

ADDIS ABABA UNIVERSITY
SCHOOL OF GRADUATE STUDIES
INSTITUTE OF BIOTECHNOLOGY



Identification and Characterization of Maize Chlorotic Mottle Virus and Sugarcane Mosaic Virus Associated with Maize Lethal Necrosis Disease in Benishangul-Gumuz and Oromia Regions of Ethiopia

Mengistu Fentahun Mekureyaw

A Thesis Submitted to School of Graduate Studies of the Addis Ababa University in Partial Fulfillment of the Requirement for the Degree of Master of Science in Biotechnology

Addis Ababa, Ethiopia

October, 2016

Declaration

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

Mengistu Fentahun

Signature.....

Date.....

The work has been done under my supervision

Advisor: Tileye Feyissa (PhD)

Signature.....

Date.....

ADDIS ABABA UNIVERSITY
SCHOOL OF GRADUATE STUDIES
INSTITUTE OF BIOTECHNOLOGY

This is to certify that the thesis prepared by, **Mengistu Fentahun** entitled **Identification and Characterization of *Maize Chlorotic Mottle Virus* and *Sugarcane Mosaic Virus* Associated with Maize Lethal Necrosis Disease in Benishangul-Gumuz and Oromia Regions of Ethiopia** and submitted in partial fulfillment of the requirements for the degree of Master of science in Biotechnology complies with the regulations of the university and meets the accepted standards with respect to originality and quality.

Signed by the Examining Committee:-

1. Dr. Tesfaye Alemu (Examiner) Signature.....Date.....
2. Dr. Kifle Dagne (Examiner) Signature.....Date.....
3. Dr. Tileye Feyissa (Advisor) Signature.....Date.....

Dr. Tesfaye Sisay Signature.....Date.....

Director, Institute of Biotechnology

Identification and Characterization of Maize Chlorotic Mottle Virus and Sugarcane Mosaic Virus Associated with Maize Lethal Necrosis Disease in Benishangul-Gumuz and Oromia Regions of Ethiopia

Mengistu Fentahun Mekureyaw
Institute of Biotechnology, Addis Ababa University, Ethiopia

ABSTRACT: Maize (*Zea mays* L.) is a staple food for over 70 million peoples in Africa, and predominantly produced and consumed directly by small scale holder families. Small scale holder farming operations in sub-Saharan encompasses over 25 million hectares. Ethiopia ranked second in Africa with annual maize production estimated to 3.98 metric tons per hectare. In 2013, Ethiopian farmers in some areas experienced high yield losses (30 to 100%) from a maize disease, later identified as Maize Lethal Necrosis Disease (MLND). The disease causes symptoms ranging from leaf tissue mottling and malformed ears to premature plant death. MLND develops from synergistic co-infection by *Sugarcane Mosaic Virus* and *Maize Chlorotic Mottle Virus*. Information on the diversity and transmission of those viruses are limited in Ethiopia. Hence, further information and understanding have valuable input on the control, diagnosis and management of MLND. No attempts were also made to characterize and identify SCMV and MCMV strains of Oromia and Benishangul-Gumuz Regions of Ethiopia. Hence, this study was designed with the aim to study MLND responsible viruses via serological, molecular and artificial inoculation techniques. A total of 174 symptomatic leaf samples were collected from over 33 fields of Oromia and Benishangul-Gumuz regions. The survey result reveals different degree of MLND incidence ranging from 10-100% infestation of study fields. Using DSMZ's Double Antibody Sandwich-ELISA (DAS-ELISA) kit for serological assay, 43 samples showed strong reaction for MCMV antibody and only 14 samples were clearly positive for SCMV. Leaf samples from Benishangul-Gumuz didn't react with the MCMV antibody used. Reverse transcriptase polymerase chain reaction (RT-PCR) technique using specific viral primers revealed the presence of MCMV and SCMV in most samples. Some samples mainly from Benishangul-Gumuz region which were negative by DAS-ELISA were also positive by RT-PCR suggesting that the method was more sensitive. Further confirmation of serological and RT-PCR based identification and analysis of the genetic diversity of representative viral strains was done by direct sequencing of the RT-PCR products. The results indicated that both SCMV and MCMV strains shows strong homology with previous East African isolates deposited in the GenBank. The phylogenetic tree indicated that the virus isolates are highly similar with each other. The haplotype networking showed there is less diversified MCMV, SCMV isolates are relatively highly diversified with almost all isolates as an individual haplotype, and MCMV isolates showed strong similarity to Chinese isolates with less number of mutations. The artificial inoculation results showed that virus strains from the study areas were able to cause mosaic and leaf streak or necrosis symptom on sweet corn planted in greenhouse. In conclusion, this study unequivocally confirmed the association of MCMV and SCMV with MLND in the study areas and for the first time provided information on the genetic variability by sequence analysis of several Ethiopian samples. Generally, symptoms, incidence, DAS-ELISA, RT-PCR and artificial inoculation results clearly showed the presence and diversity of MCMV and SCMV in the study area.

Key words: DAS-ELISA; genetic diversity; MLND; RT-PCR; *Zea mays*

Acknowledgments

Foremost, I would like to express my sincere appreciation to my advisors Dr. Tileye Feyissa and Dr. Adane Abraham for their continuous support and encouragement during my study period. Their guidance, patience, motivation, and immense knowledge really helped me in all the time of research and writing-up.

Besides my advisors, I would like to express my kind appreciation and acknowledgments for the following institutions:-Ethiopian Institute of Agricultural Research: giving me the chance to join MSc. Program, partial financial support and use of laboratory facilities; Addis Ababa University: for allowing me to enroll in MSc. program and my particular recognition goes to all my teachers at Institute of Biotechnology of Addis Ababa University; Korea Program on International Agriculture (KOPIA-Ethiopia) for material and technical support of my field based research and Addis Ababa Science and Technology University for partial financial support during field survey and sample collection.

My sincere thanks also go to individuals: Dr. Dawit Tesfaye, who gave me full support during the bioinformatics aspect of my work; Dawit Beyene, a PhD student at Queensland University, Australia, whose technical advice was really valuable.

Dedication

This is for you, Mom. Thanks for always being there for me.

Your kindness, devotion and endless support during my journey; your selflessness
will always be remembered.

You left fingerprints of grace on my life. You shan't be forgotten.

Table of Contents

	Page
Abstract.....	iii
Acknowledgment.....	iv
Dedication.....	v
List of Tables	vi
List of Figures.....	vii
List of Appendices	viii
List of Abbreviations	ix
List of Virus Acronyms and Names.....	x
1. Introduction.....	1
2. Literature Review.....	5
2.1 Maize.....	5
2.1.1 Origin and Distribution.....	5
2.1.2 Taxonomy.....	6
2.1.3 Botany.....	7
2.1.4 Global Production.....	8
2.1.5 Production Constraints.....	10
2.2 Plant Viruses.....	11
2.2.1 Viral Disease of Maize.....	12
2.2.2 Wide spread Maize Viruses.....	13
2.3 Maize Lethal Necrosis Disease.....	17
2.4 Maize Virus Detection and Diagnosis.....	18
2.4.1 Symptomatology.....	20
2.4.2 Serological Methods.....	20
2.4.3 Nucleic Acid Based Methods.....	22
2.5 Maize Lethal Necrosis Disease Management.....	24
2.5.1 Reduction of Initial Inoculums.....	24
2.5.2 Reducing the Rate of Infection.....	25

3. Objectives.....	27
4. Materials and Methods.....	28
4.1. Study Area and Collection of Samples.....	28
4.1.1 Study Area.....	28
4.1.2 Sample Collection.....	28
4.2 DAS-ELISA.....	30
4.3 Total RNA Extraction and Quality Detection.....	32
4.4 Reverse Transcriptase Polymerase Chain Reaction (RT-PCR).....	32
4.5 RT-PCR Product Direct Sequencing and Sequence Analysis.....	33
4.6 Sap Preparation for Artificial Inoculation.....	34
5. Results.....	35
5.1 Symptomatology.....	35
5.2 Incidence of Virus Infestation.....	36
5.3 DAS-ELISA Results.....	37
5.4 Reverse Transcriptase PCR Results.....	39
5.5 Sequence and Sequence Analysis Results.....	40
5.6 Artificial Inoculation Results.....	46
6. Discussion.....	48
7. Conclusion.....	52
8. Recommendation.....	53
9. References.....	54
10. Appendices.....	64

List of Tables

Page

Table 1 List of primers used for viral genome part amplification..... 33

Table 2 Summary of Average incidence of MLND Symptoms in the Study area.....37

Table 3 Summary of the DAS-ELISA result using ELISA reader.....38

List of Figures	Page
Figure 1 Location Map of MCMV and SCMV study area.....	29
Figure 2 Symptomatic maize leaf samples and fields during sample collection.....	30
Figure 3 Different maize plant samples and fields showing range of symptoms	36
Figure 4 DAS-ELISA results of different levels of color development reaction to Virus specific antibodies	38
Figure 5 One step RT-PCR screening of SCMV and MCMV infected samples of maize.....	39
Figure 6 Phylogenetic tree of nucleotide sequence of 22 MCMV field isolates from maize fields and 19 MCMV accessions from GenBank	42
Figure 7 Phylogenetic tree of nucleotide sequence of 10 SCMV field isolates from Maize fields and 23 SCMV accessions from GenBank.....	44
Figure 8 Evolutionary Divergence Networking tree of all the MCMV isolates of this Study and the 19 isolates of the GenBank using Network 4.6 software.....	44
Figure 9 Evolutionary Divergence Networking tree of all the SCMV isolates of this Study and the 23 isolates of the GenBank. using Network 4.6 software.....	45
Figure 10 Different levels of symptoms development during artificial inoculation.....	47
Figure 11 Agarose gel bands showing positive sample for MCMV.....	47

List of Appendices

Page

Appendix 1 Double Antibody Sandwich ELISA (DAS-ELISA) Protocol.....	64
Appendix 2 Pure Helix™ Total RNA Purification Kit	66
Appendix 3 HelixCRIPT™ One-Step RT-PCR Kit [Hot-Taq]	70
Appendix 4 Data Sheet for Sample Collection and Incidence	72

List of Abbreviations

AARC-Assossa Agricultural Research Center

ABI- Applied Biosystems

ASARECA- Association for Strengthening Agricultural Research in Eastern and Central Africa

BCIP-5-Bromo-4-Chloro-3'-Indolyphosphate

CIP-International Potato Center

CSA- Central Statistics Agency

CGIAR- Consortium of International Agricultural Research Centers

CIMMYT- International Maize and Wheat Improvement Center

DAC-ELISA-Direct antigen Coating-ELISA

DAS-ELISA-Double Antibody Sandwich Enzyme Linked Immunosorbent Assay

DIBA- Dot-Immuno binding assay

ELISA- Enzyme Linked Immunosorbent Assay

FAO-Food and Agriculture Organization

IgG- Immunoglobulin G

IC-RT-PCR- Immuno-capture reverse transcription-PCR

mAb- Monoclonal Antibody

MLND- Maize Lethal Necrosis Disease

NCM-ELISA-Nitrocellulose Membrane Enzyme-linked Immunosorbent Assay

NGS- Next Generation Sequencing

pAb-Polyclonal Antibody

PBS- Phosphate Buffer Saline

PBS-T- Phosphate Buffer Saline+Tween20

PCR-Polymerase Chain Reaction

PVP- Polyvinyl pyrrolidine

RT-PCR- Reverse Transcription PCR

ssRNA- Single Strand Positive Sense RNA

TAS-ELISA- Triple Antibody Sandwich Enzyme-linked Immunosorbent Assay

TBS-Tris-Buffer Saline

T-TBS- Tris-Buffer Saline-Tween20

List of Virus Acronyms

BYDV-*Barley Yellow Dwarf Virus*

CMV-*Cucumber Mosaic Virus*

JGMV-*Johnson Grass Mosaic Virus*

MCDV-*Maize Chlorotic Dwarf Virus*

MCMV-*Maize Chlorotic Mottle Virus*

MDMV-*Maize Dwarf Mosaic Virus*

MMV-*Maize Mosaic Virus*

MRDV-*Maize Rough Dwarf Virus*

MRCV-*Maize Rough Chlorotic Virus*

MSV-*Maize Streak Virus*

MSSV-*Maize Sterile Stunt Virus*

MStV-*Maize Stripe Virus*

MWLMV-*Maize White Line Mosaic Virus*

MYSV-*Maize Yellow Stripe Virus*

SrMV-*Sorghum Mosaic Virus*

SCMV-*Sugarcane Mosaic Virus*

SCSMV-*Sugarcane Streak Mosaic Virus*

SCYLV-*Sugarcane Yellow Leaf Virus*

TMV-*Tobacco Mosaic Virus*

WSMV-*Wheat Streak Mosaic Virus*

1. Introduction

Maize or corn (*Zea mays* L) belongs to the grass family *Poaceae* (*Gramineae*). It has a diploid number of twenty chromosomes ($2n = 20$) (Tito; *et al.*, 1991). It has a large genome with a total gene number of 42,000 – 56,000 (Yuan *et al.*, 2013). The use of maize varies in different countries. Maize is cultivated on nearly 150 million hectare in about 160 countries having wider diversity of soil, climate, biodiversity and management practices that contributes 36 % in the global grain production (Shaw, 1988; Dowsell *et al.*, 1996). However the major maize production areas are located in temperate regions of the globe. It is clearly indicated that the United States, China, Brazil and Mexico account for 70% of global production (Ranum *et al.*, 2014).

Maize is Ethiopia's leading cereal crop in terms of production with 6.2 million tons produced in 2013 by 9.3 million farmers across 2 million hectares of land (CSA, 2013). In terms of cultivated area, it is the second most widely cultivated crop next to tef [*Eragrostis tef* (Zucc.) Trotter]. Ethiopian farmers grow maize, primarily for subsistence with 75% of all maize output consumed by farming households, making it a key crop for overall food security and for economic development in the country (CSA, 2013).

Maize lethal necrosis disease was first identified in USA in 1976 where it was reported to be caused by double infection with *Maize Chlorotic Mottle Virus* (MCMV), genus *Machlomovirus*, family Tombusviridae, and any of cereal viruses in *Potyviridae* family; *Sugarcane Mosaic Virus* (SCMV), *Maize Dwarf Mosaic Virus* (MDMV) or *Wheat Streak Mosaic Virus* (WSMV) (Nault *et al.*, 1978). Symptoms of MLND includes; elongated yellow streaks parallel to leaf veins, streaks may coalesce to create chlorotic mottling, that may be followed by leaf necrosis (Nelson *et al.*, 2011; Makone *et al.*, 2014) which may lead to “dead heart” symptom and plant death (Wangai *et al.*, 2012), premature aging of the plants (Gordon *et al.*, 1984), failure to tassel and sterility in male plants, malformed or no ears (Uyemoto *et al.*, 1981; Gordon *et al.*, 1984), failure of cobs to put on grains and rotting of cobs (Wangai *et al.*, 2012). The viruses causing MLND are transmitted by several insect vectors which include thrips and beetles for MCMV and aphids for SCMV and MDMV (Brunt *et al.*, 2000; Wangai *et al.*, 2012). The known WSMV vector is the eriophyidae (Slykhuis, 1995). Single infection of maize with one of these viruses causes mild symptoms on maize and does not cause MLND. Low level of transmission through maize seeds has been reported for both MCMV and SCMV (Brunt *et al.*, 2000). However, double infection by these viruses cause MLND and results in yield losses ranging from 50 to

90% have been reported (Niblett and Claflin, 1978) and can reach 100% where the disease pressure is high.

The first outbreak of MLND was reported in East Africa in September 2011 along rift valley regions of Kenya (Wangai *et al.*, 2012). Regions that were reported to have the disease include; Bomet, Naivasha, Narok, Chepalungu, Sotik, Transmara, Bureti, Nakuru, Konoin, South Narok, Mathira East, Imenti South Districts and Nyeri (Wangai *et al.*, 2012) and subsequently in Rwanda (Adams *et al.* 2014), and the Democratic Republic of Congo (Lukanda *et al.* 2014). In August 2012, this disease was also reported in Tanzania around border regions especially Northern zone and along Lake Zone (Makumbi and Wangai, 2013). Northern zone includes Arusha (Karatu, Mlangarini, Longijave and Ngaramtoni), Kilimanjaro (Hai district in Nshara and lower Moshi) and Manyara in Kiru, Babati, Mbulu and Simanjiro. Lake Zone includes Mwanza, Mara and Shinyanga. In Uganda, the disease was first reported in October 2012 in Busia then in border district of Tororo, Mbale and Kapchorwa (ASARECA, 2013). MLND was first reported in February 2013 in Gisesero site, Musanze District in Northern Province of Rwanda and it then spread to western Province (Adams *et al.*, 2014; ASARECA, 2013). This disease is not reported yet in Burundi (ASARECA, 2013).

In Ethiopia, the disease was first reported in 2013/2014 cropping seasons to cause various levels of damage ranging from low infection rate to total crop failure (Mahuku *et al.*, 2015). In some maize fields in Koka area in East Shewa Zones of Oromia Region and Duguna Fango area of Wolayta zone in SNNPR, the disease has caused a total crop failure forcing farmers to replace maize with other crops. There are more reports in recent months indicating the occurrence and damage of the disease in many districts of Jinka area of SNNPR as well as in Amhara region in the north. This entails the disease is expanding in higher rate in Eastern regions of Africa within relatively shorter period of time. Therefore, it is high time to develop proper management system to halt its distribution to other parts of Africa and the world as well.

Small scale laboratory analysis of the MLND infected maize samples by standard ELISA method from Oromia region in recent months at Ambo Plant Protection Research Center (APPRC) has confirmed the association of MCMV and SCMV with MLND. It should be noted however here that, SCMV has been known to commonly occur on maize in Ethiopia since long time causing

mild mosaic disease (Abraham and Albrechtsen, 1998; Lencho *et al.*, 1997). Hence, only the MCMV component of the MLND is new to Ethiopia. MDMV occurs in Ethiopia but is less widely distributed than SCMV whereas WSMV has not been yet identified in Ethiopia (Lencho *et al.*, 1997). It is therefore, believed that like in other East African countries, MLND in Ethiopia is caused by a mixed infection of MCMV and SCMV, with the former being the new and the most important component (Mahuku *et al.*, 2015).

Although there are both field and laboratory evidences that unequivocally confirms the occurrence and importance of MLND and its main causal viruses in various parts of Ethiopia, there is no comprehensive report on the extent of its spread in the country as the disease is only recently reported. Maize plants exhibiting severe yellowing and chlorotic mottle symptoms were observed in the Upper Awash Valley of Ethiopia. From a survey of 12 farms in the Central Rift Valley, 100 symptomatic maize leaf samples were collected and, 38 were positive for MCMV, 15 for SCMV and 27 were positive for both MCMV and SCMV (Mahuku *et al.*, 2015). Given the serious potential economic consequence of the disease, there is obviously a need to conduct a comprehensive national survey to determine the spread of the disease. Its geographical distribution as well as the diversity of the viruses causing the disease needs to be identified as many viruses can synergistically interact to cause MLND.

In addition, there is a need to collect and identify the potential insect vectors associated with diseased maize or other host plants and determine whether they transmit the virus (es) in question and their transmission efficiency. Such baseline information on the geographical distribution of the disease and its vector will help in determining areas which are free from disease but may be under threat so that regulatory authorities can monitor disease outbreaks in new areas and introduce control measures such as local quarantine enforcement. In the same survey, information on farmers' perception on the disease and the indigenous knowledge of how farmers try to cope with this new disease can also be collected and used as an input in future research to manage the disease. Finally, the information generated in such a survey will help in the identification of disease hotspot areas for screening of maize MLND resistance and also in identifying areas with severe MLND incidence.

Although some precautionary regulatory steps taken to prevent possible spread of MLND with seed from one plant to the other or one location to the other in East Africa including Ethiopia are

justified, there is still a wide gap in our understanding as to whether and to what extent MCMV in East African region is transmitted by seeds, and by which insect vectors; beetle, thrips or both. Furthermore, there are reports that apart from maize, the MLND causing viruses in USA are harbored by a number of alternate grass hosts that contribute to the epidemiology of the disease by acting as overwintering hosts (Uyemoto, 1983). The potential plant hosts for MLND causing viruses and their vectors in Eastern Africa is not known. There is therefore, a critical need to generate information as to the methods of natural modes of transmission and survival of viruses causing MLND in Ethiopia to guide regulatory and management decisions.

The most desirable and feasible method in virus disease management is the use of host resistance. Preliminary screening of maize varieties for MLND in recent years in Kenya indicated that there is genetic variation in genotype response. Hence, extensive screening of maize germplasm in Ethiopia using standardized protocols is believed to lead to identification of sources of resistance. Furthermore, the knowledge of prevalent strains of MCMV and SCMV occurring in various regions of the country is important in directing breeding efforts to the prevalent strain. Previous attempts to characterize maize infecting strain of SCMV suggested the prevalence of a strain close to SCMV-MDV (Adane Abraham, 1999, unpublished data, PPRC progress report). It is however not clear if MCMV which is a new pathogen is interacting with this common SCMV strain or a new SCMV strain or other completely new potyviruses such as MDMV and WSMV.

Since MLND is a new disease to Africa in general and Ethiopia in particular, there is near complete lack of scientific findings that will lead to its effective management. For the same reason, apart from attempts to test some selected seed samples for the viruses by request of the Ministry of Agriculture based on experience of other countries, there is no much research projects initiated to date to fill the existing gap. Hence, the aim of this research is to generate scientific findings and technologies that will enable the integrated management of this newly emerged disease which is rapidly becoming a serious challenge to maize production in Ethiopia. Due to its devastating nature and lack of appropriate control options, this newly emerged disease is considered as a serious menace to maize production and hence to national food security.

2. Literature Review

2.1 Maize

Maize (*Zea mays* L.), called corn in some region of the world, is a grass of tropical origin that has become the major grain crop in the world in terms of total production, with recent production around 800 million tons per year. It is one of the oldest human-domesticated plants (Abdolreza Abbassian, 2006). Most maize grain produced is used as animal feed; in less developed countries it is, however, also a staple food. It is also used as raw material for many industrial uses, including bio-fuel production (Olawale and Tontsa, 2015). The name maize is derived from Arawak-Carib word *mahiz* and the term corn most likely originates from the Germanic ‘**korn**’ which referred to any edible grass. It is also known as corn in North America (Purse-glove, 1976). It is important staple crop in east Africa (FAOSTAT, 2013) and is one of the most widely cultivated gramineous plants in the regions due to its ability to grow in diverse climates (Agbonifo and Olufolaji, 2012).

