the most important maize production constraints in Ethiopia. In this study morphological and molecular characterization was used to determine potential insect vectors that transmit MLN causing viruses in southern and southwestern Ethiopia. Suspected insect vectors of MLN disease causing viruses collected from maize fields were initially identified to genus/species level based on the morphological features. Further taxonomic determination was carried out using DNA sequencing from cytochrome oxidase primers. Pre-identified insect vectors of MLN-causing viruses were separately tested in the greenhouse for potential transmission of *Maize chlorotic mottle virus* (MCMV) and *Sugarcane mosaic virus* (SCMV). Accordingly, maize thrips, *Franklinella* sp. and cereal leaf beetle, *Oulema* sp. transmitted MCMV, whereas corn leaf aphids, *Rhopalosiphum maidis* transmitted SCMV. The insects spp. identified in current study are considered potential vectors of the two viruses in the field. Out of 86 maize fields surveyed, Franklinella sp. were widely distributed in 22 (25.6%) fields, mostly in Arsi and West Shewa zones of Oromia region. *R. maidis* was abundant in all the areas assessed suggesting that this insect spp. is a vector for SCMV in Ethiopia. The presence of these insects as vectors of MLN causing viruses might be one of the most important factors contributing to the spread of MLN disease from plant to plant and field to field as well as to new geographical areas.

Key words: *Franklinella* sp, Maize lethal necrosis, *Oulema* sp, *Rhopalosiphum maidis,* Transmission

1. Introduction

In 2011, the outbreak of the most devastating new disease named maize lethal necrosis (MLN) has been observed in Kenya (Wangai *et al*., 2012). Since then, the disease has widely been reported and widespread in several other eastern Africa countries (Mahuku *et al*., 2015a, Adams *et al*., 2014; Lukanda *et al*., 2014). MLN is a viral maize disease caused by the co-infection of *Maize chlorotic mottle virus* (MCMV; genus *Machlomovirus*; family *Tombusviridae*) and one of any viruses from the family *Potyviridae*, such as *Sugarcane mosaic virus* (SCMV), *Maize dwarf mosaic virus* or *Wheat streak mosaic virus* (Redinbaugh and Stewart, 2018). In East Africa, MLN is caused by co-infection of MCMV and SCMV (Wangai *et al*., 2012; Adams *et al*., 2014; Mahuku*et al*., 2015b).

Major factors associated with MLN emergence include continues maize cultivation year after year on the same field, the presence of vectors transmitting the disease from plant to plant and field to field, and susceptible maize cultivars (Mahuku *et al*., 2015b; Regassa*et al*., 2020). Alternative hosts, soil, infected maize plant residue and seed transmission of MLN causing viruses also play significant roles in development and preservation of MLN epidemics (Nyvall, 1999; Jensen *et al*., 1991; Regassa *et al*., 2021a, 2022). For an insect-vectored virus disease to emerge in a crop, the virus, vector, and a susceptible host must come together in an environment conducive for the disease. The transmissions of viruses from plant to plant by vectors provide the main method of spread in the field for many viruses that cause severe economic loss (Hull, 2014). In the case of East Africa including Ethiopia, the introduction of MCMV appears to be a new factor. MCMV was reported for the first time in eastern Africa in 2012 (Wangai *et al*., 2012; Mahuku *et al*., 2015a, Fentahun *et al*., 2017), but SCMV has been prevalent worldwide for many decades. For a disease to be perpetuated there must be virus reservoirs and vector populations capable of sustaining the disease (Redinbaugh and Stewart, 2018). The development of effective disease control measures will require an understanding of the relative importance of these reservoirs in disease initiation.

In Ethiopia, MLN is first reported in 2014 cropping season and caused various levels of damage ranging from low infection rate to complete crop failure (Mahuku *et al*., 2015a) depending on the area and varieties used, and continues to constrain maize production in the country (Regassa *et al*.,

2020). Due to its devastating nature and lack of resistant maize genotypes and appropriate management options, the disease is considered as a serious threat to maize production and menace to national food security. Since its occurrence, MLN is distributed in major maize production areas of Ethiopia especially in central, western, southern and southwestern parts (Fentahun *et al*., 2017; Guadie *et al*., 2018; Regassa *et al*., 2020). Knowledge of the ways in which a virus maintains itself and spreads in the field is essential for the development of effective management measures. The transmission of viruses from plant to plant by vectors provide the main method of spread in the field for many viruses that cause severe economic loss (Hull, 2014).

In Ethiopia, the rapid spread of MLN across different regions of Ethiopia suggested that the vectors might be arthropods. Reports are available in other countries like Hawaii, in USA that MCMV is transmitted by adult maize thrips, *Frankliniella williamsi* (Jiang et al., 1992; Cabanas et al., 2013) and flower thrips, *Frankliniella occidentalis* (Zhao *et al*., 2014). Thrips, beetles and aphids were observed during the field assessment in MLN infected maize fields and were thought to be the vectors of MLN causing viruses in Ethiopia (Bekele *et al*., 2017; Demisse *et al*., 2017; Guadie *et al*., 2018; Terefe and Gudero, 2019; Regassa *et al*., 2020). A study conducted in Ethiopia revealed that the spread of MLN causing viruses are linked to free movement of insect vector and continuous availability of the host plants (Regassa *et al*., 2020). Regardless of this report, insects observed during the field assessment, such as beetles, thrips and aphids were neither identified to species level nor experimentally tested for their ability to efficiently transmit MLN causing viruses (Guide *et al*., 2018; Terefe and Gudero, 2019; Regassa *et al*., 2020). This study was conducted to identify insect vectors of MLN causing viruses and their ability to efficiently transmit the viruses in Ethiopia.

2. Materials and Methods

2.1. Field assessment, insect specimen and plant sample collection

Field assessments and collection of suspected insect vectors of MLN causing viruses were carried out in two regions of southern Ethiopia, Oromia (Jimma, East Shewa and Arsi zones) and South Nation, Nationality and Peoples (SNNP) (Sidama, Wolayita and Hadiya zones) regional states (Fig 1) during the main and off-seasons of maize production in 2017 and2018. The altitudes of surveyed maize fields ranged from 958 to 1995 m.a.s.l. and the crop was at vegetative growth stage. Oromia

and SNNPR were selected for the field assessment based on high prevalence and incidence of MLN infection (Regassa *et al*., 2020).

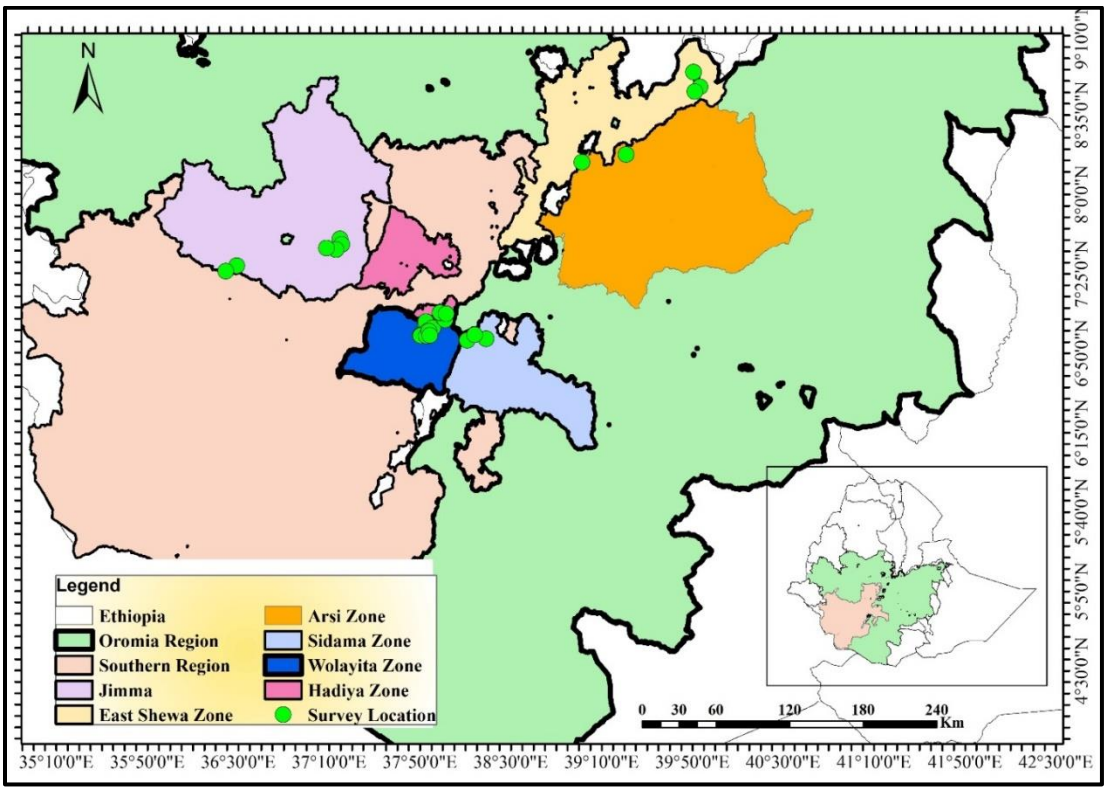

Fig 3. Map showing study site in SNNP (Sidama, Wolayita and Hadiya zones) and Oromia (Jimma, East Shewa and Arsi zones) regions of Ethiopia.

Based on MLN incidence and severity three zones from each region and 1-3 districts were purposively selected from each zone. In each of the selected areas, randomly selected maize fields were assessed at 5 to 8 km intervals along the main and accessible rural roads. A total of 86 fields were assessed, in each field, maize plants were inspected visually for the presence of insects and typical MLN-like symptoms in a diagonally manner (in an 'X' way) by counting 25 randomly selected plants at equal distance (10 m) in each transect. Within selected plants different types of suspected insect vectors including aphids, thrips and beetles which are presumed to be vectors of MLN causing viruses (MCMV or SCMV) were assessed and collected. Count of insect was done from 25 randomly selected plants and recorded as the total number of insect species present in selected plants per field as low (≤30), medium (31 to 60) and high (>60).

Insects were either aspirated directly from plants or swept from plants with a net and then aspirated. For sedentary insect vectors, plant parts containing the insects were collected and transferred by thoroughly sweeping using camel brush. Each insect type was collected in sufficient numbers live in small cage or jar to be used in transmission studies for further rearing. Part of the insect samples was preserved in 70% alcohol for identification purpose.

In addition to insect assessment and collection, maize leaf samples were also collected from each field to determine whether the collected insects were from MLN infected maize field or not. The leaf samples were selected on the basis of apparent symptoms of MLN infection. MLN disease intensity (incidence and severity) of each field was determined. Disease incidence was calculated by using maize plants with MLN-like symptoms expressed as a percentage of the total number of plants assessed in the field. Disease severity was scored on a rating scale of 1 to 5 (Regassa *et al*., 2020). The severity scales obtained were converted to percent severity index (Osunga *et al*., 2017). Collected maize leaf samples were labeled, put in plastic bags, taken to the laboratory and tested immediately or kept at 4-6 °C in the refrigerator until processed for virus detection by DAS-ELISA.

2.2. Virus detection and vector species identification

2.2.1. Virus detection in plant samples by ELISA and PCR

ELISA

The presence of MLN causing viruses were detected from the collected leaf samples and insects by DAS-ELISA. As MLN is caused by co-infection of MCMV and SCMV in Ethiopia, the same samples that were tested for MCMV were also tested for SCMV. The detection of antibodies for SCMV (AS-0166), MCMV (AS-1087) and their respective positive controls were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) Plant Virus Collection, Germany. DAS-ELISA test was done following the procedures of standard protocols at the DSMZ-Plant Virus Collection as described by Clark and Adams (1977). Since MCMV is the main cause of MLN and new to Ethiopia, further confirmation of ELISA positive samples by PCR followed by sequencing was done with selected samples from different locations (Sidam, Hadiya and Jima zones of Ethiopia) to evaluate the diversity in the coat protein genes of MCMV.

PCR

Out of 83 ELISA positive samples, six were randomly selected and tested by PCR using designed specific primers amplifying full-length coat protein genes. Total RNA was extracted from maize leaves using the BIO BASIC EZ-10 Spin Column Plant RNA Mini-Preps Kit (Cat. No.: BS82314) following the manufacturer's instructions. One step reverse transcription PCR (RT-PCR) process used with the primers for MCMV, forward (MCMV- Forb; 5′-ATGAGAGCAGTTGGGGAATGCG-3′) a reverse primer (MCMV- Revb; 5′- CGA ATC TAC ACA CAC ACA CTC CAGC-3′) which amplified 550 bp (Wangai *et al*., 2012).

RT-PCR parameters for the primers used in this study were as follows: initial cDNA synthesis of 42 °C for 50 min, followed by 30 cycles of denaturation at 94 °C for 1 min; annealing at 55 °C for 1 min and extension at 72 °C for 1 min. Final extension was done at 72°C for 10 min after the 30 cycles. The amplified DNA fragments were resolved on a 1.0% (w/v) agarose gel (1× Tris Acetate-EDTA buffer), stained with gel red and visualized with a UV light.

Purification of PCR products and sequencing

PCR amplicons of three virus (MCMV) isolates from Sidama, Hadiya and Jimma zones were randomly selected and purified using PCR purification kit (Bio Basic, Canada) following the manufacturer protocol. Ten microliters of the purified product were mixed with 5 µl of 10 µM forward or reverse primers in a separate tube and submitted to Macrogen Inc. for Sanger sequencing (Seoul, South Korea). Corresponding sequences of MCMV available in the NCBI GenBank database (www.ncbi.nlm.nih.gov) were included for comparative analysis. Sequences included are those reported from Africa.

2.2.2. Morphological and PCR-based vector identification

Insect rearing

Insects of three taxa, Homoptera aphids, Thysanoptera thrips and Coleoptera beetles collected from different location were sorted and reared on health maize seedlings for further identification and determination of their transmission of MLN causing viruses (MCMV and SCMV). The highly susceptible maize variety (Limu) to MLN (Regassa *et al*., 2021b) obtained from Pioneer Hi-Bred Seeds-Ethiopia and used for insect rearing and transmission assays were grown in 25 cm diameter plastic pots filled with a sterilized soil mixture (soil, sand and yard manure in the ratio of 2:1:1,

respectively). The seedlings were grown until 3-4 leaf stage to be used and placed inside insect-proof cages with a photoperiod of 12 h and a temperature range of 25-30°C. Both potted maize seedlings used for rearing and transmission were kept in a separated section of the cage to avoid uncontrolled insect infestation or MCMV and SCMV contamination.

Colonies of each insect species collected were individually reared on healthy maize seedlings grown inside pot and placed in insect proof cages and insect were transferred to potted maize seedlings inside cages. Leaf samples from the maize seedlings used for insect rearing were periodically tested by DAS-ELISA to confirm that the insects are free from MCMV or SCMV.

Morphological identification

The insects confirmed as vectors of MLN causing viruses (MCMV and SCMV) were identified to genus/species level. Morphological identification of the insects to species level was done by features for aphids using the key by Blackman and Eastop (2000) and Thrips Lucid Key Server by Moritz *et al*. (2017). The morphologically based on taxonomy were further supplemented by PCR and DNA sequencing.

PCR-based vector identification

DNA Extraction

Genomic DNA was extracted using Dellaporta method Dellaporta et al. (1983) from ethanol-preserved specimens. A single specimen of cereal leaf beetle and 10-15 corn leaf aphids were ground separately with a micro pestle in a sterile 1.5ml microcentrifuge tubes by adding liquid nitrogen and mixed with 500 µl of extraction buffer containing 100mM Tri-HCL pH 8.50, 10 Mm EDTA, 500MmNaCl, 1% 2-mercapthoethanol and 500µl homogenate were transferred into a microcentrifuge tube. After adding 33 µl of 20% SDS (sodium dodecyl sulphate) the tube was incubated at 65°C for 10 min and 500 µl of 5 M potassium acetate was added to it immediately and then the mixture was mixed thoroughly by vortex. The tube was kept in ice for 10 min and then centrifuged at 13,000 rpm for 10 min. Four hundred fifty micro liter of the supernatant was collected in a new microfuge and mixed with 225 µl (half the volume of the supernatant) of cold isopropanol. The tube was further incubated at -20°C for 1 hr. After centrifugation at 13,000 rpm for 15 min, the supernatant was discarded and the pellet was washed by centrifugation at 13,000 for 4 min with 500µl of 70% ethanol. The pellet was air-dried and resuspended in 100 µl of

nuclease free water. The quality of extracted DNA samples was examined by gel electrophoresis in 1% agarose gel (prepared in 1X TAE buffer, with gel red) visualized on UV-light.

