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Identification and characterization of viruses causing maize lethal necrotic disease (mlnd) in Northern Tanzania

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IDENTIFICATION AND CHARACTERIZATION OF VIRUSES CAUSING MAIZE LETHAL NECROTIC DISEASE (MLND) IN NORTHERN TANZANIA

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A Dissertation Submitted in Partial Fulfillment of the Requirements for the Degree of Master's in Life Sciences and Engineering of the Nelson Mandela African Institution of Science and Technology

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SUMMARY

Maize Lethal Necrosis (MLN) is a viral disease in maize currently reported in eastern and central Africa countries including Kenya, Rwanda, Uganda, Ethiopia, Congo and Tanzania. The disease is caused by *Maize Chlorotic Mottle Virus* (MCMV) in synergism with *Sugarcane Mosaic Virus* (SCMV), *Wheat Streak Mosaic Virus* (WSMV) or *Maize Dwarf Mosaic Virus* (MDMV). The present study aimed at assessing farmers' awareness of the spread and loss due to MLND, identify and characterize the causative viruses in Kilimanjaro, Arusha and Manyara regions in Northern Tanzania. Past experiences of the occurrences and losses due to MLND were assessed by interviewing maize farmers (n = 137) in the regions between April and June, 2015. Disease prevalence was assessed after the awareness survey based on direct observation and counts of symptomatic maize plants in quadrants within individual farms (n = 41) across villages within different agro-ecological zones (AEZs). Viruses causing MLND were detected in maize leaves by Double Antibody Sandwich-Enzyme Linked Immuno Sorbent Assay (DAS-ELISA), Reverse Transcription-Polymerase Chain Reaction (RP-PCR) and Next Generation Sequencing (NGS) - Illumina MiSeq. Based on interviews, 99% of the farmers were aware of MLND symptoms. About 51.8% of farmers had experienced MLND in three subsequent years (2013 - 2015). The disease was said to have caused total crop failure in the majority of the farms (88%) in 2014. The prevalence of MLND differed across regions ($P = 0.0012$) and villages ($P < 0.0001$) but did not differ across AEZs ($P > 0.05$). The highest prevalence was recorded in Kilimanjaro with 22% symptomatic maize plants followed by Arusha (14%) and Manyara (10%). Of all the samples collected, 65% were positive for SCMV by DAS-ELISA test and 97% positive for MCMV by RT-PCR test with a co-infection of 64%. The highest incidence of both viruses; MCMV (100%) and SCMV (98%) was recorded in Lyamungu Kati-Hai district. NGS analysis showed that, there were no *Potyviruses* other than SCMV found in the samples collected in the regions. Based on phylogenetic tree, MCMV from this study are highly similar to themselves and to the existing eastern Africa isolates (99% nt identity). However, SCMV from this study have significant genome diversity within themselves. They were found to be similar to the highly virulent SCMV isolate from Hebei-China and from Kenya (87 - 99% nt identity). Therefore, similar management practices including production of resistant maize varieties can be applied in the regions affected by MLND.

DECLARATION

I, Fatma Hussein do hereby declare to the Senate of Nelson Mandela African Institution of Science and Technology that this dissertation is my own original work and that it has neither been submitted nor being concurrently submitted for degree award in any other institution.

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Fatma Hussein **Date**

(MSc Candidate)

The above declaration is confirmed by:

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Dr. Tileye Feyissa **Date**

(Supervisor)

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CERTIFICATION

The undersigned certify that they have read the dissertation titled ***Identification and characterization of viruses causing Maize Lethal Necrotic Disease (MLND) in Northern Tanzania*** and recommend for examination in fulfillment of the requirements for the degree of Masters in Life Sciences and Engineering of the Nelson Mandela African Institution of Science and Technology.

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Dr. Tileye Feyissa **Date**

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Prof. Patrick A. Ndakidemi **Date**

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DEDICATION

I humbly dedicate this work to my beloved children Dhul-kifl Omari and Dhul-qarnayn Omari who were born during my studies.

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LIST OF ABBREVIATIONS AND SYMBOLS

CF–PCR	Competitive fluorescence polymerase chain reaction
CLN	Corn Lethal Necrosis
COSTECH	Commission for Science and Technology
DAC-ELISA	Direct antigen coating- ELISA
DAS-ELISA	Double antibody sandwich ELISA
DIBA	Dot-immuno- binding assay
ELISA	Enzyme Linked ImmunoSorbent Assay
EM	Electron microscopy
IC–PCR	Immuno-capture polymerase chain reaction
IC-RT-PCR	Immuno-capture reverse transcription-polymerase chain reaction
MCMV	*Maize Chlorotic Mottle Virus*
MCMV-KA	*Maize Chloritic Mottle Virus*-Kansas isolate
MCMV-NE	*Maize Chloritic Mottle Virus*- Nebraska isolate
MDMV	*Maize Dwarf Mosaic Virus*
MLND	*Maize Lethal Necrotic Disease*
mM	Millimolar
NGS	Next generation sequencing
pM	Picomolar
RT-PCR	Reverse transcriptase polymerase chain reaction
SCMV	*Sugarcane Chlorotic Mottle Virus*
SCSMV	*Sugarcane Streak Mosaic Virus*
SCYLV	*Sugarcane Yellow Leaf Virus*
SrMV	*Sorghum Mosaic Virus*
TAS-ELISA	Triple antibody sandwich ELISA
U/ml	Unit per milliliter
WSMV	*Wheat Streak Mosaic Virus*
µl	Microliter
µM	Micromolar

CHAPTER ONE

1.0 INTRODUCTION

This chapter describes the general introduction of the study. It mainly focuses on the background information of the study, research problem and justification, objectives, research questions, hypothesis and significance of the research.

1.1 BACKGROUND INFORMATION

Maize lethal necrotic disease (MLND) is a devastating viral based synergistic disorder in maize currently reported in eastern and central Africa countries including Kenya (Wangai *et al*., 2012a, b; Adams *et al*., 2013), Rwanda (Adams *et al*., 2014), Ethiopia (Mahuku *et al*., 2015b), Congo (Lukanda *et al*., 2014) and Tanzania. The Disease was first identified in 1976 in Kansas (Niblett *et al*., 1978). It is caused by *Maize chlorotic mottle virus* (MCMV) in combination with other *Potyviruses* family like *Sugarcane Mosaic Virus* (SCMV), *Wheat Streak Mosaic Virus* (WSMV) or *Maize Dwarf Mosaic Virus* (MDMV) (Uyemoto, 1983). In East Africa, SCMV is the only *Potyvirus* reported to synergize with MCMV causing MLND (Mahuku *et al*., 2015a).

MLND is seed borne (Jensen *et al*., 1991; Delgadillo Sánchez *et al*., 1994; Mahuku *et al*., 2015a) and vector transmitted (Mahuku *et al*., 2015a). Vectors include aphids (Brault *et al*., 2010), rootworms (Nault *et al*., 1978; Jiang *et al*., 1992; Uyemoto, 1983), thrips (Jiang *et al*., 1992) and beetles (Nault *et al*., 1978; Gordon *et al*., 1984; Jensen *et al*., 1991). Infected soil due to infected maize debris has also been reported by Nyvall (1999) and Mahuku *et al*. (2015a) to be the source of inoculum for viruses in the next seasons of maize production.

The disease is a big threat to maize production, more than 90% yield loss has been reported (Wangai *et al*., 2012c). Symptoms of MLND including chlorotic mottling on the leaves and necrosis were reported by farmers and extension staffs in Northern (Kilimanjaro, Arusha and Manyara) and Lake zones (Mwanza, Mara and Shinyanga) of Tanzania in 2012. However, viruses associated with the disease are not well studied. Knowledge of the incidence and prevalence of MLND in Tanzania is also limited. Therefore the present study aimed at investigating the disease magnitude in affected areas and identifying the viruses causing MLND in Northern Tanzania.

1.2 PROBLEM STATEMENT AND JUSTIFICATION

Maize accounts for over 75% of total cereal production in Tanzania (Suleiman *et al*., 2015). It is produced for both commercial and domestic uses. However, MLND is now considered a major threat in Arusha, Manyara, Kilimanjaro, Mwanza and Mara, causing a major loss in maize production. These regions are one of the major maize producing areas and are considered as country's grain basket (Nkonya, 1998). To safeguard maize production and to develop MLND eradication approaches, there is a need to identify viruses that are causing the disease and understand their prevalence. Currently there is a huge ongoing effort to study the viruses associated with the disease in the neighboring country of Kenya (Wangai *et al*., 2012a, b; Adams *et al*., 2013; Makone *et al*., 2014) but less effort in Tanzania. Correct identification and understanding the MLND causing viruses and their prevalence would enable maize breeders and pathologists to manage MLND including initiating cropping patterns that would reduce maize cultivation in disease hotspot areas and breeding for resistance varieties.

1.3 OBJECTIVES

1.3.1 General objective

The objective of this study was to investigate the prevalence and identify viruses causing Maize Lethal Necrotic Disease so as to develop management strategies to safeguard maize production in Northern Tanzania.

1.3.2 Specific objectives

a) To investigate the prevalence of MLND in the major maize growing areas in Northern Tanzania in order to document the current status of the disease.
b) To identify and characterize viruses causing Maize Lethal Necrotic Disease in Northern Tanzania.

1.4 RESEARCH QUESTIONS

a) What knowledge and experience on MLND do farmers have in Northern Tanzania?
b) What is the prevalence of MLND based on symptoms at individual farm levels in villages within agro-ecological zones and across regions in Northern Tanzania?

c) What viruses are associated with MLND in Northern Tanzania?

1.5 HYPOTHESIS

H_0: MLND prevalence and identity of causative viruses do not differ across regions in Northern Tanzania.

H_1: MLND prevalence and identity of causative viruses differs across regions in Northern Tanzania.

1.6 SIGNIFICANCE OF THE RESEARCH

The output from this study creates baseline information on the viruses that are causing MLND and also documents the current status of the disease in Tanzania. Better understanding of the prevalence of MLND is useful to policy makers, researchers and other concerned bodies to know and be able to develop control measures in order to avoid losses caused by the disease in the country. The output from this work may also help farmers in decision making whether to continue planting the same crop (maize), shift to other food crops that are not affected by the disease or employing a maize free zone until when the solution is found including availability of tolerant/resistant maize varieties.

CHAPTER TWO

Insights of maize lethal necrotic disease: A major constraint to maize production in East Africa[1]

SUMMARY

Maize Lethal Necrotic Disease (MLND) is a major constrain to maize production in East Africa. The disease was first reported in Kenya in 2011 and a year later in Tanzania, Uganda and Rwanda. MLND is caused by *Maize Chlorotic Mottle Virus* (MCMV) in combination with viruses of genus *Potyvirus*, mostly *Sugarcane Mosaic Virus* (SCMV). The co-infection is the one that results in intensive to complete yield loss. Diagnosis of MLND based on symptoms is reported ineffective because symptoms like stunting and chlorosis resembles nutrient deficiencies or maize mosaic. Detection and characterization of MLND causing viruses have been done with techniques such as Enzyme-Linked Immuno-Sorbent Assay (ELISA), PCR and Next Generation Sequencing (NGS). Relatively little work has been done to characterize MLND causing viruses in Tanzania prior to those techniques. The disease can be managed through the use of certified seeds, sanitation, quarantine, crop rotation, the use of resistant/tolerant maize varieties and other cultural practices. The use of resistant maize varieties is considered the most reliable, eco-friendly, effective and economical way of managing MLND.

Keywords: ELISA, Etiology, *Maize Chlorotic Mottle Virus*, Maize Lethal Necrotic Disease, Nucleic acid based methods, resistant maize varieties, *Sugarcane Chlorotic Mottle Virus.*

[1] Kiruwa et al. 2016. African Journal of Microbiology Research. DOI: 10.5897/AJMR2015.7534. Article Number: 4E22F8957518. Vol. 10(9), pp. 271-279.

2.0 INTRODUCTION

Maize (*Zea mays L.*) is an important staple crop in East Africa (FAOSTAT, 2013). It is one of the most widely cultivated gramineous plants in the regions due to its ability to grow in diverse climates (Acland, 1977; Agbonifo and Olufolaji, 2012). In 2011, a disease with virus-like symptoms (chlorotic mottle of maize leaves, mild to severe mottling and necrosis) causing dramatic maize damage in farmers' fields was reported in Kenya and Tanzania (Wangai *et al*., 2012a, b). The disease was identified as a Maize Lethal Necrotic Disease (MLND) (Wangai *et al*., 2012b; Adams *et al*., 2013). In Tanzania, this disease is present in Kilimanjaro, Arusha, Manyara, Mwanza, Mara, and Shinyanga regions. It is considered as the worst enemy of maize crops in recent times (Wangai *et al*., 2012b). This review paper discusses MLND in east Africa, including its importance, diagnostics, etiology, managements and then highlights the future research needs.