2.1.1 Origin and Distribution

Maize originated under warm, seasonally dry conditions of Mesoamerica, and was by human selection converted from a low-yielding progenitor species into its modern forms, with a large rachis (cob) of the female inflorescence bearing up to 1,000 seeds. The center of origin for *Zea mays* has been established as the Mesoamerican region, now Mexico and Central America (Watson & Dallwitz, 1992). As revealed by archaeological records and phylogenetic analysis, domestication of maize began at least 6000 years ago, occurring independently in regions of the southwestern United States, Mexico, and Central America (Matsuoka *et al.*, 2002). Maize spread around the world, particularly in temperate zones, after European discovery of the Americas in the 15th century (Farnham *et al.*, 2003). It was the staple food for a number of civilizations in the Americas over several millennia preceding the arrival of Europeans in the late 15th century. In pre-Columbian times it was grown from Chile to Northern Canada and from sea level to 3300m altitude. The early American civilizations were based on maize which made settled life possible. It was the basis of life for the Aztec and Maya people, whose life evolved around the “milpa” (cornfield) (Shaw, 1988, Dowsnell *et al.*, 1996).

The Portuguese introduced maize to Southeast-Asia from the America in the 16th century. The maize was introduced into Spain after the return of Columbus from America and from Spain it went to France, Italy and Turkey. In India, Portuguese introduced maize during the seventeenth

century. From India it went to China and later it was introduced in Philippines and the East Indies (Jean du Plessis, 2003).

There is no evidence of maize cultivation in Africa until the 16th century (Miracle 1966), when it was introduced from the Americas to Africa along the western and eastern coasts, gradually moving inward as a ration with the slave trade. Before 1965, the rise of maize production in all of African countries was propelled to a greater or lesser extent by the following driving factors: the agronomic suitability of maize, the British starch market, milling technology, the integration of Africans into the settler wage economy; and market and trade policies promoted by settler farm lobbies (Miracle, 1966).

2.1.2 Taxonomy

Maize belongs to the tribe Maydeae of the grass family *Poaceae*. “Zea” (zela) was derived from an old Greek name for a food grass. The genus *Zea* consists of five species including [*Z. diploperennis*](#), [*Z. perennis*](#), [*Z. luxurians*](#), [*Z. nicaraguensis*](#), and [*Z. mays*](#). The species *Zea mays* is divided into four subspecies: *huehuetenangensis*, *mexicana*, *parviglumis* and *mays* of which the subspecies *mays* is economically important. The other first three subspecies are teosintes which are wild grasses in Mexico and Central America (Doeblay, 1990; Mangelsdorf *et al.*, 1981). An early hypothesis on origin of maize proposed that maize was produced by natural hybridization between two wild grasses, a species of *Tripsacum* and a perennial subspecies of teosinte (*Zeadiploperennis*). Further, teosinte was crossed with wild maize and the modern maize was produced as a result (Mangelsdorf, 1974). However the hypothesis about origin of maize is not limited to this idea and different authors have different suggestions (Radu *et al.*, 1997; Purseglove, 1972; Eubanks, 1993).

2.1.3 Botany

Maize (*Zea mays*) is a thick-stemmed annual grass, usually with a single stem, one to four meters tall, with one or more tillers. It is monoecious and diclinous, with male and female inflorescences

born separately on the same plant. Maize is generally protandrous, that is, the male flower matures earlier than the female flower. Within each male flower spikelet, there are usually two functional florets, although development of the lower floret may be delayed slightly in comparison to the upper floret. Each floret contains a pair of thin scales i.e. lemma and palea, three anthers, two lodicules and rudimentary pistil. Pollen grains per anther have been reported to range from 2000 to 7500 (Dhillon and Prasanna, 2001).

Maize is an annual grass plant ranging in height from 40 cm up to five meters with a diameter of between five and six centimeters. It is woody, and filled with sweet pith, and with nodes and internodes that can commonly be around 20 cm each. There are a great number of varieties in terms of size, but the most commonly cultivated range from one to three meters. Maize has shallow, fibrous roots that grow to a maximum depth of only 50 cm. Aerial, adventitious roots also form at the nodes at the base of the stem. Maize leaves are very large (up to 10 cm wide and 1 m long) and sheath-like (at their base they wrap round the stem) with a flat, extended blade in the shape of a strip with parallel veins. Under these leaves and close to the stems, the ears grow (Jean du Plessis, 2003).

Maize has a high photosynthesis efficiency which is made possible by the specialized anatomical and biochemical features that enable a so-called “C4” photosynthesis. This trait is shared by only a few other crops, including sorghum and sugarcane. Legumes and most other grass crops have what is known as C3 photosynthesis, which renders them less responsive to high light and temperature and, hence, lower-yielding. C4 photosynthesis also confers high water use efficiency: maize can produce one kg of dry weight using only about 40 kg of water, compared to water use ratios of 60 kg or more in most C3 crops (Steven *et al.*, 2014).

Maize is among the most human-modified crops on earth. Its assumed progenitor is teosinte, a wild relative with a very small rachis that breaks at maturity to release 10 to 12 seeds enclosed in capsules. Selection produced a maize plant that can grow up to 5 m high, with a rachis on a single branch that contains as many as 800-1000 kernels covered by modified leaves (husks) that protect the kernels from desiccation (Graham *et al.*, 2004).

Human intervention in the development of maize as a crop is aided by the fact that maize is a monoecious crop, with separate male (the tassel) and female (the ear) flower structures borne on

the same plant. Most unusually, the male and female flowers are separated by a distance of one or more meters, and pollen must travel this distance in order to effect pollination. Pollens are dispersed by wind, making maize highly cross-pollinated. At the same time, it is relatively easy to capture pollen from the tassel and to prevent pollination by covering the extended pistils before they emerge. This aids in controlling pollination and enables the making of planned crosses between or within plants (Hoisington *et al.*, 1998).

Successful maize production depends on the correct application of production inputs that will sustain the environment as well as agricultural production. These inputs are, inter alia, adapted cultivars, plant population, soil tillage, fertilization, weed, insect and disease control, harvesting, marketing and financial resources. It needs 450 to 600 mm of water per season, which is mainly acquired from the soil moisture reserves. About 15 kg of grain are produced for each millimeter of water consumed. At maturity, each plant will have consumed 250 liter of water. The total leaf area at maturity may exceed one square meter per plant (Smale and Jayne, 2003).

2.1.4 Global Production

Maize is a cereal crop which is cultivated widely throughout the world and has the highest production among all the cereals. The worldwide production of maize was more than 960 MnMT in 2013/14. It is an important food staple in many countries and is also used in animal feed and many industrial applications. The crop has tremendous genetic variability, which enables it to thrive in tropical, subtropical, and temperate climates (Rosegrant *et al.*, 2008).

Maize is one of the most versatile crops having wider adaptability under varied agro-climatic conditions. Globally, maize is known as queen of cereals because it has the highest genetic yield potential among the cereals. It is cultivated on nearly 150 million ha in about 160 countries having wider diversity of soil, climate, biodiversity and management practices that contributes 36 % (782 MT) in the global grain production. USA is the largest producer of maize and contributing nearly 35 % of the total production in the world and maize is the driver of its economy.

United States has a large maize surplus, which also makes it the largest maize exporter. Brazil, Ukraine and Argentina are the other key maize producing countries after USA. The four countries, together, account for 80-85% of the total exports of maize. Maize is by far the largest component of global coarse-grain trade. Most of the maize that is traded is used for feed; smaller amounts are traded for industrial and food uses. Japan has negligible maize production and has consistently been the top maize importer in the world. About ~90 per cent of Japan's maize requirements come from USA and another 8-9 per cent from Argentina and Brazil. China and Mexico, though being amongst the top maize producing nations, are also top importers (Heisey and Edmeades, 1999).

Mexico, due to its close proximity to USA, imports 99 per cent of its maize from USA. While Mexico has consistently been amongst the top importers; China has become a maize deficit country over the past five years due to the high demand growth from feed industry. However, there has been a declining trend in maize exports from USA. The reason for decrease in exports from USA is due to maize being consumed locally for production of ethanol. The rising demand for maize and decrease in imports from USA has led to an increase in prices globally (FAOSTAT, 2010).

Maize is produced on nearly 100 million hectares in developing countries, with almost 70% of the total maize production in the developing world coming from low and lower middle income countries (FAOSTAT, 2010). By 2050, demand for maize will double in the developing world, and maize is predicted to become the crop with the greatest production in the developing world and globally by 2025 (Rosegrant *et al.*, 2008). In large parts of Africa, maize is the principal staple crop; accounting for an average of 32 % of consumed calories in Eastern and Southern Africa, rising to 51 % in some countries. Heisey and Edmeades (1999) estimated that one quarter of the global maize area is affected by drought in any given year. Additional constraints causing significant yield and economic losses annually include low soil fertility, pests and diseases. It is difficult to give an accurate figure on combined maize yield losses due to these stresses; however it is likely to be extensive.

Maize is Ethiopia's largest cereal crop in terms of total production, area planted, and number of farm holdings. Corn accounts for 20 % of the total area covered by cereal and around 30 % of the total cereal production. In addition to the highest total production per year and the highest per

hectare yield, corn is also the single most important crop in terms of the number of farmers engaged in cultivation (Abu Tefera *et al.*, 2014).

In developed countries, maize is consumed mainly as second-cycle produce, in the form of meat, eggs and dairy products. In developing countries, maize is consumed directly and serves as staple diet for some 200 million people. Most people regard maize as a breakfast cereal. However, in a processed form it is also found as fuel (ethanol) and starch. Starch in turn involves enzymatic conversion into products such as sorbitol, dextrine, sorbic and lactic acid, and appears in household items such as beer, ice cream, syrup, shoe polish, glue, fireworks, ink, batteries, mustard, cosmetics, aspirin and paint (Jean du Plessis, 2003).

2.1.5 Production Constraints

a. Abiotic Constraints

Farmers consistently singled out drought as the key abiotic constraint to maize production in all regions. Drought was nevertheless a concern even in primarily irrigated systems due to periodic uncertainty regarding water availability and timing of availability. Farmers in most groups perceived a worsening trend in drought and its impacts on maize yields. The worsening trend for early frost is also a concern in different countries, because it affects the timing of planting. Surface water logging that often has negative effects on yields was reported in different parts of the world. Farmers in different countries also identified floods, soil erosion, and soil infertility as having considerable negative impacts on maize productivity (Meng *et al.*, 2006).

Soil fertility was one of the most serious constraints to maize production. Due to a number of socio-economic factors, the primary input into maintaining and improving soil fertility is manure/compost. Farmers complained that they do not have access to adequate quantities of manure/compost because of diminishing access to quality fodder for their animals. In comparatively accessible areas, fertilizer is used to supplement manure/compost. Traditional planting, lodging and weeding practices are labor intensive and have significant impact on productivity (Paudyal *et al.*, 2001).

b. Biotic Constraints

Farmers identified a wide range of field insect pests, including corn borer, cutworm, and corn leaf aphid, which were common across all the maize agro-ecological regions. Problems with other insects such as mole cricket, wireworm, armyworm, and red spiders varied across maize systems. Caterpillars, grasshoppers, and weevils are also among the damaging insects (Meng *et al.*, 2006). White grubs (*Phyllophaga* spp. and *Cyclocephala* spp.), stem borers (*Chilo partellus*), and termites (*Microtermes* spp. and *Macrotermes* spp.) were major maize field insects in all agro-ecologies. Army worms (*Spodoptera* spp.) and cutworms (*Agrotis* spp. and other species) were also major problems in all agro-ecologies (Paudyal *et al.*, 2001).

Aphid (*Rhopalosiphum* spp.), locust, red ant, and tassel beetle were also reported by farmers due to their substantial yield loss. Stem borers can be particularly problematic in spring and summer plantings when temperatures and insect reproduction rates are high (Paudyal *et al.*, 2001). Grain weevils (*Sitophilus* spp.) and rodents were the primary cause of post harvest storage losses across all maize agro-ecological regions. Farmers in different rain fed spring/fall/winter maize systems also described storage problems caused by moths (*Sitotroga cerealella*). Maize production also faced a great obstacle due to reported more than disease causing 57 types of viruses (Meng *et al.*, 2006).

Diseases identified by farmers included head smut (*Sphacelotheca reiliana*), turcicum blight (*Helminthosporium turcicum*), downy mildew (*Sclerospora* spp.) and Banded leaf and Sheath blight (*Rhizoctonia solani*). Turcicum Leaf Blight is ubiquitous in hill environments and can cause severe losses if the variety does not have good genetic resistance.

2.2 Plant Viruses

Plant viruses are infectious, intracellular and obligate pathogens that are too small to be seen with a light microscope, but despite their small size, they can cause lethal diseases in plants.

They are found everywhere in nature or it can be said that wherever cellular life occurs, viruses also occur. Virus particles are not complete cells so they cannot carry out functions of their own. The size of most of the viruses falls in the range of 30 and 100 nm. However, there are many exceptions, like the poxviruses has oval shape virions and have size of 200 to 400 nm. The genetic material of viruses can be either DNA or RNA, occurring as either single stranded or double stranded molecules in nature and protein coat may be wrapped around the nucleic acid. The genetic material of viruses is overlapped by a protein coat. Some viruses may possess a wrap over a protein coat; other may have internal proteins (Hull, 2002).

Plant viruses do not have the molecular machinery to replicate, have to move from infected to uninfected plant for survival otherwise, they may not be able to survive. The history of plant virus diseases starts from the early sixteenth century. The first plant virus disease reported in 1576 was tulip flower breaking disease (tulip mosaic). Plant viral diseases are serious threats to crops and cause economic losses in variety of crops by reducing yield and compromising quality (Kang *et al.*, 2005). There are about 1000 plant-infecting viruses known today and most plant viruses have a broad host-range (Hull, 2002). The first plant virus discovered was *Tobacco mosaic virus* (TMV). Plant viruses have been classified in 73 different genera, of which 49 have been divided into families. On the basis of genome, the plant viruses can also be classified into two groups i.e. DNA and RNA viruses (Khan and Dijkstran, 2006).

Plant viruses are widespread and economically important plant pathogens. Virtually all plants that humans grow for food, feed, and fiber are affected by at least one virus. It is the viruses of cultivated crops that have been most studied because of the financial implications of the losses they incur. However, it is also important to recognize that many “wild” plants are also hosts to viruses. Although plant viruses do not have an immediate impact on humans to the extent that human viruses do, the damage they do to food supplies has a significant indirect effect. The study of plant viruses has led to the overall understanding of viruses in many aspects. Virus diseases have a disastrous effect on crop yields and threaten the food producing potential of Africa and other parts of the world (Gelderblom, 2008).

2.2.1 Viral Disease of maize

The sixth report of the International Committee on Virus Taxonomy (ICTV) (Murphy *et al.*, 1995) lists *maize chlorotic dwarf virus* (MCDV, *Sequiviridae*, *Waikavirus*); *maize chlorotic mottle virus* (MCMV, *Machlomovirus*); *maize dwarf mosaic virus* (MDMV, *Potyvirus*); *maize mosaic virus* (MMV) and *maize sterile stunt virus* (MSSV, *Rhabdoviridae*); *maize rayado fino virus* (MRFV, *Marafivirus*); *maize rough dwarf virus* (MRDV, *Reoviridae*, *Fijivirus*); *maize streak virus* (MSV, *Geminiviridae*, *Mastrevirus*); *maize stripe virus* (MStV, *Tenuivirus*) and *maize white line stripe viruses* (MWLMV, unassigned) as viruses mainly occurring in maize. The VIDE database (Brunt *et al.*, 1997) lists another 57 viruses as commonly infecting maize, including *cucumber mosaic virus* (CMV, *Bromoviridae*, *Cucumovirus*) and BYDV, as well as *maize yellow stripe virus* (MYSV, *Tenuivirus*). McGee (1988) gives another listing, with some overlap, but with a considerable amount of information on virus distribution and severity.

2.2.2 Wide Spread Maize Viruses

Maize Chlorotic Mottle Virus (MCMV)

MCMV is the only species in the genus *Machlomovirus* family *Tombusviridae* (Stenger and French, 2008; King *et al.*, 2011), closely related to members of the genus *Carmovirus*. It is an isometric single component particle containing 4.4 kb single stranded positive sense genomic RNA (ssRNA) (Goldberg and Brakke, 1987; Lommel *et al.*, 1991) and has a smooth spherical or hexagonal shape with a capsid protein of 25 kDa (Lommel *et al.*, 1991).

In early stages, the youngest leaves of the infected plants show fine chlorotic spots that coalesce and develop into broad chlorotic stripes along the veins. These chlorotic stripes contrast with dark green tissue when observed against the light. Leaves showing chlorosis finally die. Plants are stunted because of shortened internodes. Infected plants produce fewer and smaller ears. In most cases, the male inflorescence is malformed. The virus is transmitted mainly by several chrysomelid leaf beetles such as *Chaetocnema pulicaria* and *Diabrotica* spp., over a short period of time. Reports indicate that it is transmitted at very low rates via infected seed. When MCMV occurs in combination with *sugarcane mosaic virus* (SCMV), *maize dwarf mosaic virus* (MDMV) or *wheat streak mosaic virus* (WSMV), it produces a severe reaction and cause a disease known as maize lethal necrosis (MLN) (Carlose, 1984).

Sugarcane Mosaic Virus (SCMV)

SCMV is one of the major viruses in the genus *Potyvirus*, family *Potyviridae*. The virus is not enveloped having filamentous flexuous particles (700-760 nm long and 13-14 nm in diameter) of single stranded positive sense RNA (Teakle *et al.*, 1989).

These viruses are transmitted by several genera and species of aphids, including *Rhopalosiphum maidis* (Fitch) and also by seed at low rates. After feeding on an infected plant, the aphid can immediately transmit the virus. These pathogens can infect other grass and cereal hosts, such as sorghum, Johnson grass, and sugarcane. No infection occurs in broad-leaf plants. Infected plants develop distinct mosaic irregularities in the distribution of normal green color on the youngest leaf bases. Sometimes the mosaic appearance is enhanced by narrow chlorotic streaks extending parallel to the veins. Later on, the youngest leaves show a general chlorosis, and streaks are larger and more abundant. As plants approach maturity, the foliage can turn purple or purple-red. Depending on time of infection, there may be severe stunting of the plant. Plants infected early may produce nubbins or be totally barren (Thorat *et al.*, 2015).

Maize Chlorotic Dwarf Virus (MCDV)

Infected plants initially show small chlorotic spots developing later into a general chlorosis in the whorl leaves. Plants become stunted due to shortening of internodes, and leaves may become reddish late in the season, resembling the reddening symptoms caused by corn stunt and maize bushy stunt. MCDV is transmitted by the leafhoppers *Graminella nigrifrons* (Forbes) and *G. sonora* (Ball) for an extended period of time after they have fed on infected plants. Johnsongrass serves as a reservoir host for the virus and the vector when maize is not being grown (Carlose, 1984).

Maize Dwarf Mosaic Virus (MDMV)

MDMV belongs to genus *Potyvirus*, family *Potyviridae* (Giolitti *et al.*, 2005). The virus is a single stranded positive sense RNA (ssRNA) with a flexuous filaments viral particle of 750 nm long and 13 nm wide (Williams and Alexander, 1965; Bancroft *et al.*, 1966; Autrey, 1983).

Maize dwarf mosaic disease is one of the most important viral diseases of maize worldwide. The disease is caused by a set of related viruses classified as the “sugarcane mosaic subgroup” of the virus genus *Potyvirus* (Shukla *et al.* 1992). The major disease causing viruses are *Maize dwarf mosaic virus* (MDMV), which is distributed throughout the southern USA, Europe, and Australia, and *Sugarcane mosaic virus* (SCMV, formerly classified as MDMV-B), which is found worldwide (Jones *et al.* 2011). Two other viruses in the sugarcane mosaic subgroup also cause maize dwarf mosaic disease: *Johnson grass mosaic virus* (JGMV) and *Sorghum mosaic virus* (SrMV) (Shukla *et al.* 1992).

Maize Mosaic Virus (MMV)

The disease has been found in many countries worldwide. The vector is the *planthopper* *Peregrinus maidis*, which transmits the virus for most of its life after feeding on an infected plant. The vector also transmits maize stripe virus. Hosts for MMV include maize, sorghum, and a few other graminaceous species. Plants are most susceptible when infected 4 to 6 weeks after emergence. The most conspicuous symptoms are dwarfing and striping along the veins. Degree of dwarfing depends on plant age at infection. Because internodes are shortened, leaves appear “crowded” and erect. Fine continuous stripes develop along the veins beginning at leaf bases. Later symptoms include shorter-than-normal leaves with a rough and fleshy appearance. Stripes may be dark yellow, and may finally become necrotic. Prior to total necrosis of the tissues, foliage turns red or dark purple (CIMMYT, 2004).

Maize Stripe Virus (MStV)

This disease has been reported in tropical locations of Africa, Asia, and the Americas, including Hawaii, India, and Australia. Initial symptoms on the leaves are small chlorotic specks that later develop into narrow parallel chlorotic stripes along the younger leaves. The chlorotic bands can vary in width and extend from the base to the tip of the leaves. Infected plants usually show stunting and bending of the tassel. Normally ear development and yield are reduced. The virus is transmitted by the planthopper (leafhopper) *Peregrinus maidis*, and the vector transmits the virus

for most of its life after feeding on an infected plant. The vector can also transmit maize mosaic virus. Neither sap nor seed transmission of the disease agent has been reported (Carlose, 1984).

Maize Streak Virus (MSV)

Maize streak virus (MSV; Genus *Mastrevirus*, Family *Geminiviridae*) occurs throughout Africa, where it causes what is probably the most serious viral crop disease on the continent. It is obligately transmitted by as many as six leafhopper species in the Genus *Cicadulina*, but mainly by *C. mbila* Naudé and *C. storeyi*. In addition to maize, it can infect over 80 other species in the family *Poaceae*. Whereas 11 strains of MSV are currently known, only the MSV-A strain is known to cause economically significant streak disease in maize. Natural resistance to MSV in maize, and/or maize infections caused by non-maize-adapted MSV strains can result in narrow, interrupted streaks and no obvious yield losses. MSV epidemiology is primarily governed by environmental influences on its vector species, resulting in erratic epidemics every 3–10 years. Even in epidemic years, disease incidences can vary from a few infected plants per field, with little associated yield loss, to 100% infection rates and complete yield loss (Bua and Chelimo, 2010).

The disease, reported first from East Africa, has now extended to many other African countries. Early disease symptoms begin within a week after infection and consist of very small, round, scattered spots in the youngest leaves. The number of spots increases with plant growth; they enlarge parallel to the leaf veins. Soon spots become more profuse at leaf bases and are particularly conspicuous in the youngest leaves. Fully elongated leaves develop a chlorosis with broken yellow streaks along the veins, contrasting with the dark green color of normal foliage. Severe infection causes stunting, and plants can die prematurely or are barren. Many cereal crops and wild grasses serve as reservoirs of the virus and the vectors (Kyetere *et al.*, 1999).

