Maize Lethal Necrosis Disease in Ethiopia: A Newly Emerging Threat to Maize Production

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Abstract

The occurrence of maize lethal necrosis (MLN) disease in Ethiopia was first reported in 2014. Thereafter, consecutive surveys were carried out in all major maize growing areas across the country to understand the levels of distribution and incidence as well as potential alternate hosts of the disease. Symptomatology was used to determine the incidence and level of damage caused by MLN at the field level. Samples of maize plants and alternative grass hosts showing MLN symptoms were collected in all areas surveyed. Viruses associated with the symptoms were diagnosed in the laboratory using Enzyme-Linked Immunosorbent Assay (ELISA), Lateral Flow Assay (LFA), Real Time-Polymerase Chain Reaction (RT-PCR), Multiplex RT-PCR, and RT-PCR using porous ceramic cube. The results showed a wider distribution of MLN in Ethiopia with incidence levels reaching as high as 100% in some areas. Maize planted during the off-season were found to be severely affected by MLN as compared to the main season crop. In addition to maize, MLN viruses were found to infect various grass species indicating the presence of alternate hosts. This study confirmed seed transmission of MLN disease, but variable rates of transmission were observed that needs to be studied further. Considering the current rate of MLN disease distribution in Ethiopia, necessary management strategies should be devised and implemented before the disease causes significant damage to maize production.

Keywords: ELISA, Distribution, Incidence, LFA, Maize Lethal Necrosis, RT-PCR

Introduction

Maize Lethal Necrosis (MLN) is a viral disease of maize currently threatening maize production in East Africa including Ethiopia. A serious outbreak of unknown syndrome, later diagnosed as maize lethal necrosis (MLN) (Wangai *et al*., 2012a; Adams *et al*., 2013), was first reported in September 2011 from Bomet District of Kenya. By 2012, symptoms typical of MLN disease were observed in a number of districts in the Central, Nyanza, Western, and Rift Valley Provinces of Kenya (Wangai *et al*., 2012b). In subsequent years, MLN has been reported in most maize-growing districts in Kenya in addition to several East African countries bordering Kenya where maize is an important staple food; viz. Uganda and South Sudan (FAO, 2012), Tanzania (Wangai *et al*., 2012b), Democratic Republic of Congo (DRC) (Lukanda *et al*., 2014), Rwanda (Adams *et al*., 2014) and Ethiopia (Mahuku *et al*., 2015a). MLN is caused by synergistic infection of maize with interaction of *Maize cholortic mottle virus* (MCMV) from the genus *Machlomovirus* in the family *Tombusviridae*, and a virus from the family *Potyviridae*, especially *Wheat streak mosaic virus* (WSMV), *Maize dwarf mosaic virus* (MDMV) or *Sugarcane mosaic virus* (SCMV, formerly MDMV-B) (Niblett and Claflin, 1978; Uyemoto *et al*. 1980; Goldberg and Brakke, 1987). In eastern Africa, MLN was found to result from co-infection of maize with *Maize chlorotic mottle virus* (MCMV) and *Sugarcane mosaic virus* (SCMV). Mahuku *et al*. (2015b) indicated that under certain circumstances, maize infection with MCMV alone could cause extensive crop damage in East Africa. Besides, it was shown that several other unrelated viruses and abiotic stresses can exacerbate MCMV infection to cause MLN (Redinbaugh and Zambrano-Mendoza, 2014).

Symptoms observed vary widely depending on germplasm, time of infection, prevailing environmental conditions and ratios of the viruses infecting the plant. The symptoms can easily be confused with drought, micro- nutrient deficiency or stalk borer infestation. Thus, MLN diagnosis should be confirmed with other methods employing positive laboratory identification. Field level MLN symptoms include mosaic, mottling, necrosis and shortened internodes/stunting. In some cases maize plant produces many shoots (excessive tillering). Premature plant death; failure to tassel/sterility in male plants; malformed /no ears; premature drying or rotting of cobs, poor seed set and shriveled small ears are some of MLN symptoms. Towards maturity, the cobs shrink and do not produce any grains. Infected plants are frequently barren, especially if infection occurs at early stage of the plant (Niblett and Claflin, 1978; Uyemoto *et al*., 1981). In maize plants co-infected with MCMV and a potyvirus, the concentrations of MCMV were three to eleven times higher than in plants infected with

MCMV alone (Goldberg and Brakke, 1987; Scheets, 1998).

In Ethiopia, MLN syndrome was first noticed in the Upper Awash Valley during the 2014 cropping season, where it caused about 100% yield losses. Consecutively, reports on significant damages of maize by MLN was received from different parts of the country. As a result, the Ethiopian Institute of Agricultural Research (EIAR) established a team of researchers composed of pathologists and breeders to investigate the problem and come up with possible solutions. In 2014, the team visited maize growing areas of the rift valley and identified the cause as MLN disease (Mahuku *et al*., 2015a). In addition, surveys were conducted to monitor the disease occurrence, distribution and levels of damage during 2015 and 2016 main cropping seasons and in irrigated areas during the off-seasons. This report summarizes some MLN-related research activities conducted in Ethiopia that include assessment of MLN distribution and incidence in major maize growing areas; identification of potential alternate hosts of MCMV and SCMV; seed health testing and quantification of rate MLN seed transmission.