2.1 MAIZE LETHAL NECROTIC DISEASE

1.1.1 Causative agents/Pathogens

MLND is caused by *Maize Chlorotic Mottle Virus* (MCMV) as a single virus infection or in combination with viruses from *Potyviridae* family, such as *Sugarcane Mosaic Virus* (SCMV), *Wheat Streak Mosaic Virus* (WSMV) or *Maize Dwarf Mosaic Virus* (MDMV) (Bockelman *et al*., 1982). The double infection (co-infection) by the viruses is more severe than single infection (Nilblett and Claflin, 1978; Scheets, 1998). In East Africa, synergistic infection by MCMV and SCMV gives rise to MLND which is also referred to as Corn Lethal Necrosis (CLN) (Uyemoto *et al*., 1980; 1981).

2.1.2 History and geographical distribution of the disease

In September 2011, the first outbreak of MLND was reported in East Africa along rift valley regions of Kenya (Wangai *et al*., 2012a, b). Regions that were reported to have the disease included; Bomet, Naivasha, Narok, Chepalungu, Sotik, Transmara, Bureti, Nakuru, Konoin, South Narok, Mathira East, Imenti South Districts and Nyeri (Wangai *et al*., 2012c). In August 2012, this disease was also reported in Northern and Lake Zone regions of Tanzania (Makumbi and Wangai, 2013). In Uganda, the disease was first reported in October 2012 in Busia then in

the border district of Tororo, Mbale and Kapchorwa (ASARECA, 2013). In Rwanda, MLND was first reported in February 2013 in Gisesero site, Musanze District in Northern Province and in the western Province (ASARECA, 2013; Adams *et al*., 2014). This disease is not reported yet in Burundi (ASARECA, 2013).

This disease is new in East Africa but not new in the other parts of the world as it was identified as Corn Lethal Necrosis in 1976 in Kansas (Nilblett *et al*., 1978; Uyemoto, 1983), Peru (Castillo, 1977; Uyemoto, 1983), Hawaii (Kauai) in the early 1990s (Nelson *et al*., 2011), Nebraska in 1976 (Uyemoto, 1983), Argentina (Gordon *et al*., 1984), Texas and Brazil (Uyemoto, 1983). The possibility of spreading to other areas cannot be ruled out and hence need to quantify its distribution in a wider context.

2.1.3 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*., 2012b). Maize is susceptible to this disease at all stages of development, specifically from the seedling stage to near maturity (CGIAR Research Program MAIZE, 2012). The loss is due to the fact that, infected maize plants are barren, have small ears, distorted and set little or no grains resulting in reduced yield or no yield at all. In addition, production costs increase as farmers use herbicides and insecticides to control weeds and insect vectors transmitting the disease. Furthermore, seed production costs increase as extra cost of seed treatment is incurred by the seed companies.

2.2 DIAGNOSIS OF THE DISEASE

The best method of controlling any plant disease requires proper diagnostic tools for identification of the causative agents (Webster *et al*., 2004; Adams *et al*., 2013). Several methods have been used to diagnose plant viral diseases. They 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 (EM) (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 viz; symptomatology, serological and nucleic acids based methods mostly used in the diagnosis of plant virus diseases specifically MLND have been discussed.

2.2.1 Symptomatology

Symptoms are physiological conditions that indicate presence of a disease caused either by biotic (pests and pathogens) or abiotic (environmental conditions) factors in the plants (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

Symptoms of MLND includes; elongated yellow streaks parallel to leaf veins, chlorotic mottling, and leaf necrosis which may lead to "dead heart" symptom and plant death (Nelson *et al*., 2011; Wangai *et al*., 2012a; Makone *et al*., 2014), 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*., 2012a).

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 due to virus infection but nutrient deficiencies or maize mosaic (Nelson *et al*., 2011). In addition, factors like unfavorable environmental conditions, damage by pests, air pollution, herbicide 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 as 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).

2.2.2 Serological methods

Detection and diagnosis of plant viruses based on serological tests have been used since the 1960s (Martin *et al*., 2000). These tests are believed to be the best in the 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-immuno-binding assay (DIBA), and immuno-capture reverse transcription-polymerase chain reaction (IC-RT-PCR) by using the MAb 4B8 that was 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 methods have been used to identify WSMV in wheat (Montana *et al*., 1996; Ilbagi *et al*., 2005), MCMV, SCMV and MDMV in maize (Louie, 1980; McDaniel and Gordon, 1985; Jensen *et al*., 1991; Giolitti *et al*., 2005; Xie *et al*., 2011; Adams *et al*., 2013; Lukanda *et al*., 2014). DAS-ELISA has been used to identify MLND causing viruses in Kenya but gave negative results, probably due to low sensitivity and poor specificity for unusual or variant isolates (Adams *et al*., 2013). A 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 (Mahuku *et al*., 2015a, b).

Despite a wide use of serological methods such as ELISA in virus detection, they have been reported to be less accurate in identifying unusual or variant isolates because of being too specific to a particular species or even strain of a virus (Adams *et al*., 2013; Mezzalama *et al*., 2015). Furthermore, the methods require proper selection of good reagents and optimum level of antibodies, sensitivity and specificity toward the pathogen under study, proper handling, storage of reagents and incubation time as these factors have been reported by Hewings and D‟Arcy (1984) to affect ELISA results.

2.2.3 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 Next Generation Sequencing (NGS) are among nucleic acid based methods used in the diagnosis of many plant virus diseases including MLND (Zhang *et al*., 2011; Wangai *et al*., 2012b; Adams *et al*., 2013, Lukanda *et al*., 2014; Mahuku *et al*., 2015a, b).

2.2.3.1 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 including diagnostics of plant virus diseases (Henson and French, 1993; Hadidi *et al*., 1995; Lopez *et al*., 2003; Doughari *et al*., 2009) because it is fast, specific, sensitive, and versatile (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 TaqmanÔ 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 real- time PCR in maize seeds (Zhang *et al*., 2011) and in maize leaves (Adams *et al*., 2014). Real-time PCR has been considered as the best confirmatory test in the routine diagnosis of the MLND causing viruses (Adams *et al*., 2013). RT-PCR has been used to detect/verify MCMV and SCMV in maize (Wangai *et al*., 2012b; Mahuku *et al*., 2015a), 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 systematic contamination of PCR reagents and to ensure that the desired amplicon is produced in a 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.

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 the detection and identification of viral diseases of plants such as MLND especially in low income-developing countries including east Africa, thus, affecting proper diagnosis of viral diseases of plants in the region.

2.2.3.2 Sequencing

Sequencing is a very reliable technique for virus identification and has led to the 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 in several studies to identify and characterize plant viruses, including MLND (Adams *et al*., 2013; 2014; Mahuku *et al*., 2015a, b). Using NGS, characterization of MCMV and SCMV in Kenya showed that the MCMV had a similarity of more than 96% to the Yunnan strain from China, but different from the US strains while SCMV was found most similar to a strain from China (Adams *et al*., 2013). Other similar study indicated that, complete nucleotide sequence of MCMV Nebraska isolates

(MCMV-NE) and Kansas isolates (MCMV-KA) were closely related sharing 99.5% nucleotide sequence identity, suggesting to have a very recent common ancestor (Stenger and French, 2008). Despite the importance of NGS in detection of novel unidentified viral plant diseases, it is not used extensively because of high costs of the machine and operating costs. This has severely affected proper diagnosis MLND in East Africa leading to very low level of molecular diagnosis. Therefore, there is a need of capacity building and enhancing developing countries in plant disease diagnostics.

2.3 ETIOLOGY OF PATHOGENS CAUSING MLND

Sufficient knowledge of causative agents of a disease, their origin, their disseminations and survival properties usually results in designing adequate control methods of the disease.

2.3.1 Taxonomy of the pathogens

- *Maize Chlorotic Mottle Virus (MCMV)*

MCMV is the only species in the genus *Machlomovirus*, family *Tombusvirideae* (Stenger *et al*., 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 *et al* 1987; Lommel *et al*., 1991) and has a smooth spherical or hexagonal shape (Scheets, 2010) with a capsid protein of 25 kDa (Lommel *et al*., 1991).

- *Sugarcane Mosaic Virus (SCMV)*

SCMV is one of the major viruses in the genus *Potyvirus*, family *Potyvirideae*. 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).

- *Wheat Streak Mosaic Virus (WSMV)*

WSMV is one of the viruses in genus *Tritimovirus*, family *Potyvirideae* (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 - 700 nm long (Kumar *et al*., 2004; Wegulo *et al*., 2008).

- *Maize Dwarf Mosaic Virus (MDMV)*

Maize Dwarf Mosaic Virus belongs to genus *Potyvirus,* family *Potyvirideae* (Giolitti *et al*., 2005). The virus is a single stranded positive sense RNA (ssRNA) with a flexuous filament viral particle of 750 nm long and 13 nm wide (Williums *et al*., 1965; Bancroft *et al*., 1966; Autrey, 1983).

2.3.2 Life cycle of the pathogens

2.3.2.1 Survival between cropping seasons

MLND causing viruses can survive in infected maize residuals and contaminate soil, alternative hosts like sorghum (Toler, 1985), millet (Bockelman *et al*., 1982; ASARECA, 2013), Johnson grasses (Knoke *et al*., 1974; Toler, 1985; ASARECA, 2013) and other grasses in the family Poaceae (Scheets, 2004). These infected crop residues can harbor MLND viruses and act as source of inoculums in the next seasons of maize production.

2.3.2.2 Transmission

MCMV is transmitted by vectors mainly beetles (Nault *et al*., 1978; Gordon *et al*., 1984; Jensen *et al*., 1991) rootworms (Nault *et al*., 1978; Uyemoto, 1983; Jiang *et al*., 1992), thrips (Jiang *et al*., 1992). SCMV is transmitted by several species of aphids in non- persistent manner (Brandes, 1920; Pemberton and Charpentier, 1969; Zhang *et al*., 2008). WSMV is transmitted by mites in a persistent manner (Kumar *et al*., 2004; Wegulo *et al*., 2008). MDMV is transmitted by aphids in a non-persistent manner (Knoke *et al*., 1974; McDaniel and Gordon, 1985; Toler, 1985; Simcox *et al*., 1995). Infected soil and seeds have been reported as a reservoir and a means of viruses transmission (Jensen *et al*., 1991; Delgadillo Sánchez *et al*., 1994; Nelson *et al*., 2011). Human activities such as using tools in infected fields without thorough washing can transmit the disease causing viruses from infected to uninfected fields

2.3.2.3 Initial infection of maize plant

Generally, plant cells have a robust cell wall and viruses cannot penetrate them unaided. Therefore, they penetrate through wounds created by the feeding mode of insect vectors (Ellis *et al*., 2008) or mechanical injury by human activities. The feeding insect deposits/injects MLND causing viruses rapidly when feeding on a non-infected plant. Such a relationship is termed "non- persistent" and this is common transmission for *Potyviru*ses by aphids (Zhang *et al*., 2008; Trigiano *et al*., 2008). Beetles spread a layer of pre-digestive materials known as regargitant on the leaves as they feed, when viruliferous beetles spread this layer they also deposit virus particles in the wound at the feeding site (Trigiano *et al*., 2008). Once inside the cell, the viral protein coat is removed and nucleic acid enters the nuclear membrane and alters the maize DNA machinery so as to produce many of its copies. The viruses causing MLND first change their RNA to complementary DNA (cDNA) to mimic its host maize DNA. When more copies of viral particles have been synthesized, their movement between cells is through plasmadermata and the whole maize plant through phloem (Ellis *et al*., 2008). This results in disease manifestation and secondary cycles to alternative hosts (sorghum, millet, sugarcane and Johnson grasses e. t. c) and therefore continue repeated cycles during seasons and off seasons by the aid of vectors (Mahuku *et al*., 2015a).

2.4 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 result in are known. Disease management in this context is described based on basic principle as described by Whetzel (1929) and Maloy (2005).

2.4.1 Reduction of initial inoculums

2.4.1.1 Pathogen exclusion/strictly quarantine

Pathogen exclusion is the prevention of disease establishment in areas where it does not occur (Maloy, 2005). This is a major objective of plant quarantine procedures throughout the world.