Maize Rough Dwarf Virus (MRDV)

This virus has been known for several years in countries in Europe and Asia, as has its variant, “Mal de Rio Cuarto,” in central Argentina and Uruguay. Infected plants show stunting; secondary veins become chlorotic and thick. The leaves become leathery and younger leaves roll upwards with characteristic overgrowths (enations) on the veins on the underside. Symptoms can

be detected in seedlings at approximately one month of age. In later stages, infected plants develop a reddish color and form either no ear or simply nubbins which are often bent at the tip. The tassels and upper leaves are malformed and underdeveloped. The virus is transmitted by several delphacid planthoppers including *Laodelphax striatellus* for MRDV and *Delphacodes kuscheli* for MRCV. Transmission is for most of the life of the vector after feeding on an infected plant, and females can pass the virus to the next generation through the eggs. Mal de Rio Cuarto Virus in central Argentina and MRDV in northern China have been reported as seriously affecting maize production (Singh, 2007).

Maize Fine Stripe Virus (Maize Rayado Fino Virus, or MRFV)

“Rayado fino”, or fine stripe, is caused by a virus transmitted by the leafhopper *Dalbulus maidis*. The vector will transmit the virus for most of its life after feeding on an infected plant. *Dalbulus maidis* is also a vector of the corn stunt spiroplasma and maize bushy stunt phytoplasma. This virus is distributed from southern North America to South America, including the Caribbean, and has been observed in several Central American countries to reduce yields by as much as 43%. Leafhoppers can transmit more than one of these pathogens at a time, and mixed infections are common. Symptoms develop about two weeks after plants have been infected. They begin as small, isolated chlorotic spots easily observed by holding leaves against the light. Later, the spots become more numerous and fuse, forming 5 to 10 cm stripes that advance along the veins (CIMMYT, 2004).

Wheat Streak Mosaic Virus (WSMV)

WSMV is one of the viruses in the genus *Tritimovirus*, family *Potyviridae* (Kumar *et al.*, 2004). It is single stranded positive sense RNA (ssRNA) approximately 9.4 to 9.6 kb sizes with a 3'-poly A terminus. It has a filamentous particle of 15 nm diameter and 690 to 700 nm long (Kumar *et al.*, 2004; Wegulo *et al.*, 2008).

2.3 Maize Lethal Necrotic Disease

Maize lethal necrosis disease (MLND) is a disease that results from combined infection by two viruses: maize chlorotic mottle virus (MCMV) and either sugarcane mosaic virus (SCMV) or maize dwarf mosaic virus (MDMV) or wheat streak mosaic virus (WSMV). MLND will not develop if only MDMV and WSMV occur together. Infected plants are short. The leaves show chlorosis and die at about flowering time. There is no ear development in plants infected during early stages of growth (Wangai *et al.*, 2012).

In 2011, a disease with virus like symptoms (chlorotic mottle on maize leaves, mild to severe mottling and necrosis) were reported in East Africa causing dramatic maize damage in farmers' fields (Wangai *et al.*, 2012). The disease was identified as Maize Lethal Necrotic Disease (MLND) (Wangai *et al.*, 2012; Adams *et al.*, 2013), a new disease in Africa and perhaps the worst enemy of the maize crops in recent times.

Causative agents/pathogens

MLND is caused by *Maize Chlorotic Mottle Virus* (MCMV) as a single virus infection or in combination with other *Potyviridae* family like *Sugarcane Mosaic Virus* (SCMV), *Wheat Streak Mosaic Virus* (WSMV) or *Maize Dwarf Mosaic Virus* (MDMV) (Bockelman *et al.*, 1982). The double infection (co-infection) which is more severe than single infection (Niblett and Claflin, 1978; Scheets, 1998) occurs mostly with two viruses; MCMV and SCMV and this gives rise to what is known as MLND, also referred to as Corn Lethal Necrosis (CLN) (Uyemoto *et al.*, 1980; Uyemoto *et al.*, 1981).

The possibility of spreading to other areas cannot be ruled out and hence need to quantify its distribution in a wider context. The extent of yield loss due to the impact of the disease MLND is a big threat to maize production in East Africa as it can cause intensive to complete yield loss (Wangai *et al.*, 2012). Maize is susceptible to this disease at all stages of development specifically from seedling stage to near maturity (CGIAR Research Program MAIZE, 2012). The loss is due to infected maize plants with small ears, distorted and set little or no grains. On the other hand, maize production costs are increasing as farmers use herbicides and insecticides to control weeds and insect vectors transmitting the disease. Furthermore, seed production costs

also increase as extra cost of seed treatment is incurred by the seed companies. Therefore, proper solution must be found to properly manage the MLN disease to reduce the losses and maximize production.

2.4 Maize Virus Detection and Diagnosis

The first goal is to know the problem(s). This requires accurate diagnosis, but obtaining a correct diagnosis is often challenging. Pests, herbicide interactions and residue, plant nutrition, environmental stress, and below ground damage could confuse the diagnostician. While rusts, smuts, downy mildews, and most foliar pathogens are easily determined, interactions with virus and virus-like agents with many abiotic factors often present a challenge to anyone but an experienced diagnostician. Consequently, there is often a constant need for accurate diagnosis on a field by field basis. Missed diagnoses mean additional losses and inappropriate control strategies. Even with a correct diagnosis, the disease could be too far advanced for intervention during the current season. The list of diseases to be entered into this esoteric scheme needs to be clearly defined (Richard, 1998).

Diagnosis of MLND causative agents based on observation of symptoms has been reported to be less accurate because some of the symptoms like stunting and chlorosis may not be virus infection but nutrient deficiencies or maize mosaic (Nelson *et al.*, 2011). Additionally, factors like unfavorable environmental conditions, damage by pests, air pollution, herbicides applications, and infection by non-viral pathogen can also induce virus like symptoms (Naidu *et al.*, 2003). Furthermore, symptoms may be very slight and inconclusive. Infected plants may be symptomless (Lima *et al.*, 2012) or different viruses may cause similar symptoms in a plant (Webster *et al.*, 2004). Therefore, to be certain and to avoid misdiagnosis, other confirmatory tests must be done to ensure accurate diagnosis of virus infection (Bock, 1982).

The best method of controlling plant diseases is proper identification of the causative agents (Webster *et al.*, 2004; Adams *et al.*, 2013) and this is supported by the best diagnostic tools. Several methods have been used to diagnose plant viral diseases. These methods include; serological methods, nucleic acids based methods (Singh and Singh, 1995; Naidu *et al.*, 2003; Webster *et al.*, 2004; Punja *et al.*, 2007; Trigiano *et al.*, 2008), electron microscopy (Singh and

Singh, 1995), physical properties of a virus (that is, thermal inactivation point, dilution end point, and longevity *in vivo*) (Trigiano *et al.*, 2008), transmission tests, and symptomatology (Naidu *et al.*, 2003). In this review, only three methods via symptomatology, serological and nucleic acids based methods have been used in the diagnosis of plant virus diseases specifically MLND are discussed.

2.4.1 Symptomatology

Symptoms are one of the indications of plants being affected either by biotic (pests and pathogens) or abiotic (environmental conditions) factors in fields (Agrios, 2005). They are important in disease management as some of the management practices such as rouging are based on the observed symptoms. Symptoms of MLND include; elongated yellow streaks parallel to leaf veins, streaks may coalesce to create chlorotic mottling. Chlorotic mottling may be followed by leaf necrosis (Nelson *et al.*, 2011; Makone *et al.*, 2014) which may lead to “dead heart” symptom and plant death (Wangai *et al.*, 2012), premature aging of the plants (Gordon *et al.*, 1984), failure to tassel and sterility in male plants, malformed or no ears (Uyemoto *et al.*, 1981; Gordon *et al.*, 1984), failure of cobs to put on grains and rotting of cobs (Wangai *et al.*, 2012).

2.4.2 Serological methods

Detection and diagnosis of plant viruses have included serological tests since the 1960s (Martin *et al.*, 2000). These tests are believed to be the best in identification of large number of field samples (Wu *et al.*, 2013). They are reported as one of the most specific and easiest methods for rapid and precise identification (Naidu *et al.*, 2001; Astier *et al.*, 2007). Such tests include enzyme-linked immuno-sorbent assay (ELISA) which includes triple antibody sandwich ELISA (TAS-ELISA), double antibody sandwich ELISA (DAS-ELISA) and direct antigen coating ELISA (DAC-ELISA) (Kumar *et al.*, 2004), dot-immunobinding assay (DIBA), and immuno-capture reverse transcription-polymerase chain reaction (IC-RT-PCR) by using the MAb 4B8 that is developed for sensitive, specific, and rapid detection of MCMV in fields (Wu *et al.*, 2013). Other serological tests include; tissue blot immunoassays, immuno-electron microscopy (trapping and decoration), western blots, double immune diffusion and lateral flow rapid tests (Lima *et al.*, 2012). These serology tests are based on antigen-antibody reaction (Lima *et al.*,

2012). Among serological methods, ELISA has been extensively used in many studies to identify viral diseases of plants (Punja *et al.*, 2007). The reason being relatively high sensitivity and specificity (highly strain specific) (Lima *et al.*, 2012), low cost and simple for routine diagnosis (Webster *et al.*, 2004; Kimar *et al.*, 2004). This test is based on the basic principle in which the virus antigens are recognized by their specific antibodies (IgG) in association with colorimetric properties (Lima *et al.*, 2012).

ELISA method have been used to identify WSMV in wheat (Montana *et al.*, 1996; Ilbagi *et al.*, 2005), MCMV in maize (Jensen *et al.*, 1991; Xie *et al.*, 2011; Adams *et al.*, 2013; Lukanda *et al.*, 2014), SCMV in maize (Louie, 1980; Adams *et al.*, 2013; Lukanda *et al.*, 2014) and MDMV in maize (McDaniel and Gordon, 1985; Giolitti *et al.*, 2005). DAS-ELISA has been used to identify MLND causing viruses in Kenya but gave negative results (Adams *et al.*, 2013) probably due to low sensitivity and poor specificity for unusual or variant isolates (Adams *et al.*, 2013). Similar study was done to identify MCMV and SCMV by ELISA (DAS-ELISA and Indirect ELISA) with polyclonal antibodies produced against the East African strains of MCMV and SCMV and it was successful (Mahuku *et al.*, 2015). In spite of serological methods such as ELISA being less accurate in identifying unusual or variant isolates because of being too specific to a particular species or even strain of a virus as reported by Adams *et al.* (2013), still it can be used in identification because it is the easiest method associated with low cost. Furthermore, it is rapid and can be used in the identification of large number of samples and that is why it is intensively used in quarantine/movement of seeds and plants across countries to identify diseases of quarantine importance including MLND (Mezzalama *et al.*, 2015). However, there must be proper selection of good reagents and ensuring the level of antibodies' sensitivity and specificity toward the pathogen under study, proper handling, storage of reagents and incubation time and temperature must be done carefully as these factors have been reported by Hewings and D Arcy (1984) to affect ELISA results.

2.4.3 Nucleic acid based methods

Nucleic acid based methods have been used in identification and characterization of many viral diseases of plants (Henson and French, 1993; Hadidi *et al.*, 1995; Lopez *et al.*, 2003). Polymerase chain reaction (PCR) and sequencing are among nucleic acid based methods used in the diagnosis of many plant virus diseases including MLND (Zhang *et al.*, 2011; Wangai *et al.*, 2012; Adams *et al.*, 2013, Lukanda *et al.*, 2014; Mahuku *et al.*, 2015).

Polymerase chain reaction (PCR)

PCR is a molecular technology that facilitates the amplification of rare copies of specific nucleic acid sequences to produce a quantity of amplified product that can be analyzed (Coleman and Tsongalis, 2006). This method is used in many applications (Doughari *et al.*, 2009) including diagnostics of plant virus diseases (Henson and French, 1993; Hadidi *et al.* 1995; Lopez *et al.*, 2003) because of its speed, specificity, sensitivity, and versatility (Naidu *et al.*, 2003). Apart from detection of viruses, PCR products (amplicons) can be sequenced to provide further data on strain types (Webster *et al.*, 2004). There are several PCR variants including basic PCR, reverse-transcription-PCR (RT-PCR) common for RNA viruses, real-time PCR (Lopez *et al.*, 2003; Kumar *et al.*, 2004; Rao *et al.*, 2006; Punja *et al.*, 2007; Hardingham *et al.*, 2012), Multiplex PCR, Nested PCR (Lopez *et al.*, 2003; Webster *et al.*, 2004; Rao *et al.*, 2006; Punja *et al.*, 2007; Hardingham *et al.*, 2012), immunocapture PCR (IC-PCR), competitive fluorescence PCR (CF-PCR) and fluorescence RT-PCR using Taqman[®] technology (Webster *et al.*, 2004). These PCR variants are designed to increase sensitivity, alter specificity or allow automation of detection (Webster *et al.*, 2004).

PCR has been used in diagnosis of many viral diseases of plants including detection of MCMV by realtime PCR in maize seeds (Zhang *et al.*, 2011) and in maize leaves (Adams *et al.*, 2014). Real-time PCR is considered as the best confirmatory test and for routine diagnosis and it is species specific (Adams *et al.*, 2013). Additionally, RT-PCR has been used to detect/verify MCMV and SCMV in maize (Wangai *et al.*, 2012; Mahuku *et al.*, 2015), MCMV in sugarcane (Wang *et al.*, 2014) and in maize (Xie *et al.*, 2011), SCMV, Sorghum Mosaic Virus (SrMV), Sugarcane Streak Mosaic Virus (SCSMV) and Sugarcane Yellow Leaf Virus (SCYLV) in sugarcane (Xie *et al.*, 2009), and SCMV in maize and sorghum (Rafael *et al.*, 2014).

PCR results can be affected by a number of factors including improper handling and storage of reagents, PCR contaminants, quality of enzyme (that is, Taq polymerase), type of primers and annealing temperature and the presence of inhibitors that can affect amplification of the target DNA which may be the result of improper purification of DNA/RNA (Viljoen *et al.*, 2005). These inhibitors may lead into false negative results and contaminated amplicons may lead to false positive results. Therefore, considerable care is required throughout the process. It is essential to include proper positive and negative control reactions to guard against systemic contamination of PCR reagents and to ensure that the desired amplicon is produced in positive reaction (Coleman and Tsongalis, 2006). Moreover, Rao *et al.* (2006) reported on non-uniform distribution of most viruses in plant and even less in the plot, orchard or nursery, therefore studies on sampling methodologies and sample processing is urgently needed to avoid false negative results. Nevertheless, PCR is considered as the best confirmatory and reliable method for routine diagnosis. However, the need of expertise and high costs of reagents hinders it to be used extensively in detection and identification of viral diseases of plants such as MLND especially in low income-developing countries including east Africa.

Sequencing

Sequencing is a very reliable technique for virus identification and has led to development of strain specific probes and primers from extensive sequence data available from many viral isolates (Punja *et al.*, 2007). Next-generation sequencing (NGS) is one of modern techniques that have been used in the diagnosis of new unidentified viral plant diseases. This technique involves generation of sequences in non-specific fashion and identification is based on similarity searching against GenBank (Adams *et al.*, 2013). It has been used to identify and characterize plant viruses including MLND (Adams *et al.*, 2013, 2014; Mahuku *et al.*, 2015). Among those studies includes characterization of MCMV and SCMV in Kenya whereby MCMV showed a similarity of more than 96% to the Yunnan strain from China but different from US strains while SCMV was found most similar to a strain from China (Adams *et al.*, 2013). Other similar study, complete nucleotide sequence of MCMV isolates in Nebraska was done, whereby sequences of MCMV-NE (Nebraska isolates) and MCMV-KA (Kansas isolates) were closely related sharing 99.5% nucleotide sequence identity suggesting that the two virus isolates share a very recent common ancestor (Stenger and French, 2008).

However, in spite of NGS being the most modern and effective method for detection of novel unidentified viral plant diseases, it is not used extensively because of high cost. This has severely affected proper diagnosis of plant diseases (including MLND) in the region's leading to very low level of molecular diagnosis. Therefore, there is a need of capacity building and enhancing developing countries in plant disease diagnostics. Because of low level of molecular diagnosis of plant diseases in east Africa, virus strains causing MLND are not well known. Therefore, there is a need of using modern techniques to identify and characterize viruses causing MLND across regions of east Africa and hence set strategic plans to manage the disease and thereby secure food and alleviate poverty (Adams *et al.*, 2013).

2.5 Maize lethal Necrosis Disease Management

Disease management is the selection and use of appropriate techniques to suppress disease to a tolerable level (Fry, 2012). The goal of plant disease management is to reduce the economic and aesthetic damage caused by plant diseases (Maloy, 2005). Proper disease management is achieved when the causation and the effect that the disease could cause are known. Disease management in this context is described based on basic principles of disease control by Whetzel (1929) with modifications as explained by Maloy (2005) and other studies.

2.5.1. Reduction of initial inoculums

Pathogen exclusion/strict quarantine: Pathogen exclusion is the prevention of disease establishment in areas where it does not occur. This is a major objective of plant quarantine procedures throughout the world. Maize seeds are inspected before entering and going out of countries and within country regions to prevent transmission of the disease especially by seed transmission. Plant quarantine is a national service and is organized within the framework of Food and Agriculture Organization (FAO) (Kumar *et al.*, 2004). It is considered as one of the best procedures of controlling movement of MCMV, rather than attempting to control the endemic SCMV (Adams *et al.*, 2014). This is because MCMV is new in East Africa, reported in Kenya in 2011 (Wangai *et al.*, 2012) but SCMV was reported in East Africa in 1973 (Louie,

1980). Enforcement of this practice will have significant effects in limiting the introduction of MLND into other areas and prevent their spreading and hence reducing threats of food security.

Pathogen eradication: This method reduces pathogen from infected areas before it becomes well established (Maloy, 2005). 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 inoculums in the next season, rouging of diseased maize plants (Mawishe and Chacha, 2013), eliminating weeds and other alternative hosts (insect vectors) which serve as reservoir for viruses (Webster *et al.*, 2004; Maloy, 2005; Trigiano *et al.*, 2008). Crop rotation can be done by planting a non-host crop, this can reduce (but not eliminate) density of the viruses and manage MLND (Uyemoto, 1983). Non-host crops include Irish potatoes, sweet potatoes, cassava, beans, bulb onions, spring onions, vegetables and garlic (Wangai *et al.*, 2012). Additionally, the use of techniques that disfavor vectors/movement for example, reflective mulches for aphids and sticky cards for other insect vectors that feed on maize can be used to reduce vectors for transmission and thereby reducing density of inoculums.

2.5.2. Reducing the rate of infection

Avoidance: This method aims at avoiding contact between host (maize) and pathogen (viruses) by planting maize in field with no history of the disease, provide adequate plant spacing and avoid crowding, avoiding injury to the maize plants because viruses penetrates the plants through wounds and avoiding the use of recycled maize seeds by using certified seeds (Trigiano *et al.*, 2008; Wangai *et al.*, 2012), planting maize on the onset of the main rainy season and not during the short rain season so as to create a break in maize planting seasons (Wangai *et al.*, 2012). This will reduce the population of vectors and hence low rate of infection and disease severance.

Plant protection: This method involves protection of the host (maize) from invading pathogens (viruses). It is achieved by spraying chemicals and modification of plant nutrient (the use of manure and fertilizers) and environment. MLND viruses cannot be 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

(aphids, rootworms, stem borers, mites, thrips) that transmit MLND. Such insecticides include Imidacloprid, Thiamethoxam, Deltamethrin, Abamectin, Permethrin, Endosalphon and Dimethoate (TPRI, 2011). For effective control of vectors, appropriate insecticides must be sprayed once every 1 to 2 weeks and there should be rotation of multiple chemicals every month to avoid immunity development of the target vector (Mezzalama *et al.*, 2015). The use of chemicals has been reported insufficient in the management of plant virus diseases (Satapathy, 1998; Perring *et al.*, 1999). Other protection techniques include the use of manure, basal and top dressing fertilizers to strengthen the resistance of plants to disease and pests (Wangai *et al.*, 2012).

Resistant or tolerant varieties: This is the most reliable, effective, environmental friendly and economical way of controlling plant diseases (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. Many efforts are being done to produce resistant varieties of maize in eastern Africa (ASARECA, 2014). For example, strong collaboration between CIMMYT and national maize programs has been established to effectively tackle the MLN challenge in eastern Africa (CGIAR Research Program MAIZE, 2012). This resulted in establishment of a centralized MLN screening facility for eastern Africa at the KALRO Livestock Research Farm in Naivasha. Additionally, Ngotho (2013), reported on the funding from the Bill and Melinda Gates Foundation and Syngenta Foundation for Sustainable Agriculture that will be used to develop fast tracking maize varieties that are tolerant to the disease and drought by scientists and researchers within Pan-Africa and the eleven ASARECA countries, Kenya, Uganda, Tanzania, Rwanda, Burundi, Ethiopia, Sudan, Eritrea, DRC Congo, Madagascar and South Sudan.

3. Objectives

General Objective:

- To investigate the geographical distribution and incidence of MLND in maize fields of Oromia and Benishangul-Gumuz, characterize the causal viruses and strains using serological and molecular techniques and to identify different viral strains that infect maize by artificial inoculation.

Specific Objectives:

- To determine the geographical distribution and incidence of MLND in Oromia and Benishangul-Gumuz regions of Ethiopia;
- To identify the causal viruses and strains using serological and molecular techniques;
- To study phylogenetics relations of different isolates of MCMV and SCMV and isolates from the GenBank;
- To study individual and synergetic effects of MCMV and SCMV on sweet corn by artificial inoculation.

4. Materials and Methods

4.1 Study Area and Collection of Samples

4.1.1 Study Area

Sampling technique of this study was random sampling between 5 km distance in potential fields identified along the main road including fields in Melkassa Agricultural Research Center (MARC) and Assossa Agricultural Research Centers (AARC). Due to preliminary report, emphasis were given to areas in the rift valley and fields in Benishangul-Gumuz; where the problem is increasing from time to time and many farmers have been abandoned to grow maize due to Maize Chlorotic Mottle Virus and Sugarcane Mosaic Virus severe infestation. The collection sites were characterized by an altitude ranging from 1300 – 2000 m.a.s.l. The fields were selected systematically based on the following criteria: maize production area coverage; prevalence and high level of infestation with different viruses; potential of the area to maize production; accessibility for sampling; economic importance of yield loss caused by MLND.

Main maize growing areas of Oromia and Benishangul-Gumuz Regions were diagnostically surveyed from September 20, 2015 to January 4, 2016 maize growing main and off-seasons. The fields surveyed were varying from small yard to large hectare. East Shewa in the Rift Valley areas, where maize grows under irrigation and western Ethiopia, Benishangul-Gumuz around Assossa were given more attention for sample collection. The survey was conducted at East Shewa in Rift Valley area Melkassa, along the road from Mojo to Zwai and Awassa; Ambo to Guder in western Ethiopia; and different farmers fields around Assossa. In the survey, 43 maize fields were inspected and 174 symptomatic maize leaf samples were collected (Appendix 4). The relative locations of the fields where maize samples collected are shown in Fig. 1.

4.1.2 Sample Collection

In each field, plants were observed and carefully examined for viral symptoms. Plants showing virus disease symptoms like stunting, necrosis, mottling, streak and mosaic pattern were given more attention to pick up sample from individual plant (Fig. 2). From each field, 5-6 samples were collected and total of 174 samples were collected during the survey periods (Appendix 4). The incidence of viral infection, based on field observation, was recorded at each field.