PCR

DNA amplification was carried out by using primers which amplified 658 bp of COX 1 gene, forward primer: LCO 2518087 5′-GGTCAACAAATCATAAAGATATTG G-3′ and reverse primer: HCO 2518088 5′-TAAACTTCAGGGTGACCAAAAAATCA-3′ (Jalali *et al*., 2015).

PCR was carried out with Universal Cytochrome Oxidase primers with a total reaction volume of 20 µL using a prime thermal cycler (Prime, Fuses 6.3A HBC T 250 (2), UK). One microliters of DNA (20 ng/µL) were used with 19 µL of PCR mix containing 2µL of 10X PCR buffer, 1 µL of dNTP's (10 mM), 1 µL of Taq polymerase and 1 µL of magnesium chloride 25 mM, 1µl forward primer, 1µl reverse primer and 12µl sterile water were added to make the final volume to 20 µL. The thermocycler program used for the specific primers was 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, extension at 72 °C for 1 min. Final extension was done at 72°C for 10 min after the 30 cycles.

Purification of PCR products and sequencing

PCR amplicons of insect vectors were purified using PCR purification kit (Bio Basic, Canada) following the manufacturer protocol. Ten microliters of the purified product were mixed with 5 µl of 10 µM forward or reverse primers in a separate tube and submitted to Macrogen Inc. for Sanger sequencing company (Seoul, South Korea). Each sequence of forward and reverse primers was checked by Bioedit 7.0.2 software. DNA sequence analysis was carried out using BEAST software including sequences from genebank.

2.3. Virus maintenance and vector transmission test

2.3.1. Virus (MCMV and SCMV) maintenance

Leaves from maize plants showing typical symptoms of chlorosis, mottling and mosaic symptoms were collected from MLN disease infected maize fields during the assessment of insect vectors. For the confirmation of the target viruses, the collected samples were assayed for both viruses (MCMV and SCMV) by DAS-ELISA as described above. After the assay each virus was

transferred separately onto 3-4 leaves growth stage of maize seedlings by mechanical inoculation. For the availability of virus isolates, each virus was maintained separately by periodic mechanical inoculation to healthy maize in the insect proof greenhouse.

2.3.2. Vector transmission test

Transmission test experiment was conducted in greenhouse in 2018-2019. Healthy seedlings of maize were grown in 25 cm diameter plastic pot filled with sterilized mixtures of soil, sand and yard manure in the ratio of 2:1:1. Seven seeds were sown in each pot and later thinned to five plants. At three weeks after emergence (3-4 leaf stage), the maize seedlings were placed in insect proof cages covered with clear polyester clothing and were used for inoculation experiments.

Adult insects were used for the transmission study. Each insect species was put in a separate Petri-dish containing dry filter paper using camel hair brush and starved for two to three hours. The starved insects were transferred to Petri dishes containing leaves harvested from MLN infected maize plants. The acquisition and inoculation access periods were adjusted as previously adopted to examine the transmission of MLN causing viruses by the chrysomelid beetles, thrips and aphids (Nault *et al*., 1978; Jensen, 1985; Cabanas *et al*., 2013). The aphids were allowed acquisition access period of 20-35 minutes on the infected maize leaves while thrips and beetles were allowed a period of 2 days. After the acquisition feeding period, varying numbers of insects depending on their abundance (Aphids: 50; thrips: 40; beetles: 25 per pot) were transferred to health maize seedlings in cages using a camel hair brush and allowed an inoculation access period of 1 hour for aphids, 2 days for beetles and thrips. Maize plants mechanically inoculated with MCMV and SCMV were used as positive controls, whereas healthy maize (not infested by insect and infected by virus) as negative control. A total of 20 plants (5 plants per replication and repeated four times) were tested for each insect species. After the inoculation access period, the maize plants were sprayed with lamdex® 5% EC (Lambda cyhalothrin 50g/l) to eliminate the insects and were transferred to a greenhouse for observing possible symptom development.

Additionally, the insects were collected from MLN-infected maize in the field and transferred to pots of healthy maize without an acquisition feeding period, to determine the ability of aphids, thrips and beetles to transmit MLN when collected directly from infected plants in the field. Plants were inspected for visual symptoms (presence or absence within pot) of virus infection seven days

after initiation of transmission tests. DAS- ELISA tests were performed to confirm for both viruses (MCMV and SCMV) transmission to maize seedlings.

3. Results

3.1.MLN disease intensity, insect vector population and distribution

The intensity (incidence and severity) of MLN and insect abundance during main and off-season is presented in Tables 1 and 2. Low to high level of MLN intensity was noted, which varied from location to location and field to field. During the main cropping season (July to August), the maximum mean MLN incidence of 43% (n= 4) and severity of 54.7% (n= 4) were recorded in Arsi zone of Oromia regional state whereas the least mean incidence of 20.18% (n= 11) and severity of 33.09% (n= 11) were recorded in Sidama zone of SNNP regional state (Table 1). During the off-season (March to April), a higher mean MLN incidence (51.25%) (n= 4) and severity (66.25%) (n= 4) were recorded in Arsi zone, Jeju district (Table 2).

Maize thrips and beetles were moderately abundant (medium population) and widely distributed in Arsi and West Shewa zones of Oromia region in both main and off-seasons. In SNNP region, beetles were moderately abundant and distributed in all assessed areas within the region in both seasons, while thrips were not observed in Sidama and Hadiya zones during main season. Beetles (cereal beetles and ladybug beetles) were moderately abundant and widely distributed in all assessed maize fields during the main cropping season, whereas aphids were abundant in all assessed zones and considered to be the primary vector of SCMV (Table 1).

Table 1. Maize lethal necrosis (MLN) intensity, insect abundance and distribution during main cropping season (July to August) of 2017 in Ethiopia.

Region [a]	**Zone**	**District**	**MLN intensity (%)**						**Insect vectors and their abundance** [b]			
			Incidence			**Severity**			**Thrips**	**Aphids**	**Cereal leaf beetles**	Ladybug beetles
			Min	max	mean	Min	Max	mean				
Oromia	Arsi	Jeju	12	70	43	24	70	54.7	L-M	M	L	M-H
	East Shewa	Fantale	40	75	52.3	35	85	57	M	L-M	L	M
		Lume	26	70	51.5	35	45	40	M	L	L	M
		Adama Zuria	25	100	60	20	80	48.3	M	M	L-M	M
	Jima	Omo nada	17.7	66.9	32.4	15	65	32.8	L	M	M	M
		Seka Chekorsa	25	50	38.1	25	50	35	L	M	M	M

SNNP	Sidama	Hawassa Zuria	10	40	20	25	50	35	L	M	M	M
		Boricha	10	75	24	20	75	34.9	NA	L-M	M	M
	Walayita	Damot Gale	5	75	36.2	10	75	36.2	NA	M	M	M
		Damot Pulasa	15	65	37.1	24	50	34.9	NA	L-M	M	M
	Hadiya	Misirak Badewacho	15	75	32	15	60	36.8	NA	L-M	M	M

[a] SNNP = South Nation, Nationality and People.

[b]total number of insect species presented in selected plants per field with L = low (≤30), M = medium (31 to 60) and H = high (>60), NA= not available.

Table 2. Maize lethal necrosis (MLN) intensity, insect abundance and distribution during the off-season (March to April) of 2018 in Ethiopia.

Zone	**District**	**MLN intensity (%)**						**Insect and their abundance**			
		Incidence			**Severity**			**Thrips**	**Aphids**	**Cereal leaf beetles**	Ladybug beetles
		Min	**max**	**mean**	**min**	**Max**	**Mean**				
Arsi	Jeju	25	85	51.25	45	80	66.25	M-H	M	L	L
Eest Shewa	Fantale	20	75	37.5	35	75	52.50	M-H	M	M-H	M
Hadiya	MisirakBadewacho	20	85	41.50	25	80	56.00	M	M	M	M

Total number of insect species presented in selected plants per field with L= low (≤30), M= medium (31 to 60) and H= high (>60).

3.2. Virus detection and vector species identification

3.2.1. Virus (MCMV) detection in plant samples by PCR

The result of molecular test confirmed that the three samples sequenced from SNNP (Sidama and Hadiya zones), and Oromia (Jimma Zone) of Ethiopia confirm the samples are MCMV. Figure 2 shows the electrophoretic analysis of RT-PCR amplified products using the recommended detection parameters. The sequences used in the gene bank are given in table 3.

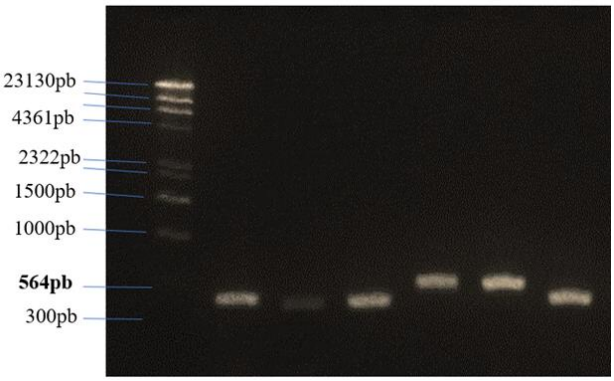

Fig. 2. Electrophoretic detection of one step RT-PCR results of MCMV infected samples

Table 3. MCMV sequence analysis and genetic variation with other East African countries isolate

No	Haplotype	Accession code	Country	No	Haplotype	Accession code	Country
1	Hap_1:	MN756483	Tanzania	53	Hap_2	MN706223	Tanzania
2	Hap_2	MN718732	Tanzania	54	Hap_2	MN706225	Tanzania
3	Hap_2	JX286709	Kenya	55	Hap_2	MN706228	Tanzania
4	Hap_2	KF744393	Rwanda	56	Hap_2	MN706229	Tanzania
5	Hap_2	KF744394	Rwanda	57	Hap_2	MN706231	Tanzania
6	Hap_2	KF744395	Rwanda	58	Hap_2	MN706232	Tanzania
7	Hap_2	KF744396	Rwanda	59	Hap_2	MN706233	Tanzania
8	Hap_2	KM926617	Kenya	60	Hap_2	MN706234	Tanzania
9	Hap_2	KP772217	Ethiopia	61	Hap_2	MN706235	Tanzania
10	Hap_2	KP798452	Ethiopia	62	Hap_2	MN706236	Tanzania
11	Hap_2	KP798453	Ethiopia	63	Hap_2	MN706237	Tanzania
12	Hap_2	KP798454	Ethiopia	64	Hap_2	MN706238	Tanzania
13	Hap_2	KP798455	Ethiopia	65	Hap_2	MN706239	Tanzania
14	Hap_2	KP851970	Rwanda	66	Hap_2	MN706242	Tanzania
15	Hap_2	MF467377	Tanzania	67	Hap_3	MN706240	Tanzania
16	Hap_2	MF467384	Tanzania	68	Hap_3	MN706226	Tanzania
17	Hap_2	MF467386	Tanzania	69	Hap_4	MK491605	Kenya
18	Hap_2	MF467387	Tanzania	70	Hap_4	MN706224	Tanzania
19	Hap_2	MF467389	Tanzania	71	Hap_5	MH238455	Kenya
20	Hap_2	MF467391	Tanzania	72	Hap_5	MN706227	Tanzania
21	Hap_2	MF510223	Kenya	73	Hap_6: 1	MF467390	Tanzania
22	Hap_2	MF510224	Kenya	74	Hap_7	MF467380	Tanzania
23	Hap_2	MF510225	Kenya	75	Hap_8	MF467378	Tanzania
24	Hap_2	MF510226	Kenya	76	Hap_9	MF467376	Tanzania
25	Hap_2	MF510227	Kenya	77	Hap_10	MF467375	Tanzania
26	Hap_2	MF510228	Kenya	78	Hap_11	MF467374	Tanzania
27	Hap_2	MF510230	Kenya	79	Hap_12	MF510238	Kenya
28	Hap_2	MF510231	Kenya	80	Hap_13	MN706218	Tanzania
29	Hap_2	MF510233	Kenya	81	Hap_13	MN706241	Tanzania
30	Hap_2	MF510236	Kenya	82	Hap_13	MN706216	Tanzania
31	Hap_2	MF510239	Kenya	83	Hap_14	MN706230	Tanzania
32	Hap_2	MF510240	Kenya	84	Hap_15	MN706220	Tanzania
33	Hap_2	MF510241	Kenya	85	Hap_16	MN706215	Tanzania
34	Hap_2	MF510242	Rwanda	86	Hap_17	KJ699379	Democratic Republic of Congo
35	Hap_2	MF510243	Ethiopia	87	Hap_18	MH238452	Kenya
36	Hap_2	MF510244	Kenya	88	Hap_19	MH238450	Kenya
37	Hap_2	MF510245	Kenya	89	Hap_19	MH238449	Kenya

No	Haplotype	Accession code	Country	No	Haplotype	Accession code	Country
38	Hap_2	MF510246	Kenya	90	Hap_20	MF467388	Tanzania
39	Hap_2	MF510247	Kenya	91	Hap_21	MF467383	Tanzania
40	Hap_2	MF510248	Rwanda	92	Hap_22	MF467382	Tanzania
41	Hap_2	MF510249	Rwanda	93	Hap_23	MF467379	Tanzania
42	Hap_2	MF510250	Kenya	94	Hap_24	MF510237	Kenya
43	Hap_2	MF510251	Rwanda	95	Hap_25	MF510235	Kenya
44	Hap_2	MH205605	Kenya	96	Hap_26	MF510234	Kenya
45	Hap_2	MH238453	Kenya	97	Hap_27	MF510232	Kenya
46	Hap_2	MH238454	Kenya	98	Hap_28	MN706221	Tanzania
47	Hap_2	MK491604	Kenya	99	Hap_29	MN706213	Tanzania
48	Hap_2	MK491606	Kenya	100	Hap_30	MH238451	Kenya
49	Hap_2	MN706214	Tanzania	101	Hap_31	D4DZAA099 1	Sidama, Ethiopia
50	Hap_2	MN706217	Tanzania	102	Hap_31	D4DZAA098 1	Hadiya, Ethiopia
51	Hap_2	MN706219	Tanzania	103	Hap_31	D4DZAA096	Jimma, Ethiopia
52	Hap_2	MN706222	Tanzania				

3.2.2. Morphological and PCR-based vector identification

Morphological identification of insects collected suggested that the most predominant species on maize are corn leaf aphid (*Rhaphalosiphum maydis)*, beetles (cereal leaf beetle and ladybug beetles), and thrips (*Franklinella)* were encountered with MLN infected plants.

Samples identified as corn leaf aphid morphologically aligned to the *Rhopalosiphum Maddis* which were previously reported from Pakistan and Bangladesh (Fig 3). However, cereal leaf beetles (*Oulema* sp.) did not show any resemblance with available sequences in the Genbank (Fig 4.) revealed that this was new records to the Genbank.