Maize seeds are inspected before entering and going out countries and within country regions to prevent transmission of seed borne diseases. 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 (Wangai *et al*., 2012a,b) compared with SCMV which has been present since 1973 in the region (Louie, 1980). Enforcement of this practice can have significant effects in limiting the introduction and spread of diseases including MLND into uninfected areas.

2.4.1.2 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 can act as source of inoculums in the next season, rouging of diseased maize plants (Mawishe *et al*., 2013) and 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 pathogen. Non-host crops include Irish potatoes, sweet potatoes, cassava, beans, bulb onions, spring onions, vegetables and garlic (Wangai *et al*., 2012a). 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 virus transmission and thereby reducing density of inoculums.

2.4.2 Reducing the rate of infection

2.4.2.1 Avoidance

This method aims at avoiding contact between host (maize plant) and pathogen (viruses) by planting maize in field with no history of the disease, providing adequate plant spacing to avoid crowding, avoiding injury to the maize plants because viruses penetrates the plants through wounds and avoiding the use of recycled maize seeds but rather using certified seeds (Trigiano *et al*., 2008; Wangai *et al*., 2012a) and 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*.,

2012a). This can reduce the population of vectors and hence low rate of infection and disease severance.

2.4.2.2 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 (e. g. aphids, rootworms, stem borers, mites, thrips) that transmit MLND. Such insecticides include Imidacloprid, Thiamethoxam, Deltamethrin, Abamectin, Permethrin, Endosalphan 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*., 2012a).

2.4.2.3 Resistant or tolerant varieties

This is the most reliable, effective, environmentally 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 made to produce resistant varieties of maize in eastern Africa (ASARECA, 2014). For example, a strong collaboration between CIMMYT and National maize programs has been established to effectively tackle the MLND challenge in eastern Africa (CGIAR Research Program MAIZE, 2012). This resulted in the establishment of a centralized MLN screening facility in eastern Africa at the KALRO Livestock Research Farm in Naivasha (CGIAR Research Program MAIZE, 2012). In addition, Ngotho (2013) has 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 of Kenya, Uganda, Tanzania, Rwanda, Burundi, Ethiopia, Sudan, Eritrea, DRC Congo, Madagascar and South Sudan.

2.5 CONCUSION

This paper reviewed MLND, an emerging viral disease in East Africa including its importance, etiology, detection methods and measures for controlling the disease. If proper management of MLND is not taken seriously, the disease will spread throughout Africa where maize is produced, resulting in serious economic impacts, food insecurity as well as affecting livelihoods and well-being of Africa.

2.6 FUTURE RESEARCH NEEDS

In order to manage MLND effectively in East Africa, the following questions needs to be answered: How do the virus strains causing MLND present in regions of east Africa differ in the rate of infection? What insect vectors are responsible for transmission of MLND causing viruses in EA? What is the relationship between MLND causing viruses and their insect vectors? How can these insect vectors be managed? How seeds contribute in transmission of the viruses causing MLND? What genes are responsible for host resistance? How can these genes be incorporated into seed stocks by breeders? What is the prevalence/incidence of MLND in each region of EA? What management practices can be used against MLND in Tanzania? And what is the contribution of climate change to the spread of MLND?

CHAPTER THREE

Status of Maize Lethal Necrotic Disease in Northern Tanzania

SUMMARY

The present study were conducted to gain insights on the farmers' awareness of the spread and loss due to Maize Lethal Necrotic Disease and to assess the magnitude of the disease in major maize growing areas in Kilimanjaro, Arusha and Manyara regions in Northern Tanzania. Past experiences of the occurrences and losses due to MLND were assessed by interviewing maize farmers (n = 137) in the regions between April and June, 2015. Disease prevalence was assessed after the awareness survey based on direct observation and counts of symptomatic maize plants in quadrants within individual farms (n = 41) across villages within different agro-ecological zones (AEZs). Prevalence of MLND in Northern Tanzania in 2015 differed across regions (p = 0.0012) and villages ($p < 0.0001$) but did not differ across agro-ecological zones ($p > 0.05$). The highest prevalence was found in Kilimanjaro with a mean of 22% symptomatic maize plants followed by Arusha (14%) and Manyara (10%). Based on interviews, 99% of the farmers were aware of MLND symptoms. About 51.8% of farmers had experienced MLND in three subsequent years (2013 - 2015). The disease was said to have caused total crop failure in the majority of the farms (88%) in 2014. Most of the farmers (54.0%) practiced roughing of infected plants as a means of managing the disease. Although farmers witnessed an annual recurrence of the disease in their farms, they lacked effective control strategies. Because maize is the staple crop to the majority of people in East Africa, there is a need to conduct robust epidemiological and genetic studies to establish an effective control of MLND.

3.1 INTRODUCTION

Maize is a staple cereal crop to the majority of people in East Africa (De Groote, 2002; USAID, 2010; Suleiman *et al*., 2015). Despite its importance, its production is currently threatened by Maize Lethal Necrotic Disease (MLND), which is currently spreading at an alarming rate. The disease was first reported in the Southern rift valley, Kenya in 2011 (Wangai *et al*., 2012), and in Mwanza and Arusha regions of Tanzania in 2012 (Makumbi *et al*., 2013). It has since spread to several other maize producing African countries (Lukanda *et al*., 2014; Adams *et al*., 2014). The necrotic symptoms occur at different stages of maize development and can lead to 100% crop loss, and no effective control methods have been identified.

Etiology has identified a combination of *Maize Chlorotic Mottle Virus* (MCMV), a *Tombusvirus* and some cereal *Potyviruses* as the causal agents of MLND (Uyemoto *et al*., 1980; 1981). The combination of several viral strains makes the disease complex because of varying favorable conditions for the virulence and transmission of the viruses. MLND viruses are known to be transmitted by insect vectors (Mahuku *et al*., 2015a). MCMV is transmitted by thrips (Jiang *et al*., 1992), rootworms (Nault *et al*., 1978; Jiang *et al*., 1992; Uyemoto, 1983) and beetles (Nault *et al*., 1978; Gordon *et al*., 1984; Jensen *et al*., 1991), while most cereal *Potyviruses* are transmitted by aphids (Brault *et al*., 2010). The disease is also known to be seed borne and soil borne (Jiang *et al*., 1992; Brault *et al*., 2010; Mahuku *et al*., 2015a).

To safeguard maize production and food security in sub-Saharan Africa, there is need to conduct research (etiological, epidemiological, and genetic studies) to facilitate identification of effective sustainable MLND control measures. To ensure utility of the research outputs, studies should be conducted in major maize producing regions, particularly where the disease has been previously recognized. The Northern Tanzanian region is one of the major maize producing areas and is considered as country's grain basket (Nkonya, 1998). MLND has been reported by farmers and agricultural extension agents of Northern Tanzania, but there has been a lack of a comprehensive information about the extent of disease spread and the incidence.

To develop MLND eradication approaches, there is a need to understand its prevalence, viral transmission mechanisms, the environmental factors, and the genetics for host resistance. There is currently a huge ongoing effort to study the disease in Kenya (Wangai *et al*., 2012a, b; Adams

et al., 2013; Makone *et al*., 2014, but less effort in Tanzania. Prevalence studies would enable the individual Tanzanian regions to initiate cropping patterns that would reduce maize cultivation in hotspot areas, and hence enhancing food security. A better understanding of the viral transmission mechanisms would enable researchers to devise vector control strategies, and to study genetics for host resistance for the target environmental conditions. The objectives of the current study were to: 1) investigate the prevalence of MLND in the major maize growing areas of Northern Tanzania in order to document the current status of the disease, 2) investigate the factors associated with the occurrence and reoccurrence of the disease and the losses associated with the disease, 3 investigate the control methods practiced by individual farmers in the regions.

3.2 METHODOLOGY

3.2.1 Study sites

Two parallel studies involving farmers interviews and direct maize farm surveys were conducted between April and June, 2015 in Arusha, Kilimanjaro and Manyara regions in Northern Tanzania (Fig. 1). The area lies between latitudes 2° to 6° S and longitudes 34° to 39° E and is characterized by bimodal annual rainfall range of 500 - 1500 mm (Nkonya, 1998). The two rain seasons include a long/heavy rainfall between March and May and a short/light rainfall between October and December. Maize production is higher in the long rainfall season than short rainfall season because in short rainfall season, rain is unreliable and not intensive. The region has high, moderate and low rainfall agro-ecological zones (AEZs). The high rainfall AEZ (high AEZ) has an altitude above 1500 masl and receives an annual rainfall ranging between 1200 and 1500 mm (Nkonya, 1998). There is little maize production in this AEZ. The moderate rainfall AEZ (moderate AEZ) lies within at altitude of 900 - 1500 masl and receives annual rainfall between 800 and 1200 mm. The low rainfall AEZ (low AEZ) lies below 900 masl and receives a rainfall between 500 and 800 mm. The temperatures and rainfall in the low and moderate AEZs are favorable for intensive maize cultivation. In this study, 41 maize farms affected by MLND were surveyed in selected eight villages (Table 2). The study also included face to face interviews with farmers who have experienced MLND in their farms. The sample farmers of whom information was collected comprised 137 farmers. At least 30 farmers from each selected areas were interviewed. The sites were selected based on the history of the disease (presence of MLND

since the first report in 2012) and based on farming practices especially irrigation farming. The study group was selected with the help of Agricultural field officers (Extension staff) on the ground that they cultivate maize and have experienced MLND in their areas.

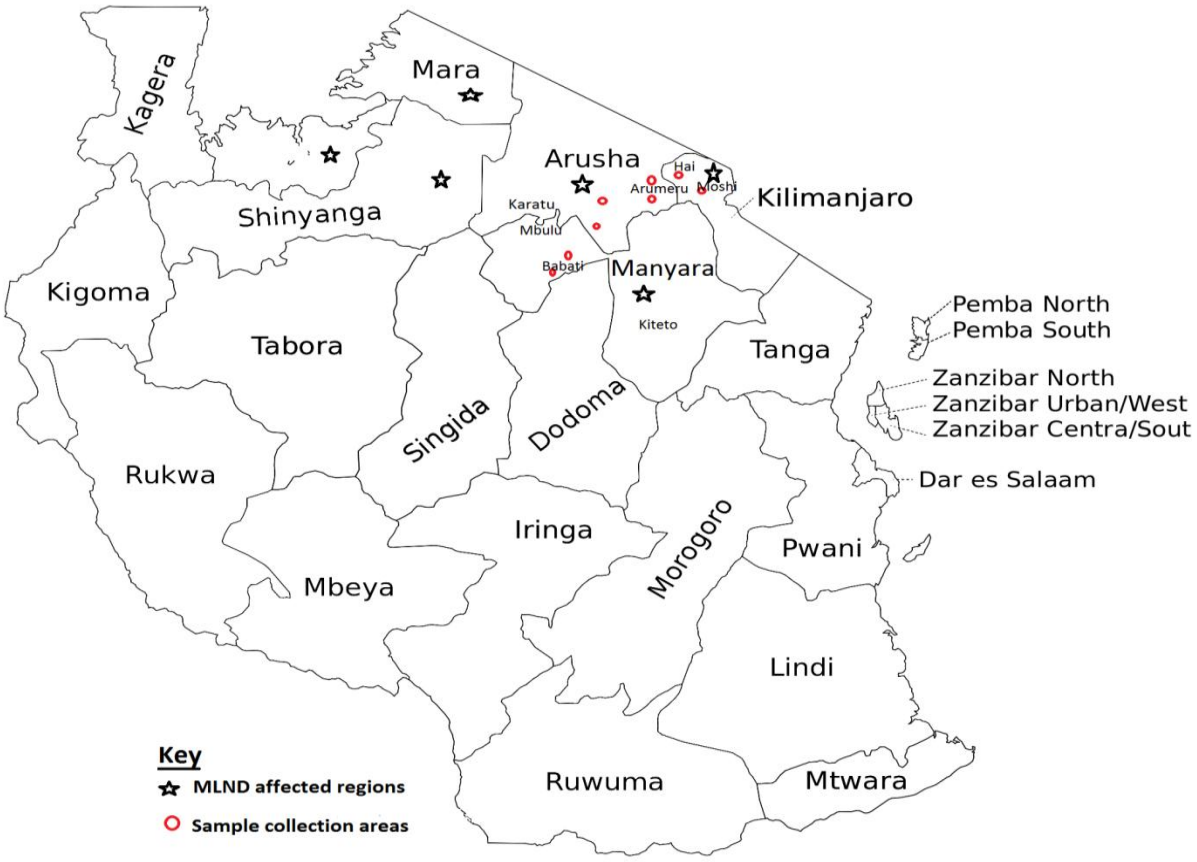

Figure 1 Map of Tanzania showing areas affected by Maize Lethal Necrotic Disease

2.2.2 Data collection and analysis

2.2.2.1 Assessment of farmers' awareness and experiences on ML ND

A questionnaire consisting of semi-structured items was designed. Purposive sampling was conducted to maize farmers in the selected five villages (Table 2) in Northern Tanzania. Data were collected through a farm survey by face to face interviews with farmers (n = 137). The questionnaire was designed in English and translated to Kiswahili, the national language which is understood by all farmers and pre-tested using small number of farmers in the same areas before using it in this study. Data collected included biographic information, MLND knowledge,

MLND prevalence, loss due to MLND and factors influencing the occurrence of the disease as shown in Table 1. Data were analyzed using IBM SPSS Statistics v.21 and Excel.