Approximately 2-3 cm long maize leaf samples were subjected to be dried in approximately 5 g of silica gel or Calcium Chloride (CaCl₂) simply by placing silica gel or CaCl₂ powder in collecting tubes which can be tightly sealed to make the sample leaf to be dried before nucleic acids degraded. To minimize cross contamination of samples surgical latex glove and ethanol (70%) was used to wash hand.

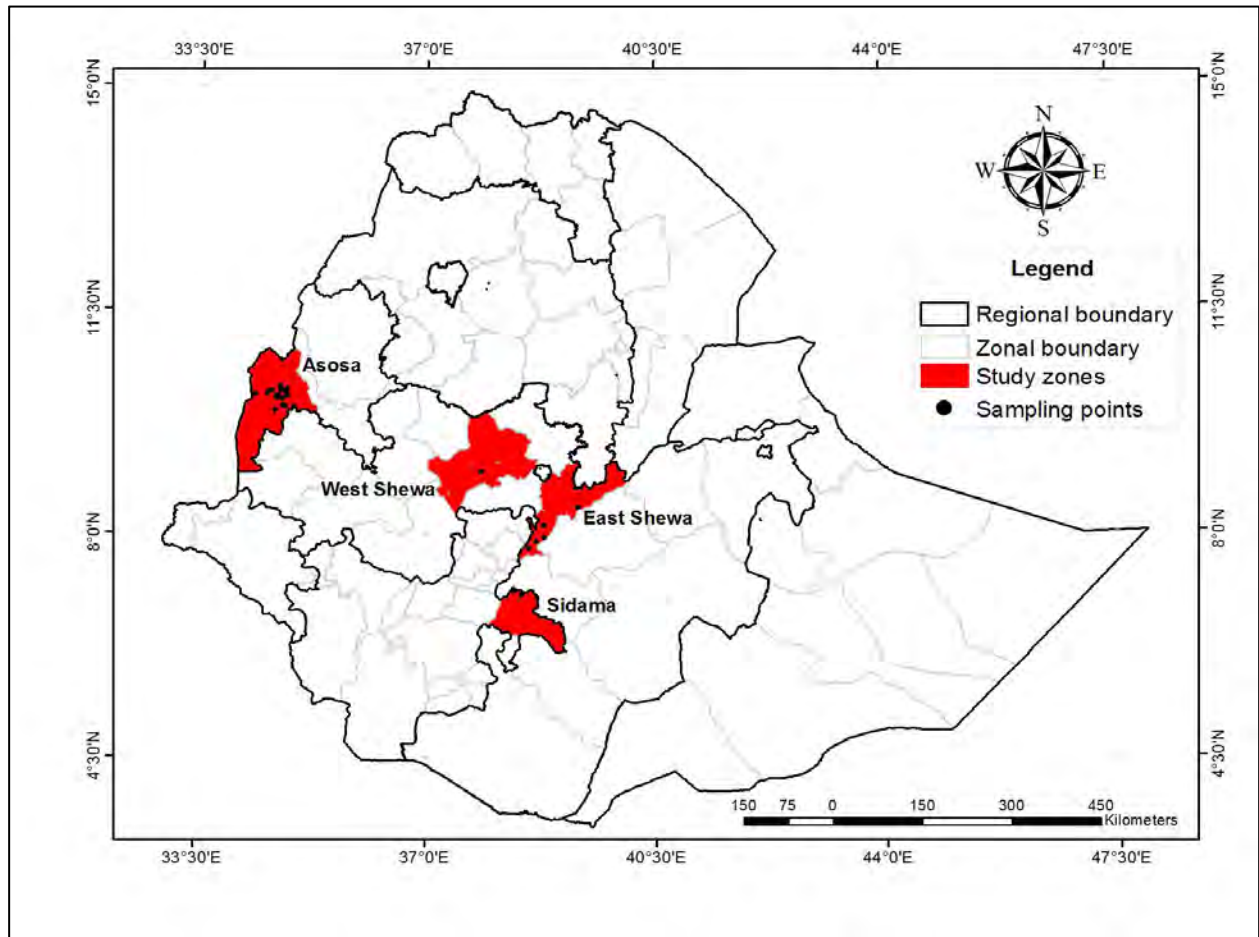


Figure 1. Location Map of MCMV and SCMV study area

The fields were examined using an “X” shaped sampling path. Virus disease symptom incidence was calculated according to James (1974) as the percentage of plants showing maize virus symptoms to the total number of plants observed in the field.

$$\text{Disease incidence (\%)} = \frac{\text{Number of symptomatic (infected) plants}}{\text{Number of plants in the field}} \times 100$$

The virus disease incidence calculated was recorded and estimated as percentage infection, Where by 1-20% =low incidence; 21-49% moderate incidence; and 50-100 % =high incidence.

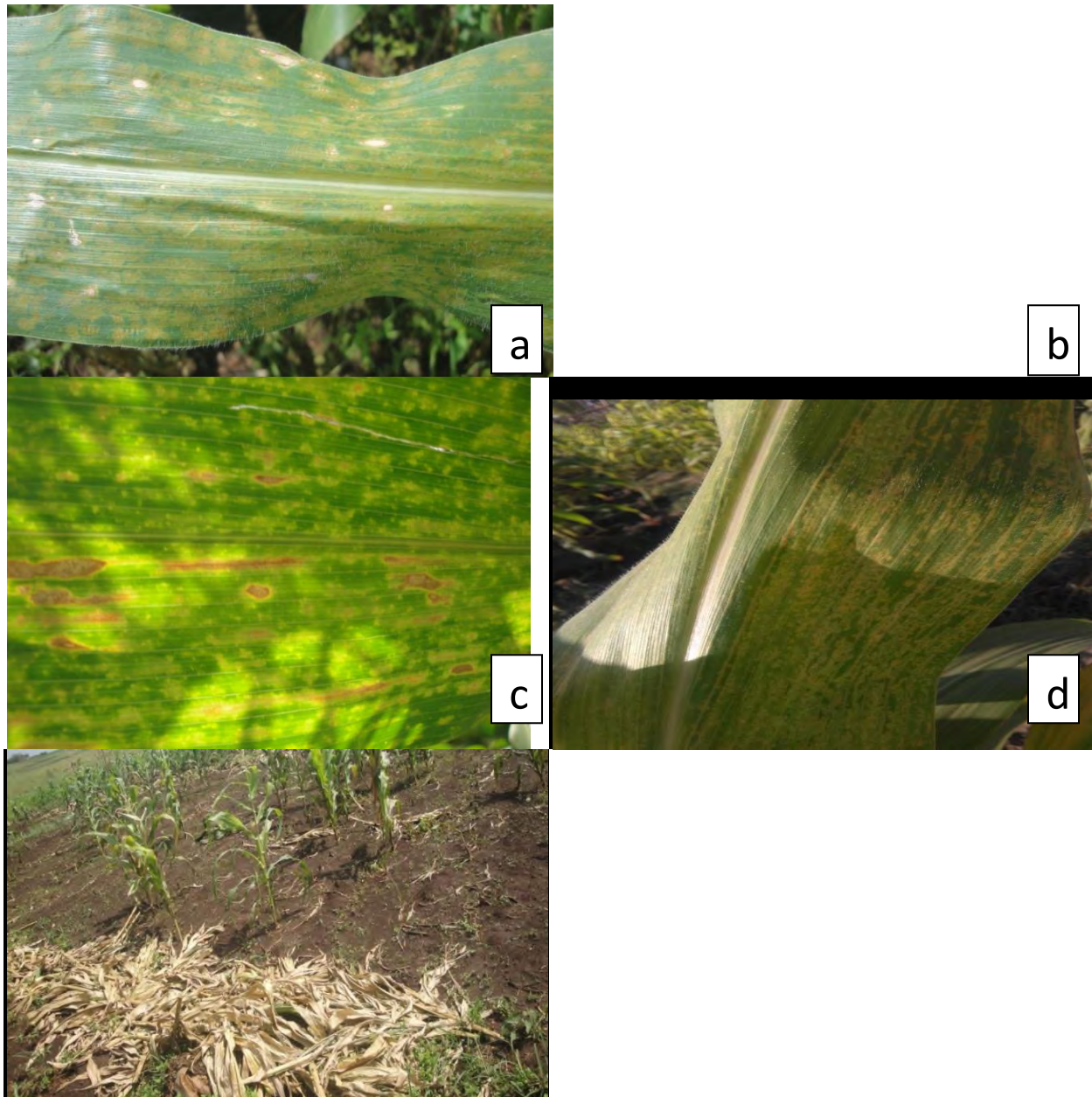


Figure 2. Symptomatic maize leaf samples and fields during sample collection, a. mosaic symptom, b. mosaic and necrotic symptom, c. streak symptoms and d. fields replacing by other crops

4.2 DAS-ELISA

All 174 samples in two rounds (102 and 72) were tested using double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) to examine the occurrence of MCMV and SCMV, and to see whether those samples obtained show mixed infection of both viruses during the DAS-ELISA assay. The DAS-ELISA was done at the Plant Molecular Biology Laboratory of National Agricultural Biotechnology Research Center, Holetta, Ethiopia by using a kit from DSMZ (Appendix 1) in 96 well ELISA plate (Nunc polysorp).

Specific coating antibody (IgG) of the respective viruses (MCMV and SCMV) was diluted with coating buffer; i.e. 1 μ l of IgG in 1 ml of coating buffer with a recommended dilution of 1:1000 according to the manual with the kit. Then 100 μ l of diluted IgG was added to each wells of the ELISA plate by leaving both upper and lower borders to prevent border effects. Each plate of the respective viruses were sealed with parafilm and incubated for 2 hrs at 37°C. This was followed by three times washing with washing buffer (PBS).

Approximately 0.05 g of dried leaf sample were put into a plastic bag and 2500 μ l of extraction buffer added for crashing using the bottom of test tube/a pistil until we get clear plant sap. After drying the plates with tissue paper 100 μ l of sample antigen (plant sap) were added to each well in replicate. Positive and negative controls from the kit were also added in each plate with a similar fashion as the samples. All the plates sealed with parafilm and incubated for overnight at 4°C.

Specific enzyme conjugated (IgG-AP) was diluted in conjugate buffer (dilution 1:1000). The plates taken from overnight incubation washed 3 times with in 3 minute interval and dried with a tissue paper. One hundred μ l of specific enzyme conjugate solution was added to each well. Plates of similar viruses sealed together and incubated for 2 hrs at 37°C. Then Sigma PNP substrate tablet was dissolved in substrate buffer. After washing and drying all the plates 100 μ l of substrate solution was added to each well of all the plates and incubated for 60 minutes at room temperature.

The result was assessed by visual observation for color development and quantitative measurement was made by determining absorbance at 405nm (A_{405nm}) using an ELISA plate reader model (Human, Germany), an hour after substrate addition. The mean absorbance reading of negative controls was determined and two times to the mean values of negative control was

used as the positive/negative thresholds. A sample was regarded as positive when showed clear color development and if its average absorbance readings exceeded two times the mean of the negative control values after 60 minutes of incubation, i.e., when $A_{405nm} \times 1 + x_2 / 2$ of infected sample $> A_{450nm} \times 1 + x_2 / 2$ of negative control and fairly comparable with positive control reading value.

4.3 Total RNA Extraction and Quality Detection

Total RNA of all the samples was extracted using Inclone biotech RNA Mini Extraction Kit (Appendix 2). RNA extraction was performed at Microbiology Laboratory of Agriculture and Nutrition Research Laboratory at Ethiopian Institute of Agricultural Research, Addis Ababa. Approximately 50 mg of Silica gel/ $CaCl_2$ -dried leaf samples was taken and 1500 μ l of Lysis/Extraction buffer was added and then crashed using mortar and pestle. This was followed by centrifugation and the supernatant was transferred into a microcentrifuge tube. Then 500 μ l of Chloroform added and centrifuge at 12,000 rpm for 10 min. And 300 μ l of the aqueous phase and 400 μ l of Isopropanol was transferred to spin column centrifuge and the lystate was discarded and then the column was washed with Washing Buffer 1 (WB1) and centrifuged for another one min then again the column was washed with Washing Buffer 2 (WB2) and centrifuged for one minute. The column was centrifuged for 2 minutes without adding anything. The column was transferred to a new RNase free microcentrifuge tube and 50 μ l of Elution Buffer (EB) was added into the spin column followed by incubation at room temperature for one min and then centrifuged at 12,000 rpm for one minute. Finally, the RNA was stored at $-20^\circ C$ for further use.

The quality of the total RNA extracted was detected using 2% (w/v) agarose gel. To prepare 2% agarose gel; 2 g agarose powder was added in 100 ml TAE buffer followed by heating in microwave. To detect RNA on UV light Safe gel stain was added instead of ethidium bromide. The melted agarose was poured in to the gel Cast tray and allowed to solidify for 1 hr. A total of 2 μ l RNA was mixed with 2 μ l of 6x loading dye and electrophoresed at a voltage of 135V for 30 min. Visualization and image capturing were done by using Bio-Rad gel doc system. From the gel images all samples were shown good bands which indicates the presence of good quality and amounts of total RNA.

4.4 Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

The RT-PCR was done at Microbiology Laboratory of Ethiopian Institute of Agricultural Research Head Quarter, Addis Ababa. PCR amplification was carried out in 25 µl reaction volume containing 1µl template RNA, 12.5µl of 2x reaction mix, 1 µl of enzyme, 1 µl of each primer and 8.5 µl of distilled water (Appendix 3).

Amplification was performed by using one step RT-PCR program on Applied Biosystems; 96 wells Thermal Cycler, which consisted of an initial cDNA synthesis of 42 °C for 50 min, followed by one cycle of 95 °C for 12 min to inactivate the reverse-transcriptase. Then denaturation at 95 °C for 30 seconds followed by annealing at 55 °C for 30 seconds and followed by extension at 72 °C for one min for 35 cycles with a final extension of 10 min at 72 °C with holding at 10 °C.

For all primers PCR amplification was optimized by adjusting reaction mixtures to varying concentration of primer, RNA and reaction mixes with the same PCR program. Primers used in this study are summarized in Table 1.

Table 1. List of primers used for viral genome part amplification.

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

Amplified PCR products were separated on 1 % agarose gels in 0.5x TAE buffer with safe gel stain incorporated in the gel and 100 bp DNA Ladder was used as a standard molecular weight marker to compare amplicons with their expected size. Positive and negative controls for each virus was loaded accordingly after mixing 3 µl PCR products with 2 µl loading dye. The agarose gel electrophoresis running condition was adjusted to 135 V for 30 min.

4.5 RT- PCR Products Direct Sequencing and Sequence Analysis

After checking the quality of PCR products on 1 % agarose gel, around 20 µl of each sample PCR products were sent to Genotech Co., which is a commercial sequencing company in South Korea. PCR products generated from 24 samples for SCMV and 33 samples for MCMV and the primers used were sent. The sequencing was performed using ABI BigDye terminator kit (ABI). The sequencing PCR reaction program was 96 °C for 10 seconds, 50 °C for 10 seconds and 60 °C for 3 min of 30 cycles. After PCR, the unreacted fluorescent-labeled ddNTPs were removed using Ethanol. Then the labeled products were dissolved in distilled water or HDF (Hi-Di Formamide) before they were loaded into the sequencing instrument (ABI 3730 x1 DNA analyzer).

All the sequences were edited using “BioEdit Sequence Alignment Editor” and “CLC main Workbench” software. Percentage of identities between sequences from this study and sequences obtained from the GenBank was revealed by blasting online. Phylogenetic and networking tree construction and sequence analysis was done by using tree view X and MEGA 5 software.

4.6 Sap Preparation for Artificial Inoculation

To study the virulence in synergetic and individual effect of MCMV and SCMV by artificial inoculation the method of Goldberg *et al.* (1987) was used. Sweet corn plants at 3 leaf stage were used which was planted in National Agricultural Biotechnology Research Center, Holetta green house. A total of 27 pots of sweet corn for inoculation and control were used (five plants per each pot).

The sap was extracted using K_2HPO_4 buffer by taking 10 gram of DAS-ELISA and RT-PCR positive leaf sample and crashing in mortar and pistil with 10 ml buffer then by adding carborandom. The plants were covered with dark plastic before 24 hr of inoculation to increase their susceptibility. Inoculum was prepared from both Assossa and Zeway area samples of MCMV only, SCMV only and MCMV+SCMV for three pots, each pot containing five plants for the first round of inoculation. Then after; symptoms of MLND were developed and a new pop corn plants were inoculated by extracting a sap from the diseased plantlets. Pictures of the inoculated plants were taken in a week interval for a month. Around 25 Leaf samples from all the inoculated plantlets were crosschecked for individual and synergetic infection of MCMV and SCMV by RT-PCR.

5. Results

5.1 Symptomatology

The most commonly observed symptoms were severe chlorotic mottle, streak, dead heart, mosaic, necrosis, stunting, premature drying of the husks and no grain (Fig. 3). Symptoms on plants that were co-infected with both viruses were typically more severe than on plants infected with a single virus. Maize plants that were found to be positive only for SCMV had characteristic clear streak purple symptom (Fig. 3. a and b), and some maize plants exhibited no symptoms (Fig 3 l). Plant with symptoms associated with only SCMV infected plants showed yellowish streak leaves and general overall stunting of the plants (Fig. 3. b and c). Plant samples that were infected with only MCMV exhibited clear mosaic and necrosis symptoms (Fig. 3. d, e and f). Plant samples that were positive for both MCMV + SCMV showed severe symptoms including clear leaf necrosis, mosaic, leaf drying and rolling, stunting of the plant and yellowish leaves and vein clearing (Fig. 3. g, h and i). Also in some fields there was evidence that farmers were removing the infected maize plants from the whole field and replacing with other late crops like teff and prepare the land for off-season cropping (Fig. 3. J and k).



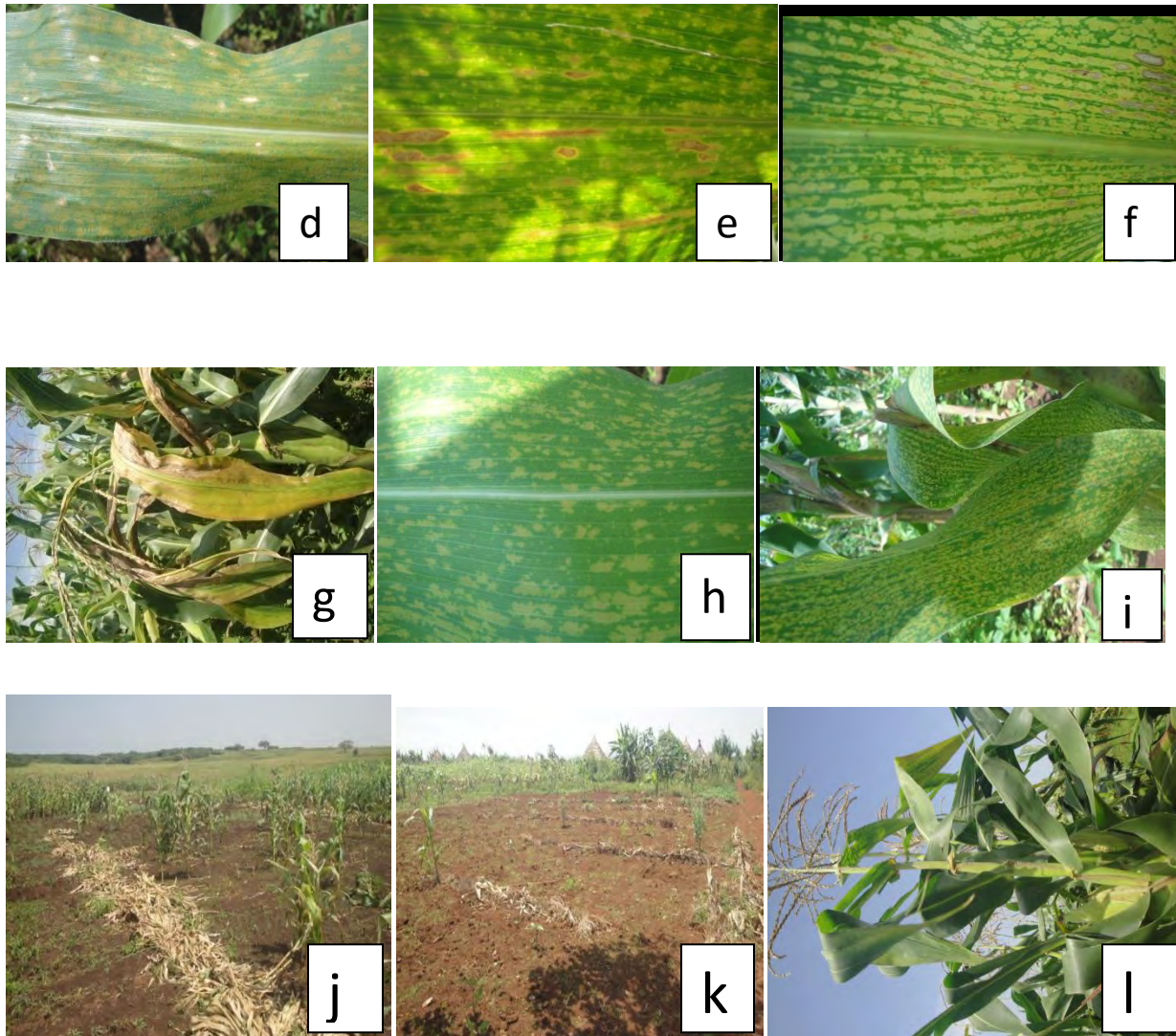


Figure 3. Different maize plant samples and fields showing range of symptoms (a and b showing streak symptom, c showing dead heart symptom, d, e and f showing Mosaic and Chlorosis symptom, g, h and I showing yellowish and Mosaic symptom, j and k showing fields replacing with other crops and l shows maize plants without any symptom).

5.2 Incidence of Virus Infection

The survey result revealed that the incidence of the disease was at different rate ranging from low incidence (10%) to high incidence (100%) and replacement of the maize fields with other crops in some fields. The highest incidence of MLND symptom were recorded in both Regions benishangul-Gumuz, Assossa Zone Assossa Woreda, Agusha kebele and Oromia West Shewa Zone, Ambo farm ranges up to 100%. The Second highest incidence was recorded at Melkassa field of Oromia and Komshga 28 of Benishangul-Gumuz ranges up to 95%. Both fields in

Melkassa Agricultural Research Center (MARC) and Assossa Agricultural Research Center (AARC) were in third position with incidence ranges up to 90% followed by fields in East Shewa Dugda Woreda, Gressa with incidence of 85% and Adamitulu of Abingermama with 80% of infestation. The lowest incidence was recorded at both fields of Holetta and Awassa with 10% incidence (Table 2).