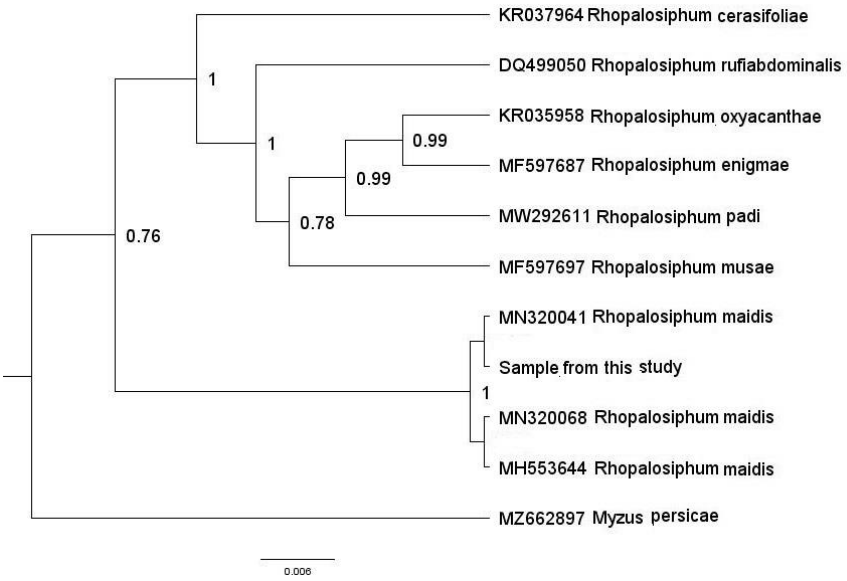

Fig 3. Phylogenetic tree of *Rhopalosiphum maidis* based on COX gene sequences.

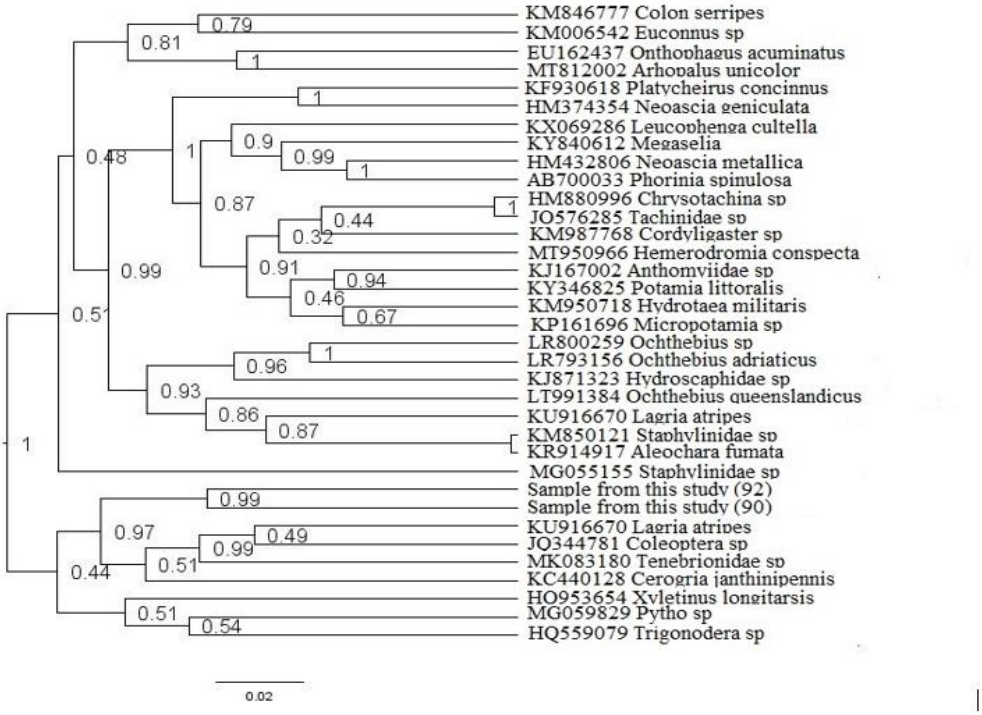

Fig 4. Morphologically *Oulema* sp but based on COX gene sequences no any resemblance with insects sequence in the gene bank.

3.3.Vector transmission test

The first evaluation was done on the basis of presence or absence of symptoms of virus infection within the pot. Among insects collected from MLN-infected maize field and directly caged on plants without an acquisition feeding period, *Oulema* sp. and *Franklinella* sp. transmitted MCMV to healthy plants (Table 4).

Healthy maize plants developed symptoms of MCMV infection were ELISA positive after being caged with thrips (*Franklinella* sp.*)* and cereal leaf beetles (*Oulema* sp.) that had previously fed on MCMV infected maize (Table 4). True bugs (*Stink bugs* sp.), ladybug beetles (*Hippodamia quindecimmaculata, Hyperaspis bigeminata* and *Paranaemia vittigera)* and *Graptostethus servus*) and *Graptostethus* spp. after feeding on MCMV and SCMV-infected plants, did not transmit the virus to healthy maize seedlings, while *Rhapolosiphum maidis* transmit SCMV to healthy maize seedlings (Table 5). Plants inoculated with MCMV and SCMV homogenates developed similar symptoms and tested positive by DAS-ELISA. Plant sap extracted from uninfected (healthy maize) did not produce symptoms and also tested negative for both viruses by DAS-ELISA.

Table 4. *Maize chlorotic mottle virus* (MCMV) and *Sugarcane mosaic virus* (SCMV) symptoms and ELISA readings results for insects collected from MLN-infected maize in the field and transferred to pots of healthy maize without an acquisition feeding period**.**

Insect collection and acquisition condition	**Common name**	**Species**	**Plants with symptoms/total plants**	**DAS-ELISA result***	
				MCMV	**SCMV**
Field-collected without acquisition feeding	Cereal leaf beetles	*Oulema sp.*	8/20	+	-
	True Bugs	*Stink bugs sp.*	0/20	-	-
	Ladybug beetles	*Hippodamia quindecimmaculata*	0/20	-	-
		Hyperaspis bigeminata	0/20	-	-
		Paranaemia vittigera	0/20	-	-
	Thirps	*Franklinella sp.*	8/20	+	-
	Aphids	*Rhapolosiphum maidis*	0/20	-	-
Negative control		Uninfected plants	0/20	-	-
Positive control		MCMV	20/20	+	-
		SCMV	20/20	-	+

*- = negative (MCMV/SCMV was not detected by DAS-ELISA

Table 5. Insect species tested as vectors for maize lethal necrosis (MLN) causing viruses (MCMV and SCMV) and their transmission rate

Species	Number of insects per plant	Plants infected/plants tested (% infected)	ELISA reading result*		Transmission rate
			MCMV	SCMV	
Thysanoptera					
Franklinella sp.	8	13/20 (65%)	+	-	0.65
Homoptera					
Rhapolosiphum maidis	10	10/20 (50%)	-	+	0.50
Beetles (Coleoptera)					
Oulema sp.	5	11/20 (55%)	+	-	0.55
Hippodamia quindecimmaculata	5	0/20 (0%)	-	-	0
Hyperaspis bigeminata	5	0/20 (0%)	-	-	0
Paranaemia vittigera	5	0/20 (0%)	-	-	0
Hemiptera					
Stink bugs sp.	10	0/20 (0%)	-	-	0
Graptostethus spp	5	0/20	-	-	0

*- = negative (MCMV/SCMV was not detected by DAS-ELISA

Ten out of twenty (50%) plants inoculated mechanically from the homogenized of field-collected *Oulema* sp. and 11 out of 20 (55%) of *Franklinella* sp., produced typical symptoms of MCMV and positive DAS-ELISA results (Table 6). MCMV was recovered both by transmission feeding and by mechanical inoculation from *Oulema* sp.and *Franklinella* sp. Plants inoculated with MCMV homogenate developed similar symptoms and tested positive for MCMV by DAS-ELISA. Plant sap extracted from healthy maize did not produce symptoms of MCMV infection in healthy maize seedlings when mechanically inoculated into the seedlings and these seedlings also tested negative for MCMV by DAS-ELISA.

Table 6. Symptoms and ELISA results after mechanical inoculations of insect homogenate from samples tested positive by ELISA for *Maize chlorotic mottle virus* (MCMV).

Treatment	Inoculation	Presence of symptoms /total plants	DAS-ELISA result*
Inoculated with insect homogenate	*Oulema* sp.	10/20	+
	Franklinella sp.	11/20	+
Control inoculations	Sap extract from healthy maize	0/20	-
	MCMV	20/20	+

*- = negative (MCMV/SCMV was not detected by DAS-ELISA

4. Discussion

MLN disease in Eastern Africa including Ethiopia was caused by double infection of MCMV, and SCMV (Wangai *et al*., 2012; Adams *et al*., 2014; Mahuku *et al.*, 2015a, b). In Ethiopia, MCMV is a newly introduced virus that is the most important component of MLN while SCMV is known to commonly occur on maize and other related crops in the country for long period of time (Lencho *et al*., 1997). This study confirmed that the samples sequenced and analyzed from Sidama, Hadiya and Jima zones confirmed the samples are MCMV. As also reported by Braidwood *et al*. (2017) there is very little variation between the geographic areas both in African countries and worldwide.

The spread of MLN causing viruses is linked to the movement of insect vectors and continuous availability of the host plants in the field (Regassa *et al*., 2020). Continuous mono-cropping of maize season after season or year after years promotes carryover of vector population from one cropping season to another. This situation favors buildup of insect-vectors and increased infestation that results in severe crop damage.

The MLN intensity and thrips population were higher in Arsi zone (Jeju), East Shewa (Fantale) of Oromia and Hadiya zone of SNNP regional states than in the other zones during both main and off-seasons (Tables 1 and 2). Most maize growers in these areas experienced continuous maize production throughout the year due to availability of residual moisture and water for irrigation (Regassa *et al*., 2020). This cropping practices and presence of maize plant in the field from season to season provides conducive environment for the presence, reproduction and spread of the insect vector population and crop infection by MLN causing viruses by serving as a bridge between cropping seasons. Although, Arsi (Jeju) and East Shewa zones of Oromia region had higher MLN disease intensity than the other surveyed zones, the areas were characterized by moderate rainfall amounts and higher temperatures (Abate *et al*., 2015). Such environments are characterized by warm and semi-humid weather conditions, which could be advantageous for insect vectors reproduction, development and spread that in turn resulted in increased incidence and severity of MLN. Vector populations of most plant viruses were built up faster in parts with high temperature and high relative humidity, and decline at low temperature and high rainfalls (Islam *et al*., 2017).

The study identified *Rhopalosiphum maidis* as the vector of SCMV, while maize thrips (*Franklinella* sp.) and cereal leaf beetle (*Oulema* sp.) were identified as vector of MCMV. Thrips (*Franklinella* sp.) was the most important and widely distributed insect vector in most of the

surveyed areas, especially in Arsi and West Shewa zones of Oromiya region (Tables 1 and 2) where MLN was first identified in 2014 (Mahuku *et al*., 2015). Aphids (*R. maidis*) were abundant in all assessed zones in the two regions indicating the importance of these insects as vectors of SCMV in Ethiopian maize growing areas (Table 1).

The finding of these study was in agreement with reports of Sahi *et al*. (2003) who reported that *R. maidis* were the potential vectors of SCMV in maize. The results are also comparable with that of Wakman *et al*. (2001) who found that *R. maidis* as a very effective vector of SCMV from mature maize plants to maize seedlings. In addition to by *R. maids*, transmission of SCMV from maize to maize by other several aphid species including *Myzus persicae, Schizaphis graminum, Aphis gossypii,* and *R. padi* (Sahi *et al*., 2003). The aphid *(R. maidis*) was also known to be the most capable vector of SCMV in other crops like sugarcane and sorghum (Singh *et al*., 2005; Perera *et al*., 2012; Klein and Smith, 2020). It has been also reported that MCMV is transmitted by the vector activity of adult maize thrips, *Frankliniella williamsi* (Jiang *et al*., 1992; Cabanas *et al*., 2013). Studies conducted by Zhao *et al*. (2014) also showed that flower thrips, *Frankliniella occidentalis* can transmit MCMV.

SCMV is vectored with a non-persistent transmission mode. In non-persistent mode of transmission of plant viruses, the vector acquires the virus from infected host within seconds, retains it and inoculates another host within a few minutes (Ng and Perry, 2004; Gandhi and Murali, 2017). Similar to the current finding, the maize leaf aphid (*R. maidis)* transmits SCMV to maize in a non-persistent manner where acquisition and inoculation requires only very brief stylet penetration of less than one minute (Ng and Falk 2006; Adams *et al*., 2014). The maize leaf aphid is regarded as a specific insect pest for *Poaceae* family (Kuo *et al*., 2006; Razmjou and Golizadelo, 2010) and mainly distributed in areas where sorghum and maize are cultivated. The thrips semi persistently transmits MCMV by acquiring it from infected plants for a maximum of three hours without the latent period (inability to inoculate immediately following acquisition). There after the infected maize thrips inoculates the virus to healthy plants (Cabanas *et al*., 2013). Thrips have piercing-sucking mouth parts and use stylets to pierce the epidermis (Nault, 1997; Ullman *et al*., 1992; Cabanas *et al*., 2013). MCMV is also transmitted in a semi persistent manner by six different species of chrysomelid beetles including cereal leaf beetle (*Oulema melanopa*), maize flea beetle (*Chaetocnema pulicaria*), flea beetle (*Systena frontalis*), southern maize rootworm beetle

(*Diabrotica undecimpunctata*), Northern maize rootworm (*D. longicornis*) and western maize rootworm (*D. virgifera*) (Nault *et al*., 1978; Jensen, 1985). Beetle transmitted viruses enter plant tissues through the wound created by beetle chewing and rapidly translocate far from the wounded sites through the plant xylem (Cabanas *et al*., 2013).

In conclusion, the current investigation provided evidence that SCMV is transmitted by maize leaf aphid (*P. maids*) and MCMV by thrips (*Franklinella* sp.) and cereal leaf beetle (*Oulema* sp.) in Ethiopia. The presence of these insects as vectors of MLN causing viruses on maize plants is believed to contribute to widespread of MLN viruses from plant to plant, field to field and to new geographical areas. Further investigation is required to answer questions like which insect life stages are more capable of acquiring and transmitting the viruses and how long the viruses persist in the insect after acquisition. Virus transmitting vectors are likely to survive on weeds and use them as reservoirs for the viruses that they vector. Future research should also include other possible vectors and their importance in transmission of MLN causing viruses. Since the best approach for the management of MLN and its vectors is to use integrated pest management practices including rouging of virus infected/symptomatic plants, good field sanitation such as weed control measures to eliminate alternate hosts for potential buildup of MLN causing viruses and vectors; seed dressing with systemic insecticide that would remain effective when the plants are at susceptible stages; and developing/use of maize varieties resistant to MLN. Additional information is also needed on the potential of alternate hosts and the seasonal population dynamics of the identified vectors.

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Paper V

Evaluation of Seed Dressing Insecticides for the Control of Maize Lethal Necrosis Vectors

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Abstract

Most of the overwhelming plant diseases caused by viruses including maize lethal necrosis (MLN) are attributed to viruses transmitted by vectors. The transmission of viruses from plant to plant by vectors provide the main means of spread in the field for many viruses that cause severe economic loss. Methods to control the vectors of plant virus diseases are intended at eliminating or altering one or more of the primary contributors (vector, virus, and host plant) in the transmission process or at preventing their coming together. The current experiments were conducted to evaluate seed-treatment insecticides for their efficacy on early season control of insect vectors of MLN causing viruses. The study on the effects of insecticide treatment on germination revealed that it does not significantly influence the germination of maize seed even up to six months storage before planting. Among the tested seed treatment insecticides, thiamethoxam 25% at 2.0g/kg seed and imidalm T 450 at rate 1.5 showed superior control efficacy against maize thrips (*Frankliniella* sp.) with 96.06% and 95% population reduction respectively, and maize leaf aphid (*Rhopalosiphum maidis*) with 97.37% and 96.67% reduction percentage respectively. Hence, dressing of maize seeds before planting with such insecticides can be used for early-stage protection against potential vectors of the MLN causing viruses.

Keywords: Aphids, Insecticides, Seed dressing, Thrips, Vector

1. Introduction

The emergence and rapid spread of plant virus disease can result in high epidemics and huge crop losses. Maize lethal necrosis (MLN) was emerged as a serious threat to maize production in eastern Africa in 2011 and since then the disease is causing from low to complete loss of maize production in the region (Wangai *et al*., 2012; Mahuku *et al*., 2015a). MLN is a viral disease caused by double infection of *Maize chlorotic mottle virus* (MCMV) with any one of potyviruses, namely *Maize dwarf mosaic virus* (MDMV), *Wheat streak mosaic virus* (WSMV), Sugarcane mosaic virus (SCMV), or the recently described *Johnsongrass mosaic virus* (JGMV) (Stewart *et al*., 2014, 2017).