2.2.2.2 Assessment of the prevalence of MLND in northern Tanzania

Based on responses from the farmers' interviews and information from agricultural extension agents, farms (n = 41) were selected for direct observation and sampling of infected maize plants. The farms were within eight villages which were stratified across the maize producing AEZs in Northern Tanzania (Table 2). Within each farm, MLND symptoms were observed in quadrants of one-hundred maize plants. The number of maize plants with apparent MLND symptoms were counted in three random quadrants of each quarter acre of the farm. The mean of the counts of the symptomatic plants (%) was considered as the magnitude of the disease in the sampled farm. MLND prevalence across the villages within AEZs and the regions were compared based on the percentages of symptomatic plants from the sampled farms. The analysis were performed in a nested linear regression model using JMP Pro v.12 (SAS Institute Inc., 2013, Cary, NC.).

Table 1 Type of information collected during this survey

Type of information	Specific data collected in the questionnaire
General information	Age, gender, education level, maize farm acreage and duration for maize cultivation
Farmer's knowledge on MLND	Knowledge on MLND Date/year of first recognition Symptoms of MLND Location of farms with MLND Seed types used (local/recycled) or commercial/certified seeds Presence of vectors for transmission and their names (e.g. beetles, thrips, rootworms, aphids) Measures taken to avoid more MLND spread Re-occurrence in the same farm
Yield loss due to MLND	Difference in the maize yield before and after MLND occurrence, its description Loss extent
Prevalence of MLND	Area affected with MLND and its status in year 2015 e.g. low, average, high or no disease Reasons for that status Year with high MLND incidence since the first report in 2012
Factors influencing MLND	Seasons and their effect on MLND occurrence

3.3 RESULTS AND DISCUSSION

3.3.1 Prevalence of MLND based on symptomatic maize plants in Northern Tanzania

Prevalence of MLND in Northern Tanzania in 2015 differed across regions (p = 0.0012) and villages ($p < 0.0001$). The highest prevalence was recorded in Kilimanjaro with a mean of 22% symptomatic maize plants followed by Arusha (14%) and Manyara (10%) (Table 2). The prevalence did not differ across agro-ecological zones ($p > 0.05$) (Table 2).

Table 2 Prevalence of Maize Lethal Necrotic Disease in three regions of Northern Tanzania

Regions	Villages	Altitudes (masl)[a]	Rainfall (mm/year)[a]	AEZs codes[a]	Sampled farms (n)	Maize plants with MLND symptoms (%)[b]
Kilimanjaro	Lyamungu Kati	900 - 3500	500 - 1400	N4	8	20.6 ± 2.4A
	Mandaka Mnono	500 - 1200	800 - 1000	E2	5	24.0 ± 2.9A
Mean	-	-	-	-	-	22.0 ± 1.9A
Arusha	Ngaramtoni	1300 - 1700	600 - 1200	N5	14	19.1 ± 1.6A
	Madira-Sing'isi	1300 - 1700	600 - 1200	N5	3	16.0 ±3.4AB
	Tengeru	1300 - 1700	600 - 1200	N5	6	4.7 ± 2.6B
	Mlangarini	1300 - 1700	600 - 1200	N5	3	2.8 ± 4.2B
Mean	-	-	-	-	-	14.0 ± 1.6B
Manyara	Ayasanda	500 - 1200	800 - 1000	E2	1	10.0 ± 5.2AB
	Nyunguni	500 - 1200	800 - 1000	E2	2	9.9 ± 4.2AB
Mean	-	-	-	-	-	10.0 ± 3.3B

[a]Agro-ecological Zones (AEZs), and altitudes were obtained from the Ministry of Agriculture, Livestock and Fisheries. N4, E2 and N5 are agro-ecological zone codes respective to the villages [http://www.kilimo.go.tz/agricultural%20maps/Tanzania%20Soil%20Maps/Soil%20maps.htm].

[b]Areas connected with common letter A or B do not differ statistically and vice versa.

The study revealed that, MLND is more persistent in areas under irrigation where maize is grown throughout the year (no break between seasons). Examples of such areas in Kilimanjaro includes: Lyamungu Kati and Mandaka Mnono. This is probably due to the presence of infected maize debris that is incorporated in the soil which act as source of viruses (Nyvall, 1999; Mahuku *et al*., 2015a). Presence of the viruses in the soil as the result of inadequate tillage to bury infected maize debris and improper field sanitation become source of inoculums. It may also be due to environmental conditions such as high temperature and wet weather conditions especially during dry seasons favor the increase of both vectors and virus population. Creating breaks between seasons has been reported by Wangai *et al*. (2012a) to reduce vector population and hence low MLND incidence. There is a possibility of irrigation water to carry MLND viruses as the process involves movement of water from one point of the field to another that may contain soil and debris (Mahuku *et al*., 2015a).

However, some places of Arusha for example in Mlangarini, MLND prevalence was very low (2.8%), different from year 2014. This was explained by extension staffs to be due to early planting, removal of infected maize debris that could act as source of inoculum, the use of certified seeds, insecticide application and that proper education was given concerning the disease management. In Manyara, maize farms were badly affected by drought, that is why very few farms (3 farms) planted late were visited and thus counts to the lowest (10%) MLND prevailed region. Late planting is one of the factors that favor the occurrence of MLND. Early planting is one of the disease management systems since it allows plants to escape infection or reduce severity of the disease (Maloy, 2005).

3.3.2 Socio-demographic data

The Majority (39.4%) of maize farmers in Northern Tanzania are between the age of 31 - 50 years followed by the age of 15 - 30 years (Table 3). This age is energetic as opposed to old age and therefore it is involved in high production activities despite agricultural constrains such as MLND. Of the interviewed farmers, male gender were 57.7% and female were 42.3%. The highest education of most farmers (70.8%) interviewed were primary education (Table 4). Education of farmers has influence in agricultural activities especially on adoption of new technologies and management practices of crop diseases. The least they are educated the least they can handle and adopt technologies and therefore bad farming practices (Schreinemachers *et*

al., 2015). Most farmers (90.5%) are small scale farmers with cultivation land of not more than 5 acres while only 9.5% cultivate maize in more than 5 acres (Table 5). This may be attributed by land shortage in Northern Tanzania. Most land is used by large-scale commercial farms and state-protected areas devoted to wildlife and tourism (Ujamaa Community Resource Team, 2010). About 76.6% of the respondents have cultivated maize for more than five years (Table 6).

Table 3 Age of respondents in Northern Tanzania

Age (years)	Frequency	Percent (%)
15 - 30	38	27.7
31 - 50	54	39.4
51 - 60	24	17.5
>60	21	15.3
Total	137	100.0

Table 4 Education levels of the respondents in Northern Tanzania

Levels of education	Frequency	Percent (%)
Illiterate/no schooling	15	10.9
Incomplete primary school	10	7.3
Primary school education	97	70.8
Secondary school	10	7.3
High school	3	2.2
College and university	2	1.5
Total	137	100.0

Table 5 Distribution table of land used for maize by farmers in Northern Tanzania

Acres used for maize cultivation	Frequency	Percent (%)
< 1 acre	32	23.4
1 acre	37	27.0
2 acres	26	19.0
3-5 acres	29	21.2
> 5 acres	13	9.5
Total	137	100.0

Table 6 Duration of maize production by respondents in Northern Tanzania

Number of years of maize cultivation	Frequency	Percent (%)
1year	5	3.6
2years	5	3.6
3-5 years	22	16.1
> 5 years	105	76.6
Total	137	100.0

3.3.3 Farmers' knowledge on MLND

The present study found that 51.8% of farmers in Northern Tanzania particularly Kilimanjaro, Arusha and Manyara regions recognized MLND in 2013 and 99.3% were aware of MLND based on symptoms but not on disease transmission and management (Table 6). These results concur with the study by Makone *et al*. (2014) who also found that farmers are unaware of MLND transmission and its management. Among MLND management activities, rouging of infected maize plants were practiced by 54.0% farmers followed by insecticides application (10.7%) while 28.7% farmers took no measures to avoid more spread of the disease (Table 7). When infected maize plants are left on farms, there is greater chance to more spreading of the disease

and its re-occurrence. That may probably be the reason for the re-occurrence of MLND in 50.4% farmers' farms.

Table 7 Farmer's awareness on Maize Lethal Necrotic Disease in Northern Tanzania

Measures taken by farmers to avoid spreading of MLND	Percent (%)	First year of MLND recognition by farmers	Percent (%)
Roughing of diseased plant	54.0	2015	5.8
Weed elimination	2.7	2014	33.6
Insecticide application	10.7	2013	51.8
No any measures taken	28.7	2012	7.3
Addition of fertilizer and watering	4.00	2011	1.5
Total	100.1		100.0

3.3.4 Prevalence of MLND in Northern Tanzania from 2012 - 2015

Assessment of the farmers regarding MLND prevalence from year 2012 of the first report to 2015 revealed that MLND prevalence and incidence was higher in 2014 (54.7%) as compared with other years (Fig. 2) which resulted in total crop failure to 88.3% farmers (Table 8). The trend of MLND prevalence was found to be very low (2.2%) in 2012 with a sharp rise to 22.6% and 54.7% in 2013 and 2014 consecutively and then dropped in 2015 as shown in Fig. 2. Similar scenario was reported in Kenya where MLND was found to spread slowly in early years but progressed rapidly in 2012 and 2013 (De Groote *et al*., 2016). This can be explained by disease establishment factors such as; the ability of the pathogen to adapt to new environment, ability to disperse rapidly over long distances causing distractive disease epidemics (as for MLND presence of vectors that can transmit viruses between and within maize farms) and the ability to survive between seasons (Keane *et al*., 1997).

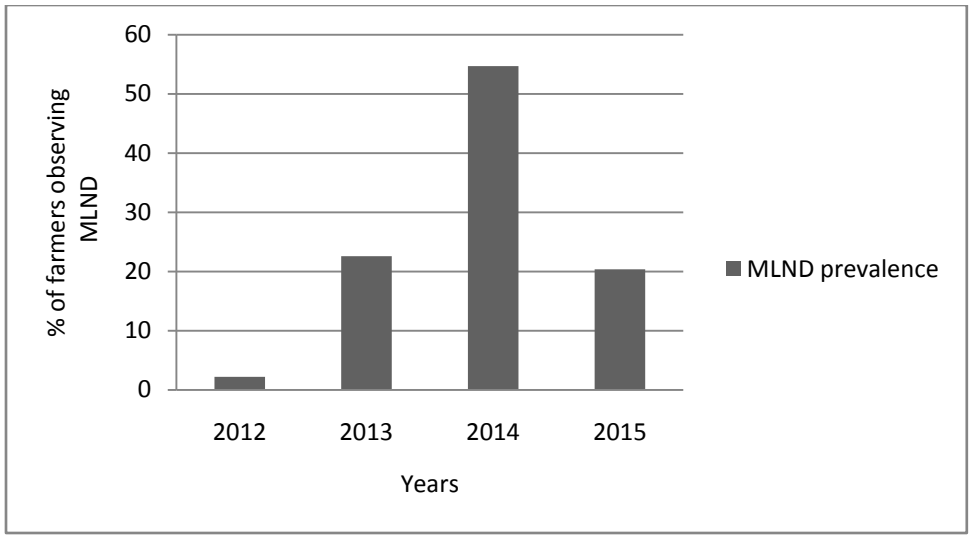

Figure 2 The prevalence of Maize Lethal Necrotic Disease in Northern Tanzania from 2012 to 2015

3.3.5 Yield Loss due to MLND in 2014

Farmers of Northern Tanzania are experiencing a greater loss due to MLND despite their effort and capital invested in maize production. The most affected are small scale farmers whose livelihood depends mostly in agriculture. These farmers contribute to over 80% of Tanzania's total maize production (Nkonya, 1998; FAO, 2015). About 88.3% farmers in Northern Tanzania reported to have complete yield loss of maize cultivated in year 2014 production (Table 8**)**. A loss of 100% by 88% farmers is such a big loss for Tanzanian per capital income and food security at large.