Table 2. Summary of average incidence of MLND symptoms in the study area

Region	Zone	District/ Woreda	Kebele	No of Fields Surveyed	No of Samples taken	Average Incidence of MLND (%)
Benishangul- Gumuz	Assossa	AARC	AARC fields	4	22	90%
		Assossa	Komeshg 28	2	8	95%
			14 Gambela	1	5	55%
			Amba 14	2	5	40%
			Amba 5	1	4	60%
			Baro	3	9	30%
			Agusha	1	8	100%
		Bambassie	Ataferbavare	3	6	75%
			Dabus	4	17	50%
			Keshmando	2	9	70%
		Selga	24	2	8	45%
			22	1	3	45%
Oromia	East Shewa	Adamitulu	01	3	12	55%
			Bochesa	3	12	65%
			A/Germama	3	13	80%
		Dugda	Gressa	3	11	85%
		Melkassa	MARC field	1	6	90%
			Awash	1	6	95%
	West Shewa	Ambo	African Jucie	1	8	100%
		Holetta	HARC field	1	1	10%
SNNP	Awassa	Awassa	Awassa	1	1	10%

5.3 DAS-ELISA Results

DAS-ELISA tests were carried out for both MCMV and SCMV. Using a positive threshold of two times greater than the mean of the negative control 43 samples (25%) were positive for MCMV, 14 samples (8%) were positive for SCMV and 9 (6%) samples were positive for both

MCMV and SCMV (Table 3). Among 102 maize plant samples of Assossa tested by DAS-ELISA, only 3 samples showed strong reaction to the antibody of SCMV and no samples were positive for MCMV as well as for both MCMV+SCMV during the DAS-ELISA assay (Fig 4.). It was found that 42 samples were positive for MCMV from samples collected in different fields of Oromia and 11 samples out of 71 were positive for SCMV and 9 were positive for both SCMV+MCMV. The single sample from Awassa was positive only for MCMV.

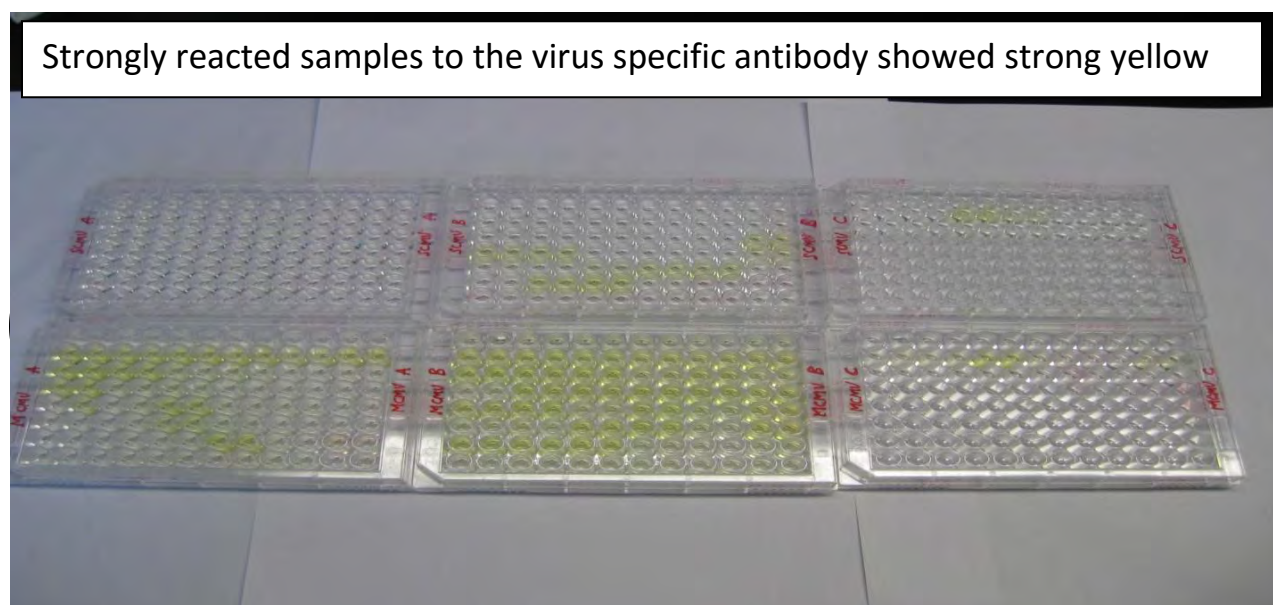


Figure 4. DAS-ELISA results of different levels of color development and reaction to virus specific antibodies

Table 3. Summary of the DAS-ELISA result after reading the result on ELISA reader

Region	Zone	District	No of samples assayed	Viruses Detected			
				MCMV Only	SCMV Only	MCMV+SCMV	None
Benishangul-Gumuz	Assossa	AARC	22	0	1	0	21
		Assossa	32	0	1	0	31
		Bambassie	37	0	1	0	36
		Selga	11	0	0	0	11
Oromia	East	Adamitulu	37	16	0	0	21

	Shewa	Dugda	13	12	0	0	1
		Melkassa	12	6	5	3	1
	West Shewa	Ambo	8	8	6	6	0
		Holetta	1	0	0	0	1
SNNP	Sidama	Hawassa	1	1	0	0	0
Total			174	43 (25%)	14 (8%)	9 (6%)	123 (71%)

5.4 Reverse Transcriptase PCR Results

In the PCR, the band formed shows most of the samples were positive for both MCMV and SCMV according to the specific primers used. From 102 samples collected from Asososa, 85 maize plant samples were positive for MCMV, 67 samples were positive for SCMV and 67 samples were positive for both SCMV+MCMV. From samples of the other areas, 58 samples were positive for MCMV, 47 samples were positive for SCMV and 41 samples were positive for MCMV+SCMV.

From 10 random samples taken from Assossa, the result of RT-PCR showed, all the samples were positive for MCMV and 7 of them were positive for SCMV and both SCMV+MCMV. The band were confirmed by comparing with the 1Kbp DNA ladder and the size of primers used for both viruses (478 bp for MCMV and 319 bp for SCMV). That means the specific bands are at a position corresponding to the expected (Fig 5). The quality of total RNA extracted was checked using 2% (w/v) agarose gel.

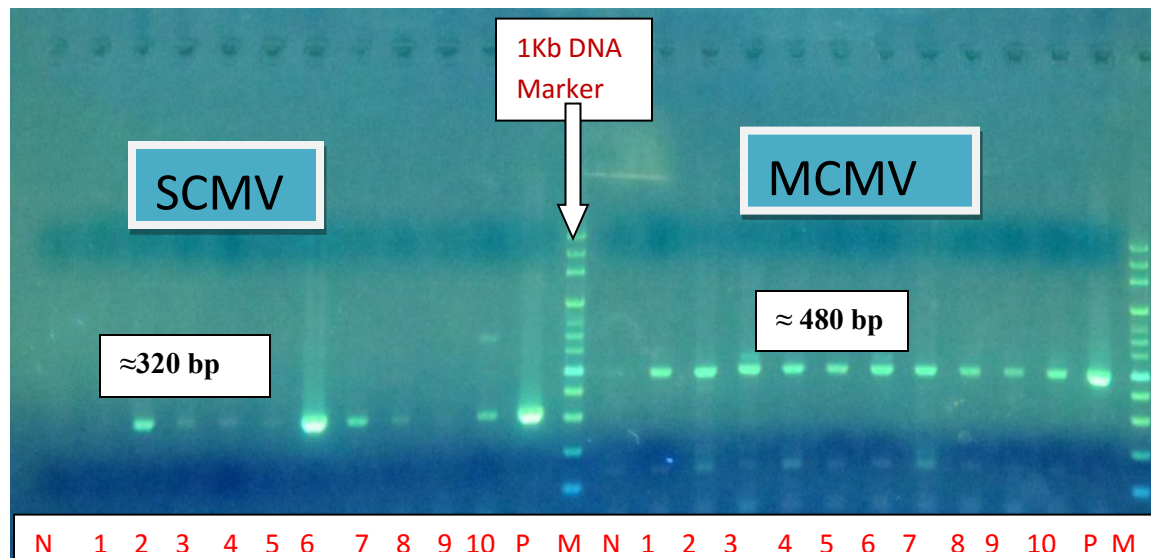


Figure 5. One step RT-PCR screening of SCMV and MCMV infected samples of maize. (M- 1 kb DNA ladder, 1 to 10- similar samples for detection of SCMV and MCMV. N- negative control and P- positive control)

5.5 Sequence and Sequence Analysis Results

After all the raw sequences were edited, the BLAST result showed only 22 samples showed significant similarity of 97-99% with MCMV in the GenBank and 10 samples showed significant similarity of 95-98% with SCMV in the GenBank. All the MCMV sequences showed more similarity with isolates from Kenya, Ethiopia and Rwanda. The MCMV-8 showed the highest similarity of 99% with KT250549 of Kenyan isolate.

All SCMV partial genome sequences showed more significant similarity with isolates of Kenya and Ethiopia. Isolates from Assossa, Ambo and Melkassa showed similarity of 98 % whereas another isolate from Ambo showed less similarity of 95% with an isolate from Kenya in the GenBank. Phylogenetic reconstruction based on nucleotide sequences of the isolates was obtained using the GenBank alignment. The tree was generated through MEGA package version 5.1 (Gouy *et al.*, 2010) with the Neighbor-joining method using the Kimura 2-parameter model (Kimura, 1980) for distance estimation and bootstrap analyses with 1,000 replications. Cluster definitions were based on monophyletic groups from a fixed level indicated by the topology of the tree and with similarity cutoff point of 50% and scale length of 0.002.

The optimal tree with the sum of branch length = 0.09101430 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The tree is drawn to scale length of 0.002, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method and are in the units of the number of base substitutions per site. The analysis involved 41 nucleotide sequences by excluding values that showed less than 50% similarity and a branch length of 0.002. All

positions containing gaps and missing data were eliminated. There were a total of 471 positions in the final dataset. Evolutionary analyses were conducted in MEGA5.

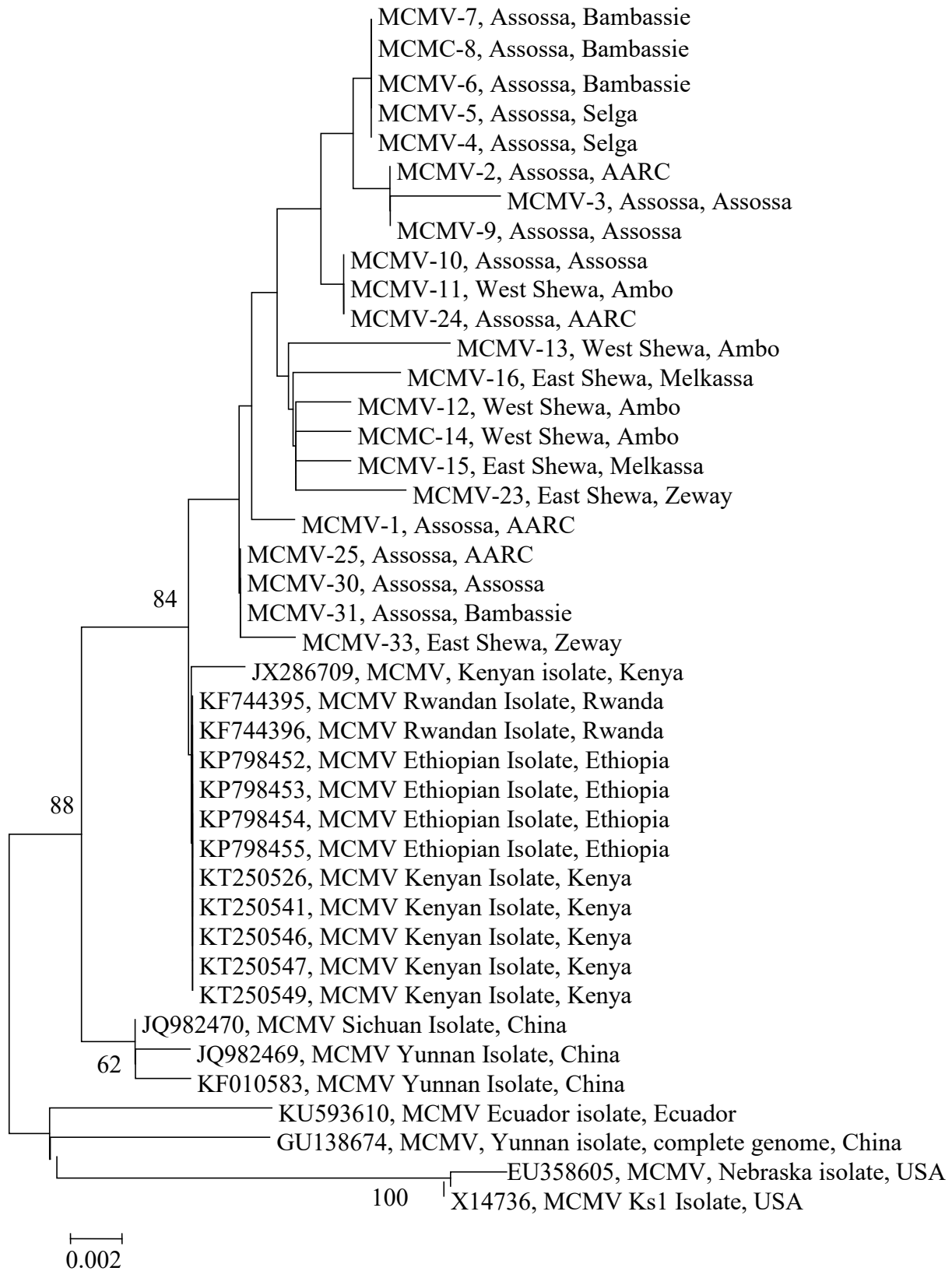
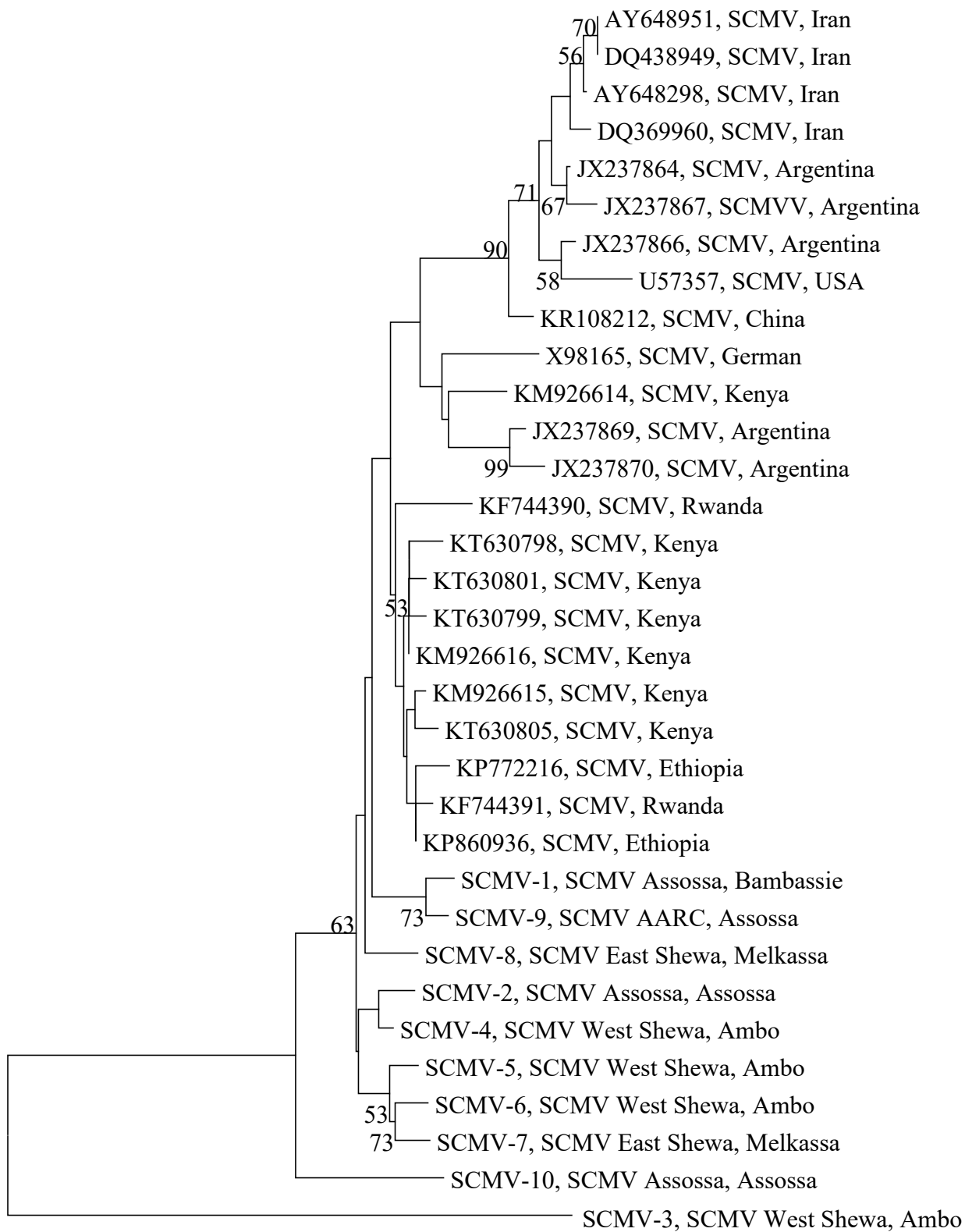


Figure 6. Phylogenetic tree of nucleotide sequence of 22 MCMV field isolates from maize fields of the study area and 19 MCMV accessions from GenBank. KP798452, KP798453, KP798454 and KP798455 were Ethiopian Isolates. KF744395 and KF744396 are from Rwanda. KT250526, KT250541, KT250546, KT250547, KT250549 and JX286709 were Kenyan isolates. JQ982470, JQ982469, GU138674 and KF010583 were from China. EU358605 and X14736 were from USA and KU593610 was from Ecuador.

Haplotype networking of both MCMV and SCMV isolates and the sequences from the GenBank were used using network software. The network was used to show the genetic divergence among isolates used in the study and the GenBank isolates.



0.002

Figure 7. Phylogenetic tree of nucleotide sequence of 10 SCMV field isolates from maize fields of the study area and 23 SCMV accessions from GenBank. KP772216 and KP860936 were Ethiopian Isolates. KF744391 and KF744390 are from Rwanda. KM926615, KT630801, KM926616, KT630798, KT630799, KT630805 and KM926614 were Kenyan isolates. JX237869, JX237870, JX237864, JX237866 and JX237867 are from Argentina. DQ369960, DQ438949, AY648298 and AY648951 were Iranian Isolates. X98165 from Germany, KR108212 from China and U57357 was from USA.

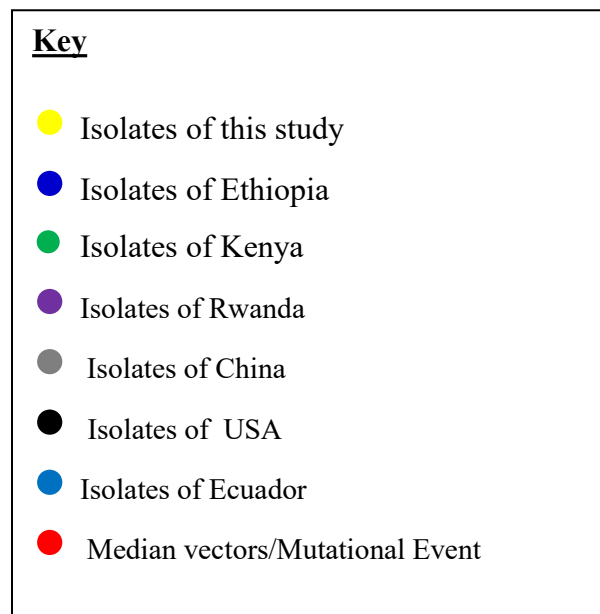


Figure 8. Evolutionary Divergence Networking tree of all the MCMV isolates of this study and the 19 isolates of the GenBank Using Network 4.6 software.

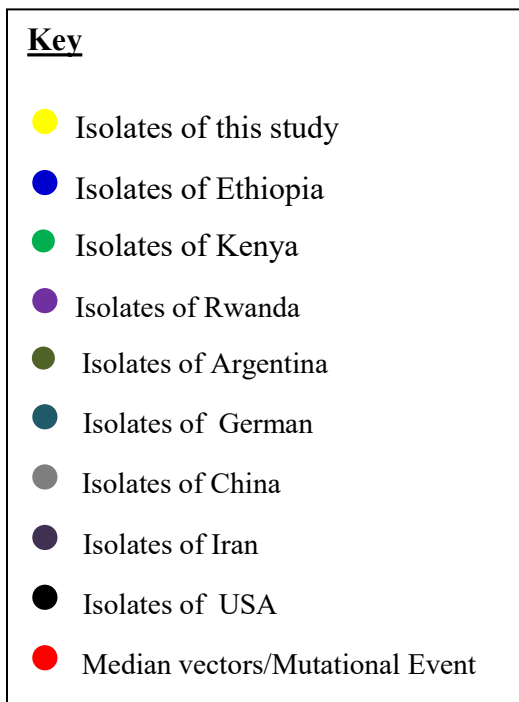


Figure 9. Evolutionary Divergence Networking tree of all the SCMV isolates of this study and the 23 isolates of the GenBank using Network 4.6 software.

5.6 Artificial Inoculation Results

Symptom Development

After two weeks of the first round of inoculation only the pop corn plants inoculated with viral sap from Ambo sample developed clear mosaic symptom on all the three pots. All the sweet corn seeds sowed showed 100 % germination rate. After two weeks of inoculation, the clear mosaic pattern on the leaves was visible (Fig 10. a and b). The symptom was very clear also after three weeks (Fig 10. c and d) and after one month (Fig 10. e), whereas the controls appear healthy (Fig 10. g). after one month of inoculation, some of the plants started to show a mosaic symptom (Fig 10. f). Plants inoculated during the second round by using the sap from the first round inoculated and that showed the symptom, all developed the mosaic symptom.