The outbreak of MLN caused by co-infection of MCMV and SCMV in Ethiopia has been first observed and reported in the Upper Awash Valley of Oromia region in 2014 (Mahuku *et al*., 2015b). The disease has since spread to other major maize producing regions of the country including Oromia, South Nation, Nationality and Peoples (SNNP), Amhara, Benishangul-Gumuz and Tigray (Bekele *et al*., Fentahun *et al*., 2017; Guadie *et al*., 2019; Regassa *et al*., 2020).

MLN causing viruses have insect vectors that spread into, between and within crops and also transmitted thorough seed at low rate (Jensen *et al*., 1991, Regassa *et al*., 2021). MCMV is transmitted by many insects, by thrips being the major vector, and combines with the aphid-borne *Sugarcane mosaic virus* (SCMV) to cause MLN (Jiang *et al*., 1992; Cabanas *et al*., 2013; Regassa *et al*., unpublished data).

Various weed and cultivated plants identified as alternate hosts, insect vectors, the transmissibility from infected seed and infested soil to newly raised maize seedlings, and the persistence in soil and maize residue of MLN causing viruses (MCMV and SCMV), are epidemiologically important and maintain the virus inoculum in the absence of maize crop in the field, and support the survival of the virus for continuous infection (Regassa *et al*., 2021, 2022).

Most of the devastating virus caused plant diseases including MLN are attributed to viruses transmitted by vectors. In the absence of vectors, these diseases would be of little importance. Most methods to control the vectors of plant virus diseases are intended at eliminating or altering one or more of the primary contributors (vector, virus, and host plant) in the transmission process or at preventing their coming together. Pesticide seed treatments are insecticides or fungicides,

applied to seed, to control diseases of seeds and seedlings; insecticides are used to control insect pests. Plant viruses including MLN cannot be directly controlled by the use of pesticides, however, certain systemic insecticides can control the insect vectors that carry viruses from host to host. Several insecticides, formulated either as granules or spray applications can be used to manage vectors.

Seed dressing in agriculture involves the treatment of seeds with insecticides and/or fungicides in order to fight above-and belowground insects and soil-borne fungal diseases (Taylor *et al*., 2001). Before planting, application of seed treatment using systemic insecticides can provide early-stage protection against thrips, aphids and other potential vectors of the MLN including beetles (Alford, 2000). Thiamethoxam and imidacloprid singly or with mixed other pesticides are commonly used as a systemic seed treatment to protect seeds and seedlings against injury by early season insects (Wilde 1997; Tharp *et al*., 2000; Wilde *et al*., 2001). Both imidacloprid and thiamethoxam have the potential to provide long-term residual control of a broad spectrum of insect pests (Maienfisch *et al*., 2001; Wilde *et al*., 2001). However, available and registered seed dressing insecticides were not evaluated against insect vectors of MLN causing viruses in Ethiopia. Therefore, the objective of this study was to evaluate seed-treatment insecticides for their efficacy on early season insect vectors of MLN causing viruses.

2. Materials and Methods

The experiment was conducted at the Ambo Agricultural Research Center under greenhouse condition at 25–30 °C Day temperature. Corn leaf aphid (*Rhopalosiphum maidis*) adults used in this study had been first identified as vector of SCMV, and maize thrips (*Frankliniella* sp.) identified as vector for MCMV. *Frankliniella* sp. and *R. maidis* were reared separately on healthy maize seedlings grown inside pot size soil condition) and placed in insect proof cages and insect were transferred to potted maize seedlings inside cages.

2.1. Plant materials and seed treatment

The treatments consisted of untreated controls and four systemic insecticides selected for their ability to taken up from the treated seed coat and translocate to all parts of the plant during germination, and untreated controls. Registered systemic seed dressing insecticides in Ethiopia either singly or with combination of fungicide were used. The systemic insecticides were Apron star 42 WS, Thiamethoxam 25% WG, Imidalm T 450 WS and Proseed Plus 63 WS. MLN

susceptible maize seeds (BH661) were obtained from Bako Agricultural research center. The seeds were dressed uniformly with three rates of application for each treatment (recommended, lower and above respective dosage of insecticides Table 1). The solution volume used (product + water) was 8 ml, 10 ml, 7 ml, and 10 ml per 1 kg of seeds for Apron star 42 WS, Thiamethoxam 25% WG, Imidalm T 450 WS and Imidacloprid, respectively. The product slurry was distributed over 1kg of seeds with respective dosage/application rate of insecticide in the bowls and stirred for 5-10 minutes to coat seed uniformly with the insecticide slurry. Then the treated seeds were allowed to air-dry in the laboratory. The dried seeds treated with respective dosage of insecticides were packed in separate polythene bags and kept under laboratory condition until sowing. After one- and six-month's storage its effect on seed germination and vector management were evaluated. Untreated seeds were used as a control.

Table 6. Descriptions of insecticides (treatment) used as seed treatment for control of maize lethal necrosis transmitting insect vectors with application rate.

Trade name	**Common name**	**Treatment with code**	**Application rate (g/kg seed)**
Apron Star 42 WS	thiamethoxam 20% + metalaxyl - 20% + difenoconazole 2%	T_1 = Apron star 42 WS	2.5
		T_2 = Apron star 42 WS	2.0
		T_3 = Apron star 42 WS	3.0
Imidalm T 450 WS	imidacloprid + thram	T_4 = Imidalm T 450 WS	1.0
		T_5 = Imidalm T 450 WS	0.5
		T_6 = Imidalm T 450 WS	1.5
Proseed Plus 63 WS	Imidacloprid + Thiram + Carboxin	T_7 = Imidacloprid + Thiram + Carboxin	3.0
		T_8 = Imidacloprid + Thiram + Carboxin	2.0
		T_9 = Imidacloprid + Thiram + Carboxin	1.0
Evident 25 % WG	Thiamethoxam 25% WG	T_{10} = Thiamethoxam 25% WG	2.0
		T_{11} = Thiamethoxam 25% WG	1.0
		T_{12} = Thiamethoxam 25% WG	3.0
		T_{13} = Untreated	-

2.2. Germination test of insecticide-treated seeds

The effect of insecticide seed treatment on the germination rate and storage time was evaluated two times after 1- and 6-month storage. Randomly selected 20 seeds from each treatment of the stored at one and six months were placed on water moist double layer of filter paper. Water was added into each plate as required to keep the filter paper moist. The experiment was conducted in CRD with three replications. The test was conducted in greenhouse at temperature of 25-30°C during day and 18°C at night. Following standard seed germination determination by Gorim and Asch (2012), a seed with visible radicle (longer than 2 mm) was considered as germination. Number of newly germinating seeds was recorded daily for 8 consecutive days, and the cumulative germination rate was calculated.

2.3. Exposing the insects to seedlings developed from treated seeds

The effect of different seed treatments on vectors of MCMV (*Frankliniella* sp.) and SCMV (*R. maidis*) were assessed in an insect proof cage at room temperature ranging from 25-35 $^{\circ}$C. Treated seeds were planted in sterilized soil mixture inside 25 cm diameter plastic pots (five seed per pot). After plants were germinated and reached two leaf stages, each colonies vectors from rearing cage were introduced/transferred (20 *Frankliniella* sp. and 30 *R. maidis* per plant) on to the maize seedlings developed from seeds treated with insecticides. Adult and immature thrips were exposed to seedlings developed from treated seeds treated. Three pots (replications) were established for each application rate of each insecticide. Treatments were evaluated by counting the number of live vectors staring from the 3rd day after insects transferred to maize seedlings at seven days intervals for two consecutive weeks (i.e., at 3, 10 and 17 days).

2.4. Data collection and analysis

Seed germination was recorded daily up to 8 consecutive days, after the start of the experiment. Germination percentage (GP) was calculated according to the International Seed Testing Association (ISTA) method

$$\mathrm{GP} = \frac{\text{Number of normally germinated seeds}}{\text{Total number of seeds sown}}$$

The percentage of the reduction (% R) of the insect population was calculated according to the following equation (El-Naggar and Zidan, 2013).

% R = [(NIC – NIT)/ NIC] × 100, where NIC = number of insects in the control and NIT = number of insects in the treatment.

Statistical analyses were carried out using the SAS procedure of GLM (SAS Institute, Cary, NC, USA). Least significant differences were calculated with an analysis of variance using the LS means statement in the general linear model procedure of SAS software (SAS Institute 9.4 Cary, NC, USA).

3. Results

3.1. Effect of insecticidal seed treatments on maize seed germination

The germination percentage of maize seed after being treated with four systemic insecticides at different rates is presented in Table 2, show that threre not significantly diffirences between the treatments. The insecticides did not influence the germination percentage even up to six months of storage after treatments. However, germination was higher (100%) for Apron star 42 WS @2g/kg, Imidalm T 450 WS @ 1g/kg, Imidacloprid + Thiram + carboxin @2g/kg, Apron star 42 WS @ 2.5g/kg treated seeds and untreated seeds, and lower for Thiamethoxam 25% WG (95-96.67%). Irrespective of insecticides used in seed treatment the germination percentage did not differ significantly over different periods of storage. It slightly decreased from 100 - 96.67% in treated seed stored for 1 month and 98.33 – 95% for treated seed stored for six months before planting (Table 2). These levels of reduced germination were comparatively small, never exceeding 4% of the controls in the final evaluation. Comparing all tests, the germination percentage ranged from 95 to 100%.

Table 7. Influence of seed treatment on the germination (%) of maize seeds stored for one and six months after treated by insecticides.

Treatment	Rate (product in g per 1kg seed)	Germination (%)	
		After 1-month storage	After 6-month storage
Apron star 42 WS	2.5	100.00	100.00
	2.0	100.00	98.33
	3.0	98.33	98.33
Imidalm T 450 WS	1.0	100.00	98.33
	0.5	98.33	98.33
	1.5	98.33	98.33
Thiamethoxam 25% WG	2.0	98.33	95.00

	1.0	96.67	96.67
	3.0	96.67	95.00
Imidacloprid + Thiram +	2.0	100.00	98.33
Carboxin	1.0	98.33	98.33
	3.0	98.33	98.33
Untreated seed	used as control	100.00	98.33

3.2. Effect of insecticidal seed treatments on control efficacy against thrips

The population of the thrips *(Frankliniella* sp.) were significantly lower on maize seedling developed from the treated seeds than those seedlings developed from the untreated control ($F_{12, 38.22} = 76.37$, $p < 0.0001$). All the insecticidal treatments reduced *Frankliniella* sp. population over untreated control and the reduction varied from 90.67 to 99.67% for treated seeds stored for one months before planting and 60.00 to 95.33% for six months stored.

In these seed-treatment tests against MCMV vector (*Frankliniella* sp.), all the tested dosages of thiamethoxam 25 WG and Imidacloprid + Thiram + Carboxin@1g/kg (at low rate) were found more effective in reducing *Frankliniella* sp. than the other treatments and untreated control. While Imidalm T 450 WS@1.5g/kg (at high dosage) showed lower *Frankliniella* sp. reduction percentage on both maize seedlings derived from treated seeds stored for one and six months before planting (Fig 1).

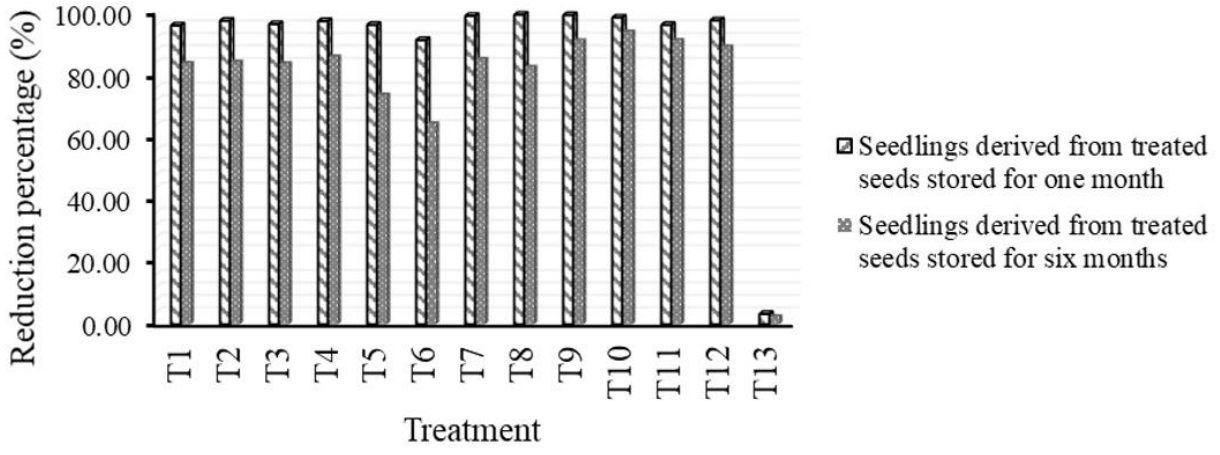

Fig. 1. Effect of insecticidal maize seed treatment on thrips *(Frankliniella* sp.) population reduction on maize seedlings developed from one-month and six-months storage after treatment.

$T_{1,}$ =Apron star @2.5g/kg, T_2 = Apron star@2g/k, T_3 = Apron star @3g/k, T_4 = Imidalm T 450 WS @1g/k, T_5 = Imidalm T 450 WS@0.5g/kg, T_6 = Imidalm T 450 WS@1.5g/kg, T_7 = Imidacloprid + Thiram + Carboxin@3g/kg, T_8 = Imidacloprid + Thiram + Carboxin@2g/kg, T_9 = Imidacloprid + Thiram + Carboxin@1g/kg, T_{10} = Thiamethoxam 25% WG@2g/kg, T_{11} = Thiamethoxam 25% WG@1g/kg, T_{12} = Thiamethoxam 25% WG@3g/kg, T_{13} = untreated.

3.3. Effect of seed treatments on control efficacy against *R. maidis*

The effectiveness of the seed treatments showed that aphid number on maize seedlings decreased from 99.48 -96.15% in seedlings developed from treated seeds after one-month of storage after treatment and 95.85-80.59% in seedlings developed from treated seeds after six-months of storage after treatment per application rate. This data indicated that *R. maidis* population reduction in the treated over untreated control ranged from 80.5 to 99.48%, and seed treatment was found most effective against *R. maidis* when Thiamethoxam 25% WG applied @2g/kg and Imidalm T 450 WS @ 1.5g/kg were used.

As that of *Frankliniella* sp., thiamethoxam 25% WG at all dosage and storage time were effective and showed insecticidal activity against *R. maidis*. Imidalm T 450 WS at high rate (1.5g/kg) was also found effective against *R. maidis*, while Imidacloprid + Thiram + Carboxin at 1.0 and 2.0 g/kg (lower and medium dosage) were showed lower reduction percentage on both maize seedlings developed from treated seeds stored for one and six months before planting (Fig 2).