Materials and Methods

MLN Disease Survey

Since the first report of MLN disease in the early season the 2014 cropping season, consecutive surveys were carried out in major maize growing regions of Ethiopia; namely, Amhara, Benishangul-Gumuz (BSG), Oromia, Southern Nation, Nationality and Peoples' (SNNP) and Tigray regions. The surveys were conducted during the main and off-seasons of 2014 – 2016. In all areas, the off-season maize are grown under irrigated conditions. During the surveys, MLN disease incidences were determined based on visual assessment of the symptoms.

During the main and off-seasons of 2014, field expedition for MLN assessment was focused on east Shewa zone (mainly. the central rift valley) of Oromia region and a total of 110 maize and 26 suspected alternate grass host samples were collected while few representative samples were obtained from SNNPR. Double Antibody Sandwich-Enzyme Linked Immuno-sorbent Assay (DAS-ELISA) and Lateral Flow Assay (LFA) test was used to detect viruses associated with the symptoms. Most of the samples were collected from fields owned by private seed companies while 10 samples were collected from irrigated smallholder maize farmers' fields, mainly in Kawa area (Boset district of east Shewa zone). Suspected alternate hosts samples including Johnson grass (9), couch grass (1), *Digitaria* sp. (1), sedge (2), sorghum (2), *Setaria* sp. (2), sugar cane (5) and unidentified grass sp. (4) were collected. In the main cropping season of 2015, 157 suspected maize samples were

collected from surveyed areas of eastern and western parts of Oromia region. In the off-season of 2015, most irrigated areas of Oromia and SNNP regions that area known to grow maize were assessed for MLN incidence. Accordingly, 22 and 24 composite samples (each from one location) were collected from Oromia and SNNP regions, respectively. During the 2016 main cropping season, two separate surveys were carried out. The first survey focused on Amhara and Tigray regions, and field assessments were made during both main and off-seasons, in which a total of 62 samples were collected. In the second survey, SNNP and Oromia regions were assessed and a total of 133 samples were collected.

All the surveys were organized to take place at appropriate stages of crop growth when the disease symptoms could be clearly observed. Hence, most maize fields visited were at booting, milking or silking stages. Sampling was done following simple random sampling technique, and the samples were collected from plants with MLND-like symptoms as the main purpose of the surveys was to quantify the existence and distribution of MLN disease in maize growing areas through identification of the type of viruses associated with the apparent symptoms. The number of samples collected varied depending on the diversity of symptoms observed in a given field. Plant samples were collected in labeled plastic bags and put in ice boxes and transported to the laboratory where it is directly processed for testing or stored at -20 °c for later processing. Data collected from the fields assessed include incidence of MLN disease-like symptoms, maize variety, growth stage, identity of grass weeds in maize fields and other related crop variables.

Maize seed health testing

After the occurrence of MLN disease in Ethiopia was confirmed, the national Ad-hoc committee in charge of MLN disease assessment and management requested for rigorous seed health testing of certified seeds produced in 2014 before distribution for planting in 2015 as well as seeds imported from abroad. In response to the requests, a total of 133 seed samples were collected across the country following seed sampling procedures of the International Seed Testing Association (ISTA) and the Association of Official Seed Analysts (AOSA) (Miles, 1963; Morrison, 1999). The Federal Ministry of Agriculture and Natural Resource (MoANR) was responsible for collecting the samples from private seed companies, and public regional and federal seed enterprises. From each sample, 400 randomly selected seeds per sample (a total of 133* 400 = 53200 seeds) were planted for growing-out test in the greenhouse and tested in the laboratory for MCMV following the sampling procedures and testing protocols indicated above. In addition, seeds of imported popcorn and sorghum (one sample each), popcorn samples collected from local market and maize seed samples

obtained from seed lots produced by the Ethiopian Seed Enterprise (ESE) at Hora Koma and Uke farms located in East Wellega zone were raised in the greenhouse and tested for MCMV during the off-season of 2016.

Laboratory Testing Procedures

i) DAS-ELISA and LFA

For serological analysis of MCMV and SCMV in the leaf tissue and seeds of maize, sorghum and popcorn, the collected samples were ground in the extraction buffer using baked mortar and pestle or put in a clean polyethylene bags and stumbled with glass rods for subsequent use in Double Antibody Sandwich-Enzyme Linked Immunosorbent Assay (DAS-ELISA) and Lateral Flow Assay (LFA) tests. In the case of direct testing of dry seeds, an extraction step was modified by prior overnight soaking of hard dry seeds in water. DSMZ diagnostic kits for DAS-ELISA (Figure 1) as essentially described by Clark and Adams (1977) and LFA (Leibniz Institut DSMZ GmbH, Plant Virus Department, Inhoffenstraße 7 b, 38124 Braunschweig, Germany) (Figure 2) were employed according to manufacturer's instructions using antibodies developed against the east African isolates of MCMV and SCMV. For DAS-ELISA test, the samples are considered positive to a given virus when it shows yellowish reaction, similar to that of the positive control (Figure 1). For the LFA, positive samples should show double red lines (Figure 2).

ii) Reverse transcription-polymerase chain reaction (RT-PCR)

Maize seeds and fresh young leaf tissues (100 mg) with symptoms suggestive of MCMV and SCMV and virus-free healthy controls were ground to fine powder in liquid nitrogen using baked mortar and pestle or Genogrinder. Total RNA was extracted with RNeasy Plant Mini Kit (Inclone, Korea) according to the manufacturer's instruction. The integrity of the extracted RNA was visually verified after electrophoresis in 1% agarose gel stained with ethidium bromide.