Table 8 Description of maize yield loss encountered by farmers due to MLND

Description of maize yield loss	Frequency	Percent (%)
Complete yield loss (100%)	121	88.3
< 25% loss	6	4.4
No yield loss (0%)	5	3.6
Unaware of the loss	5	3.6
Total	137	100.0

3.3.6 Farmers' perception on the role of insect vectors in MLND transmission

There are several reports that MLND viruses are transmitted by vectors (Nault *et al*., 1978; Uyemoto, 1983; Gordon *et al*., 1984; Jensen *et al*., 1991; Scheets, 1998; Jiang *et al*., 1992) but results of the present study proved that most farmers are not aware of the roles of vectors especially those involved in MLND transmission. Few farmers (29.9%) were aware of the presence of vectors, 11.7% were unaware and 58.40% farmers observed no vectors in their farms (Table 9). Reason for 11.7% and 58.4% could be just unaware of their presence because of lack of curiosity or the part in the maize plant on which vectors feed may affect their visibility. For example, rootworms feeds in roots therefore if a farmer is not curious enough she/he will not be able to notice them. Additionally, probably farmers were not able to notice vectors in their field because of vectors' small body sizes. For example thrips are not easily noticed in farms because they have minute body sizes less than 0.05 inch long (Bethke *et al*., 2014). It could also be due to seed transmission and not vectors because MLND has been reported as seed born disease (Jensen *et al*., 1991; Delgadillo Sánchez *et al*., 1994). In this study, insect vectors that were observed by farmers include; beetles, rootworms, aphids, thrips, mites, stem borers and leaf hoppers. However, not all insect vectors (e. g. leafhoppers and stem borers) witnessed in farmers' maize farms affected by MLND have been reported to transmit the disease.

Table 9 Farmers response on presence of insect vectors in their farms affected by MLND

Responses	Frequency	Percent (%)
Insects present	41	29.9
No insects	58.4	58.4
Unaware	11.7	11.7
Total	137	100.0

3.3.7 Seed varieties used by farmers

Farmers in Northern Tanzania reported to have been using both certified and recycled maize seeds. A total of 87.1% farmers used certified seeds, with Seed CO (24.1%) and Stuka (17.0%) leading while 12.9% used local/recycled seeds (Fig. 3). Despite using these seeds, farmers reported re-occurrence of MLND in their farms. Similarly, Mahuku *et al*. (2015a) reported that

MLND affect almost all commercial varieties in Kenya. Because of the complex nature of MLND that involves interaction of more than one virus (Nilblett *et al*., 1978; Uyemoto *et al*., 1980; Uyemoto *et al*., 1981; Scheets, 1998; Lamichhane *et al*., 2015), having seeds that are resistant to both viruses is a bit complicated and thus affecting disease management.

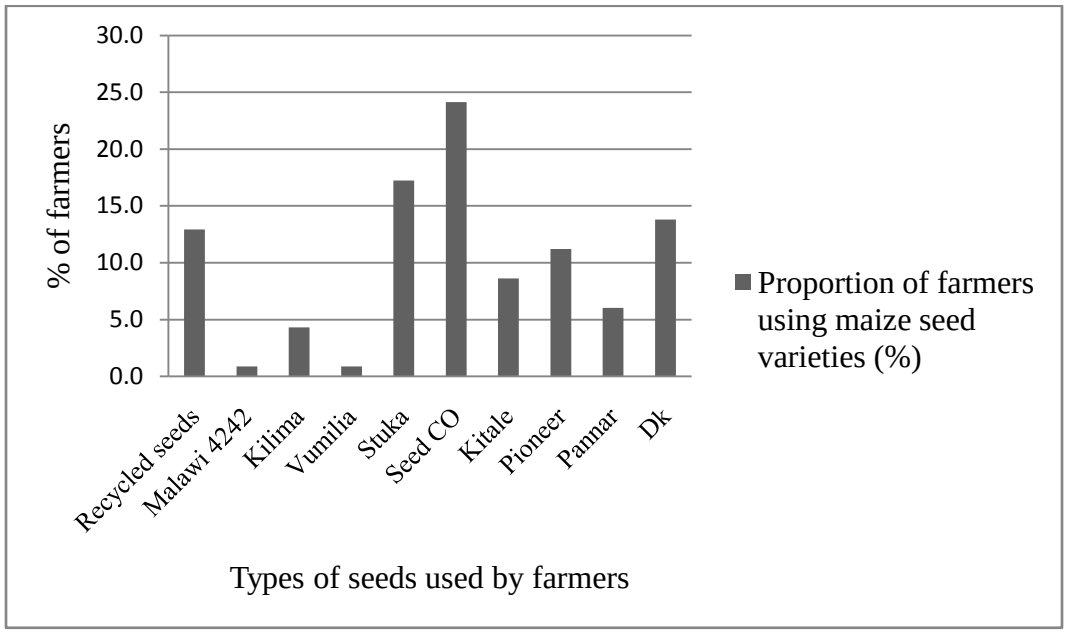

Figure 3 Maize varieties grown by farmers in Northern Tanzania in 2015

3.3.8 Seasons with high MLND occurrence

Despite other environmental factors, MLND occurrence in Northern Tanzania was found to be higher (67.9%) in long rain seasons than other seasons as shown in Fig. 4. This implies that maize production activities in Northern Tanzania during long rain seasons can easily collide with favorable conditions for occurrence of MLND. Short rainfall (*Vuli)* between October and December are unreliable (Kabanda *et al*., 1999) while irrigation which could be used during drought is underdeveloped and expensive to operate (Mmbaga *et al*., 2001).

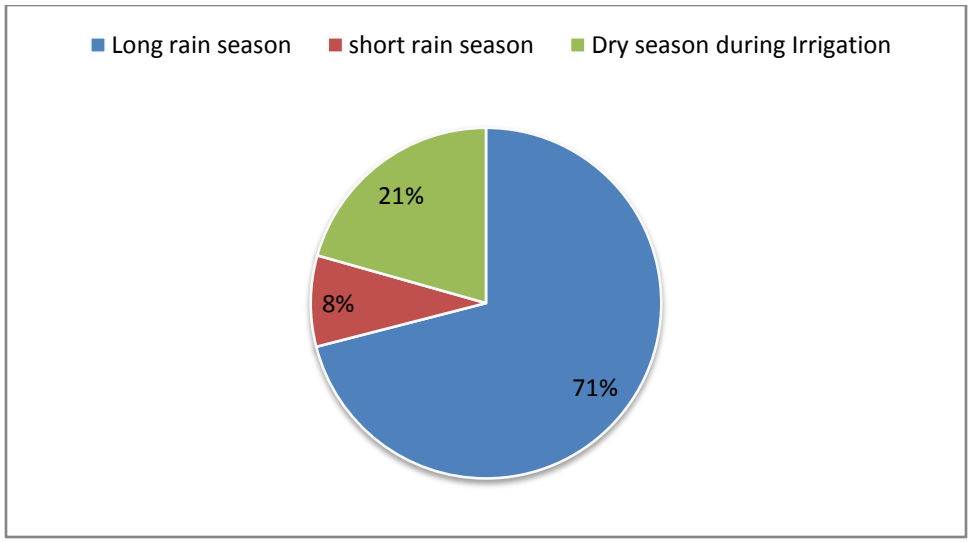

Figure 4 Occurrence of MLND in Northern Tanzania in each season

3.3.9 Areas affected by MLND in Northern Tanzania

According to farmers and personal observations, MLND in 2015 seemed to dominate and prevail to new places in Northern Tanzania that include; Lyamungu Kati-Ureni KNCU (Machame Narumu), Mandaka Mnono (Old Moshi West), Mabogini, Sambarai (Kindi), Chekereni, Kimashuku, Mwanamasota, Shirimatunda and Njia panda Machame in Kilimanjaro region. Ngaramtoni, Ekenywa, Kimyaki, Olmotonyi, Kilima Moto, Oldonyosambu, Mlangarini, Usa River, Madira (Seela-Sing'isi) and Nduruma, in Arusha region and Ayasanda, Bonga, Himiti, Riroda, Nyunguni (Babati-town), Karatu, Kiteto in Manyara region (Fig. 1). The prevalence may be the result of monoculture practices by farmers as observed in the present study. Only 2.2% of farmer cultivated other food crops like tomatoes and common beans while 97.8% of farmers' repeated maize cultivation which led to re-occurrence of MLND in 50.4% farmers' fields. Availability of susceptible maize crop all the time enhances the increase of vectors population and viruses. Crop rotation to non-susceptible plants has been reported by Maloy (2005) as one of the options for disease management especially soil borne diseases since it helps in reducing vector population and viruses density.

3.4 CONCLUSION AND RECOMMENDATIONS

The results showed that MLND in 2015 is relatively low. It also shows that, farmers lack proper education on the disease management practices. As a result, re-occurrence of MLND on the same farm and spread to other maize farms causing major crop damage and yield loss. Given the importance of maize to most of the people in Tanzania and the damage that MLND is causing, urgent solution must be developed including production of resistance/tolerant maize seeds to MLND and providing proper education to farmers on the disease management. Having 22% maize plants symptomatic to MLND in Kilimanjaro for example, cannot be ignored given that MLND is soil borne and vector transmitted.

CHAPTER FOUR

Identification and Characterization of viruses causing Maize Lethal Necrotic Disease in Northern Tanzania

SUMMARY

Maize is the most important cereal crop and staple food for majority of Tanzanians. Its production has significantly increased over the past 10 years based on planted areas rather than increased yields. The production in Lake and Northern zone is affected by a highly devastating disease called Maize Lethal Necrosis (MLND). A total of 223 symptomatic and randomly selected maize leaves were collected between April and June, 2015 in Kilimanjaro, Arusha and Manyara regions in Northern Tanzania. Detection of the causative agents in all samples collected was performed using Double Antibody Sandwich-Enzyme linked Immuno Sorbent Assay (DAS-ELISA) and Reverse Transcription-Polymerase Chain Reaction (RT-PCR). Based on the DAS-ELISA and RT-PCR results, a subset samples (n = 48) consisted of RNA samples (n = 30) positive for MCMV and SCMV, negative for both (n = 1), and negatives (n = 17) for SCMV was used for viruses characterization using Next generation sequencing (NGS) Illumina Miseq. Of all the samples collected, 65% were positive for SCMV by DAS-ELISA test and 97% positive for MCMV by RT-PCR test with a co-infection (MCMV and SCMV together) of 64%. The highest incidence of MCMV (100%) and SCMV (98%) was recorded in Lyamungu Kati-Hai district. The lowest incidence of MCMV (80%) and SCMV (16%) was recorded in Mlangarini, Arumeru district and Mandaka Mnono, Moshi district respectively. NGS analysis showed that, there were no *Potyviruses* other than SCMV found in the samples collected in the regions, which means only SCMV and MCMV are involved in causing MLND. Based on phylogenetic tree, MCMV from this study are highly similar to themselves and to the existing eastern Africa isolates (99% nt identity). However, SCMV from this study have significant genome diversity within themselves. They were found to be similar to the highly virulent SCMV isolate from Hebei-China and from Kenya with nucleotide identity ranging from 87 - 99%. Therefore, similar management practices including production of resistant maize varieties can be applied in the regions affected by MLND.

4.1 INTRODUCTION

Maize is the most important cereal crop and staple food for about 1.2 billion people in sub Saharan Africa (IITA, 2009). Among the countries of SSA, Tanzania is a major maize producer having the largest planted area in all Southern and Eastern Africa (FAO, 2015). Over 5 million hectares of Tanzanian's land are used for maize production (FAO, 2015). The production has significantly increased over the past 10 years based on planted areas rather than increased yields which counts up to 6 million metric tons annually (FAO, 2015). Per capital consumption of maize is about 128 kg (Suleiman *et al*., 2015). Maize is also a commercial crop, source of fuel and animal feed (Davis, 2003; Suleiman *et al*., 2015). It is intensively grown in three agro-ecological zones in Tanzania namely; Southern Highland, Lake and Northern zones (Nkonya, 1998). Currently maize production in the Lake and Northern zone is affected by a highly devastating disease called Maize Lethal Necrosis (MLN). This disease was first reported in Kenya in 2011 and one year later in Tanzania (Wangai *et al*., 2012b). The disease is reported to be caused by a complex of viruses causing complete crop failure and yield reduction (Wangai *et al*., 2012b; Makone *et al*., 2014).