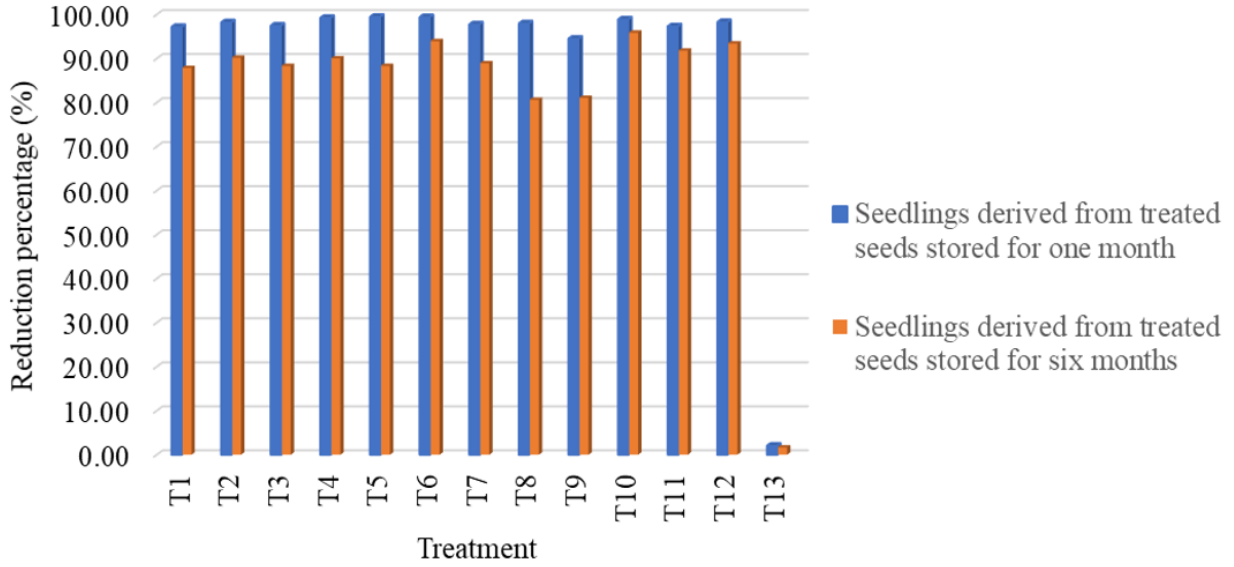

Fig. 1. Effect of insecticidal maize seed treatment on maize leaf aphid *(Rhopalosiphum maidis)* population reduction on maize seedlings developed from one-month and six-months storage after treatment. $T_{1,}$ =Apron star @2.5g/kg, T_2 = Apron star@2g/k, T_3 = Apron star @3g/k, T_4 = Imidalm T 450 WS @1g/k, T_5 = Imidalm T 450 WS@0.5g/kg, T_6 = Imidalm T 450 WS@1.5g/kg, T_7 = Imidacloprid + Thiram + Carboxin@3g/kg, T_8 = Imidacloprid + Thiram + Carboxin@2g/kg, T_9 = Imidacloprid + Thiram + Carboxin@1g/kg, T_{10} = Thiamethoxam 25% WG@2g/kg, T_{11} = Thiamethoxam 25% WG@1g/kg, T_{12} = Thiamethoxam 25% WG@3g/kg, T_{13} = Untreated

3.4. Cumulative effect of different seed dressers on MLN causing virus vectors

The cumulative effect of different seed treatments on *Frankliniella* sp. (vector of MCMV) and *R. maidis* (vector of SCMV) population exposed to maize seedlings developed from treated seeds stored for up to six months is given in Tables 3 and 4. Significant reduction of both vector species population was indicated in all treatments over control (untreated). The results revealed that the treatments thiamethoxam 25% at 2.0g/kg seed and imidalm T 450 at rate 1.5 were significantly superior in reduction of both species population when compared with the others. While Imidacloprid + Thiram + Carboxin@1g/kg (at low rate) is the lowest in reduction of *Frankliniella* sp. (Table 3) and *R. maidis* (Table 4) population.

Among different dosages seed treatments tested against *Frankliniella* sp., thiamethoxam 25% WG@ 2.0 g/kg seed was superior over thiamethoxam 25% WG@ 1.0 and 3.0 g/kg seed. Among dosages of imidalm T 450 WG, imidalm T 450 WG @ 1.5 was significantly superior imidalm T 450 WG @ 0.5 and 1.0 g/kg seed (Table 3). Similar trend was also indicated for *R. maidis* (Table 4).

Table 8. Cumulative effect of different insecticidal seed dressers on MCMV vector thrip (Frankliniella sp.).

Treatment	**Rate (g/kg maize seed)**	**Mean population reduction**
Thiamethoxam 25% WG	2.0	96.06a
Imidalm T 450WS	1.5	95.00ab
Thiamethoxam 25% WG	1.0	93.50bc
Thiamethoxam 25% WG	3.0	93.22bc
Imidalm T 450 WS	1.0	91.95dc
Imidacloprid + Thiram + Carboxin	3.0	91.50de
Imidalm T 450 WS	0.5	90.83def
Apron star	2.0	90.72def
Apron star	3.0	89.89ef

Apron star	2.5	89.72f
Imidacloprid + Thiram + Carboxin	2.0	84.78g
Imidacloprid + Thiram + Carboxin	1.0	77.72h
Untreated		2.89i

Means are from both two treated seeds stored for one and six months. Means followed by the same letters are not significantly different ($P < 0.05$).

Table 9. Cumulative effect of different insecticidal seed dressers on *Sugarcane mosaic virus* aphid (*Rhopalosiphum maidis)* vector.

Treatment	**Rate (g/kg maize seed)**	**Mean population reduction**
Thiamethoxam 25% WG	2.0	97.37a
Imidalm T 450 WS	1.5	96.67ab
Thiamethoxam 25% WG	3.0	95.85abc
Imidalm T 450 WS	1.0	94.63abc
Thiamethoxam 25% WG	1.0	94.56abc
Apron star	2.0	94.18abc
Imidalm T 450 WS	0.5	93.89bc
Imidacloprid + Thiram + Carboxin	3.0	93.33bc
Apron star	3.0	92.89bcd
Apron star	2.5	92.52cd
Imidacloprid + Thiram + Carboxin	2.0	89.30de
Imidacloprid + Thiram + Carboxin	1.0	87.77e
Untreated		1.85f

Means are from both two treated seeds stored for one and six months. Means followed by the same letters are not significantly different ($P < 0.05$).

4. Discussion

Maize is attacked by various sucking insect pests and chewing species during the growing season. Furthermore, most of these insects can carry and spread viral diseases among plants via feeding from infected plant to healthy one. Plant virus diseases including MLN causing viruses (MCMV and SCMV) can be controlled to some level by controlling the vectors that transmits the virus. In Ethiopia, maize thrips (*Frankliniella* sp.) and Corn leaf aphid (*Rhopalosiphum maidis*) are the major vectors of MCMV and SCMV, respectively (Regassa *et al*. unpublished data). The vectors especially thrips breed quickly at high temperatures and continually migrate to newly emerged maize leaves and are also too small to be easily identified and are usually not directly exposed to foliar sprays, as they are mostly concentrated on internal leaves (Ding *et al*., 2018). As compared with foliar sprays, systemic seed treatments provide a good solution for such problem because the strong upward passage allows insecticides on seeds to be continuously absorbed and transferred to new leaves throughout the seedling level (Elbert *et al*., 2008, Alford and Krupke, 2017).

Insecticide seed treatment did not significantly influence the germination of maize seed even up to six months storage before planting. The slight decline in germination percentage may be due to ageing effect leading to depletion of food reserves (Laxman *et al*., 2017).

The current study conducted on the vectors of MLN causing viruses indicated that the control efficacy differed among seed dressing insecticides with different dose. More satisfactory reduction levels of maize thrips *(Frankliniella* sp.) and corn aphids *(Rhopalosiphum maidis)* were achieved using thiamethoxam 25% WG @ 2.0 g/ kg seed than other insecticides used in this study at the same dose. In similar of this study, Ding *et al*. (2018) demonstrated that treating maize seeds with thiamethoxam (1.0 and 2.0 g/kg of seeds) reduced thrips infestations on maize under field condition. However, compared with other tested seed dressing insecticides, Imidacloprid + Thiram + Carboxin (1.0 g/kg of seeds) had a lower control effect for both vectors. The differences in efficacy may be associated to the toxicity of the different insecticides to thrips. As reported by Byrne *et al*. (2007) other than maize plant, thiamethoxam and imidacloprid provide good control of avocado thrips in bioassays. The toxicities of thiamethoxam to larvae and adult females of western flower thrips (*Frankliniella occidentalis*) were higher than those of other tested neonicotinoids (nitenpyram, imidacloprid, and thiacloprid) (Shan *et al*., 2012).

The findings from the current study indicate that insecticide seed dressing of maize seeds before planting with thiamethoxam 25% WG @ 2.0 g/ kg seed or Imidalm T 450@ 1.0 g/kg of seeds) can be usedfor early-stage protection against potential vectors of the MLN causing viruses in Ethiopia.

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Paper VI

Tropical Plant Pathology
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ORIGINAL ARTICLE

Screening maize genotypes for resistance to maize lethal necrosis disease in Ethiopia

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Abstract
Maize lethal necrosis (MLN) is becoming a great challenge to maize production that threatens food security for most households in East Africa since 2011. MLN in East Africa, including Ethiopia, is caused by co-infection of maize chlorotic mottle virus (MCMV) and any other potyvirus, often sugarcane mosaic virus (SCMV). A greenhouse study was conducted at Ambo Agricultural Research Center to determine the reactions of various maize genotypes to MLN to identify resistant genotypes that can be either utilized in breeding programs or recommended for commercial production. A total of 306 maize genotypes (275 inbred lines and 31 commercial varieties) collected from various research centers and seed companies were evaluated in the greenhouse under artificial MLN inoculation. Weekly MLN severity score, pooled mean of weekly severity score, area under disease progress curve (AUDPC), final disease severity, and disease incidence were used to assess reactions of the maize genotypes to MLN. Double antibody sandwich enzyme-linked immunosorbent assay test showed that only 2% of the highland maize inbred lines were resistant to MLN, whereas none of the lowland and mid-altitude inbred lines were resistant to the disease. Roughly 7%, 16.7%, and 5.5% of the inbred lines from highland, mid-altitude, and lowland maize breeding programs, respectively, showed moderately resistant reactions to MLN. However, higher proportion of the inbred lines from all the breeding programs revealed susceptible to highly susceptible reactions. Most of the maize varieties evaluated were infected by MLN with different levels of reactions that ranged from moderately susceptible to highly susceptible. Only two highland varieties (Wenchi and Kolba) showed moderately resistant reaction. In general, while most of the maize genotypes evaluated were infected by the viruses, considerable number of inbred lines and varieties showed resistant/tolerant reactions to MLN. Inbred lines that showed good level of resistance to the disease can be used as sources of desirable genes in maize breeding programs, while MLN resistant or tolerant varieties could be recommended for extensive commercial production.

Keywords AUDPC · Incidence · Inbred line · Genotype · MLN severity · Variety

Screening Maize Genotypes for Resistance to Maize Lethal Necrosis Disease in Ethiopia

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Abstract

Maize lethal necrosis (MLN) is becoming a great challenge to maize production that threatens food security for most households in East Africa since 2011. MLN in East Africa, including Ethiopia, is caused by co-infection of maize chlorotic mottle virus (MCMV) and any other potyvirus, often sugarcane mosaicvirus (SCMV). Agreenhouse study was conducted at Ambo Agricultural Research Center to determine the reactions of various maize genotypes to MLN to identify resistant genotypes that can be either utilized in breeding programs or recommended for commercial production. A total of 306 maize genotypes (275 inbred lines and 31commercial varieties) collected from various research centers and seed companies were evaluated in the greenhouse under artificial MLN inoculation. Weekly MLN severity score, pooled mean of weekly severity score, area under disease progress curve (AUDPC), final disease severity, and disease incidence were used to assess reactions of the maize genotypes to MLN. Double antibody sandwich enzyme-linked immuno sorbent assay test showed that only 2% of the highland maize inbred lines were resistant to MLN, whereas none of the lowland and mid-altitude inbred lines were resistant to the disease. Roughly 7%, 16.7%, and 5.5% of the inbred lines from highland, mid-altitude, and lowland maize breeding programs, respectively, showed moderately resistant reactions to MLN. However, higher proportion of the inbred lines from all the breeding programs revealed susceptible to highly susceptible reactions. Most of the maize varieties evaluated were infected by MLN with different levels of reactions that ranged from moderately susceptible to highly susceptible. Only two highland varieties (Wenchi and Kolba) showed moderately resistant reaction. Ingeneral, while most of the maize genotypes evaluated were infected by the viruses, considerable number of inbred lines and varieties showed resistant/ tolerant reactions to MLN. Inbred lines that showed good level of resistance to the disease can be used as sources of desirable genes in maize breeding programs, while MLN resistant or tolerant varieties could be recommended fo rextensive commercial production.

Keywords: AUDPC, Incidence, Inbred line, Genotype, MLN severity, Variety

Introduction

Maize (*Zea mays* L.,2n=2x=20) is one of the most important food security crops produced in Africa. In Ethiopia, maize has a significant share among cereal crops in terms of production, yield, distribution, and adaptation. Among all cereals, maize is second to teff (*Eragrostistef*) in area coverage with 2.3 million ha (17.7% area allocated to allcereals) land planted to the crop, but first in productivity (4.7 t ha^{-1}) with total annual production of 10.6 million tons (CSA, 2020).

Maize lethal necrosis (MLN) is a viral disease of maize caused by the co-infection of two viruses, maize chlorotic mottle virus (MCMV) and any of the cereal viruses from the *Potyviridae* family, such as sugarcane mosaic virus (SCMV), maize dwarf mosaic virus (MDMV), or wheat streak mosaic virus (WSMV). MLN was first reported in KS, USA, in1976 (Niblett and Claflin, 1978) and in Africa in 2011 in Kenya (Wangai et al.2012).In Africa, including Ethiopia, the main cause of MLN is the co-infection of MCMV and SCMV (Wangai et al., 2012; Adams et al., 2014; Lukanda et al., 2014; Mahuku et al., 2015a; Fentahun et al., 2017; Guade et al., 2019; Regassa et al., 2020).

Soon after its emergence in Kenya, MLN was reported in other East African countries including Uganda, Tanzania, Rwanda, Democratic Republic of Congo, and Ethiopia during2012–2014(Adamsetal.,2014; Lukanda et al.,2014; Mahuku et al., 2015a, b). MLN has been reported to cause up to 100% yield loss, making it a serious threat to food security in major maize producing countries of East Africa (Redinbaugh and Stewart,2018), and continues to challenge maize production in the regions (Boddupalliet al., 2020; Regassaet al., 2020). In 2013, estimated national losses of maize production due to MLN in Kenya were reported as 17–58% depending on maize production ecologies (De Groote et al., 2016). Since its occurrence in 2014 in the upper Rift Valley of Ethiopia (Mahukuet al., 2015a, b), MLN spread into other major maize producing areas of the country, especially in central, western, southern, and southwestern parts of the country and caused from low to complete maize crop failure (Fentahun et al., 2017; Guadie et al., 2019; Regassa et al.,2020).

Maize is susceptible to MLN disease at all stages of its growth and development, from seedling to nearmaturity (Beyeneetal., 2017). Under field conditions, if a maize plant is infected at the early growing stage, complete yield loss may occur (Uyemoto, 1983; Wangai et al., 2012). MLN-causing viruses are

commonly transmitted from plant to plant and field to field by insect vectors; MCMV is transmitted by thrips (Cabanas et al., 2013) or chrysomelid beetles (Nault ,1978), and SCMV is transmitted by aphids (Braultetal.,2010). Both viruses can also be transmitted by contaminated seeds at low rate, which can contribute to rapid and long-range dissemination of the disease (Jensen et al., 1991; Zhang et al., 2011; Regassa et al., 2021).

Due to its rapid spread; interaction with local viruses; transmission by different mechanisms including insect vectors, contaminated seed, and soil; and the absence of resistant commercial maize varieties in the production system, MLN has caused significant negative impact to maize production. In Africa, including Ethiopia, where MCMV is considered a new virus, there is no sufficient information available on the management of MLN. In other regions, for example in Hawaii, integration of cultural practice, hostresistance/tolerance, and suitable insecticides have been used (Nelson et al., 2011). Most smallholder farmers in East Africa cannot afford to apply pesticides to control the vector populations, and recycling of seed that may be infected with MLN causing viruses is common in the region (Beyene et al., 2017). In Ethiopia, field observations suggested that under a natural disease pressure, local and improved maize varieties grown by farmers are not resistant to MLN (Regassa et al., 2020). Development of MLN resistant varieties is economically feasible and environmentally sustainable approach for disease management. However, it requires identification of resistant genotypesand incorporation of disease resistance into agronomically desirable varieties or promotion of such genotypes for direct commercial use. Effective screening of different maize genotypes is vital in identifying genetic resistance for MLN among locally adapted maize germplasm.