To amplify MCMV and SCMV viruses from suspected maize leaf and seed samples, various derivatives of RT-PCR were employed, viz. RT-PCR using porous ceramic cube, multiplex RT-PCR and normal RT-PCR. Primer pairs used in each case are summarized in Tables 1a and b.

Table 1a. Primer pairs and sets used in RT-PCR and RT-PCR using porous ceramic cube for the detection of MCMV and SCMV from infected maize seeds and leaf tissues.

Virus	Primer name	Sequence (5' - 3')	Product size	Tm
MCMV	MCMV-F	GTCCTGGCCTCAGTGGTTAAGG	478 bp	55 °c
	MCMV-R_1	CGCACAGAGTTGAACACAATTGT		
SCMV	SCMV-F1	AGCTAAG(a)GAAGCCCACATGCAG	319 bp	55 °c
	SCMV-R	AGAAGACACTGGGTCCAACCCTG		
MCMV	2681F	5'-ATGAGAGCAGTTGGGGAATGCG	550bp	
	3226R	5'-CGAATCTACACACACACACTCCAGC		
SCMV	8679F	5'-GCAATGTCGAAGAAAATGCG	900bp	
	9595R	5'-GTCTCTCACCAAGAGACTCGCAGC		

Table 1b. Primer pairs and sets used in Multiplex RT-PCR for the detection of MCMV and SCMV from infected maize seeds and leaf tissues.

Multiplex set	Virus	Primer name	Sequence (5' – 3')	Size (bp)	T_m
Set 1	SCMV	SCMV-F	GCG(a)TGGCTTC(t)TC(g)GAAATGCAACC	1307 bp	55 °c
		SCMV-R	AGAAGACACTGGGTCCAACCCTG		
	MCMV	MCMV-F	GTCCTGGCCTCAGTGGTTAAGG	988 bp	
		MCMV-R_2	TCTCCAGTCATGGTCATCACGC		
Set 2	SCMV	SCMV-F	GCG(a)TGGCTTC(t)TC(g)GAAATGCAACC	1307 bp	55 °c
		SCMV-R	AGAAGACACTGGGTCCAACCCTG		
	MCMV	MCMV-F	GTCCTGGCCTCAGTGGTTAAGG	478 bp	
		MCMV-R_1	CGCACAGAGTTGAACACAATTGT		

For RT-PCR and RT-PCR employing porous ceramic cube, a one-step RT-PCR 20 µl reaction (Genetbio, Korea) containing RT-PCR premix (10 µl), primer F (10 pmol) (1µl), primer R (10 pmol) (1µl), distilled water (8 µl) and Template (1 µl for RT-PCR or RNA absorbed ceramic cube) was used. The RT-PCR condition was as follows: 42 °C for 50 min, initial denaturation at 95°c for 12 min, followed by 35 cycles at 95 °C for 30 sec for denaturation, 55 °C (primer 'TM') for 30 sec, and 72°c for 1 min 30 sec for extension, and final extension at 72 °C for 10 min.

For multiplex RT-PCR, a one-step RT-PCR of 20 µl reaction (Genetbio, Korea) was containing RT-PCR premix (10 µl), primer set for MCMV and SCMV (20 pmole) (0.5µl x 4 primers), Template (2 µl) and distilled water (6µl). The multiplex RT-PCR condition was same as for normal RT-PCR and RT-PCR using porous ceramic cube. The PCR products were separated using 1% agarose stained with ethidium bromide and visualized with UV light and amplicon size was measured using 100 bp DNA size ladder (Figure 3).