The reported causative agents of MLND in Eastern and Central Africa are *Maize Chlorotic Mottle Virus* (MCMV) and *Sugarcane Mosaic Virus* (SCMV) (Wangai *et al*., 2012b; Adams *et al*., 2014; Luanda *et al*., 2014; Makone *et al*., 2014; Mahuku *et al*., 2015a, b). MCMV from family *Tombusvirideae* is a single stranded positive sense RNA with genome size of 4.4 kb (Lommel *et al*., 1991) while SCMV from family *Potyvirideae* is a single stranded positive sense RNA with genome size of 9.6 kb (Gell *et al*., 2015). MCMV alone can infect maize causing mild with moderate stunting and leaf mosaic symptom. However, mixed infection of MCMV and SCMV results in severe symptoms of stunting, general chlorosis and necrosis (Makone *et al*., 2014). Other *Potyviruses* such as *Wheat Streak Mosaic Virus* (WSMV) and *Maize Dwarf Mosaic Virus* (MDMV) have been reported to synergize with MCMV causing MLND (Uyemoto *et al*., 1980; 1981). Both MCMV and SCMV are vector transmitted (Nault *et al*., 1978; Uyemoto, 1983; Gordon *et al*., 1984; Jiang *et al*., 1992; Brault *et al*., 2010) seed-borne (Jensen *et al*., 1991; Delgadillo Sánchez *et al*., 1994; Mahuku *et al*., 2015a) and soil borne (Mahuku *et al*., 2015a).

Appropriate disease diagnosis is key to its proper management (Adams *et al*., 2013). This is particularly true for entirely new diseases where novel control strategies need to be developed

alongside characterization of novel agents (Kreuze *et al*., 2009). Virus detection is based on species-specific tests such as ELISA and PCR which require knowledge of the organism in question. Recently, Next Generation Sequencing (NGS) has been used to identify new viruses in disease complexes, identify variants in species and indicate frequency of viruses found in the infected samples (Coetzee *et al*., 2010). NGS has been successfully used in identification of MLND viruses (Adams *et al*., 2013; 2014). SCMV is reported to have significant genomic variation in East Africa while MCMV is similar, having a shared origin (Adams *et al*., 2014; Mahuku *et al*., 2015a). According to Adams *et al*. (2014), these findings are expected because MCMV is new in Africa, reported in 2011 (Wangai *et al*., 2012b) while SCMV existed since the 1970s (Louie, 1980).

Since the first report of MLND in Tanzania in 2012 (Makumbi and Wangai, 2013), there has been a dramatic yield reduction in affected maize production areas of Northern Tanzania. It is a big threat to food security, considering the importance of maize as a food crop in Tanzania. Despite the economic loss caused by MLND, viruses associated with it are not well studied. The incidence and genetic diversity of the viruses are unclear. Information on the incidence and genetic diversity of the causative viruses is crucial for MLND management. Therefore, the present study was conducted to identify and describe genetic diversity of viruses causing MLND in Northern Tanzania using Next Generation Sequencing (NGS) technology Illumina Miseq and Bioinformatics tools.

4.2 METHODOLOGY

4.2.1 Sample collection

Following the report of Maize Lethal Necrotic Disease of 2012 in the Lake (Mwanza, Shinyanga) and Northern zone of Tanzania, the present study is based in the Northern part of Tanzania namely; Arusha, Manyara and Kilimanjaro regions. They are located between latitudes 2° to 6° S and longitudes 34° to 39° E. A total of 223 symptomatic (Fig. 5) and randomly selected maize leaves were collected between April and June, 2015 from the three regions for laboratory tests of the causative viruses. The number of maize leaf samples from each of the sites collected is shown in Table 10. Samples were dried and stored in silica gel prior to laboratory

analysis. Maize leaf samples were tested for the MLND causing viruses using three different detection techniques; DAS-ELISA, RT-PCR and Next generation sequencing-Illumina Miseq.

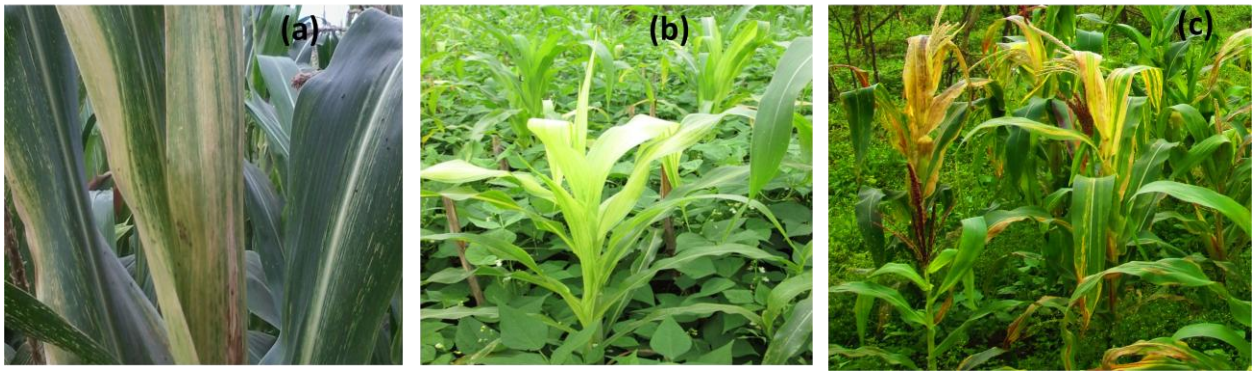

Figure 5 Maize leaves with symptoms of MLND

(a) Maize crop from Madira-Arusha with chlorotic mottling, (b) maize crop from Mandaka Mnono in Moshi-Kilimanjaro and (c) maize crops from Lyamungu Kati in Hai-Kilimanjaro with dead-heart symptom.

4.2.2 Viral detection using Double Antibody Sandwich Enzyme-Linked Immuno Sorbent assay (DAS-ELISA)

To screen for known viruses reported to cause MLND, DAS-ELISA was performed as per standard methods from manufacturer (Agdia Inc. USA) using antibodies raised/specific to MCMV and SCMV. The absorbance values of the samples at 405 nm were measured after incubation for one hour at room temperature. Appropriate positive and negative control samples were used with each test. A positive threshold was scored if the absorbance reading was 3X the negative control (Adams *et al*., 2013).

4.2.3 Viral detection using Reverse Transcriptase polymerase chain reaction (RT-PCR)

Maize leaves were ground in a sterile mortar and pestle in liquid nitrogen, RNA was then extracted from individual grounded maize sample with the ZR Plant RNA MinPrep from Zymo Research as per manufacturer's instructions. Prior to PCR, RNA quality was checked using formamide-denatured agarose gel electrophoresis on 1X TAE (Masek *et al*., 2005) and RNA quantity was measured with Qubit® 2.0 Fluorometer RNA broad range. Complementary DNA (cDNA) synthesis was performed by using Thermo Scientific Maxima First Strand cDNA

synthesis Kit # K1642 followed by PCR with reactions that consisted of 10 µl of one Taq master mix with standard buffer from New England BioLabs *Inc*. (20 mM Tris-HCl, 1.8 mM NH_4Cl, 22 mM KCl, 0.2 mM dNTPs, 5% gycerol, 0.06% IGEPAL CA-630, 0.05% Tween 20 and 25 U/ml One Taq DNA Polymerase), 0.1 µl (10 µM) for each forward 5' -CGCGGCTGACAAGCAAAT-3' and reverse primers 5' -ACTGGTTGTTCCGGTCTTG -3', 2 µl cDNA and 7.8 µl of sterile water to give a final volume of 20 µl as per manufacture's protocol. The cycling conditions were; initial denaturation at 95°C for 3 min, 30 cycles of denaturation at 95°C for 30 sec, annealing at 49.4°C for 30 sec, and elongation at 72°C for 1 min and a final extension step at 72°C for 15 min. The PCR product was detected on a gel red stained 1.2% agarose gel electrophoresis under UV light. The negative control was water instead of the template and positive control was from a known infected plant.

4.2.4 Characterization of MLND viruses by Next Generation Sequencing

A random subset of samples from Kilimanjaro (n = 15), Arusha (n = 22) and Manyara (n = 11) were selected for characterization of the viruses. Based on the ELISA and RT-PCR tests, the subset samples (n = 48) consisted of RNA samples (n = 30) positive for MCMV and SCMV, negative for both (n=1), and negatives (n=17) for SCMV. The corresponding RNA samples were used for libraries construction (preparation of samples for sequencing) using the Illumina TruSeq RNA kit (Illumina Inc. USA) following manufacturer's instructions. RNA samples with a concentration of 0.1 - 1µg were fragmented followed by first strand and second strand cDNA synthesis. Double stranded cDNA was purified using Agencourt AMPure XP magnetic beads (Beckman Coulter, Inc.) (1.8X ration beads to the volume of cDNA) followed by end repair and adapters ligation. The Double stranded cDNA with adapters were amplified using universal primer and index/adapter primer. PCR products (libraries) were then purified using Agencourt AMPure XP magnetic beads (Beckman Coulter, Inc.). Quality control analysis of the libraries was completed using Qubit® 2.0 Fluorometer (Thermo Fisher Scientific Inc.) and Agilent Tape Station 2200 systems (Agilent Technologies USA). Libraries were then normalized to 10nM and pooled. Pooled libraries with a final concentration of 6.5 pM were sequenced on the Illumina MiSeq System at the BecA-ILRI Hub generating 151 paired-end reads.

4.2.5 Sequencing data analysis

Quality control of the sequence data generated was performed using Fastqc v0.11.2 (Andrew, 2010), FASTX_toolkit (Patel *et al*., 2012) and SolexaQA (Cox *et al*., 2010) to remove adapters and poor quality sequences. *De novo* assembly of the reads was performed using Trinity v2.2.1 (Grabherr *et al*., 2011; Haas *et al*., 2013). Assembled sequences were then blasted against locally installed virus-database using BLASTN 2.2.30+, TBLASTX 2.2.30+ (Shiryev *et al*., 2007). Krona (Ondov *et al*., 2011) was used to visualize viruses present in each sample. Reference mapping was done for individual samples to the most similar reference genome downloaded from NCBI [http://www.ncbi.nlm.nih.gov/gquery/], using CLC Genomics Workbench 5.5.1 software. Sequences obtained were compared to the De *novo* assembled contigs to confirm that no artifacts had been introduced during reference assembly. These sequences were then screened for homology to known viruses by blasting against the GenBank [Ret Seq; http://ww.ncbi.nlm.nih.gov/]. Multiple sequence alignment of the viruses was performed using CLC Genomics Workbench 5.5.1 software. Nucleotide sequences of MCMV and SCMV coat proteins were used for phylogeny using Mega 6.06 (Tamura *et al*., 2013) where, maximum likelihood method based on the Kimura 2-parameter model (Kimura, 1980) was used with 1000 bootstrap replicates. A complete set of two-way comparisons were performed between SCMV sequences from this study and the closely related SCMV isolate from China (JN021933.1) for the entire polyprotein/ORF and for each gene by using Species Demarcation Tool (SDTv1.2) software (Muhire *et al*., 2014).

4.3 RESULTS AND DISCUSSION

The diagnostic results of the present study showed that, 65% of all the samples collected were positive for SCMV. High incidence (98%) of SCMV was recorded in Lyamungu Kati-Hai district and lowest incidence (16%) was recorded in Mandaka Mnono in Moshi Rural district (Table 10). However, while screening for MCMV, all samples including negative controls showed a positive response in the DAS-ELISA screen indicating false positives. The apparent failure of DAS-ELISA may be attributed by its poor specificity for the unusual or new variant isolates (Adams *et al*., 2013).

RT-PCR revealed that MCMV is a major virus for MLND with an incidence of 97%. MCMV alone can cause significant crop failure and hence yield loss (Mahuku *et al*., 2015a). A high incidence of MCMV was recorded in Kilimanjaro (100%) followed by Manyara (94%) (Table 10). Some of asymptomatic samples (data not shown) tested positive for MCMV indicating that PCR can be useful for identification of MLND even from plants with no symptoms provided the virus exists (Lima *et al*., 2012). SCMV however, was found to synergize with MCMV causing severe symptoms and eventually plant death with the co- infection of 64% in Northern Tanzania. Higher incidences of both MCMV and SCMV were recorded in Lyamungu Kati, Hai district followed by Madira, Arumeru district. In these areas, maize is cultivated throughout the year using both rain fed water and irrigation. Presence of host plant (maize) all the time may be the reason for high MLND incidence in these areas. Creating break between seasons has been reported to reduce MLND incidence (Wangai *et al*., 2012a).