Screening plants for virus resistance have been conducted commonly through artificial inoculation (Zambrano et al.,2014). Based on the nature of the experiment, this method has been done in the field, greenhouse, or in growth chambers. It is also more advantageous relative to natural infection interms of efficiency of producing sufficientv iralinoculum, uniform infection, high transmission rates, and clear separation among resistant and susceptible plants (Redinbaugh and Zambrano, 2014).

In Eastern Africa, International Maize and Wheat Improvement Center (CIMMYT) and its partners have been undertaking extensive germplasm screening since 2013 at MLN screening facility in Naivasha, Kenya, under artificial inoculation (Boddupalli et al., 2020). Evaluation of

a large collection of maize germplasm for MLN resistance showed that very few of the elite germplasm used in various public and private sector breeding programs have resist- ance reaction to MLN causing viruses, mainly to MCMV (Gowda et al., 2015; Beyene et al., 2017; Sitta et al., 2017). Studies from 2012 to 2013 confirmed that approximately 98% of commercial maize varieties in Kenya were susceptible to MLN (Boddupalli et al., 2020; Marenya et al., 2018). In Ethiopia, there is no published report on the reaction of locally adapted maize genotypes to MLN disease. Hence, the objective of this study was to screen Ethiopian maize genotypes under artificial inoculation conditions to identify MLN resistant maize genotypes which can be utilized in maize breeding programs or recommended for commercial maize production for the management of MLN disease.

Materials and methods

Genotypes and experimental design

A total of 306 maize genotypes that included 275 inbred lines and 31 commercial varieties were used for the study. The inbred lines were collected from major maize breeding programs in Ethiopia. One hundred inbred lines were obtained from Ambo highland, 66 from Bako mid-altitude, and 109 from Melkassa lowland adapted maize breeding programs. The lines were developed from diverse source germplasm and are widely used in the respective breeding programs. The commercial varieties were collected from various National Agricultural Research Centers, Dupot Pioneer Hi-Bred Seeds in Ethiopia, and Ethiopian Seed Enterprise. Two MLN susceptible maize varieties Limu and Melkassa-2, which were identified by our previous study (Regassa et al., 2020), and six MLN resistant maize cultivars CKMLN150075, CKMLN150088, CKMLN150076, CKMLN150074, CKMLN15150, and WE5135 were used as controls. The resistant cultivars were developed by CIMMYT in Kenya and are currently evaluated across locations in Ethiopia by Bako National Maize Breeding Program for possible release as commercial hybrids.

The experiment was conducted in a greenhouse at 25–35 °C Day temperature at Ambo Agricultural Research Center using artificial inoculations. Two separate sets of experiments that involved 275 inbred lines and 31 varie- ties were conducted using a completely randomized design with three replications. Each experimental unit consisted of five maize plants grown in a 30-cm diameter plastic pot. Fertilizers were applied at the rates of 100-kg N and 100-kg P_2O_5 in the forms of DAP at planting and urea 3 weeks after emergence as side dressing, respectively.

MLN causing viruses sources, inoculum preparation, and inoculation

Stock isolates of MCMV and SCMV were collected from MLN affected areas in hotter Rift Valley areas of East Shewa (Awash Melkassa district) and Arsi (Jeju district) zones of Oromia region in Ethiopia. As described by Gowda et al. (2015), after con- firming the presence of SCMV or MCMV by double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA), the viruses were propagated on maize plants and maintained in separate greenhouses periodically by mechanical inoculations. Symptomatic leaves for each virus isolate were collected separately and cut into small pieces. An extraction buffer of phosphate buffer 0.1 M was made by mixing potassium phosphate dibasic (anhydrous) and potassium dihydrogen orthophosphate (potassium phosphate monobasic) at pH 7.0 using the following ratios: KH_2PO_4 = 10.8 g, K_2HPO_4 = 4.8 g, and Na_2SO_3 = 1.26 (Sitta et al., 2017).

Symptomatic leaves for each virus were weighed and ground using sterile mortar and pestle to obtain homogenate solution or extract (1:20; 1 g of leaf materials to 20-ml extraction buffer). In order to keep uniform MLN disease pressure, optimized combinations of preparation of MLN inoculum for artificial inoculation was done using the optimized protocols as described by Gowda et al. (2015). Thus, the inoculum extracts were mixed at a ratio of 1:4 (adding one part of MCMV and four parts of SCMV) in one container to obtain an optimized virus combination known to cause MLN in East Africa.

The combination of MCMV and SCMV inoculum was then rubbed onto 4–6 leaf stage of maize seedlings in the greenhouse. Carborundum (SiC) powder, which is an abrasive agent, was used to cause microscopic injury of the leaves for easy penetration of the virus into the plant cells (Orawu et al., 2013). A second inoculation was done a week after the first inoculation to ensure effective viral pressure and spread among the genotypes and avoid disease escapes.

Viral detection

After inoculation, maize inbred lines or varieties that showed minor symptoms and asymptomatic genotypes were tested serologically for both MCMV and SCMV using DAS-ELISA. The antibodies for the test of MCMV (AS-1087), SCMV (AS- 0166), and their respective positives controls were obtained from Deutsche Sammlung von Mikroorganismen und Zellkul- turen (DSMZ) plant virus collection, Germany. The tests were conducted following the method described by Clark and Adams (1977) and the standard protocols of the DSMZ- Plant Virus Collection. Leaf extracts from

healthy maize were used as a negative control. All samples were assayed in duplicate, and the results were considered valid when the positive controls gave positive results (yellow color) and negative control wells were colorless. The recorded ELISA results were qualitatively grouped as positive and negative by comparing with the positive and negative controls.

Data collection and analysis

MLN symptoms were assessed for disease severity and incidence to obtain an indication of the disease intensity over time. Weekly MLN severity was scored using 1 to 5 scale (Shekhar and Kumar, 2012; Gowda et al., 2015), where 1 means no visible MLN symptom on leaves (resistant); 2 means symptoms on < 25% of infected leaves showing chlorotic mottling on the lower leaves (moderately resistant); 3 means symptoms on 25 to 50% of infected leaves showing chlorotic mottling and mosaic throughout the whole plant (moderately susceptible); 4 means symptoms on 50 to 75% of infected leaves showing excessive chlorotic mottling, mosaic, plant necrosis, and/ or dead heart (susceptible); and 5 means symptoms on 75 to 100% of infected leaves with severe chlorotic mottling, mosaic, and necrosis (highly susceptible) (Fig. 1). Disease severity scoring began 1 week after the last inoculation and continued for seven consecutive weeks. Delayed scoring of MLN is important to detect late-developing infections as indicated for maize rayado fino virus by Zambrano et al. (2013). MLN incidence was measured as the percentage of the number of leaves with MLN infection.

The inbred lines evaluated in this study were classified into five groups (resistant, moderately resistant, moderately susceptible, susceptible, and highly susceptible) based on reaction to MLN as assessed based on final disease severity rating. Resistant inbred lines had final MLN severity score of 1.0, with no disease symptoms. Moderately resistant inbred lines showed mild symptoms with final disease severity score of 2.0 or lower. Moderately susceptible inbred lines had final severity scores of 2–3. Susceptible and highly susceptible inbred lines showed final disease severity scores of 3–4 and higher than 4.0, respectively.

Fig. 1. Maize lethal necrosis (MLN) severity score (1 to 5 scales) used to evaluate the response of maize inbred linens and varieties to the disease. 1 = plant with no visible MLN symptoms on the leaves; 2 = symptoms on <25% of infected leaves; 3 = symptoms on 25 to 50% of infected leaves; 4 = symptoms on 50 to 75% of infected leaves and 5 = symptoms on 75 to 100% of infected leaves.

Severity scores were used to generate area under disease progress curve (AUDPC) values, which were analyzed to measure differences among treatments (maize inbred lines and varieties). The disease progress data were summarized into one value by AUDPC, which is suited when host damage and the amount and duration of the disease are proportional (Xu, 2006). AUDPC was used to make a comparison between treatments (Xu, 2006) to evaluate the resistance of host genotypes against pathogen species (Mukherjee et al., 1995), and to compare among the components as well as parameters of resistance (Mohapatra et al., 2014). AUDPC was calculated as follows:

$$\text{AUDPC} = \sum_{i=1}^{n-1} [\text{Yi} + \text{Yi} + 1]/2 * [\big((ti + 1) - ti\big)]$$

where n is the total number of observations, Yi was disease assessment (score), at the ith observation, and ti was the time of observation (days) at the ith observation.

Before calculating AUDPC, the severity scales obtained were converted to percent severity index (Osunga et al., 2017).

$$\text{Percent severity index} = \frac{\text{sum of all disease rating}}{\text{total number of rating} \times \text{maximum disease grade}} \times 100$$

AUDPC values for the pooled mean of weekly MLN severity scores, final severity, and MLN incidence were subjected to the analysis of variance (ANOVA). The genotypic mean differences were assessed using Fisher's protected least significant differences (LSD) test at 5% significance level. Pearson correlation analysis was used to determine the relationships among the variables in pair- wise comparisons.

Results

Analysis of variance

Analysis of variance revealed the presence of highly significant differences (P <0.01) among the tested inbred lines (Table 1) and varieties (Table 2) for various MLNdisease parameters studied. Neither significant variation type (Tables 1 and 2) nor among the five plants in each experimental unit, implying the repeatability and reproducibility of genotypic performances for reaction to MLN.

Table 10. Mean squares for maize lethal necrosis (MLN) disease parameters for maize inbred lines collected from highland, mid-altitude and lowland maize breeding programs in Ethiopia and evaluated in greenhouse at Ambo, Ethiopia.

Source of variation	Df	DI	PMS	FS	AUDPC
Highland					
Inbred lines	99	1078.1**	1.42**	1.91**	3219.8**
Replications	2	4.775	0.004	0.021	13.452
Residual	198	28.88	0.01	0.03	11.62
Mid-altitude					
Inbred lines	65	664.7**	1.68**	2.56**	3772.25**
Replications	2	4.677	0.001	0.019	2.049
Residual	130	24.88	0.003	0.021	5.15
Lowland					
Inbred lines	108	569.5**	1.38**	1.89**	3032.05**
Replications	2	10.85	0.038	0.001	93.72
Residual	216	16.25	0.007	0.003	14.68

** = significant at 1% probability level, df = degree of freedom, DI = disease incidence, PMS = pooled mean of weekly MLN severity, FS = final severity score, AUDPC = area under disease progress curve.

Table 2. Mean squares for maize lethal necrosis (MLN) disease parameters for maize varieties evaluated in greenhouse at Ambo, Ethiopia.

Source of variation	Df	DI	PMS	FS	AUDPC
Genotypes	30	3690.28**	2.43**	3.79**	5427.64**
Replications	2	0.685	0.002	0.025	1.85
Residual	60	1.981	0.003	0.031	6.46

** = significant at 1% probability level, df = degree of freedom, DI = disease incidence, PMS = pooled mean of weekly MLN severity, FS = final severity score, AUDPC = area under disease progress curve.

Reaction of maize inbred lines to MLN

The inbred lines evaluated in this study showed highly variable levels of reactions to MLN disease as assessed based on various disease parameters. Most inbred lines showed MLN disease symptoms within a week after inoculations while some other lines developed symptoms after 2 weeks. Commonly observed symptoms were leaf chlorosis and mottling that started from the base of the leaves and extended upwards to the leaf tips. As the disease progressed with time, severe chlorosis and mottling were observed and turned the plants to bright yellow followed by necrosis from the leaf margins. The necrosis progressed inwards to the midribs leading to the death of the whole leaf.

Mean, minimum, and maximum values of weeklyseverity scores and other disease parameters are presented in Table 3. As depicted by various disease parameters, inbred lines from lowland maize program were moresusceptible to MLN disease as compared to inbred lines from highland and mid-altitude maize breeding programs (Table 3). Highland maize inbred lines had relatively lower values of all disease parameters, and hence, these lines were more resistant to MLN disease than inbred lines from other programs.

Table 3. Mean, minimum and maximum weekly maize lethal necrosis (MLN) disease progress and other MLN disease parameters in maize inbred lines collected from various maize breeding program in Ethiopia

Source	Value	MLN severity score (based on 1-5)							Disease parameter			
		W1	W2	W3	W4	W5	W6	W7	FS	AUDPC	PMS	DI
Highland	Mean	1.64	2.11	2.34	2.74	2.99	3.18	3.37	3.37	122.11	2.62	77.23
	Minimum	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	45.50	1.00	0.00
	Maximum	3.08	4.00	4.50	5.00	5.00	5.00	5.00	5.00	204.79	4.30	100.00
Mid-altitude	Mean	1.91	2.59	3.16	3.30	3.30	3.30	3.36	3.36	139.54	2.99	83.25
	Minimum	1.00	1.00	1.00	1.00	1.00	1.50	1.50	1.50	72.63	1.54	25.00
	Maximum	3.00	4.25	5.00	5.00	4.84	5.00	5.00	5.00	205.33	4.35	100.00
Lowland	Mean	2.10	2.79	3.30	3.53	3.63	3.71	3.83	3.83	152.26	3.27	84.94
	Minimum	1.00	1.00	1.00	1.00	1.50	1.75	2.00	2.00	60.38	1.32	30.00
	Maximum	3.34	4.75	5.00	5.00	5.00	5.00	5.00	5.00	213.50	4.54	100.00

*W1-W7 = weekly MLN severity scores from first to seventh week after inoculation, FS = final severity score, AUDPC = area under disease progressive curve, PMS = pooled mean of weekly MLN severity, DI = disease incidence.

Based on final MLN severity rating, only 2% of the highland maize inbred lines showed resistant reaction to the disease, whereas none of the inbred lines from mid- altitude and lowland maize breeding programs had resist- ant reaction to the disease (Fig. 2). Nearly, 7% of the highland, 17% of the mid-altitude, and 6% of the lowland adapted inbred lines were moderately resistant to the dis- ease. Larger proportion of the inbred lines from all breeding programs showed susceptible and highly susceptible reactions to MLN. Overall, among the 275 inbred linesabout 1%, 9%, 20%, 44%, and 27% showed resistant, moderately resistant, moderately susceptible, susceptible, and highly susceptible reactions to the disease, respectively (Fig. 2). Except two highland inbred lines (ABL-91 and AMB17KN20-1), all the inbred lines evaluated showed MLN symptoms.

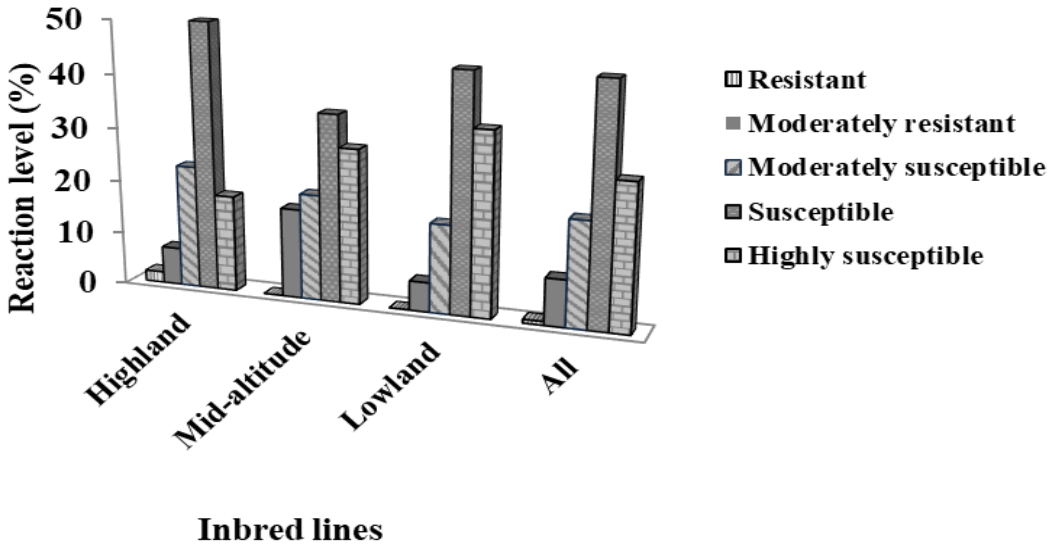

Fig. 4. Reaction of maize inbred lines collected from various Ethiopian breeding programs to maize lethal necrosis (MLN) as assessed by final disease severity score.