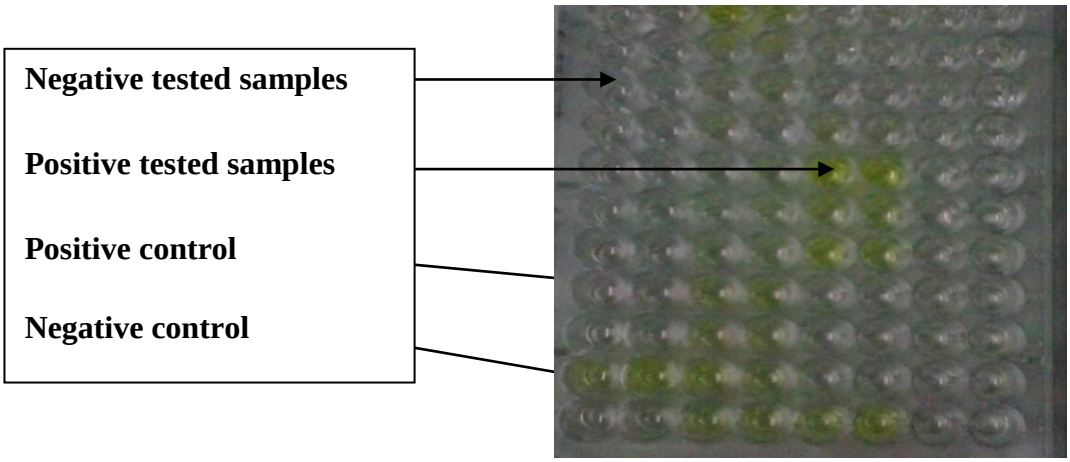

Figure 1. DAS-ELISA showing positive (yellowish) and negative (colorless) tested samples, and positive and negative controls.

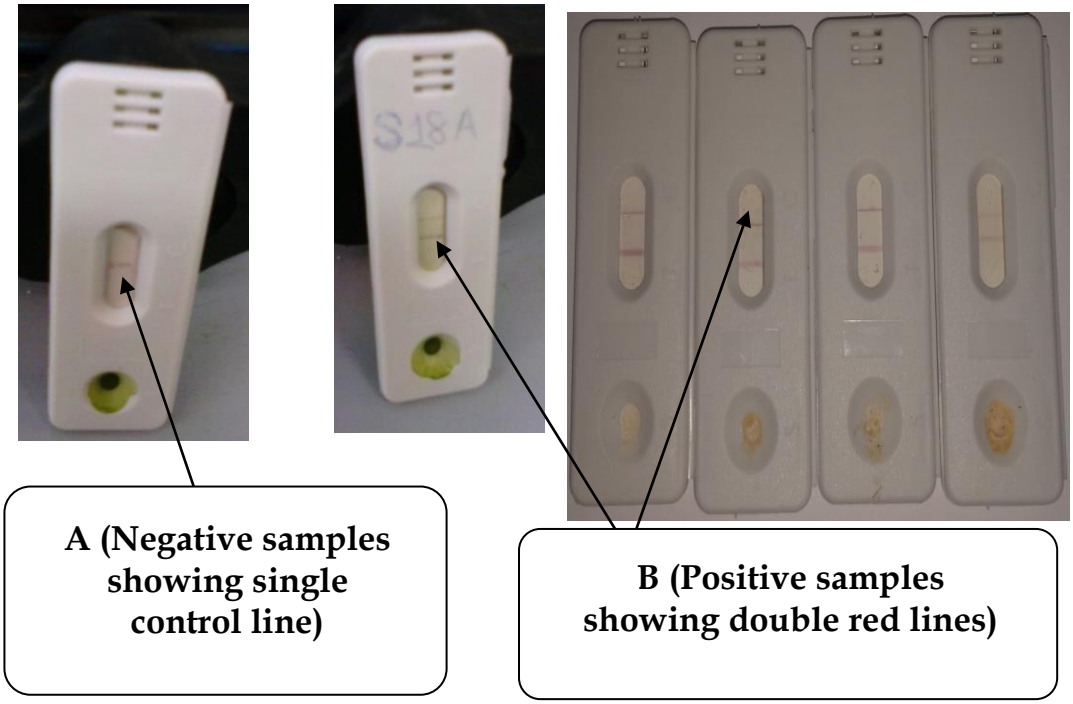

Figure 2. Lateral flow assay (LFA) results showing MLN negative (A) and positive (B) tests