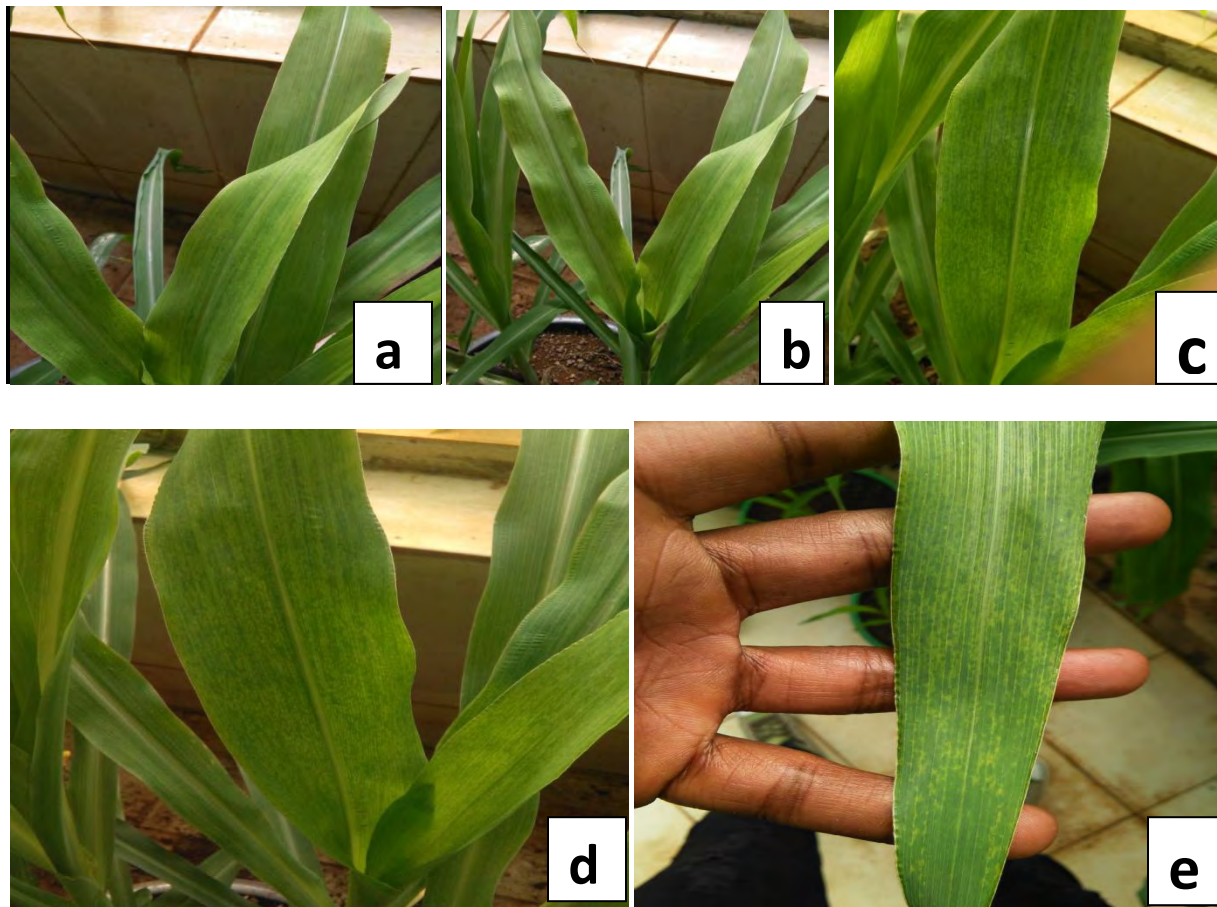




Figure 10. Pictures that show different levels of symptom development during artificial inoculation (Figure A, B,C, D and E showed symptoms of Chlorosis and Mosaic after one, two, three and four weeks of inoculation respectively, F showed Chlorosis after four weeks of inoculation and G showed the positive controls).

RT-PCR Confirmation

In order to confirm the symptom developed by the inoculation by the respective viruses, RT-PCR was performed using the same primers. The total RNA was extracted from 26 representative samples. After the PCR, the RT-PCR result showed that all the inoculated plants were positive for both SCMV and MCMV and figure 11 clearly shows positive samples after RT-PCR amplification.

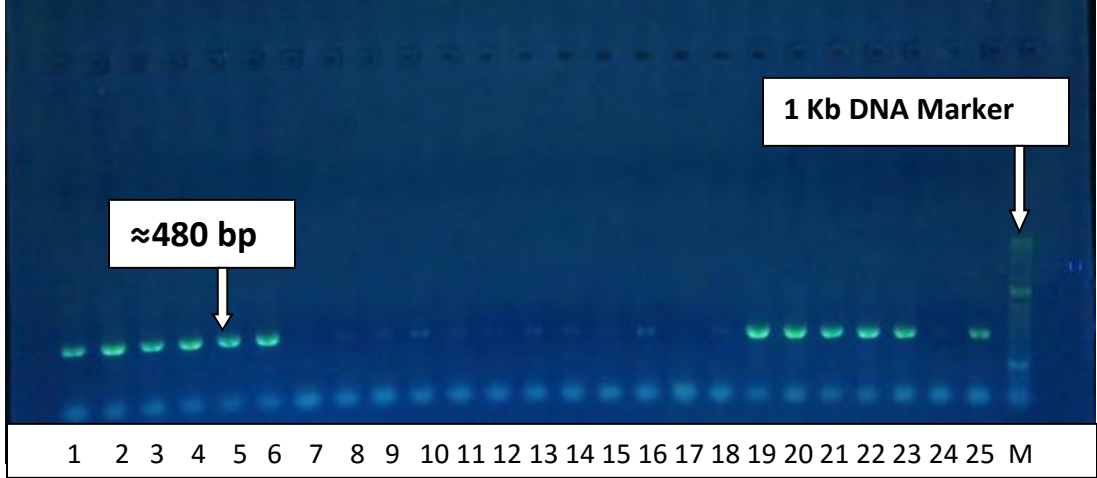


Figure 11 Agarose gel bands showing positive sample for MCMV (1-25 samples and M represents the marker used).

6. Discussion

The MLND symptoms observed in the maize fields of this study like mottling, mosaic, necrosis, leaf deformation and local lesions are similar to those earlier reported by Makumbi and Wangai (2012). Adams *et al.* (2012) also reported that samples from Kenya displayed a range of leaf symptoms including spotting ('early-stage'), streaking ('mid-stage') and necrosis on the margin ('late-stage'). Maize plants exhibiting severe yellowing and chlorotic mottle symptoms were observed in the Upper Awash Valley of Ethiopia (Mahuku *et al.*, 2015). A similar MLND symptom was also reported in Rwanda (Adams *et al.*, 2014), and the Democratic Republic of Congo (Lukanda *et al.*, 2014). However, mild mosaic or mottling symptoms and a moderate reduction of growth might be due to single infections of MCMV or SCMV.

Other symptoms of MLND includes, elongated yellow streaks parallel to leaf veins, streaks may coalesce to create chlorotic mottling, chlorotic mottling followed by leaf necrosis were also reported in the study area and which is similar with the report of Makone *et al.* 2014. According to Wangai *et al.* 2012; dead heart symptom, plant death, premature aging, failure to tassel and sterility in male plants, malformed or no ears, failure of cobs to put on grains and rotting of cobs were clearly observed in the study area.

According to Kagoda *et al.* (2016), in the case of mixed infections, early infected plants appeared stunted and showed a general chlorosis, leaf bleaching and necrosis. In addition, from the results, MLND can attack at any stage of maize growth since at every stage, right from two weeks after planting, farmers observed the MLND symptoms in Uganda. Niblett and Claflin (1978) also reported a similar range of symptoms in the US like mosaic on leaves and husks, leaf necrosis progressing inward from the margins, premature plant death, tassel necrosis, poor ear development and no seed setting.

Diagnosis of MLND causative agents based on observation of symptoms has been reported be less accurate by Nelson *et al.*, 2011; because some of the symptoms like stunting and chlorosis may not be virus infection but nutrient deficiencies or maize mosaic. Additionally as reported by Naidu *et al.*, 2003 unfavorable environmental factors, damage by pests, air pollution, herbicide application and infection by non-viral pathogen can also induce virus like symptoms.

In the study fields the incidence of the disease were ranging 10-100% and yields were severely affected. According to Mahuku *et al.* 2015 the symptoms and incidence of MLND previously described (Uyemote *et al.* 1981; Goldberg & Brakke, 1987) which were observed in the study fields are remarkably similar to those exhibited in the fields of Kenya and Rwanda, which, taken with the absence of any other pathogen known to cause these symptoms.

Prior to progressing to the sequencing approach and artificial inoculation, standard plant pathology techniques had been applied with inconclusive results. Positive serological reactions with SCMV and MCMV confirmed that infection of Ethiopian maize samples with these viruses and thereby the occurrence of MLND in the study area. Results from DAS-ELISA assay of samples from rift valley regions of Ethiopia showed that samples; collected from Zeway, Melkassa and Hwassa, showed very strong reaction to MCMV and SCMV which is in line with the report of Mahuku *et al.* (2015).

According to Souza *et al.* (2012), the weak reactions of samples from Assosa against SCMV antibody suggest there is a difference between this virus and the SCMV from other area. Although some weak reaction and mostly no reaction with the MCMV for tested samples of Assossa has been verified. These results indicate the presence of unusual or variant isolates, thereby, limiting the use of serological tests for sensitive and specific identification of these viruses in maize (Adams *et al.*, 2012; Souza *et al.*, 2012).

This may be due to the polyclonal nature of the antibodies (Souza *et al.*, 2012). According to Adams *et al.* (2012), the apparent failure of ELISA may be attributed to a number of causes, amongst them, low sensitivity and poor specificity for unusual or variant isolates. Information was not provided on the range of isolates the antibodies tested were specific to, and thus reactivity (they are broad spectrum) was assumed when purchasing the ELISA reagents. Hence, more sensitive and specific detection techniques like PCR may be necessary for samples that show negative results using DAS-ELISA.

Recently, the application of the RT-PCR technique has resulted in clear and fully reproducible method for diagnostic assay of virus infected leaf material. This is well ensured for its eventual

application as a reliable and definitive test for SCMV and MCMV regardless of strain. Hence, to validate virus free nature of different varieties and clones of maize plants through molecular method, one step RT-PCR was employed. MCMV isolates from Assosa, that do not show any kind of reaction during the DAS-ELISA assay, were clearly shown on the RT-PCR result. RT-PCR has some advantages over other serological analysis like ELISA because it requires only small amount of samples and full analysis including PCR can be done in less than 24 Hours (Thorat *et al.*, 2015).

Considering the nucleotide sequence, the Ethiopian samples showed the highest similarities, 97-99% for MCMV and 95-98% for SCMV, with the Ethiopian accessions having maize as host and with other isolates from Kenya, Rwanda, USA, China, Iran and Argentina. The high degree of sequence variation identified may also explain why many isolates described in the current study was not detected by the DAS-ELISA assay. These results are in agreement with the studies of (Mahuku *et al.*, 2015a, b, c; Adams *et al.*, 2013; Wangai *et al.*, 2012). Phylogenetic analysis showed some correlation between the phylogenetic groups and the geographical origin of the SCMV and MCMV isolates that could be seen in the groups or subgroups which is in line with the results of Souza *et al.* (2012) and Thorat *et al.* (2015). Correlations between SCMV and MCMV phylogenetic groups and geographical origin were also found by Alegria *et al.* (2003).

The genetic evolutionary network based on the number of mutational events drawn shows that MCMV isolates of the study area shares some range of similarity and results in less number of haplotypes. Relatively the map shows the MCMV isolates undergone less number of mutational event which contributes to their diversity. MCMV isolates of East Africa are clearly very close to Chinese isolates (Fig. 10). In the SCMV (Fig. 11) each isolates were shown as an individual haplotype, which suggests SCMV is more diversified than MCMV in the study area. Some isolates share haplotype similarity with Ethiopian, Kenyan and Rwandan isolates, which may be an indication where the virus originated from (Souza *et al.*, 2012).

Sequencing and phylogenetic analysis have shown that the SCMV and MCMV of maize from Ethiopia constitute a distinct monophyletic group in relation to SCMV and MCMV from several geographic regions of the world. This genetic difference can possibly be attributed to the virus

evolution on a wide range of hosts in Ethiopia, provided by many weed and cultivated species growing throughout the year and a great diversity of aphid species-vectors, conditions that predominate in tropical and subtropical areas (Souza *et al.*, 2012).

The RT-PCR product clearly revealed that SCMV and MCMV isolates from the study samples were able to cause the MLND. Some of the symptoms were not clear for visual identification and the PCR confirmation was done. Both SCMV and MCMV strains were highly virulent to cause significant level of disease on sweet corn. Also strains isolated from previous inoculation were also able to cause the disease and developing clear symptoms. Plants inoculated with only MCMV strain showed the dead heart symptoms after two weeks of inoculation.

According to Adams *et al.*, 2012 after 5 weeks of inoculation, systemic chlorotic speckling symptoms were observed on the sweet corn plants. No symptoms were seen on non-inoculated control sweet corns used. Over the next 2–5 weeks further systemic symptoms of chlorotic streaking, mottling and finally striping and necrosis developed on the inoculated maize leaves. The work shows that both MCMV and SCMV, responsible viruses for MLND can be transmitted mechanically (through wounding) by carborandom. Both the results, symptom developments and RT-PCR confirmation were in line with the results reported by Zhang *et al.*, (1991).

7. Conclusion

- As per the objective, this research has shown the presence of the two viruses, MCMV and SCMV individually and in combination to cause MLND in the study fields.
- The symptoms of MLND in the study area are remarkably similar to those exhibited in the fields of Kenya, Rwanda and other reports from Eastern Africa.
- MLND incidence in the surveyed fields clearly indicated range of infestation; which is low to high incidence and further replacement of maize fields with other crops.
- DAS-ELISA assay clearly showed that there are variant or unusual isolates of both MCMV and SCMV in the study areas.
- The overall result showed RT-PCR is more sensitive than DAS-ELISA for detection and identification of maize viruses.
- MCMV is less diversified than SCMV in the study area and the findings are in agreement with other studies.
- This work further demonstrates the power of nucleotide sequencing for allowing the rapid identification of new and unusual plant viruses and facilitating the rapid development of high throughput diagnostic assay.

8. Recommendation

- Sequencing of more samples collected from all over Ethiopia will be helpful in understanding the spread of the disease and devise control and management strategy.
- It is highly recommended that to investigate the parts of the country if there are MLND free parts.
- It is advisable to screen different genotypes of maize to obtain MLND resistant genotypes.
- It is also very important to test the extent of transmission of both MCMV and SCMV via seed, insect vectors, soil and plant debris
- It is also good to investigate alternative hosts of MLND responsible viruses
- Clear understanding is required about the full range of other viruses in the maize growing system

9. References

- Abdolreza Abbassian. (2006). Maize International Market Profile Competitive Commercial Agriculture in Sub-Saharan Africa (CCAA) Study *Trade and Markets Division Food and Agriculture Organization of the United Nation*.
- Acland J. D. (1977). East African Crops, *FAO, Longma*. P. 252.
- Adams, A.N., (1978). The detection of plum pox virus in *Prunus* species by enzyme-linked immunosorbent assay (ELISA). *Annals of Applied Biology*, **90**: 215–221.
- Adams, I. P., Miano, D. W., Kinyua, Z. M., Wangai, A., Kimani, E., Phiri, N., Reeder, R., Harju, V., Glover, R., Hany, U., Souza-Richards, R., Deb Nath, P., Nixon, T., Fox, A., Barnes, A., Smith, J., Skelton, A., Thwaites, R., Mumford, R., and Boonham, N. (2013). Use of next generation sequencing for the identification and characterization of *Maize chlorotic mottle virus* and *Sugarcane mosaic virus* causing maize lethal necrosis in Kenya. *Plant Pathol.* **62**:741-749.
- Adane Abraham and Albrechtsen, S.E. (1998). Seed-borne viruses infecting some important crops in Ethiopia. *Pest Management Journal of Ethiopia*. **2**:94-101.
- Agrios G. N. (2005). Plant Pathology. 5th edtn. *Elsevier Academic Press*, 952 pp
- Alemu Lencho, Adane Abraham, Berhanu Bekele and MesfinTessera. (1997). Identification of viruses from maize and grass hosts. *Pest Management Journal of Ethiopia* **1**:73-76
- Alegria, O. M.; Royer, M.; Bousalem, M.; Chatenet, M.; Peterschmitt, M.; Girard, J. C.; Rott, P. (2003). Genetic diversity in the coat protein coding region of eighty-six *Sugarcane mosaic virus* isolates from eight countries, particularly from Cameroon and Congo. *Archives of Virology*, **148**:357-372
- Arumuganathan, K. and Earle, E.D. (1991). Nuclear DNA content of some important plantspecies. *Plant Mol. Biol. Rep.***9**:208-218
- ASARECA (2013). Regional Conference on Fighting Against Maize Lethal Necrosis (MLN) disease to boost maize production. pp
- ASARECA (2014). Taking on the maize monster. Pp.

- Astier S, Albouy J, Maury Y, Robaglia C, Lecoq H. (2007). Principles of Plant Virology: Genome, Pathogenicity, Virus Ecology, *Science Publishers*, ISBN: 1578083168, New Hampshire, USA
- Autrey, L. J. C. (1983). *Maize Mosaic Virus* and other maize virus diseases in the islands of the western Indian Ocean. In: Proceedings of the International Maize Virus Diseases Colloquium and Workshop. The Ohio State University, Ohio Agricultural Research and Development Center, Wooster, Ohio, **44691**: 167-181.
- Bancroft J. B, Ullstrup AJ, Messieha M, Bracker CE, Snazelle TE (1966). Some biological and physical properties of a mid western isolate of *Maize Dwarf Mosaic Virus*. *Phytopathology* **56(5)**:474-478.
- Bock, K.R. (1982). The identification and partial characterization of plant viruses in the tropics. *Trop. Pest Manage.* **28**:399-411.
- Bockelman DL, Claflin LE, Uyemoto JK (1982). Host range and seed-transmission studies of *Maize Chlorotic Mottle Virus* in grasses and corn. *Plant Dis.* **66(3)**:216-218.
- Bua, B. and Chelimo, B.M. (2010). The reaction of maize genotypes to maize streak virus disease in central Uganda. *Second RUFORUM Biennial Meeting 20 - 24 September 2010, Entebbe, Uganda*
- Brunt, A.A., Crabtree, K., Dallwitz, M.J., Gibbs, M.J. and Watson, L. (2000). Viruses' of Plants. Description and Lists from the VIDE Database, 1484 pp.
- Carlos De Leon (1984). Maize Diseases: A Guide for Field Identification. Maize Program, CIMMYT.
- Castillo-Loayza J. (1977) Maize virus and virus-like diseases in Peru. In: Williams LE, Gordon DT, Nault LR. (eds) Proc Int Maize Virus Disease Colloquim Workshop, 16-19 August 1976. Wooster, OH, pp 40-44.
- CGIAR Research Program MAIZE 2012 Annual Report (2012). Annual Report. Mexico, D.F.: CIMMYT. Available at: <http://libcatalog.cimmyt.org/download/cim/98018.pdf>.

- CSA (2013). Agricultural Sample Survey 2012/2013: Report on area and production of major crops (private, peasant holdings, 'Meher' season). *Statistical Bulletin*. Vol.1, Addis Ababa: CSA.
- The CIMMYT Maize Program. (2004). *Maize Diseases: A Guide for Field Identification*. 4th edition. Mexico, D.F.: CIMMYT.
- Coleman WB, Tsongalis GJ. (2006). Molecular diagnostics for the Clinical Laboratorian. 2nd edition. *Humana press* pp.47-54
- David Hoisington, Mireille Khairallah, Timothy Reeves, Jean-Marcel Ribaut, Bent Skovmand, Suketoshi Taba, and Marilyn Warburton (1998). Plant genetic resources: What can they contribute toward increased crop productivity? *Proc. Natl. Acad. Sci. USA*. **96**: 5937–5943
- Dhillon, B.S. and Prasanna, B.M. (2001).Maize; In "Breeding Food Crops." Ed. Chopra V.L. Oxford & IBH, New Delhi. pp 147-185
- Doebley, J. (1990). Molecular evidence for gene flow among *Zea* species. *Bio.Science*. **40**(6):443-448.
- Doughari JH, Ndakidemi PA, Human IS, Bennade S (2009). Shiga toxins (Verocytotoxins). *Afr. J. Microbiol. Res.* **3**(11):681-693.
- Dowswell, C.R., Paliwal, R.L. and Cantrell, R.P. (1996). Maize in the Third World. *Westview Press*, Boulder, USA.
- Erika C.H. Meng, Ruifa Hu, Xiaohua Shi and Shihuang Zhang. (2006). Maize in China: Production Systems, Constraints, and Research Priorities. CIMMYT.
- FAOSTAT. (2010). Statistical databases and data sets of the Food and Agriculture Organization of the United Nations. Online publication.
- Goldberg, Karen-Beth and Brakke, Myron K. (1987). Concentration of Maize Chlorotic Mottle Virus Increased in Mixed Infections with Maize Dwarf Mosaic Virus, Strain B *Papers in Plant Pathology*. Paper 177.
- Gordon DT, Bradfute OE, Gingery RE, Nault LR, Uyemoto JK (1984). Maize Chlorotic Mottle Virus. CMI/AAB Description of plant viruses, p.284.
- Giolitti, F.; Herrera, M. G.; Madariaga, M.; Lenardon, S. L. (2005). Detection of *Maize dwarf mosaic virus* (MDMV) on maize in Chile. *Maydica*, Bergamo, **50**:101-104.

- Hadidi A, Levy L, Podleckis EV (1995). Polymerase chain reaction technology in plant pathology. Singh RP, Singh US (Eds.). CRC Press, Boca Raton, Florida, USA. *Mol. Meth. Plant Pathol.* pp. 167-187.
- Hardingham JE, Chua A, Wrin JW, Shivasami A, Kanter I, Tebbutt NC, Price TJ (2012). BRAF V600E Mutation Detection Using High Resolution Probe Melting Analysis, Polymerase Chain Reaction. Hernandez-Rodriguez P (Eds.). ISBN: 978-953-51-0612-8, In Tech.
- Hebert, T.T. and Castillo, J. (1973). A new virus disease of maize in Peru. *Minneapolis. 2nd Int. Congr. Pl. Path.* 72.
- Henson JM, French R (1993). The polymerase chain reaction and plant disease diagnosis. *Annu. Rev. Phytopathol.* **31**:81–109.
- Hewings, A.D. and C.J. D'Arcy. 1984. Maximizing the detection capability of a beet western yellows virus ELISA system. *Journal of Virological Methods* 9: 131–142.
- Heisey, P. W., and Edmeades, G. O. (1999). Maize production in drought-stressed environments: technical options and research resource allocation. Part 1 of CIMMYT 1997/ 1998 World Facts and Trends. CIMMYT, Mexico D.F., Mexico.
- Hull, R. Mathew. (2002). Plant Virology. Fourth edition, Academic press, New York, USA.
- Ilbagi, H., A. Çıtır, and U. Yorgancı. (2005). Occurrence of virus infection on cereal crops and their identifications in the Trakya region of Turkey. *J. Plant Dis. Protec.* **112** : 313-320.
- Jean du Plessis. (2003). Maize production. Department: Agriculture Republic of South Africa.
- Jensen, S., Wysong, D., Ball, E., Higley, P., 1991. Seed transmission of maize chlorotic mottle virus. *Plant Dis.*, **75**(5):497-498.
- Kang, Byoung, C. Inhwa Y., & Molly M. J, (2005). Genetics of plant virus resistance. *Annual Review of Phytopathology* **43**: 581-621.
- Karban, R. and Baldwin, I.T. (1997). Induced Responses to Herbivory. *U. of Chicago P.*, Chicago 319 pp.
- King, A.M.Q., Lefkowitz, E., Adams, M.J., Carstens, E.B. (2011). Virus Taxonomy: Ninth Report of the International Committee on Taxonomy of Viruses. San Diego. *Elsevier Academic Press* **9**:256-267.