Among the top 20 MLN resistant highland inbred lines, two inbred lines showed resistant and another seven moderately resistant reaction to MLN, with lower values of mean weekly and final disease severity scores (1.0–2.0), pooled mean of weekly disease severity (1.0–2.1), AUDPC (45.5–99.7), and disease incidence (0.0–54.3%) (Table 4). Inbred line ABL-91 did not show MLN disease symptom, and no virus was detected when tested with DAS-ELISA. On the other hand, AMB17KN20-1 was symptomless but tested positive for MCMV. Some in bredlines showed MLN disease symptoms in the later weeks. For example, inbred lines AMB12N33-84, AMB14N17-2–6, ABL-35, andABL-64 showed MLN symptoms in the third week; Breeder-k, AMB17N17-8 and ABL-77 in the fourth week; ABL-44 in the fifth and Breeder-w in the sixth week after inoculation. Inbred lines ABL-11, ABL-19, ABL-67, ABL-10, and ABL-42 were severely infected by MLN (Table4), and also tested positive for both MCMV and SCMV.

Table 4. Maize lethal necrosis (MLN) disease severity scores and other disease parameters of selected 20 most resistant and five most susceptible highland adapted maize inbred lines evaluated in the greenhouse at Ambo, Ethiopia.

		Weekly MLN severity score							Disease parameter				Reaction
Source	Genotype	W1	W2	W3	W4	W5	W6	W7	PMS	FS	AUDPC	DI	
Most resistant 20													
Ambo	ABL-91	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	45.50	0.00	R
CIMMYT	AMB17KN20-1	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	45.50	0.00	R
CIMMYT	Breeder-W	1.00	1.00	1.00	1.00	1.00	1.50	1.50	1.14	1.50	52.50	33.89	MR
CIMMYT	AMB17EN18-13	1.67	1.00	1.00	1.67	1.00	1.50	2.00	1.40	2.00	61.25	59.26	MR
CIMMYT	Breeder-K	1.00	1.00	1.00	2.00	2.00	1.00	2.00	1.43	2.00	63.00	41.67	MR
Ambo	ABL-77	1.00	1.00	1.00	2.00	2.00	1.67	2.00	1.52	2.00	70.00	63.52	MR
CIMMYT	AMB14N17-2-6	1.00	1.00	1.25	1.50	2.25	2.25	2.00	1.61	2.00	76.13	66.67	MR
CIMMYT	AMB17N19-4	1.84	1.00	1.00	2.00	2.00	2.00	2.00	1.69	2.00	76.42	55.47	MR
Ambo	ABL-85	2.00	2.00	2.00	2.00	2.50	2.50	2.00	2.14	2.00	99.75	54.26	MR
Ambo	ABL-80	1.00	1.25	1.42	1.58	2.00	2.00	2.25	1.64	2.25	76.13	67.10	MS
Ambo	ABL-84	1.67	2.00	2.00	2.00	2.50	2.50	2.33	2.14	2.33	99.75	76.67	MS
Ambo	ABL-83	1.13	1.25	1.42	1.46	1.54	2.04	2.37	1.60	2.37	73.35	45.00	MS
Ambo	ABL-28	1.12	1.37	1.58	1.58	2.30	2.33	2.58	1.84	2.58	85.38	55.89	MS
Ambo	ABL-36	1.00	1.25	1.29	1.29	1.67	2.38	2.75	1.66	2.75	76.56	61.25	MS
Ambo	ABL-94	1.16	1.54	1.62	1.62	1.75	1.75	2.75	1.74	2.75	77.77	74.37	MS
Ambo	ABL-39	1.55	1.63	1.25	1.67	2.08	2.42	2.92	1.93	2.92	87.38	52.73	MS
Ambo	ABL-44	1.00	1.00	1.00	1.00	1.83	2.56	3.00	1.63	3.00	74.68	75.93	MS
Ambo	ABL-40	1.13	1.25	1.50	1.50	2.00	2.50	3.00	1.84	3.00	84.44	73.34	MS
CIMMYT	AMB17N17--9	1.75	1.50	1.50	2.00	2.00	2.50	3.00	2.04	3.00	91.88	63.54	MS
CIMMYT	AMB17N17-8	1.00	1.00	1.00	2.21	2.25	2.75	3.12	1.90	3.12	88.54	74.86	S
Ambo	ABL-75	1.33	1.50	1.58	1.92	1.92	2.25	3.50	2.00	3.50	88.96	81.12	S
Most susceptible five													
Ambo	ABL-42	2.54	3.33	4.00	4.00	4.08	4.17	4.25	3.77	4.25	175.43	94.44	HS
Ambo	ABL-10	2.00	3.88	4.18	4.00	4.58	4.54	4.54	3.96	4.54	187.07	96.53	HS
Ambo	ABL-67	1.67	2.92	3.33	4.92	5.00	5.00	5.00	3.98	5.00	189.00	97.50	HS
Ambo	ABL-19	3.42	4.00	4.15	4.42	4.44	4.50	4.64	4.22	4.64	194.51	94.05	HS
Ambo	ABL-11	1.92	2.75	4.67	5.00	5.00	5.00	5.00	4.19	5.00	198.63	100.0	HS

W1-W7 = weekly MLN severity scores from first to seventh week after inoculation, PMS = Pooled Mean of weekly MLN severity, FS = Final Severity score, AUDPC = Area under Disease

Progressive Curve, DI = disease incidence, R = resistant, MR= moderately resistant, MS = moderately susceptible, S = susceptible, HS = highly susceptible.

Among the top 20 most resistant inbred lines collected from mid-altitude maize breeding program, CML204, CML464, TZMI754, TZMI751, and TZMI717 showed moderately resistant reactions to MLN with 1.0–2.0 weekly dis- ease severity score and lower AUDPC (79.9–86.8), pooled mean of weekly disease severity (1.7–1.9), final disease severity (2.0), and disease incidence (25.0–66.7%). Delayed occurrences of MLN disease symptoms were observed in CZLQ2 that showed the symptoms in the third week, and TZMI730 and CML159, which showed the symptoms in the fourth week after inoculation (Table 5). Among the inbred lines from lowland maize breeding program, MKL17K0422, MKL17K0597, MKL17K0343, MKL17K0343, and MKL1K0716 showed relatively lower values of weekly severity scores, AUDPC, and final MLN severity. These inbred lines also showed delayed occurrence of MLN symptoms (Table 6).

Table 5. Maize lethal necrosis (MLN) disease severity scores and other disease parameters of selected 20 most resistant and five most susceptible mid-altitude adapted maize inbred lines evaluated in the greenhouse at Ambo, Ethiopia.

Entry	Pedigree	Weekly MLN severity score							Disease parameter				Reaction
		W1	W2	W3	W4	W5	W6	W7	PMS	AUDPC	DI	FS	
	Most resistant 20												
1	TZMI730	1.00	1.00	1.00	1.00	1.92	2.58	2.58	1.58	74.08	60.84	2.58	MS
2	CML 464	1.00	1.67	2.00	2.00	2.00	1.50	2.00	1.74	79.92	25.00	2.00	MR
3	CML 204	1.92	2.00	2.00	2.00	1.33	1.50	2.00	1.82	80.79	51.94	2.00	MR
4	TZMI751	1.75	1.50	2.00	1.50	2.13	2.00	2.00	1.84	84.00	59.10	2.00	MR
5	TZMI754	1.54	1.67	1.83	1.83	2.00	1.96	2.08	1.85	84.59	59.09	2.00	MR
6	TZMI717	1.00	1.79	2.00	2.08	2.00	2.00	2.05	1.85	86.80	66.67	2.05	MS
7	CZLQ2	1.00	1.00	2.17	2.17	2.54	2.42	2.58	1.98	93.04	72.73	2.58	MS
8	CML159	1.00	1.00	1.00	2.42	2.58	2.83	3.08	1.99	93.05	70.00	3.00	MS
9	CML161	2.00	2.00	2.00	2.00	1.75	2.25	2.50	2.07	93.63	65.00	2.50	MS
10	TZMI766	2.00	2.75	2.00	2.00	2.00	2.00	2.00	2.11	96.25	86.81	2.00	MR
11	TZMI 733	2.00	2.25	2.25	2.00	2.42	2.00	2.00	2.13	97.41	67.73	2.00	MR
12	TZMI755	2.00	2.00	3.00	2.00	2.00	2.00	2.00	2.14	98.00	93.46	2.00	MR
13	30H83-56	1.00	1.83	2.00	2.33	2.29	2.50	2.83	2.11	98.88	90.91	2.83	MS
14	CZLQ3	1.88	2.00	1.63	3.00	2.75	2.00	2.00	2.18	100.19	71.25	2.00	MR
15	CML176/KULEN..	1.50	1.50	2.17	2.67	3.00	2.25	2.50	2.23	102.94	77.28	2.50	MS
16	TZMI746	2.00	3.00	3.00	2.00	2.00	2.00	2.00	2.29	105.00	92.44	2.00	MR
17	TZMI750	1.67	1.92	2.30	2.92	2.50	2.50	3.08	2.41	110.34	63.64	3.08	S
18	CZLQ5	1.00	1.92	2.17	2.50	2.83	3.00	3.08	2.36	111.71	80.00	3.08	S
19	MBRCF108-2-3-1	1.00	2.00	2.25	2.58	2.83	3.00	3.33	2.43	114.34	65.00	3.33	S
20	CML 543	1.00	1.50	1.50	2.84	3.00	3.59	3.67	2.44	115.83	82.50	3.67	S
	Most susceptible five												
1	124b (113)	3.00	3.50	4.00	4.33	4.50	4.50	4.64	4.07	188.33	94.88	4.64	HS
2	30H83-7	2.00	2.00	5.00	4.50	4.75	5.00	5.00	4.04	190.75	100.0	5.00	HS
3	CKL05019-#	2.00	3.50	4.00	4.75	4.75	4.67	5.00	4.10	192.48	100.0	5.00	HS

Entry	Pedigree	Weekly MLN severity score							Disease parameter				Reaction
		W1	W2	W3	W4	W5	W6	W7	PMS	AUDPC	DI	FS	
4	TZMI764	2.84	3.92	5.00	4.00	4.25	5.00	4.88	4.27	199.66	95.06	4.88	HS
5	30G 19F2-54-1-1-1-#-#	2.92	3.92	4.92	5.00	4.83	4.96	4.96	4.50	210.29	91.43	4.96	HS

W1-W7 = weekly MLN severity scores from first to seventh week after inoculation, PMS = pooled Mean of weekly MLN severity, AUDPC = area under disease progressive Curve, DI = disease incidence FS = final severity score, MR= moderately resistant, MS= moderately susceptible, S= susceptible, HS = highly susceptible.

Table 6. Maize lethal necrosis (MLN) disease severity scores and other diseases parameters of selected 20 most resistant and five most susceptible lowland adapted maize inbred lines evaluated in the greenhouse at Ambo, Ethiopia.

Entry	Genotype Name	MLN severity score							Disease parameter				Reaction
		W1	W2	W3	W4	W5	W6	W7	PMS	AUDPC	DI	FS	
	Most resistant 20												
1	MKL17K0422	1.00	1.00	1.00	1.00	1.50	1.75	2.00	1.32	60.38	54.55	2.00	MR
2	MKL17K0597	1.00	1.00	1.00	2.00	2.33	2.00	2.00	1.62	75.81	63.64	2.00	MR
3	MKL17K0343	1.00	1.00	1.50	2.00	2.00	2.25	2.50	1.75	81.38	63.64	2.50	MS
4	MKL1K0716	1.00	2.00	2.00	2.00	2.00	2.00	2.00	1.86	87.50	30.00	2.00	MR
5	MKL17K0236	1.00	1.50	2.13	2.25	2.00	2.38	2.50	1.96	92.31	72.73	2.50	MS
6	MKL17K0486	1.00	1.00	2.00	2.00	2.00	3.00	3.00	2.00	94.50	72.73	3.00	MS
7	MKL17K0581	1.00	1.00	1.00	2.00	3.00	3.00	3.00	2.00	94.50	72.73	3.00	MS
8	MKL1K0252	2.00	2.50	2.00	2.00	2.00	2.00	2.00	2.07	94.50	45.00	2.00	MR
9	MKL1K0577	2.00	2.50	2.00	2.00	2.00	2.00	2.00	2.07	94.50	40.00	2.00	MR
10	MKL17K0578	1.75	2.25	2.00	2.50	2.25	2.00	2.00	2.11	97.13	81.25	2.00	MR
11	MKL17K0028	1.33	1.00	1.00	2.00	3.00	3.00	4.00	2.19	99.16	100.0	4.00	S
12	MKL17K0457	1.50	1.50	2.63	2.00	2.00	2.75	3.00	2.20	101.50	83.33	3.00	MS
13	MKL1K0038	2.00	2.50	2.25	2.25	2.00	2.25	2.25	2.21	101.50	40.00	2.25	MS
14	MKL17K0049	1.75	1.50	2.50	3.00	2.25	3.17	2.50	2.38	112.86	71.37	2.50	MS
15	MKL17K0459	2.00	1.00	2.00	2.00	3.75	3.25	4.00	2.57	116.38	85.91	4.00	S
16	MKL1K0291	1.00	2.00	2.00	2.50	3.00	3.25	3.50	2.46	116.38	65.00	3.50	S
17	MKL17K0239	1.00	2.00	3.00	3.00	3.00	3.00	2.50	2.50	120.75	80.00	2.50	MS
18	MKL17K0504	1.67	2.00	3.00	2.50	3.50	3.00	3.00	2.67	124.85	77.78	3.00	MS
19	MKL17K0018	2.00	2.00	3.00	2.00	2.50	4.00	3.00	2.64	126.00	72.73	3.00	MS
20	MKL17K0043	1.00	2.00	3.00	3.00	3.50	3.00	3.00	2.64	126.00	83.33	3.00	MS
	Most susceptible five												
1	MKL17K0132	3.34	4.75	4.34	4.50	4.34	4.59	4.67	4.36	201.60	86.95	4.67	HS
2	MKL17K0094	2.00	3.75	4.25	5.00	5.00	5.00	5.00	4.29	203.00	100.0	5.00	HS
3	MKL17K0078	2.50	3.00	5.00	5.00	5.00	5.00	5.00	4.36	204.75	100.0	5.00	HS
4	MKL17K0632	3.25	4.50	4.38	4.88	4.75	4.75	4.67	4.45	207.08	92.88	4.67	HS
5	MKL17K0092	2.50	4.50	4.75	5.00	5.00	5.00	5.00	4.54	213.50	100.0	5.00	HS

W1-W7 = weekly MLN severity scores from first to seventh week after inoculation, PMS = pooled mean of weekly MLN severity, AUDPC = area under disease progressive curve, DI = disease incidence FS = final severity score, MR = moderately resistant, MS = moderately susceptible, S = susceptible, HS = highly susceptible.

Reaction of maize varieties to MLN

Maize varieties evaluated in this study showed significantly variable levels of resistance to MLN disease. Weekly disease severity score, other disease parameters, and reaction of the varieties to MLN were presented in Table 7. Among the disease parameters, AUDPC ranged from 45.5 to 173.8 with a mean of 116.4. Final severity score ranged from 1.0 to 4.4 with a mean of 3.0. Pooled mean of weekly MLN severity ranged from 1.0 to 3.7 with a mean of 2.5, while disease incidence ranged from 0.0 to 95.1% with a mean of 69.5. As expected, the susceptible control varieties had higher AUDPC values of 135.0 (Melkassa-2) and 154.6 (Limu), whilst the resistant control varieties had low AUDPC values ranging from 45.5 to 78.9. The six varieties from CIMMYT-Kenya CKMLN150075, CKMLN150088, CKMLN150076, CKMLN150074, CKMLN15150, and WE5135 that were used as resistant controls did not show MLN disease symptom, except WE5135 which had weekly severity rating ranging between 1.0 and 2.0 and AUDPC of 78.9). Serological test using DAS-ELISA, however, showed that all the varieties were positive for MCMV, which is the main cause of MLN disease.