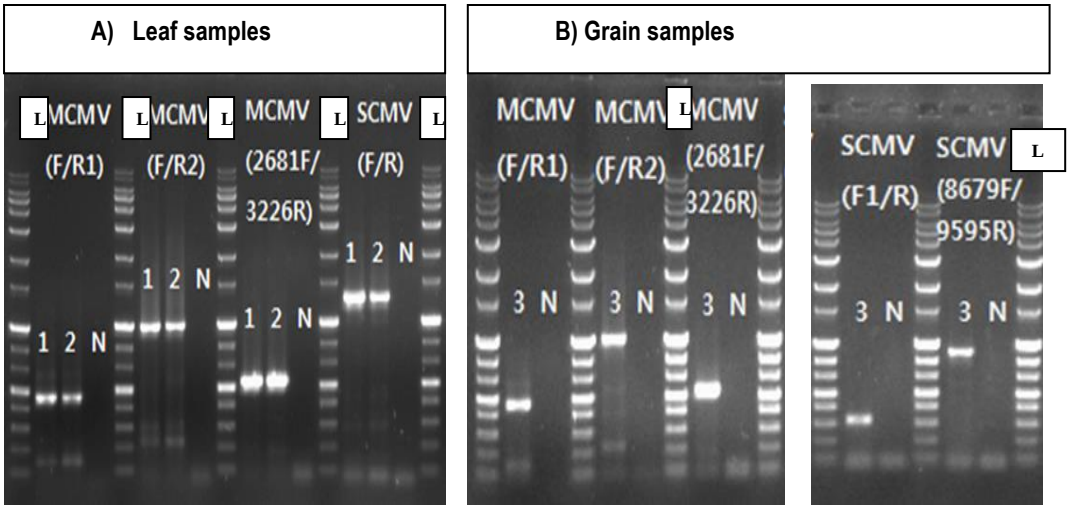

Figure 3. Multiplex RT-PCR, and RT-PCR using porous ceramic cube, testing of maize leaf (A) and seed (B) samples from Ethiopia substantiate the presence of MLN. Details of primers used for respective methods are shown in Table 1. For respective pairs of primers, numbers 1, 2 and 3 indicate test samples. N=healthy maize plant used as negative control. L= DNA size marker (100bp).

Results

Incidence and distribution of MLN in Ethiopia

Commonly observed MLND-like symptoms in the field include typical chlorosis, mosaic, mild mosaic at seedling stage, severe chlorosis, marginal leaf necrosis, chlorosis and necrosis, dead heart, tassel blasting and flower abortion, premature drying of cobs and poor or no grain filling (Figure 4). Out of the 136 samples collected from maize and suspected alternate hosts in 2014, ELISA test showed that 105 (77%) of the samples were positive for MCMV, SCMV or the combination of both (Table 2). MCMV was the most prevalent that was detected in 64% of the samples either alone or in combination with SCMV, whereas SCMV was detected in 38% of the samples. The proportion of samples with MCMV alone were 39% compared to 13% for SCMV only. The combination of both MCMV and SCMV were detected in 25% of the samples. MCMV was also detected in 65% of non-maize (alternate host) samples either alone or in combination with SCMV, whereas SCMV was detected in 19% of the samples (Table 2). The samples that were found to harbor MCMV alone were 50%, whereas only 4% of the samples were found to carry SCMV alone. MCMV was detected in several suspected alternate host plants including Johnson grass, unidentified grass species, *Digitaria* sp., sedge grass, *Setaria* sp. and sugarcane, whereas SCMV was detected only in Johnson grass and *Setaria* sp.

During the extensive MLN survey of 2014 that was carried out in Oromia, SNNPR, Benshanul-Gumuz and Amhara regions, incidence scores ranging from 10% to 100% were observed in the areas assessed (Table 3). Awi zone of Amhara region showed MLN disease incidence ranging from 30 to 60%, with estimated mean incidence of 40% as documented from a 124 ha of maize field planted to basic and certified seeds (Table 3) on Ayehu Farm. In severely infected fields of Benshangule-Gumze region, incidences ranging from 40 to 100% were recorded. Some seed production fields in this region, for example, Tsega Gebre-Hiwot Hulegeb Private Farm (TGHHPF) experienced a total crop failure. In Oromia region, incidences ranging from 10 to 100% were observed, and severe infections (100%) were recorded at Robani and Tibila farms of upper Awash basin. These farms totally abandoned the maize crop and replaced with other crops. Comparable levels of damages were also recorded around Koka in East Shewa and at Lugo in East Wellega zones (Table 3). Average incidence observed during the assessment in Oromia region ranged from 50 to 60%, except in Horo-Guduru Wollega (represented by Amuru district) where MLN incidence of less than 3% was recorded. In SNNPR, Wolayta zone showed MLN incidence ranging from 80 to100% with a mean of 85% (Table 3).