Table 10 Incidence of viruses causing Maize Lethal Necrotic Disease in regions located in Northern Tanzania

Region	District	Village	Samples collected	SCMV incidence %	MCMV incidence %	Synergism (SCMV&MCMV) %
Arusha	Arumeru	Ngaramtoni	58	53	98	53
		Mlangarini	20	45	80	45
		Madira	35	94	100	94
Kilimanjaro	Hai	Lyamungu Kati	44	98	100	98
	Moshi (V)	Mandaka Mnono	31	16	100	16
Manyara	Babati	Nyunguni	35	66	94	63
Total			223			

A total of 48 libraries were sequenced using Illumina Miseq- sequencing by synthesis method with 151 paired-end cycles. The paired-end cycles yielded 97 468 804 reads with an average length of 35 - 151 bp. After trimming/cleaning and removing duplicates 46 361 174 reads of length 17 - 122 bp were attained. GC content was between 48 to 50%. The reads were assembled and compared to locally installed plant virus-database using BLASTN+ and TBLASTX, and the results visualized using Krona. The Krona results displayed many plant viruses having very low e-values (not significant) with exception of MCMV, SCMV and Maize Streak Virus (MSV). Further analysis of *De-novo* assembly and reference assembly revealed that no artifacts were introduced during reference assembly and sequences for MCMV, SCMV and MSV (Table 12 supplementary data) had significant genome coverage while the rest has been just small sequence fragments. Table 11 shows genome coverage and depth of MCMV and SCMV representatives.

Table 11 Read counts and genome coverage of *Maize Chlorotic Mottle Virus* and *Sugarcane Mosaic Virus* obtained from reference assembly

Virus	Sample number	Read mapped	% read mapped	Average depth of sequence	% Genome coverage	Genome length (nt)
MCMV	5	578 660	65.7	15 057	99.9	4 432
	7	408 433	82.8	10 668	99.4	4 410
	11	433 159	69.5	11 176	99.7	4 421
	14	731 053	80.4	18 797	99.9	4 431
	16	429 822	37.3	10 795	99.9	4 431
	19	466 171	38.9	11 955	99.8	4 428
	21	480 071	50.1	11 738	99.5	4 416
	23	710 295	52.9	16 979	100	4 436
	27	548 930	64.9	13 866	99.8	4 427
	28	442 863	40.2	11 291	99.8	4 429
	12	453 118	73.7	11 767	99.75	4 425
	34	498 579	49.2	12 741	99.8	4 428
	24	663 071	73.4	16 529	99.9	4 431
	47	38 431	2.04	928	99.7	4 423
	17	496 273	59.3	12 672	99.5	4 415
	6	397 723	75.7	10 425	99.5	4 416
	41	448 926	58.5	11 521	99.9	4 432
	44	657 532	60.3	16 422	99.6	4 418
	43	452 173	38.5	11 294	99.9	4 431
SCMV	23	27 976	2.1	309	99.8	9 557
	24	14 378	1.6	165	99.7	9 551
	43	14 677	1.3	168	100	9 575
	46	11 321	0.8	131	99.7	9 543
	15	17 531	2.0	209	99.4	9 520
	41	18 657	2.4	211	99.7	9 552
	10	8 756	1.5	106	99.6	9 537
	27	13 241	1.6	152	99.7	9 545
	11	9 473	1.5	113	99.7	9 552
	35	9 292	1.8	115	99.9	9 563
	44	12 483	1.1	138	100	9 576
	17	9 743	1.5	115	99.8	9 556
	25	14 343	1.9	164	99.7	9 549
	5	11 621	1.3	137	99.7	9 546
	13	10 510	1.4	125	99.7	9 544
	16	12 468	1.1	144	99.7	9 549

Complete nucleotide sequence of MCMV genome (sample 23) has been obtained during assembly. It was found to be 4436 nt long with six Open Reading Frames (ORFs) similar to other MCMV isolates (Nutter *et al*., 1989; Lommel *et al*., 1991; Stenger *et al*., 2008). The BLASTn search of the full-length nucleotide sequence against the NCBI database indicated that the virus is very closely related to MCMV isolates from eastern Africa (accession KP851970.3, KP798454.1, KP772217.1, KT250543.1, KT250546.1) sharing 99% nucleotide sequence identity. This high degree of sequence identity between samples from this study and MCMV isolates from eastern Africa suggests that these virus isolates share a very recent common ancestor different from US isolates (Nebraska 97% and Ohio 97%).

Phylogenetic analysis of the coat protein (CP) sequences of MCMV from this study and existing MCMV isolates reveals that, the virus is similar to eastern African isolates while differing from Nebraska and Kansas isolates (Fig. 6). However, sample 43 from Madira (Arusha region) seem to be somehow distinct from the rest of eastern African MCMV isolates and also far from other existing isolates (Fig. 6). Two way comparison of the entire genome (sample 43) indicated to have 99% identity to eastern Africa isolates. These findings show that, MCMV from this study are highly similar.

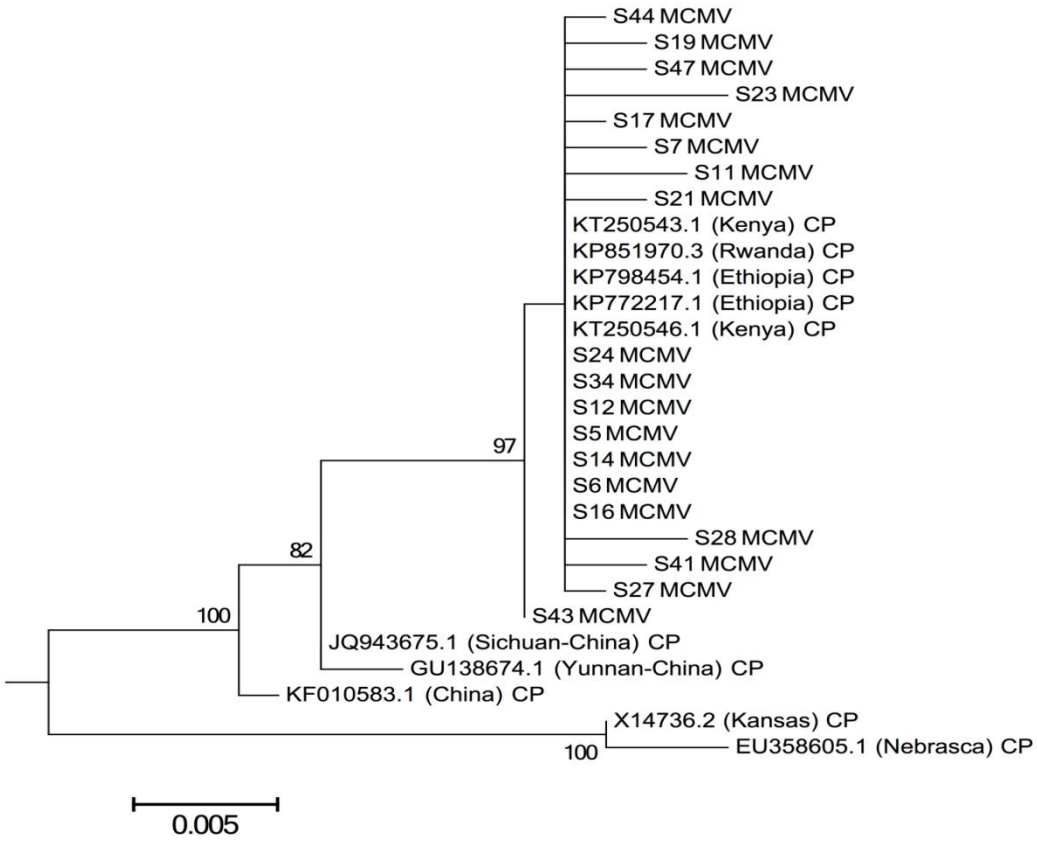

Figure 6 A maximum likelihood tree constructed with MEGA 6 using 1000 bootstrap replicates for *Maize Chlorotic Mottle Virus* coat proteins from this study and from GenBank

Assembly of SCMV sequences produced almost complete genomes with 9520 nt to 9576 nt long (sample 5,41,13,27,35,25,16,15,43,17 and 11). The genome is translated via a large polyprotein precursor containing 10 mature proteins similar to other viruses of *Potyvirideae* family (Adams *et al*., 2005) which play different roles in the life cycle of SCMV including infection, replication, movement, and transmission (Gemechu *et al*., 2006; Zhang *et al*., 2008; Liu *et al*., 2009; Wu *et al*., 2013). The BLASTn search of the full-length nucleotide sequence against the NCBI database indicated that the virus from this study had significant similarity to SCMV isolates belonging to China, a highly virulent isolate (Gao *et al*., 2011) sharing the closest relationship with 87 - 99% identity.

Subsequently, comparison of the CP nucleotide sequences with other existing SCMV CP (Fig. 7) revealed that, SCMV from this study are closely related to isolates from Kenya and Hebei-China (Gao *et al*, 2011; Adams *et al*., 2013) suggesting to have a common ancestor. However, SCMV

from this study have significant genome diversity. Five isolates (sample 23, 24 and 46 from Mandaka Mnono-Kilimanjaro region and sample 10 and 44 Ngaramtoni-Arusha region) were distant from Hebei-China isolate and far from other existing isolates (Fig. 7). The variability of SCMV is not unexpected as it has been reported in other studies (Elena *et al*., 2005; Goncalves *et al*., 2011; Padhi *et al*., 2011; Adams *et al*., 2013; Li *et al*., 2013). It is due to the lack of proofreading activity of RNA-dependent RNA polymerases, short generation time, and large population size as a result new viral genetic variants are created (Elena *et al*., 2005; Xie *et al*., 2016).

Two-way comparison of the entire polyprotein/ORF of the earlier mentioned five distinct SCMV isolates to closely related SCMV from Hebei-China (JN021933.1) ranged from 87 - 98% nt identity (Table 12). At individual gene level, the lowest nucleotide identity of 78% was recorded in 6K1 gene. Generally, sample 44 had the lowest nucleotide identity in most of its genes (Table 12). According to Adams *et al*. (2005), the species demarcation criteria for *Potyviruses* are < 76% nt identity for the entire ORF, 58% nt identity for P1 gene and 74–78% nt identity for other genes. For that case, the nucleotide identity found in the five distinct SCMV ORFs and in their individual genes are insignificant to be demarcated as new species.