- Kumar PL, Jones AT, Waliyar F (2004). Serological and nucleic acid based methods for the detection of plant viruses. Manual. International Crops Research Institute for the Semi-Arid Tropics, Patancheru, Andhra Pradesh.
- Kyeterere, D.T., Ming, R., McMullen, M.D., Pratt, R.C., Brewbaker, J. and Musket, T. (1999). Genetic analysis of tolerance to maize streak virus in maize. *Genome* **42**:20-26.
- Lima J. A. A, Nascimento A. K. Q, Radaelli, P, Purcifull D. E (2012). Serology applied to plant virology. Serological diagnosis of certain human, animal and plant diseases. Rijeka Croácia. In Tech pp. 71-94.
- Lommel, S., Kendall, T., Siu, N., Nutter, R., (199). Characterization of maize chlorotic mottle virus. *Phytopathology*, **81**(8):819-823.
- López MM, Bertolini E, Olmos A, Caruso P, Gorris MT, Llop P, Cambra M (2003). Innovative tools for detection of plant pathogenic viruses and bacteria. *Int. Microbiol.* **6**(4):233-243.
- Louie, R., and Darrah, L. L. (1980). Disease resistance and yield loss to sugarcane mosaic virus in East African-adapted maize. *Crop Sci.* **20**:638-640.
- Lukanda M, Owati A, Ogunsanya P, Valimunzigha K, Katsongo K, Ndemere H, Kumar PL (2014). First report of *Maize Chlorotic Mottle Virus* infecting maize in the Democratic Republic of the Congo. *Plant Dis.* **98**(10):1448-1448.
- Makone SM, Menge D, Basweti E (2014). Impact of Maize Lethal Necrosis Disease on maize yield: A Case of Kisii, Kenya. *Int. J. Agric. Ext.* **2**(3):211-218.
- Mahuku, G., Lockhart, B. E., Wanjala, B., Jones, M. W., Kimunye, J. N., Stewart, L. R., Cassone, B. J., Sevgan, S., Nyasani, J. O., Kusia, E., Kumar, P. L., Niblett, C. L., Kiggundu, A., Asea, G., Pappu, H. R., Wangai, A., Prasanna, B. M., and Redinbaugh, M. G. (2015a). Maize lethal necrosis (MLN), an emerging threat to maize-based food security in sub-Saharan Africa. *Phytopathology* **105**:956-965.
- Mahuku G, Wangai AW, Sadessa K, Teklewold A, Wegary D, Adams I, Smith J, Braidwood L, Feyissa B, Regassa B, Wanjala B, Kimunye JN, Mugambi C, BoTtomley E, Bryce S, Ayalneh D, Prasanna, BM (2015b). First report of Maize chlorotic mottle virus and Maize lethal necrosis on maize in Ethiopia. *Plant Dis.*
- Makumbi D, Wangai A (2013). Maize Lethal Necrosis (MLN) disease in Kenya and Tanzania: Facts and actions. CIMMYT- KARI.
- Maloy O. C. (2005). Plant Disease Management. The Plant Health Instructor.

- Mangelsdorf, P. C., Roberts, L.M. and Rogers, J.S. (1981). The Probable Origin of Annual Teosintes. *Bussey Inst. Harvard Univ. Publ.* **10**:1- 69.
- Martin, D. P., and Rybicki, E. P. (2000). RDP: Detection of recombination amongst aligned sequences. *Bioinformatics*, **16**:562-563.
- Mawishe R, Chacha E (2013). Uproot maize plants with lethal necrosis disease. Plantwise Factsheets for Farmers, CABI.
- McDaniel LL, Gordon DT (1985). Identification of a new strain of *Maize Dwarf Mosaic Virus*. *Plant Dis.* **69**(7):602-607.
- McGee, D. C. (1980). Maize disease. A reference source for seed technologists. The American *Phytopathological Society*. 150 pages
- Mexico, D.F. (1994). Maize seed industries revisited: emerging roles of the public and private sectors. World Maize facts and trends.1993/94.CIMMYT.
- Mezzalama M, Das B, Prasanna B. M, (2015). MLN Pathogen diagnosis, MLN free seed production and safe exchange to non-endemic countries.(CIMMYT brochure) Mexico, D.F.: CIMMYT.
- Murphy F. A., C. M. Fauquet, D. H. L. Bishop, S. A. Ghabrial, A. W. Jarvis, G. P. Martelli, Mayo, M. D. (1995). Viruses Taxonomy: The classification and Nomenclature of Viruses. The 6th report of the International committee on Taxonomy of viruses. *Springer-verbg, Vienna*
- Naidu RA, Hughes JDA (2001). Methods for the detection of plant virus diseases. Hughes J. D. A, Odu B. O. (Eds.). Proceedings of a Conference Organized by IITA, International Institute of Tropical Agriculture, ISBN 9781312149, Nigeria. Plant Virology in Sub-Saharan Africa, pp. 233-260.
- Naidu RA, Hughes JDA (2003). Methods for the detection of plant viral diseases in plant virology in sub-Saharan Africa, Proceedings of plant virology, IITA, Ibadan, Nigeria. Eds. Hughes JDA, Odu B, pp. 233-260.
- Nault, L.R., Styer, W.E., Coffey, M.E., Gordon, D.T., Negi, L.S. and Niblett, C.L. (1978).

- Transmission of *maize chlorotic mottle virus* by *Chrysomelid* beetles. *Phytopathology* **68**:1071–1074.
- Nelson SJ, Brewbaker, Hu J (2011). Maize Chlorotic Mottle Virus. *Plant Dis.* **79**:1-6.
- Ngotho A (2013). Kenya: New KARI Centre Fights Lethal Maize Disease.
- Niblett, C.L. and Claflin, L.E. (1978). Corn lethal necrosis – a new virus disease of corn in Kansas. *Plant Dis. Rep.* **62**:15–19.
- Nutter, R.C., Scheets, K., Panganiban, L., Lommel, S.(1989).The complete nucleotide sequence of the *maize chlorotic mottle virus* genome. *Nucleic Acids Res.* **17**(8):3163-3177.
- Nyvall, R. (1999). Field crop diseases, 3rdedn. *Iowa State University Press*, Ames, Iowa.
- Paudyal, K. R., Ransom, J. K., Rajbhadari, N. P., Adhikari, K., Gerpacio, R. V. and Pingali, P. L. (2001). Maize in Nepal: Production Systems, Constraints and Priorities for Research. Kathmandu: Nepal Agricultural Research Council and International Maize and Wheat Improvement Center.
- Perring T M, Gruenhagen NM, Farrar CA (1999). Management of plant viral diseases through chemical control of insect vectors. *Annu. Rev. Entomol.* **44**:457-481
- Punja ZK, De Boer SH, Sanfaçon H (Eds.) (2007). Biotechnology and plant disease management. CABI, 227.
- Rafael HA, Souza IRP, Barros BA, Pinto MO, Moreira RO, Gonçalves IAM, Carvalho SGM (2014). Molecular detection of the *Sugarcane Mosaic Virus* causing mosaic disease in maize and sorghum in Brazil. In: *Embrapa Milho e Sorgo-Resumoemanais de congresso (ALICE)*. In: CONGRESSO BRASILEIRO DE GENÉTICA, 59, 2013, Águas de Lindóia. Resumos. Ribeirão Preto: SBG, 2013. P 94.
- Rao JR, Fleming CC, Moore JE (Eds.) (2006). Molecular diagnostics, Current Technology and Application. *Horizon Bioscience Press*, pp. 1-19.
- Rosegrant, M. W., Msangi, S., Ringler, C., Sulser, T. B., Zhu, T., and Cline, S. A. (2008). International Model for Policy Analysis of Agricultural Commodities and Trade

- (IMPACT): Model Description. International Food Policy Research Institute, Washington, D.C.
- Satapathy MK (1998). Chemical control of insect and nematode vectors of plant viruses. *Plant Virus Control*. The American Phytopathological Society, St. Paul, Minnesota, pp. 188-195
- Scheets K (1998). Maize Chlorotic Mottle Machlomovirus and Wheat Streak Mosaic Rymovirus. Concentrations increase in the synergistic disease Corn Lethal Necrosis. *Virology* **242**(1):28-38.
- Scheets, K. (2010). *Machlomovirus*. In: *Desk encyclopedia of plant and fungal virology*, Mahy, B.W.J., and Van Regenmortel, M.H.V. (eds). *Oxford Academic Press* pp 204-209.
- Shukla, D. D.; Lauricella, R.; Ward, C. W. (1992). Serology of potyviruses: current problems and some solutions. *Archives of Virology*, New York, **5**: 57-59.
- Shukla, D.D., Ward, C.W. and Burnt, A.A. (1994). "*The Potyviridae*," CAB International, Wallingford, UK.
- Singh, M. and R.P. Singh. (1995). Digoxigenin-labelled cDNA probes for the detection of potato virus Y in dormant potato tubers. *Journal of Virological Methods* **52**: 133–143.
- Slykhuis, J.T. (1995). *Aceriatulipae* Keifer (Acarina: Eriophyidae) in relation to the spread of wheat streak mosaic. *Phytopath.*, **45**:116-128.
- Smale, M. and T.S. Jayne. (2003). —Maize in Eastern and Southern Africa: —Seeds of Success in Retrospect. EPTD Discussion Paper 97. IFPRI, Washington, D.C.
- Stenger, Drake C. and Roy French (2008). Complete nucleotide sequence of a maize chlorotic mottle virus isolate from Nebraska. *Arch Virol*, **153**:995–997
- Steven Naveed, M. Aslam, M.A. Maqbool, S. Bano, Q.U. Zaman and R. M. Ahmad (2014). *Physiology of high temperature stress tolerance at*

reproductive stages in maize. *Journal of Animal & Plant science* **24(4)**:
1141-1145

Teakle, D.S., D.D.Shukla and R.E.Ford. (1989). Sugarcane mosaic virus. Descriptions of Plant Viruses.aab 342.

Thorat, V. S , R.K. Pal, P.R. Shingote, S.B Kharte, V.M. Nalavade, D.R. Dhumale, B.H. Pawar and K.H. Babu. (2015). Detection of Sugarcane Mosaic Virus in Diseased Sugarcane using ELISA and RT-PCR Technique. *Journal of pure and applied microbiology*, Vol. 9(1), p

Tito, C.M., Poggio, L. and Naranjo, C.A. (1991). Cytogenetic studies in the genus *Zea*. 3. DNA content and heterochromatin in species and hybrids. *Theor. Appl. Genet.* **83**:58-64.

Trigiano RN, Windham MT, Windhan AS (Eds.) (2008). Plant pathology, concepts and laboratory exercises. *CRC Press* 21:269.

Tropical Pesticides Research Institute (TPRI) (2011). List of registered pesticides in Tanzania.

Uyemoto JK (1980). Detection of maize chlorotic mottle virus serotypes by enzyme-linked immunosorbent assay. *Phytopathology*, **70**: 290-292.

Uyemoto, J. K., Claflin, L. E., and Wilson, D. L. (1981). *Maize chlorotic mottle and maize dwarf mosaic* viruses: Effect of single and double inoculations on symptomatology and yield. *Plant Dis.* **65**:39-41.

Uyemoto, J. (1983). Biology and control of *maize chlorotic mottle virus*. *Plant Dis.* **67**:7-10

Van den Boogaart, T., Lomonosoff, G.P. and Davies, J.W. (1998). Can we explain RNA-mediated virus resistance by homology-dependent gene silencing? *Mol. Plant-Microbe Interact.***11**:717–723.

Viljoen GR, Nel LH, Crowther JR (2005). Molecular diagnostics PCR Handbook. Springer Science and Business Media, p.63.

Wang Q, Zhou XP, Wu JX (2014). First Report of *Maize Chlorotic Mottle Virus* Infecting Sugarcane (*Saccharum officinarum*). *Plant Dis.* **98(4)**:572-572.

- Wangai, A.W., Redinbaugh, M.G., Kinyua, Z.M., Miano, D.W., Leley, P.K., Kasina, M., Mahuku, G. and Jeffers, D. (2012a). First Report of *Maize chlorotic mottle virus* and Maize Lethal Necrosis in Kenya. *Plant Dis.* **96**:1582
- Wangai, A. W., Redinbaugh, M. G., Kinyua, Z. M., Mahuku, G., Sheets, K., and Jeffers, D. 2012b. First report of *Maize chlorotic mottle virus* and maize lethal necrosis in Kenya. *Plant Dis.* **96**:1582.
- Wangai A, Sikinyi E, Ochieng J, Miyogo S, Karanja T, Odour H, Kimani E, Irungu J, Kinyua Z, Ngaruiya P, Ligeyo D, Kipkemboi S (2012c). Joint assessment report: Report on status of maize lethal necrosis disease and general maize performance. Ministry of Agriculture, Kenya. Webster CG, Wylie SJ, Jones MG (2004). Diagnosis of plant viral pathogens. *Curr. Sci.* **86**(12):1604-1607.
- Wegulo SN, Hein GL, Klein RN, French RC (2008). Managing *Wheat Streak Mosaic*. The board of Regents of the University of Nebraska. EC 1871
- Whetzel HH (1929). The terminology of plant pathology. Proc. Int. Cong. Plant Science, Ithaca, NY, **1926**:1204-1215
- Williams, L. E. and Alexander, L. J. (1965). Maize dwarf mosaic, a new corn disease. *Phytopathology* **55**:802-804.
- Wu JX, Wang Q, Liu H, Qian YJ, Xie Y, Zhou XP (2013). Monoclonal antibody-based serological methods for *Maize Chlorotic Mottle Virus* detection in China. *J. Zhejiang Univ. Sci. B* **14**(7):555-562.
- Xie, Y., Wang, M., Xu, D., Li, R., Zhou, G. (2009). Simultaneous detection and identification of four sugarcane viruses by one-step RT-PCR. *J. Virol. Methods*,; **162**: 64-68.
- Xie, L., Zhang, J. Z., Wang, Q. A., Meng, C. M., Hong, J. A., and Zhou, X. P. (2011). Characterization of *Maize chlorotic mottle virus* associated with maize lethal necrosis disease in China. *J. Phytopathol.* **159**:191-193.
- Yinan Yuan, Phillip J. SanMiguel and Jefferey L. Bennetzen (2003). High-cost sequence Analysis of the maize genome. *The Plant Journal*, **34**: 249-255.
- Zhang, Y., Zhao, W., Li, M., Chen, H., Zhu, S., Fan, Z., (2011). Real-time Taqman RT-PCR for detection of maize chlorotic mottle virus in maize seeds. *J. Virol. Methods*, **171**(1):292-294.

10. Appendices

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

The ELISA reagents are optimized using greiner bio-one microplates, medium binding. Before opening the tubes containing coating antibody (IgG) and IgG-AP-Conjugate please spin down all the liquid by a short centrifuge.

Steps

1. Dilute specific coating antibody (IgG) in **coating buffer**; i.e. 20µl in 20ml buffer at a recommended dilution of 1:1000. Add 100 µl to each well of the microtiter plate.
2. Cover the plates and incubate at 37⁰C for 2-4 hr.
3. Wash the plates with **PBS-Tween** using wash bottle, soak for a few minutes and repeat washing two times. Blot plates by tapping upside down on tissue paper.
4. Add 100 µl of aliquots of the test sample (extracted fresh leaf samples in **Sample extraction buffer** 1:20 weight/volume) to duplicate wells.
5. Cover the plates and incubate overnight at 4⁰C.
6. Wash three times as in step 3.
7. Dilute specific enzyme conjugate (IgG-AP) in **conjugate buffer**; i.e. 20µl in 20ml buffer at a recommended dilution of 1:1000. Add 100µl to each well of the microtiter plate.
8. Cover the plates and incubate at 37 ⁰C for 2-4 hr.
9. Wash three times as in step 3.
10. Add 100µl aliquots of freshly prepared **PNP substrate** to each well.
11. Cover the plates and incubate at 37 ⁰C for 30-60 min, or as long as necessary to obtain clear reactions.
12. **Assess results by:**
 - a) Visual observation

b) Spectrophotometer measurement of absorbance at 405 nm

Buffers used in ELISA prepared from buffer packs:

1. *Coating Buffer*

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

2. *PBS-Tween*

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

3. *Sample Extraction Buffer*

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

4. *Conjugate Buffer*

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

5. *PNP substrate*

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

❖ Buffers can be stored at 40C for at least 2 months. Warm to room temperature before use.

Appendix 2. PureHelix™ Total RNA Purification Kit [Virus, Animals, Plants, Bacteria] (Column Type)

Kit contents

Column set (with cap) 50ea/**Blue Box** 7 box

Buffer RCLB 25 ml, 50 ml, 100 ml

Buffer RWB1 32 ml (**Add 8 ml ethanol**) 64 ml (**Add 16 ml ethanol**) 128 ml (**Add 32 ml ethanol**)

Buffer RWB2 8 ml (**Add 32 ml ethanol**) 16 ml (**Add 64 ml ethanol**) 32 ml (**Add 128 ml ethanol**)

MaxBinder™ solution 5 ml, 10 ml, 20 ml

EB (RNase-free) 5 ml, 10 ml, 10 ml x 2ea

Certificate Analysis 1

Description

PureHelix™ Total RNA Purification Kit [Virus, Animals, Plants, Bacteria] is designed for rapid, pure, and high yield isolation of total RNA from small amounts of various samples including blood, animal and plant tissues, bacteria and viruses. This kit is suitable to the rapid preparation of nucleic acids for molecular diagnostics using conventional and real-time RT-PCR technologies. Due to elimination of phenol, handling of the kit is safe and no harmful waste is produced. The purified total RNA can be used in a number of downstream applications.

Applications

- Preparation of total RNA for RT-PCR or quantitative RT-PCR
- Preparation of nucleic acid sample for molecular diagnostics

Store

- Ambient temperature

Quality control assay data

Functional analysis

PureHelix™ Total RNA Purification Kit [Virus, Animal, Plant, Bacteria] was tested for the isolation of total RNA from blood, animal tissue, plant leaf tissue and bacterial cell.

Protocol

Important things to do before starting

- **β-Mercaptoethanol** (not provided in this kit) must be added to Buffer RCLB before use. **Add 10 μl of β-Mercaptoethanol per 1 ml of Buffer RCLB.** The β-Mercaptoethanol(2-ME) containing Buffer RCLB is stable for 1 week at room temperature.
- Prepare **100% Isopropanol** (not provided in this kit)
- Before using for the first time, **add absolute ethanol into the Buffer RWB1** as indicated on the above to obtain a working solution.
- Before using for the first time, **add 4 volumes of absolute ethanol into the Buffer RWB2** to obtain a working solution.

※ **If you need more Buffer RWB2, you may use 80% ethanol (RNase-free).**

1. Sample Preparation and Cell Lysis.

Fresh tissue sample – Animals or plants

- 1) Add **300 μl of Buffer RCLB (2-ME added)** to **20 ~ 50 mg fresh tissue sample** in a microcentrifuge tube, and homogenize using an appropriate apparatus, such as hand-operated pellet pestle or motor-driven grinder.
- 2) Add **additional 200 μl of Buffer RCLB (2-ME added)** to the homogenized sample and vortex for 15 ~ 30 sec.

※ **Sample volume should not exceed 10% volume of Buffer RCLB.**

- 3) Centrifuge at 12,000 rpm for 10 min and transfer the supernatant into a microcentrifuge tube.
- 4) [Optional] In case that debris still remains in the supernatant, add 500 μl chloroform and vortex for 15 ~ 30 sec. Centrifuge at 12,000 rpm for 10 min and transfer the upper aqueous phase to a microcentrifuge tube.

※ Chloroform is not provided in this kit.

Blood

- 1) Transfer **100 µl** of **non-coagulating blood** to a microcentrifuge tube.
- 2) Add **500 µl of Buffer RCLB (2-ME added)** and vortex for 10 sec.

Cells from Nasal or Throat Swabs

- 1) Add **500 µl of Buffer RCLB (2-ME added)** to a microcentrifuge tube.
- 2) Brush a sterile, single-use cotton swab or Buccal Swab Brush inside the nose or mouth of the subject.
- 3) Cut the cotton tip where the nasal or throat cells were collected and place into the microcentrifuge tube containing the Buffer RCLB (2-ME added). Close the tube. Vortex and incubate at room temperature for 5 min.

Cells grown in monolayer

- 1) Put off culture media.
- 2) Add **500 µl of Buffer RCLB (2-ME added) per 1 ~ 5 x 10⁶ cells.**
- 3) Lyse cells and homogenize the sample by pipetting up and down several times.

Cells grown in suspension

- 1) Pellet **1 ~ 5 x 10⁶ animal, plant, or yeast cells, or 1 x 10⁷ bacterial cells.**
Occasionally, enzymatic lysis or mechanical disruption is required for the cell-wall disruption of some yeast and bacterial cells.
- 2) Discard the supernatant and then add **500 µl of Buffer RCLB (2-ME added).**
- 3) Lyse the sample by repetitive pipetting or vortexing for 10 sec.

2. Column Activation

- 1) Place a Spin Column into a 2 ml collection tube.
 - 2) Add **100 µl of MaxBinder™ solution** into the Spin Column.
 - 3) Centrifuge at 12,000 rpm for 30 sec and immediately proceed to next step.
- You need not discard the flow-through from the collection tube.

※ These steps are required for the best yield.

3. Column Loading

- 1) Add **300 µl** (or 0.6 x volume of the cell lysate) of **Isopropanol** to the prepared cell lysate and vortex.
- 2) Transfer the mixture directly into a spin column sitting in a 2 ml collection tube and

centrifuge at 12,000 rpm for 30 sec. Discard the flow-through.

※ [Optional] RNase-free DNase can be applied with on-column DNase digestion for the elimination of remaining genomic DNA.

4. Column Washing

- 1) Add **700 µl** of **RWB1** (ethanol added) into the spin column, and centrifuge at 12,000 rpm for 30 sec. discard the flow-through.
- 2) Add **700 µl** of **RWB2** (ethanol added) or 80% ethanol (RNase-free) into the spin-column and centrifuge at 12,000 rpm for 30 sec. Discard the flow-through.
- 3) Centrifuge again at 12,000 rpm for 2 min to remove residual ethanol.

5. Elution of RNA

- 1) Place the spin column into an RNase-free microcentrifuge tube.
- 2) Add **40 ~ 50 µl** of **EB** to the center of the column membrane, and incubate at room temperature for 1 min.
- 3) Centrifuge at 12,000 rpm for 1 min, and store RNA at -20 or -70°C.