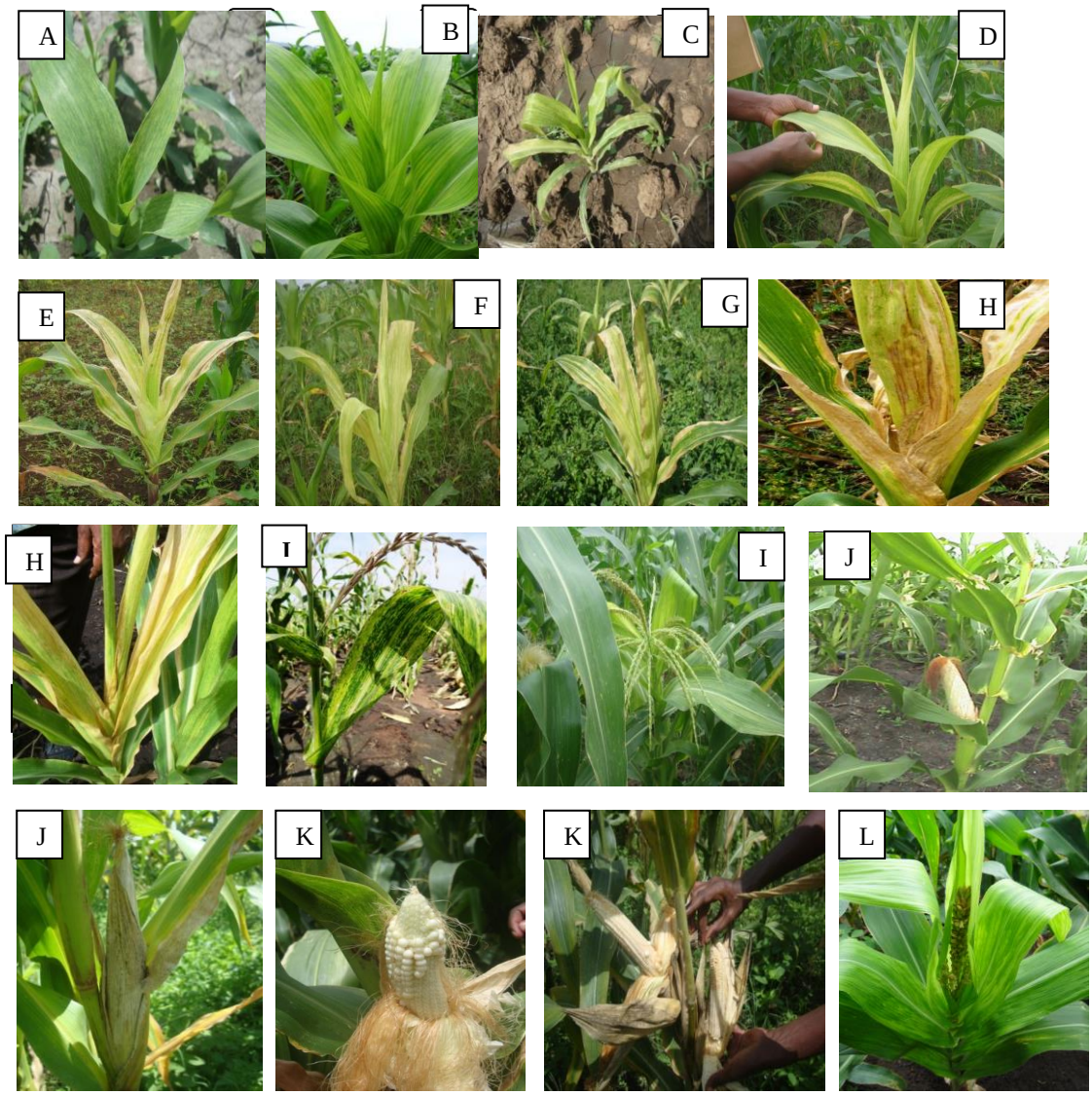

Figure 4. Commonly observed MLN disease symptoms under field conditions in Ethiopia. The leaf symptoms above represent, typical chlorosis (A), mosaic (B), mild mosaic at seedling stage (C), severe chlorosis (D), leaf necrosis starting from margins (E), severe chlorosis (F), necrosis (G), dead heart (H), tassel blasting and flower abortion (I), premature drying of cobs (J), poor or no grain filling (K), and Stunting/shortened internodes (L).

Table 2. Detection of MCMV and SCMV in samples collected from maize and suspected alternate grass hosts during the main season of 2014.

Sl No.	Host sampled	Total samples tested	Viruses detected either singly or in combination			
			MCMV	SCMV	No virus	MCMV + SCMV
1.0	Maize	110	40	17	23	30
2.0	Non-maize	26	13	1	8	4
2.1	Johnson grass	9	5	0	1	3
2.2	Coach grass	1	0	0	1	0
2.3	Grass spp.	4	2	0	2	0
2.4	Digitaria sp.	1	1	0	0	0
2.5	Sedge grass	2	2	0	0	0
2.6	Sorghum	2	0	0	2	0
2.7	Setaria spp.	2	0	1	0	1
2.8	Sugar cane	5	3	0	2	0
Total		136	53	18	31	34
Proportion of samples tested positive (%)			39	13	23	25

Table 3. Distribution and incidences of MLN disease in different areas assessed during the main season of 2014.

Region	Zone	No. of districts	Farms Assessed	Varieties	Incidence (%)	
					Range	Average
Amhara	Awi	1	2	BH660 and BH540	10 - 60	40
Benishangul Gumuz	Kamashi	3	3	BH540 and BH140	40 -100	75
Oromia	East Wollega	1	4	BH540, BH140, Limu and Jabi	30 - 100	60
Oromia	East Shewa	5	6	BH543, BH660, BH661, Limu, Shone and Agar	10 – 100	56
Oromia	West Arsi	2	2	Melkassa 2 and Melkassa 4	20 - 70	50
Oromia	Horro Guduru Wollega	1	1	BH661	-	< 3
SNNPR	Wolayta	1	2	Shone	80 - 100	85

SNNPR = South Nation, Nationalities and Peoples' Region

During the extensive surveys of the 2015 main season, 157 samples were collected from different areas of Oromia region and tested for MCMV and SCMV using DAS-ELISA. About 86% of the samples were tested positive for MCMV while 53% and 52%, respectively, were tested positive for SCMV and the combination of both viruses (Table 4). MCMV and MLN were highly distributed in east and west Shewa, Arsi and Jimma zones as compared to West Arsi and east Wollega. Among all the areas assessed, east Wollega had limited distribution of MLN causing viruses. In addition to the main season, MLN survey was conducted during the off-season of 2015 in irrigated maize fields of Oromia and SNNP regions. All the 46 composite samples with symptoms suggestive of MLN disease infection collected during the offseason were tested for MCMV; and all the samples were found to be positive for this specific virus (Table 5). All the varieties planted by the farmers in the areas assessed, including local landraces and improved varieties from various seed companies were infected by the virus. The samples collected during the off-season of 2015 were not tested for SCMV due to short supply of antiserum.