All the maize varieties evaluated in this study were infected by MLN but had different levels of reaction to the disease. Most varieties showed moderately susceptible to highly susceptible reactions. Two varieties, which were collected from Ambo highland maize breeding program (Wenchi and Kolba), showed moderately resistant reactions to the disease.

Table 7. Maize lethal necrosis (MLN) disease severity scores and other disease parameters for 31 commercial and pre-commercial maize varieties evaluated in the green house at Ambo, Ethiopia.

Entry	Genotype name	Source	MLN severity score							PMS	AUDPC	DI	FS	Reaction
			W1	W2	W3	W4	W5	W6	W7					
1	CKMLN150075	BAR	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	45.50	0.00	1.00	R
2	CKMLN150088	BAR	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	45.50	0.00	1.00	R
3	CKMLN150076	BAR	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	45.50	0.00	1.00	R
4	CKMLN150074	BAR	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	45.50	0.00	1.00	R
5	CKMLN15150	BAR	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	45.50	0.00	1.00	R
6	Wenchi	AAR	1.00	1.97	1.00	1.00	1.00	1.00	1.00	1.14	52.27	52.50	2.00	MR
7	Kolba	AAR	2.00	2.25	1.25	2.00	1.25	1.00	1.00	1.54	68.25	45.00	2.00	MR
8	WE5135	BAR	1.00	1.00	1.75	2.00	2.00	2.00	2.00	1.69	78.93	74.25	2.00	MR
9	MH138	ESE	2.00	2.00	2.42	2.92	2.33	2.00	2.42	2.30	104.13	75.00	2.42	MS
10	Damot (P3506W)	PHSE	2.00	2.92	3.00	3.00	3.00	3.50	3.50	2.99	139.42	80.00	3.00	MS

Entry	Genotype name	Source	MLN severity score							PMS	AUDPC	DI	FS	Reaction
			W1	W2	W3	W4	W5	W6	W7					
11	Gibe 2	ESE	1.92	3.00	3.33	3.70	2.67	3.08	3.08	2.97	138.75	75.00	3.08	S
12	Melkassa-1	MAR	1.25	2.50	2.61	2.44	2.58	3.00	3.08	2.50	117.65	83.89	3.08	S
13	Huluka	AAR	2.00	3.63	3.50	3.00	3.50	3.00	2.63	3.04	143.06	72.28	3.25	S
14	SPRH	BAR	1.67	1.75	1.83	2.75	2.58	2.75	3.33	2.38	108.81	80.00	3.33	S
15	MH 130	ESE	1.33	1.92	2.00	2.50	3.33	3.33	3.47	2.55	120.01	63.64	3.47	S
16	BHQP548	BAR	2.00	2.17	2.22	2.39	2.67	3.39	3.50	2.62	120.94	95.10	3.50	S
17	Hawasa 1	ESE	2.00	3.50	4.00	4.00	4.00	3.25	3.50	3.46	161.88	90.91	3.50	S
18	Shone (30G19)	PHSE	2.00	2.71	3.13	3.58	3.67	3.83	3.92	3.26	152.54	92.31	3.50	S
19	Kortu (P2809W)	PHSE	2.67	3.92	4.00	4.00	4.00	3.83	3.67	3.73	173.83	91.67	3.50	S
20	Melkassa-2	MAR	1.00	2.42	2.58	3.17	3.30	3.70	3.56	2.82	135.03	88.06	3.56	S
21	BH546	BAR	2.00	2.00	2.17	2.25	2.62	3.62	3.60	2.61	120.88	94.89	3.60	S
22	BH661	BAR	1.70	2.00	2.16	2.41	2.61	3.33	3.61	2.55	117.82	93.31	3.61	S
23	Melkassa-1Q	MAR	1.00	2.50	2.58	2.75	3.50	3.61	3.64	2.80	133.52	86.67	3.61	S
24	Jibat	AAR	2.50	3.50	3.63	3.25	2.88	3.38	3.63	3.25	149.63	91.31	3.63	S
25	Melkassa-7	MAR	1.67	2.75	2.83	2.75	3.42	3.50	3.82	2.96	138.19	92.82	3.82	S
26	Melkassa-3	MAR	1.25	2.50	3.25	3.58	3.50	3.67	3.92	3.10	146.46	91.43	3.92	S
27	BH540	BAR	1.00	1.00	2.00	3.50	3.50	4.00	4.00	2.71	129.50	85.71	4.00	S
28	BH547	BAR	2.50	2.50	2.75	3.25	3.25	4.00	4.00	3.18	147.00	88.89	4.00	S
29	Argane	AAR	3.00	3.58	3.50	3.50	3.42	3.75	4.08	3.55	162.17	92.31	4.08	HS
30	LIMU (P3812W)	PHSE	2.00	3.00	3.00	3.33	3.67	4.00	4.33	3.33	154.59	92.86	4.33	HS
31	Morka	JAR	2.25	2.50	2.50	4.25	4.40	4.40	4.40	3.53	165.03	83.84	4.40	HS
Mean			**1.67**	**2.27**	**2.39**	**2.65**	**2.70**	**2.87**	**2.96**	**2.50**	**116.38**	**69.47**	**3.01**	

AAR = Ambo Agricultural Research Center, BAR = Bako Agricultural Research Center, ESE = Ethiopia Seed Enterprise, JAR = Jimma Agricultural Research Center, MAR = Melkassa Agricultural Research Center, PHSE = Pioneer Hi-Bred Seeds-Ethiopia, W = week, PMS =pooled mean severity, AUDPC = area under disease progressive curve, FS = final severity score, DI = disease incidence, R = resistant, MR = moderately resistant, MS = moderately susceptible, S = susceptible, HS = highly susceptible.

Correlations among MLN disease parameters

Positive and highly significant correlation coefficients were observed among each of the weekly disease severity scores and among other MLN disease parameters recorded, indicating the usefulness of these parameters in evaluating the reaction of maize inbred lines and varieties to MLN (Table 8). Disease incidence, final severity, pooled mean severity, and AUDPC were strongly correlated ($p < 0.001$), with correlation coefficient values ranging from 0.70 to 0.99 for

inbred lines and 0.87 to 0.99 for varieties. Particularly, the correlation between AUDPC and pooled mean severity was very strong ($r = 0.99$, $p < 0.0001$).

Table 8. Pair-wise Pearson correlation coefficient among maize lethal necrosis (MLN) disease parameters used to evaluate 275 maize inbred lines and 31 varieties under greenhouse conditions

MLN parameter	W1	W2	W3	W4	W5	W6	W7	PMS	FS	AUDPC	DI
					Varieties						
W1		0.76**	0.69**	0.63**	0.53**	0.52**	0.53**	0.69**	0.52**	0.67**	0.59**
W2	0.76**		0.91**	0.80**	0.75**	0.68**	0.66**	0.86**	0.67**	0.85**	0.71**
W3	0.66**	0.77**		0.93**	0.88**	0.81**	0.80**	0.94**	0.77**	0.95**	0.77**
W4	0.61**	0.73**	0.87**		0.93**	0.87**	0.88**	0.96**	0.86**	0.96**	0.83**
W5	0.52**	0.64**	0.79**	0.86**		0.94**	0.94**	0.96**	0.92**	0.97**	0.84**
W6	0.50**	0.61**	0.74**	0.81**	0.89**		0.98**	0.94**	0.96**	0.95**	0.87**
W7	0.46**	0.53**	0.66**	0.73**	0.82**	0.90**		0.94**	0.98**	0.94**	0.87**
PMS	0.72**	0.83**	0.91**	0.93**	0.92**	0.90**	0.84**		0.92**	0.99**	0.87**
FS	0.46**	0.53**	0.66**	0.73**	0.82**	0.90**	0.99**	0.84**		0.92**	0.91**
AUDPC	0.69**	0.82**	0.91**	0.94**	0.93**	0.92**	0.84**	0.99**	0.84**		0.87**
DI	0.40**	0.47**	0.60**	0.64**	0.69**	0.71**	0.73**	0.70**	0.73**	0.70**	
					Inbred lines						

** = significant difference at 1% probability level; W1-W7 = weekly MLN severity scores (1 to 5 scales), PMS = pooled mean of weekly MLN severity from first to seventh week after inoculation, FS= final severity, AUDPC = area under disease progress curve and DI = disease incidence (percentage of the number of leaves with MLN infection).

Discussion

MLN is one of the most important viral diseases affecting maize, and its impact is greater in the moisture stress and mid-altitude maize producing areas in Ethiopia than in the highland areas (Regassa et al., 2020). The use of resistant or tolerant maize varieties is possibly the most durable means of managing MLN disease (Boddupalli et al., 2020). In the current study, maize inbred lines and varieties evaluated revealed divergent reactions to MLN disease that ranged from

resistant to susceptible, indicating the possibility of identifying resistant genotypes for future breeding activities or commercial production. No considerable variations were observed among the replicates of each genotype as well as among individual plants in each experimental unit, indicating that maize plants of the same genotype had simi- lar reaction to MLN and showed similar expression of MLN symptoms. Generally, most of the inbred lines and varieties evaluated had susceptible reactions to MLN. High AUDPC values and high MLN severity scores were obtained as the disease progressed with time. Our previous findings also showed that most commercial maize varieties including BH540, BH140, BH661, Shone, Limu, and Melkassa-2 were susceptible to MLN (Regassa et al., 2020).

The rate of development in MLN disease symptoms indicated the susceptibility or resistance of the genotypes to MLN disease. The outcome for plant virus interaction is due to the absence of infection (incompatible interaction) or the establishment of infection (compatible interaction) (Garcia-Ruiz, 2018). Symptoms result from interactions of the host with the virus and as a result of compatible interaction which leads to the change of assimilates from the host plant to favor the virus infection process such as its replication, multiplication, and movement (Revers et al., 1999). As the disease progressed, severe chlorosis and mottling symptoms were developed that led to bright yellow followed by necrosis from the leaf margins of the maize plant. In the process of the infection, there is a disruption of the chloroplast function, which leads to inadequate chlorophyll production consequently mottling and mosaic symptom development (Mbega et al., 2016). The infected cell also has little starch in the chloroplast and disrupted mitochondria leading to reduced respiration and photosynthesis. The reduced respiration rate and photo- synthesis lead to leaf necrosis and eventual plant death (Wang et al. 2017).

Delayed symptom development or extended incubation periods observed in some inbred lines and varieties was an indication of either the presence of resistance genes or absence of susceptible genes in those genotypes that were providing a certain degree of MLN disease resistance. Similar observations were reported by Karanja et al. (2018) who screened different maize inbred lines to MLN in Kenya. Antiviral defense restricts viral RNA translation, virus rep- lication, movement, or virion assembly, resulting in reduced virus accumulation and/or a delay in virus movement with or without a hypersensitive response (Soosaar et al., 2005; Sanfacon, 2015).

Genotypes that showed resistant reaction and restricted development of disease symptoms might

carry desirable genes for MLN resistance. Ingvardsen et al. (2010) indicated that plant defense mechanism against viruses could be mediated by resistance genes which are observed as complete resistance or extreme resistance and that the virus replication could be hindered or gone undetectable among the infected cells. In some cases, viruses may not be detected in infected plant cells due to low titer of the virus that are not at the concentration of detectable by a less-sensitive DAS-ELISA test and requires further investigation by a molecular method using real-time quantitative PCR. A resistant highland maize inbred line (AMB17KN20-1) and CIMMYT-Kenyan resistant control varieties (CKMLN150075, CKMLN150088, CKMLN150076, CKMLN150074, and CKMLN15150) did not shown MLN symptoms, but MCMV was detected when tested with DAS-ELISA, indicating that these genotypes may exhibit MLN tolerance.

Although several inbred lines and varieties evaluated in this study showed resistant to moderately resistant reaction to MLN (1–2 scores), none of the genotypes were totally immune from MLN based on DAS-ELISA tests, except one highland maize inbred line (ABL-93). This study represents the first screening of large number of maize genotypes from Ethiopian breeding programs, and hence, lack of resistance gene in most of the genotypes evaluated is expected. Similar to the current findings, the first screening of commercial maize varieties marketed in Kenya showed high levels of susceptibility to MLN under artificial inoculation (Semagn et al., 2015). Since elite maize germplasm from the Ethiopian breeding programs was not developed for MLN resistance, the identification and utilization of sources of MLN resist- ance can help to manage the disease through the develop- ment of resistant varieties. CIMMYT has already developed a considerable number of African adapted MLN resistant inbred lines and hybrids under artificial inoculation at Naiva- sha MLN screening facility (Semagn et al., 2015, Beyene et al., 2017; Boddupalli et al., 2020). The resistant hybrids from CIMMYT can be introduced to Ethiopia for immediate deployment while the inbred lines can be used in hybrid combination or to introgress MLN resistance into locally adapted germplasm using conventional and marker assisted backcross breeding.

MLN-associated viruses cause systemic infections and the virus translocation from the point of inoculation depends on the cell-to-cell movement of its particles after the viral replication and establishment (Salaudeen and Aguguom, 2014). The establishment of infection is genetically deter- mined by the availability of host factors necessary for virus replication and movement and

by the balance between plant defense and viral suppression of defense responses. During the plant virus infection process, viral factors interact with host factors antiviral or proviral activities. Proviral factors condition susceptibility to viruses by participating in processes essential to the virus infection process, such as viral RNA translation, virus replication, movement, or virion formation. On the contrary, host factors with antiviral activity restrict viral RNA translation, virus replication, movement, or virion formation (Garcia-Ruiz, 2018). Plant viruses move cell-to-cell through plasmodesmata (Heinlein, 2015), and at the initial infection site, cell-to-cell movement results in the formation of local infection, after reaching the vascular system, viruses move long-distance and infect roots and young leaves and this leading to further infection and spread (Revers et al., 1999; Garcia- Ruiz, 2018).

Similar to the current finding, our previous study (Regassa et al., 2020) identified Melkassa-2 and Limu as susceptible varieties to MLN disease. This confirms the accuracy of these studies and reproducibility of the results. On the other hand, most of the resistant control hybrids, maintained the resistance status, except WE5135, which developed mild symptoms. This discrepancy might be attributed to environ- mental factor since the current study was conducted under greenhouse conditions and previous studies in Kenya were conducted under field condition at Naivasha as described by Gowda et al. (2015) and Sitonik et al. (2019). Mahuku et al. (2015a, b) and Guadie et al. (2019) reported that MCMV isolates found in Ethiopia are highly similar to those found in Kenya, and hence, the variation is likely not attributable to the virus strain used.

Significant and positive correlation coefficients observed among the MLN disease parameters indicated that most susceptible and most resistant maize genotypes could be successfully identified using one or few of these disease parameters. Zambrano et al. (2013) and Sitta et al. (2017) have identified susceptible and resistant maize genotypes using the same parameters used in the current study.

In conclusion, the use of genetic resistance is considered as the most economically and environmentally sustainable approach for plant virus disease management. Resistant inbred lines identified in this study might serve as sources of MLN disease resistant gene (s) in maize breeding programs, while the resistant varieties could be recommended for commercial production. In addition, the inbred lines can be used in the hybridization programs to study the mode of inheritance and gene actions for MLN resistance/tolerance. Further evaluation of these

inbred lines and varieties for responses to individual MLN causing viruses would help to establish information on the genetics of MLN resistance for effective utilization of the genotypes in the breeding programs. Ethiopian breeding programs need to introduce promising MLN resistant genotypes, mainly from CIMMYT, and introgress resistance genes into locally adapted, widely used and commercially important inbred lines using conventional, and marker assisted backcross breeding.

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