Appendix 3. HelixCRIPT™ One-Step RT-PCR Kit [Hot-Taq]

Kit contents

- Enzyme Mix [Hot-Taq] 0.2 ml
- 2x Reaction Mix [Hot-Taq] (containing dNTP mix, MgCl₂) 2 x 1.25 ml
- Certificate Analysis 1

*** Store at -20**

Description

HelixCRIPT™ One-Step RT-PCR Kit is designed for sensitive amplification of the target gene in one-tube reaction from total transcripts. A reverse transcriptase, HelixCRIPT™ Thermo Reverse Transcriptase, and a thermostable DNA polymerase, HelixAmp™ *HyperSense-Taq* polymerase, HelixAmp™ *Speed-Pfu* polymerase, or HelixAmp™ *Hot-Taq* polymerase are supplied as an enzyme mixture. You can choose one of the enzymes according to your purpose.

HelixCRIPT™ One-Step RT-PCR Kit [Hot-Taq]: High specific and sensitive RT-PCR

One-step RT-PCR system provide the several advantages.

- Synthesis of cDNA and PCR amplification corresponding to target gene in one-tube reaction
- Obtain the reproductive data in the repetitive experiment(s)
- Can save the time and cost for preparation of RT-PCR
- Amplification of low-copy transcripts by RT-PCR

Application

Detection of target gene transcript from RNA

Semi-quantitative, quantitative analysis of RNA transcription level

Quality control assay data

Functional analysis

The activity for cDNA synthesis and PCR amplification of target gene transcript using HelixCript™ One-Step RT-PCR Kit was evaluated by Limit-of Detection (LOD) assay and long range of gene constitutively expressed in human total transcripts.

Protocol

1. Program the thermal cycler as follows in order to synthesize cDNA using HelixCript™ One-Step RT-PCR Kit with HelixAmp™ *Hot-Taq*.

cDNA Synthesis and Pre-denaturation (1 cycle)

50 min at 42 ~ 55°C

12 ~ 15 min at 95°C

PCR Amplification (30 ~ 40 cycles)

Denaturation: 20 sec at 95°C

Primer Annealing: 40 sec at AT°C

Extension: 1 min/kb at 72°C

Post extension (1 cycle)

5 min at 72°C

2. Add the following components into 0.2 or 0.5 ml micro-tube.

<u>Components</u>	<u>Volumes</u>
2x Reaction Mix [Hot-Taq]	25 µl
RNA Template (1 ng ~ 5 µg)	X µl
Forward primer (10 pmoles/µl)	2 µl
Reverse primer (10 pmoles/µl)	2 µl
HelixCript™ Thermo RT / HelixAmp™ Hot-Taq DNA polymerase enzyme mix	2 µl

※ RNAs : total RNA : 10 ng ~ 5 μ g, Poly(A)+ RNA : 1 ng ~ 500 ng

3. Gently mix and immediately centrifuge the reaction mix.

4. Perform the one-step RT-PCR.

Appendix 4. Data Sheet for Sample Collection and Incidence

No	Symptom	Region	Zone	Woreda	Keble	Lat.(N)	Lon.(E)	Alt.(M)
1	Stunt, Necrosis	BeniShangul-Gumuz	Assossa	Assossa	AARC, field	10.19	34.58	1551
2	Clear, Necrosis	BeniShangul-Gumuz	Assossa	Assossa	AARC, field	10.19	34.58	1551
3	Stunt, Necrosis	BeniShangul-Gumuz	Assossa	Assossa	AARC, field	10.19	34.58	1551
4	Vein clearing	BeniShangul-Gumuz	Assossa	Assossa	AARC, field	10.19	34.58	1551
5	Dwarf & Necrosis	BeniShangul-Gumuz	Assossa	Assossa	AARC, field	10.19	34.58	1551
6	Stunt & Necrosis	BeniShangul-Gumuz	Assossa	Assossa	AARC, field	10.19	34.58	1551
7	Vein clearing	BeniShangul-Gumuz	Assossa	Assossa	AARC, field	10.19	34.58	1551
8	Stunting	BeniShangul-Gumuz	Assossa	Assossa	AARC, field	10.19	34.58	1551
9	Stunt, Necrosis	BeniShangul-Gumuz	Assossa	Assossa	AARC, field	10.19	34.58	1551
10	Healthy	BeniShangul-Gumuz	Assossa	Assossa	AARC, field	10.19	34.58	1551
11	Streak & Necrosis	BeniShangul-Gumuz	Assossa	Assossa	AARC, field	10.22	34.59	1547
12	Moderate Streak	BeniShangul-Gumuz	Assossa	Assossa	AARC, field	10.22	34.59	1547
13	Streak & Necrosis	BeniShangul-Gumuz	Assossa	Assossa	AARC, field	10.22	34.59	1547
14	Red ip leaves	BeniShangul-Gumuz	Assossa	Assossa	AARC, field	10.23	34.26	1549
15	Stunting	BeniShangul-Gumuz	Assossa	Assossa	AARC, field	10.23	34.26	1549
16	Short & Necrosis	BeniShangul-Gumuz	Assossa	Assossa	AARC, field	10.23	34.26	1549
17	Streak & Necrosis	BeniShangul-Gumuz	Assossa	Assossa	AARC, field	10.23	34.26	1549
18	Necrosis & Streak	BeniShangul-Gumuz	Assossa	Assossa	AARC, field	10.23	34.26	1549
19	Dwarf & Streak	BeniShangul-Gumuz	Assossa	Assossa	AARC, field	10.23	34.26	1549
20	Necrosis & Streak	BeniShangul-Gumuz	Assossa	Assossa	AARC, field	10.23	34.26	1549
21	Premature drying	BeniShangul-Gumuz	Assossa	Assossa	AARC, field	10.23	34.26	1549
22	Streak & Necrosis	BeniShangul-Gumuz	Assossa	Assossa	AARC, field	10.23	34.26	1552
23	Streak & Necrosis	BeniShangul-Gumuz	Assossa	Assossa	Komeshg-28	10.12	34.72	1472
24	Healthy & Streak	BeniShangul-Gumuz	Assossa	Assossa	Komeshg-28	10.12	34.72	1472
25	Healthy Streak	BeniShangul-Gumuz	Assossa	Assossa	Komeshg-28	10.12	34.72	1472
26	Stunt & Necrosis	BeniShangul-Gumuz	Assossa	Assossa	Komeshg-28	10.12	34.72	1472
27	Necrosis & Streak	BeniShangul-Gumuz	Assossa	Assossa	Komeshg-28	10.12	34.72	1472

28	Necrosis & Streak	BeniShangul-Gumuz	Assossa	Assossa	Komeshg-28	10.12	34.72	1472
29	Healthy, Maturing	BeniShangul-Gumuz	Assossa	Assossa	Komeshg-28	10.12	34.72	1472
30	Stunt & Streak	BeniShangul-Gumuz	Assossa	Assossa	Komeshg-28	10.12	34.72	1472
31	White Streak	BeniShangul-Gumuz	Assossa	Bambassie	Ataferbavare	10.17	34.89	1477
32	Very Stunt	BeniShangul-Gumuz	Assossa	Bambassie	Ataferbavare	10.17	34.89	1477
33	Streak & Stunt	BeniShangul-Gumuz	Assossa	Bambassie	Ataferbavare	9.99	34.86	1475
34	Streak & Stunt	BeniShangul-Gumuz	Assossa	Bambassie	Ataferbavare	9.99	34.86	1475
35	Very Streak	BeniShangul-Gumuz	Assossa	Bambassie	Ataferbavare	10.11	34.81	1482
36	Necrosis	BeniShangul-Gumuz	Assossa	Bambassie	Ataferbavare	10.11	34.81	1482
37	Healthy & Streak	BeniShangul-Gumuz	Assossa	Assossa	14 Gambela	10.17	34.75	1477
38	Looks healthy	BeniShangul-Gumuz	Assossa	Assossa	14 Gambela	10.17	34.75	1477
39	Looks healthy	BeniShangul-Gumuz	Assossa	Assossa	14 Gambela	10.17	34.75	1477
40	White Streak	BeniShangul-Gumuz	Assossa	Assossa	14 Gambela	10.17	34.75	1477
41	Healthy	BeniShangul-Gumuz	Assossa	Assossa	14 Gambela	10.17	34.75	1477
42	Streak & Dwarf	BeniShangul-Gumuz	Assossa	Assossa	Amba-14	10.17	34.75	1453
43	Streak & Dwarf	BeniShangul-Gumuz	Assossa	Assossa	Amba-14	10.17	34.75	1453
44	Streak & Dwarf	BeniShangul-Gumuz	Assossa	Assossa	Amba-14	10.17	34.75	1453
45	White Streak	BeniShangul-Gumuz	Assossa	Assossa	Amba-14	10.12	34.82	1492
46	White Streak	BeniShangul-Gumuz	Assossa	Assossa	Amba-14	10.12	34.82	1492
47	Clear Streak	BeniShangul-Gumuz	Assossa	Assossa	Amba-5	10.16	34.73	1602
48	Clear Streak	BeniShangul-Gumuz	Assossa	Assossa	Amba-5	10.16	34.73	1602
49	Clear Streak	BeniShangul-Gumuz	Assossa	Assossa	Amba-5	10.16	34.73	1602
50	Stunt & Necrosis	BeniShangul-Gumuz	Assossa	Assossa	Amba-5	10.16	34.73	1602
51	Clear Necrosis	BeniShangul-Gumuz	Assossa	Assossa	Baro	10.24	34.66	1596
52	Clear Streak	BeniShangul-Gumuz	Assossa	Assossa	Baro	10.24	34.66	1596
53	Streak	BeniShangul-Gumuz	Assossa	Assossa	Baro	10.24	34.66	1596
54	Streak	BeniShangul-Gumuz	Assossa	Assossa	Baro	10.24	34.66	1596
55	Stunt & Streak	BeniShangul-Gumuz	Assossa	Assossa	Baro	10.31	34.77	1554
56	Very Stunted	BeniShangul-Gumuz	Assossa	Assossa	Baro	10.31	34.77	1554
57	Very Streak	BeniShangul-Gumuz	Assossa	Assossa	Baro	10.31	34.77	1554

58	Clear Necrosis	BeniShangul-Gumuz	Assossa	Selga	24	10.27	34.88	1558
59	Streak & stunt	BeniShangul-Gumuz	Assossa	Selga	24	10.27	34.88	1558
60	Very Necrosis	BeniShangul-Gumuz	Assossa	Selga	24	10.27	34.88	1558
61	Very Streak	BeniShangul-Gumuz	Assossa	Selga	24	10.27	34.88	1558
62	Very Strreak	BeniShangul-Gumuz	Assossa	Selga	24	10.22	34.86	1548
63	White Streak	BeniShangul-Gumuz	Assossa	Selga	24	10.22	34.86	1548
64	Stunt & Streak	BeniShangul-Gumuz	Assossa	Selga	24	10.22	34.86	1548
65	Clear Streak	BeniShangul-Gumuz	Assossa	Selga	24	10.22	34.86	1548
66	Very Streak	BeniShangul-Gumuz	Assossa	Selga	22	10.14	34.73	1541
67	Very Stunt	BeniShangul-Gumuz	Assossa	Selga	22	10.14	34.73	1541
68	Moderate Height	BeniShangul-Gumuz	Assossa	Selga	22	10.14	34.73	1541
69	Red Streak	BeniShangul-Gumuz	Assossa	Assossa	Baro	10.24	34.82	1553
70	Red Streak	BeniShangul-Gumuz	Assossa	Assossa	Baro	10.24	34.82	1553
71	Streak & Stunt	BeniShangul-Gumuz	Assossa	Assossa	Baro	10.24	34.82	1553
72	Streak & Necrosis	BeniShangul-Gumuz	Assossa	Bambassie	Dabus	10.02	34.83	1391
73	Very streak	BeniShangul-Gumuz	Assossa	Bambassie	Dabus	10.02	34.83	1391
74	Streak & Necrosis	BeniShangul-Gumuz	Assossa	Bambassie	Dabus	10.02	34.83	1391
75	Stunt & Necrosis	BeniShangul-Gumuz	Assossa	Bambassie	Dabus	10.02	34.83	1391
76	Dead Heart	BeniShangul-Gumuz	Assossa	Bambassie	Dabus	10.02	34.83	1391
77	Stunt & Necrosis	BeniShangul-Gumuz	Assossa	Bambassie	Dabus	10.01	34.81	1391
78	Stunt & Streak	BeniShangul-Gumuz	Assossa	Bambassie	Dabus	10.01	34.81	1391
79	Moderate height	BeniShangul-Gumuz	Assossa	Bambassie	Dabus	10.01	34.81	1391
80	Stunt & NoPod	BeniShangul-Gumuz	Assossa	Bambassie	Dabus	9.98	34.98	1404
81	Moderate height	BeniShangul-Gumuz	Assossa	Bambassie	Dabus	9.98	34.98	1404
82	Moderate height	BeniShangul-Gumuz	Assossa	Bambassie	Dabus	9.98	34.98	1404
83	Streak & Low Pod	BeniShangul-Gumuz	Assossa	Bambassie	Dabus	9.98	34.98	1404
84	Some Streak	BeniShangul-Gumuz	Assossa	Bambassie	Dabus	9.98	34.98	1404
85	Good height	BeniShangul-Gumuz	Assossa	Bambassie	Dabus	9.98	34.98	1404
86	Very Streak	BeniShangul-Gumuz	Assossa	Bambassie	Keshemando	9.93	34.7	1450
87	Very Necrosis	BeniShangul-Gumuz	Assossa	Bambassie	Keshemando	9.93	34.7	1450

88	Good Height	BeniShangul-Gumuz	Assossa	Bambassie	Keshemando	9.93	34.7	1450
89	Reddish Necrotic	BeniShangul-Gumuz	Assossa	Bambassie	Keshemando	9.93	34.7	1450
90	Stunt & Streak	BeniShangul-Gumuz	Assossa	Bambassie	Keshemando	9.93	34.7	1450
91	Stunt,Dead Heart	BeniShangul-Gumuz	Assossa	Bambassie	Keshemando	9.93	34.7	1450
92	Yellowish Streak	BeniShangul-Gumuz	Assossa	Bambassie	Keshemando	9.93	34.7	1450
93	Stunt & Streak	BeniShangul-Gumuz	Assossa	Bambassie	Keshemando	9.93	34.7	1450
94	Reddish Necrotic	BeniShangul-Gumuz	Assossa	Bambassie	Keshemando	9.93	34.7	1450
95	Stunt & Streak	BeniShangul-Gumuz	Assossa	Assossa	Agusha	10.26	34.78	1524
96	Yellow, Necrotic	BeniShangul-Gumuz	Assossa	Assossa	Agusha	10.26	34.78	1524
97	Stunt & Streak	BeniShangul-Gumuz	Assossa	Assossa	Agusha	10.26	34.78	1524
98	Stunt & Streak	BeniShangul-Gumuz	Assossa	Assossa	Agusha	10.26	34.78	1524
99	Clear Streak	BeniShangul-Gumuz	Assossa	Assossa	Agusha	10.26	34.78	1524
100	Clear Streak	BeniShangul-Gumuz	Assossa	Assossa	Agusha	10.26	34.78	1524
101	Short & Streak	BeniShangul-Gumuz	Assossa	Assossa	Agusha	10.26	34.78	1524
102	Very Streak	BeniShangul-Gumuz	Assossa	Assossa	Agusha	10.26	34.78	1524
103	Stunt, Yellowish	Oromia	East Shewa	Adamitulu	01	8.15	39.73	1640
104	Yellow, Necrosis	Oromia	East Shewa	Adamitulu	01	8.15	39.73	1640
105	Leaf tip drying	Oromia	East Shewa	Adamitulu	01	8.15	39.73	1640
106	Yellowish Mosaic	Oromia	East Shewa	Adamitulu	01	8.15	39.73	1640
107	StunVein Clearing	Oromia	East Shewa	Adamitulu	01	8.15	39.73	1640
108	Streak & Mosaic	Oromia	East Shewa	Adamitulu	01	8.15	39.73	1640
109	Mosaic Necrosis	Oromia	East Shewa	Adamitulu	01	8.15	39.73	1640
110	Moderate Height	Oromia	East Shewa	Adamitulu	01	8.15	39.73	1640
111	Yellowish, Stunt	Oromia	East Shewa	Adamitulu	01	8.15	39.73	1640
112	Yellowish, Stunt	Oromia	East Shewa	Adamitulu	01	8.15	39.73	1640
113	Yellow, Necrosis	Oromia	East Shewa	Adamitulu	01	8.15	39.73	1640
114	Necrosis & Streak	Oromia	East Shewa	Adamitulu	01	8.15	39.73	1640
115	Mosaic	Oromia	East Shewa	Adamitulu	Bochessa	7.95	38.81	1643
116	Stunt & Streak	Oromia	East Shewa	Adamitulu	Bochessa	7.95	38.81	1643
117	Stunt & Necrosis	Oromia	East Shewa	Adamitulu	Bochessa	7.95	38.81	1643

118	Very Necrosis	Oromia	East Shewa	Adamitulu	Bochessa	7.95	38.81	1643
119	Stunt, Yellowish	Oromia	East Shewa	Adamitulu	Bochessa	7.95	38.81	1643
120	Stunt & Reddish	Oromia	East Shewa	Adamitulu	Bochessa	7.95	38.81	1643
121	Yellow, Necrosis	Oromia	East Shewa	Adamitulu	Bochessa	7.95	38.81	1643
122	Leaf tip drying	Oromia	East Shewa	Adamitulu	Bochessa	7.95	38.81	1643
123	Stunt & Necrosis	Oromia	East Shewa	Adamitulu	Bochessa	7.95	38.81	1643
124	Stunt & Necrosis	Oromia	East Shewa	Adamitulu	Bochessa	7.95	38.81	1643
125	Stunt & Necrosis	Oromia	East Shewa	Adamitulu	Bochessa	7.95	38.81	1643
126	Stunt & tip drying	Oromia	East Shewa	Adamitulu	Bochessa	7.95	38.81	1643
127	Stunt & tip drying	Oromia	East Shewa	Adamitulu	Abinagermama	8.11	39.03	1625
128	Good & Healthy	Oromia	East Shewa	Adamitulu	Abinagermama	8.11	39.03	1625
129	Yellow, Necrosis	Oromia	East Shewa	Adamitulu	Abinagermama	8.11	39.03	1625
130	Yellow, Necrosis	Oromia	East Shewa	Adamitulu	Abinagermama	8.11	39.03	1625
131	Drying, Necrosis	Oromia	East Shewa	Adamitulu	Abinagermama	8.11	39.03	1625
132	Yellowish, Streak	Oromia	East Shewa	Adamitulu	Abinagermama	8.11	39.03	1625
133	Drying, Necrosis	Oromia	East Shewa	Adamitulu	Abinagermama	8.11	39.03	1625
134	Yellowish, Drying	Oromia	East Shewa	Adamitulu	Abinagermama	8.11	39.03	1625
135	Necrosis & Streak	Oromia	East Shewa	Adamitulu	Abinagermama	8.11	39.03	1625
136	Vein Clearing	Oromia	East Shewa	Adamitulu	Abinagermama	8.11	39.03	1625
137	Stunt & Mosaic	Oromia	East Shewa	Adamitulu	Abinagermama	8.11	39.03	1625
138	Mosaic & Stunt	Oromia	East Shewa	Adamitulu	Abinagermama	8.11	39.03	1625
139	Leaf tip Yellow	Oromia	East Shewa	Adamitulu	Abinagermama	8.11	39.03	1625
140	Yellow, Necrosis	Oromia	East Shewa	Dugda	Gressa	7.86	38.96	1650
141	Clear Mosaic	Oromia	East Shewa	Dugda	Gressa	7.86	38.96	1650
142	Mosaic, Yellowish	Oromia	East Shewa	Dugda	Gressa	7.86	38.96	1650
143	Necrosis & Streak	Oromia	East Shewa	Dugda	Gressa	7.86	38.96	1650
144	Stunt & Necrosis	Oromia	East Shewa	Dugda	Gressa	7.86	38.96	1650
145	Mosaic & Drying	Oromia	East Shewa	Dugda	Gressa	7.86	38.96	1650
146	Yellowish, Drying	Oromia	East Shewa	Dugda	Gressa	7.86	38.96	1650
147	Yellowish, Drying	Oromia	East Shewa	Dugda	Gressa	7.86	38.96	1650

148	Yellowish	Oromia	East Shewa	Dugda	Gressa	7.86	38.96	1650
149	Yellow Necrosis	Oromia	East Shewa	Dugda	Gressa	7.86	38.96	1650
150	Yellow & Mosaic	Oromia	East Shewa	Dugda	Gressa	7.86	38.96	1650
151	Mosaic, Necrosis	Oromia	East Shewa	Dugda	Gressa	7.86	38.96	1650
152	Mosaic, Necrosis	Oromia	East Shewa	Dugda	Gressa	7.86	38.96	1650
153	Dwarf & Necrosis	Oromia	East Shewa	Adama Zuria	Melkassa	8.42	39.32	1550
154	Mosaic Pattern	Oromia	East Shewa	Adama Zuria	Melkassa	8.42	39.32	1550
155	Dried comb	Oromia	East Shewa	Adama Zuria	Melkassa	8.42	39.32	1550
156	Yellow Necrosis	Oromia	East Shewa	Adama Zuria	Melkassa	8.42	39.32	1550
157	Streak & Stripe	Oromia	East Shewa	Adama Zuria	Melkassa	8.42	39.32	1550
158	Clear Necrosis	Oromia	East Shewa	Adama Zuria	Melkassa	8.42	39.32	1550
159	Dwarf/Stunting	Oromia	East Shewa	Adama Zuria	Melkassa	8.42	39.32	1550
160	Streak & Mosaic	Oromia	East Shewa	Adama Zuria	Melkassa	8.42	39.32	1550
161	Streak	Oromia	East Shewa	Adama Zuria	Melkassa	8.42	39.32	1550
162	Stunting	Oromia	East Shewa	Adama Zuria	Melkassa	8.42	39.32	1550
163	MCMV	Oromia	East Shewa	Adama Zuria	Melkassa	8.42	39.32	1550
164	Mosaic & Streak	Oromia	East Shewa	Adama Zuria	Melkassa	8.42	39.32	1550
165	Mosaic	Oromia	West Shewa	Ambo	African Juice	8.98	37.85	2101
166	Mosaic Necrosis	Oromia	West Shewa	Ambo	African Juice	8.98	37.85	2101
167	Mosaic Necrosis	Oromia	West Shewa	Ambo	African Juice	8.98	37.85	2101
168	Yellow Necrosis	Oromia	West Shewa	Ambo	African Juice	8.98	37.85	2101
169	Necrosis, Mosaic	Oromia	West Shewa	Ambo	African Juice	8.98	37.85	2101
170	Clear Mosaic	Oromia	West Shewa	Ambo	African Juice	8.98	37.85	2101
171	Yellow Necrosis	Oromia	West Shewa	Ambo	African Juice	8.98	37.85	2101
172	drying Leaf	Oromia	West Shewa	Ambo	African Juice	8.98	37.85	2101
173	Stunt	Oromia	AA Zuria	Wolmera	Holetta	9.05	38.5	2391
174	Yellowish leaf	SNNP	Sidama	Awasa	Awasa	7.05	38.47	1708