Table 4. Distribution of Maize Lethal Necrosis disease causing viruses in different maize growing zones of Oromia region during the main growing season of 2015.

Zone	No. of districts	Locations assessed	No. of samples	Samples tested positive for MLN viruses		
				MCMV	SCMV	MCMV + SCMV
West Arsi	3	4	9	3	3	3
East Shewa	3	6	108	106	75	74
Arsi	1	1	2	2	1	1
West Shewa	2	3	8	8	3	3
East Wollega	3	5	15	1	1	1
Jimma	3	5	15	15	NT	-
Total	15	24	157	135	83	82
Proportion of samples with positive tests (%)				86	53	52

NT = Not tested for MLN disease causing viruses

Table 5. Symptomatic composite leaf samples collected from irrigated maize fields in Oromia and South regions of Ethiopia and tested positive for MCMV during the off-season of 2015.

Region	Zone	No. of District	Locations assessed	Varieties used
SNNP	Sidama	1	1	Melkasa-2
	Silti	2	3	Shone
	Alaba Liyu Woreda	1	1	Shone, Limu
	Hadiya	1	2	Limu, BH540
	Wolayta	4	5	BH140, Jabi, Limu
	Gamo Gofa	3	3	BH140
	Konta Liyu Woreda	3	4	BH140, Local
	Dawro	2	3	BH660, BH543, BH540
Oromia	East Shewa	4	7	BH540, BH661, Jibat, MH140, Shone, recycled seeds
	West Arsi	2	4	BH661, BH540, Katumani, unknown
	Jima	4	6	Local, BH543, BH661
	East Wollega	2	3	BH661, Limu, Shone
	Total	29	42	

SNNPR = South Nation, Nationalities and Peoples' Region

To monitor further spread of MLN disease in the country, additional surveys were carried out in Amhara, Oromia, Tigray and SNNP regions during the 2016 main and off-seasons. In Tigray region, two zones (south and central) were assessed during the off-season and a total of 41 samples were collected. DAS-ELISA test showed that all the samples were MCMV positive (Table 6). The varieties used by the farmers during this season include local, Melkassa 2 and Shone. None of the varieties were found to be immune to the virus. DAS-ELISA testing of another 21 samples collected from Amhara (Awi and west Gojam zones) and Tigray (South zone) regions during the main rainy season of 2016 showed that 89% of the samples were MCMV positive while 26% were SCMV positive. Higher level of MCMV distribution was observed in Tigray region during the main and off-seasons as compared to Amhara region. As indicated in Table 7, among 53 samples collected from six maize growing zones of SNNPR in the main season of 2016, 75% were tested positive for MCMV using DAS-ELISA. Besides, 12 of these samples were tested again using Lateral Flow Assay (LFA) and again 75% were found to be positive for MCMV. In the same season, 80 samples were collected from seven zones of Oromia region, and 59% were observed to be positive for MCMV when tested using DAS-ELISA. Wolayta, Dawro and Kefa zones of SNNR, and west Arsi, east Shewa and west Shewa zones of Oromia regions were found to be heavily infected by the disease. Overall assessment of the two regions showed that among 133 samples collected and tested using DAS-ELISA, 65% were found to be positive for MCMV.

Table 6. Prevalence of Maize Lethal Necrosis disease causing virus in certain maize growing areas of Amhara and Tigray regions during the main and off-seasons of 2016.

Season	Region	Zone	**No. of District**	**Locations assessed**	Variety used	No. of samples	Samples tested positive for MLN viruses	
							MCMV	SCMV
Offseason	Tigray	South	2	4	Local, shone	22	22	NT
		Central	1	1	Melkassa-2, shone, unknown	19	19	NT
Main Season	Amhara	Awi	1	1	BH540	9	4	5
		West Gojjam	1	1	BH540	6	4	6
	Tigray	South	1	3	Local	6	6	5
Total			6	10		62	55	16
Proportion of samples with positive test (%)							89	26

Table 7. Maize Lethal Necrosis assessment and detection of causal virus in maize growing zones of Oromia and South regions of Ethiopia during the main seasons of 2016.