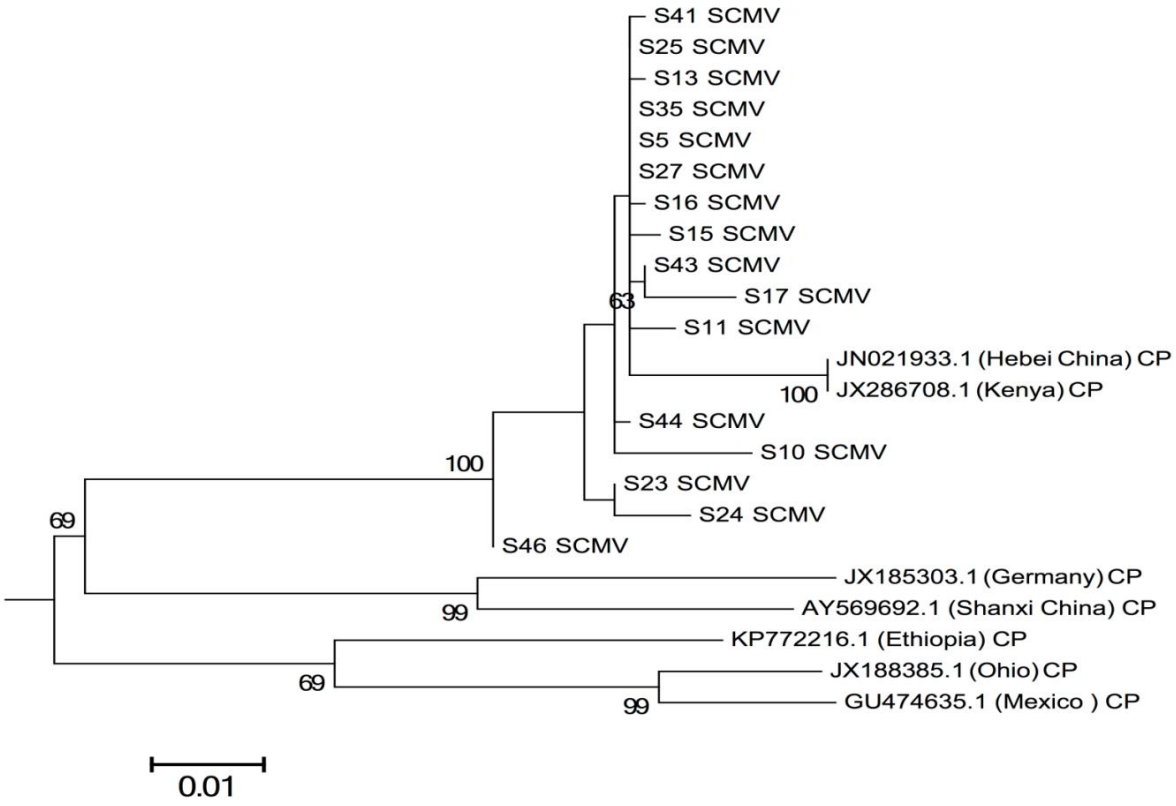

Figure 7 A maximum likelihood tree constructed with MEGA 6 using 1000 bootstrap replicates for *Sugarcane Mosaic Virus* coat proteins from this study and from GenBank

Table 12 Comparison of nucleotide identity of SCMV (JN021933) genes to five SCMV distant isolates from Tanzania

Sample number	Gene % identity*										
	Polyprotein (ORF)	P1	HC-Pro	P3	6K1	CI	6K2	VPg	NIa-Pro	NIb	CP
10	94	95	96	92	100	91	98	93	95	92	97
23	98	99	99	99	98	99	99	99	98	98	98
24	98	99	98	99	100	99	99	99	98	98	98
44	87	95	87	84	78	85	81	86	80	88	98
46	96	98	96	95	100	96	99	96	95	95	98

* Percentage identity was calculated by Species Demarcation Tool (SDT v 1.2)

Closer assessment of the sequenced data did not reveal any other *Potyviruses* (apart from SCMV) reported to associate with MLND (Uyemoto *et al*., 1980; 1981). Therefore, MLND in Tanzania is identified to be caused by MCMV and SCMV the same as other African countries with MLND report (Wangai *et al*., 2012b; Adams *et al*., 2013; Adams *et al*., 2014; Lukanda *et al*., 2014; Mahuku *et al*., 2015a, b). SCMV has been reported in East Africa since 1970s (Loue, 1980); the introduction of MCMV in 2012 prompted MLND (Wangai *et al*., 2012b). According to Adams *et al*. (2014), quarantine measures to control the movement of MCMV could be more effective than controlling the endemic SCMV.

3.4 CONCLUSION AND RECOMMENDATION

MLND is identified in Tanzania to be caused by MCMV and SCMV. The unique genomes of MCMV and SCMV (Tanzanian isolates) not only add new information to the database but also challenges our understanding of their interaction, gene expression and recombination. Having detected MSV in samples which also MCMV and SCMV were detected, gives us a question on the complex nature of infection and host's reaction. We therefore suggest further studies on the role of SCMV in synergism, co-infection complexes and gene expression, vector biology, host range and resistance to MLND.

CHAPTER FIVE

5.1 GENERAL DISCUSSION

In the present study, farmers' interviews, direct field observations, and multiple diagnostic tools have been used to demonstrate the current status of MLND and the associated viruses in Kilimanjaro, Arusha and Manyara regions in Northern Tanzania. The survey showed that, majority of farmers were aware of the disease based on symptom but do not know how to manage it. This is similarly true for farmers in Kisii, Kenya (Makone *et al*., 2014). While farmers are trying different methods to salvage their maize crop, the disease still remains unmanageable. The high incidence of MLND was associated with complete yield loss in 88% of the surveyed farms. And this represents a huge threat for Tanzanian per capital income and food security at large. Measures to intervene MLND spread including training of farmers on proper agronomic practices and crop protection is required to minimize disease pressure. These farmers contribute to over 80% of Tanzania's total maize production (Nkonya, 1998; FAO, 2015).

Farmers in Northern Tanzania were using both certified and recycled maize seeds. However, most seeds used were reported by them to be susceptible to MLND. Similarly, Mahuku *et al*. (2015a) reported that MLND affect almost all commercial varieties. Because of the complex nature of MLND that involves interaction of more than one virus (Nilblett *et al*., 1978; Uyemoto *et al*., 1980; Uyemoto *et al*., 1981; Scheets, 1998; Lamichhane *et al*., 2015), having seeds that are resistant to both viruses is a bit complicated and thus affecting disease management.

The study showed that, prevalence of MLND in 2015 in Northern Tanzania was relatively low. Despite the low prevalence, there was high incidence of both MCMV and SCMV, specifically Lyamungu Kati, Hai district and Madira, Arumeru district. MCMV was recorded to be the major virus for MLND in Northern Tanzania. This is clearly indicated from test results from samples taken in Mandaka Mnono, Kilimanjaro, where farms were badly affected with very high MCMV incidence yet SCMV incidence was low. According to Mahuku *et al*. (2015a), MCMV alone can cause significant disease development and hence crop failure. Large number of thrips, a potential vector for MCMV (Jiang *et al*., 1992) where observed in these locations which could be the reason for the high MCMV incidence.

NGS analysis has shown the presence of two viruses, MCMV and SCMV that had previously been reported to cause MLND (Xie *et al*., 2011; Adams *et al*., 2013). The findings revealed that, MCMV isolates detected in this study have high degree of sequence identity to MCMV isolates from eastern Africa suggesting to have a shared recent common ancestor, different from MCMV isolates from Nebraska (Stenger and French, 2008) and Ohio (Nutter *et al*., 1989). Since MCMV was first reported in 2011 in Kenya (Wangai *et al*., 2012b) and SCMV in 1970s (Loue, 1980), I hypothesize that the MCMV was acquired from Kenya. The analysis also indicated that, SCMV from this study are closely related to highly virulent isolates from Kenya and Hebei-China, suggesting to have a common ancestor (Adams *et al*., 2013; Gao *et al*., 2011).

Despite the closeness, SCMV isolates from this study have significant genome diversity. However, the diversity found in SCMV genome (Tanzanian isolates) is insignificant to be demarcated as new species based on Adams *et al*. (2005) criteria. The variability of SCMV is not unexpected as it has been reported in other studies (Elena and Sanjuán, 2005; Gonçalves *et al*., 2011; Padhi and Ramu, 2011; Adams *et al*., 2013; Li *et al*., 2013). It is due to the lack of proofreading activity of RNA-dependent RNA polymerases, short generation time, and large population size as a result new viral genetic variants are created which are heritable (Elena *et al*., 2005; Xie *et al*., 2016). According to Xie *et al*. (2016), the difference found in SCMV genes may be due to their roles in the life cycle. Additionally, Host and geographical conditions have been reported as a source of variability in SCMV (Xie *et al*., 2016).

Lately, no report of MDMV and WSMV in MLND cases in East Africa. Measures including quarantine to limit introduction of these two *Potyviruses*, local seed inspection and controlling their vectors could be of paramount importance because co-infection with these viruses will worsen the condition and hence massive losses in maize production. These findings provide a foundation for evaluating the epidemiological characteristics of MCMV and SCMV in Tanzania and can be useful in designing long-term, sustainable management strategies for MLND.

5.2 CONCLUSION

The study showed that, MLND in 2015 is relatively low. It also shows that, farmers lack proper education on the disease management practices. As a result, re-occurrence of MLND on the same farm and spread to other maize farms causing major crop damage and yield loss. MLND in Tanzania is identified to be caused by MCMV and SCMV similar to other eastern African countries. Therefore, similar management practices including production of resistant varieties can be applied within the region. The unique genomes of MCMV and SCMV (Tanzanian isolates) not only add new information to the database but also challenges my understanding of their interaction, gene expression and recombination. Having detected MSV in samples which also MCMV and SCMV were detected, gives me a question on the complex nature of infection and host's reaction.

5.3 RECOMMENDATION

To manage MLND, extension staffs should train farmers regarding MLND management practices including farm sanitation, application of pesticides to control MLND vectors and the use of certified seeds. Therefore, there is a need for seed companies and breeders to screen and hybridize resistant/tolerant seeds so as to manage MLND because this method is considered to be most effective, ideal, economical and eco-friendly way of managing viral diseases of plants. Additionally, further studies are recommended on the disease including vector biology because vectors play important role in disease transmission, viruses synergism, gene expression and recombination, host resistance and tolerance, host range and biological tests as these will provide proper information and sustainable solution to MLND and thereby secure food in Tanzania.

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APPENDICES

Appendix 1 Questionnaire on investigation of the status of Maize Lethal Necrotic Disease in Northern Tanzania

Date of visit…… Name of interviewee…….....
Village…….District…….Region……

Declaration

I declare that any information provided by the interviewee is confidential and will be used only for the purpose of this research.

A. GENERAL INFORMATION (*tick or circle the correct answer)*

1. Gender
   a) Male b) Female

2. Age of the respondent
   a) 15–20 b) 21–30 c) 31–40
   d) 41–50 e) 51–60 f) > 60

3. Are you able to read and write? a) Yes b) No

4. What is you education level?
   a) Illiterate/no schooling b) Incomplete primary school c) Primary school education
   d) Secondary school e) High school f) College and university

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5. Do you own land for cultivation? a) Yes b) No

6. How many acres do you have for maize plantation? a) Less than 1 acre b) 1 acre c) 2 acres d) 3-5 acres e) more than 5 acres

7. For how long have you cultivated maize? a) 1 year b) 2 years c) 3-5 years d) more than 5 years

8. Do you know MLND? a) Yes b) No ……
9. What is the first time you heard about MLND? a) This year 2015 b) 2014 c) 2013 d) 2012 e) 2011 f) others (*please mention*) …….

10. What are the symptoms of MLND?

a) Light greenish mottling (alternating light and dark green areas) of the leaves.
b) Bright greenish-yellow mottling of leaves.
c) Dying of the leaves inward from the margins with eventual death of mature plants
d) Dead heart
e) Small cobs with little or no grain set
f) Dying of plants before teaseling
g) Poorly filled ear with premature drying of the husks.

11. Did it happen in a) your farm b) your neighbor's farm?

12. Where is the farm that had MLND?
……..
13. When MLND happen in year 2013 and 2014, what type of seeds did you use? a) Local seeds b) company seeds (please mention their names)
…….
14. When this disease happened, were there vectors (e. g insects) in the farm? a) many vectors b) very little c) no vectors
15. What were those vectors?
    a) Beetles
    b) Rootworms
    c) Aphids
    d) Stem borers
    e) Mites
    f) Thrips
16. What measures were taken to rescue the plants?
    a) Rouging of diseased plants
    b) Insecticide application to manage vectors
    c) Weeds were eliminated
    d) No any measures taken
    e) Other measure (*please mention them*)
    ……
    ……..
17. Did MLND happen in the followed season on the same farm? a) Yes b) No (*if yes, mention type of seeds used*) a) previous harvested maize used as seeds b) other seeds (*Please mention them*)
……..
.....
.....
18. Was there any difference in the maize yield before and after MLND happened? a) Yes b) No *(if yes describe the differences)*

……
……

19. Describe the losses a) small b) big c) average .....
20. Where do you think MLND is present this year? Mention places

……
……

21. What is the status of MLND this year? a) Low b) High c) No MLND
22. If there is low or no MLND at all, what do you think is the cause?
    a) Early planting
    b) The use of certified seeds
    c) Removal of infected maize plant debris
    d) Insect vector management
    e) Elimination of weeds
    f) Proper education was given to farmers concerning the disease
    g) Others *(please mention)*

    ……
    ……
23. Which year had the highest MLND prevalence a) 2015 b) 2014 c) 2013
    d) 2012 e) 2011
24. What do you think favors the occurrence of MLND? .....
25. In what season MLND is high? a) Long rain season b) short rain season
    c) dry season during irrigation

26. What are your opinions?

.....
.....

THANK YOU_____END_____

Appendix 2 Supplementary data

Table 13 Read counts and genome coverage of *Maize Streak Virus* obtained from reference assembly

Virus	Sample#	Read mapped	% read mapped	Average depth of sequence	% Genome coverage	Genome length (nt)
MSV	15	8,937	0.4	377	100	2689
	26	992	0.1	37	100	2689
	39	795	0.04	8	96.7	2607
	44	733	0.03	11	99.4	2673