Region	Zone	No. of Districts	No. of Locations	Crop Variety	No. of samples	Incidence (%)		Samples tested +ve for MCMV	
						Range	Average	DAS-ELISA	LFA
SNNP	Wolayta	3	6	BH540, Jabi, Limu, Shone	12	40 - 100	86	12	9
	Hadiya	1	3	BH540,Limu	4	5 - 40	21	4	NT
	Guraghe	1	2	Limu, Shone	9	-	0	2	NT
	Dawro	1	2	BH540, BH660, Shone	9	60 - 100	90	9	NT
	Silte	2	3	BH540, Shone	5	-	0	0	NT
	Kefa	2	4	BH540, BH661, Shone	14	30 - 80	67	13	NT
Oromia	West Arsi	3	3	BH540, BH543	7	5 - 80	33	5	NT
	East Shewa	2	2	BH140, BH540, BH543	10	5 - 80	33	7	NT
	West Shewa	2	2	Limu, unknown	6	-	85	6	6
	East Wollega	3	3	BH140, BH540, BH546, Jabi, Limu	21	0 - 85	16	10	NT
	Jimma	5	11	BH660, Agar, Damot, Limu, Shone	24	0 - 30	10	14	NT
	South West Shewa	1	2	BH660, Shone	6	0 - 50	25	3	NT
	Illubabor	2	3	Shone, Unknown	6	2 - 20	14	2	NT
Total		28	46		133			87	15
Proportion of samples with positive test (%)								65	11

DAS-ELISA = Double Antibody Sandwich-Enzyme Linked Immunosorbent Assay; LFA = Lateral Flow Assay; NT = Not tested; SNNP = SNNPR = South Nation, Nationalities and Peoples' Region.

Maize seed health testing

A total of 133 seed samples were collected from the 2014 cropping season and tested for MLN disease causing viruses during the 2015 off-season at APPRC laboratory. MCMV was detected in only two of these samples, each represented by 400 seeds. From each sample found to be MCNV positive, only one seed (i.e. 0.25%) carried MCMV. In addition to DAS-ELISA and LFA assays, seed transmission of MCMV was further confirmed and verified using RT-PCR in collaboration with the Korea Project on International Agriculture (KOPIA), in Ethiopia (KOPIA-Ethiopia). The RT-PCR showed that seed samples collected from MLN infected field of the Rift valley did carry MCMV, which is the most important virus for the disease. In addition, ELISA testing of imported popcorn samples revealed that MCMV was detected in 25% of 100 seed samples tested. MCMV was not detected from imported sorghum seeds that were tested using DAS-ELISA. Popcorn samples collected from local markets and tested for MLN causing virus indicated that none of the samples did harbor the virus. During field surveys, two seed samples, each containing 460 seeds, were collected from MLN affected fields (Hora Koma and Uke farms) of east Wollega and tested for MCMV using DAS-ELISA. All the seed samples obtained from Hora Koma were found to be free from MCMV whereas 6.5% of the samples from Uke were positive to MCMV.

Discussion

MLN is a disease currently threatening maize production in east Africa, first reported in September 2011 from maize growing areas of Kenya (Wangai *et al*., 2012a). Since then, the disease spread into many east African countries bordering Kenya at an alarming rate (Wangai *et al*., 2012b; Adams *et al*., 2014; Lukanda *et al*., 2014; Mahuku *et al*., 2015a). In Ethiopia, the disease was first observed in 2013 in the Central Rift Valley of Ethiopia and officially reported in 2014 (Mahuku *et al*., 2015a). Assessment studies on the effect of MLND on maize yield in Kenya suggested that the disease affected almost all commercial maize varieties, bringing about an estimated yield losses of 30% to 100% depending on the stage of disease onset and severity. Similarly, the current study showed MLN incidences as high as 100% in east Shewa, east Wollega and Kamashi zones in 2014 (Table 3) and in Wolayta and Dawro zones in 2016 (Table 7) of Ethiopia. In certain areas, the disease caused a total crop damage, which resulted into 100% yield loss; for example, at Robani and Tibila farms of Upper Awash River Basin and Tsega Gebre-Hiwot Hulegeb Private Farm (TGHHPF) located in Benishangul-Gumuz region. The disease was found to affect all improved commercial and local varieties; and all fields of commercial and smallholder farmers either for grain or seed production.

This indicates that none of the varieties currently used by the farmers and seed growers are resistant to the disease. This should be expected as the disease is a new entry to the country and none of the varieties under production and the source germplasm have previously been screened for the disease. Similar to the current observation, Mahuku *et al*. (2015b) indicated that all currently available commercial maize varieties in Kenya are susceptible to MLN disease. Although expected national yield loss due MLN disease in Ethiopia has not been estimated and documented, In Kenya, MLN affected 77,000 ha in 2012 that translates into an estimated yield loss of 126 million metric tons valued at $52 million USD (Wangai *et al*., 2012b).

Results of field assessments, sample collection and virus detection activities revealed that MLN-causing viruses were distributed in major maize growing areas of Ethiopia surveyed under this study. Besides maize, several alternate hosts, mainly grass family, were found to carry MLN causing viruses. This indicated that maize is not the only host plant for MLN disease, and other plant species such as those identified in this study (Table 2) can spread the disease. Earlier, Bockelman *et al*. (1982) reported that at least 19 grass species belonging to the family *Poaceae* are experimental hosts for MCMV. Recently, in addition to maize, several natural hosts of MCMV were identified in east Africa (Kenya and Uganda); namely, sugarcane, finger millet (*Eleusine coracana*), proso millet (*Panicum miliaceum*), foxtail millet (*Setaria italica*), sorghum, Napier grass, Kikuyu grass (*P. clandestinium*) when tested by ELISA and RT-PCR (Wang *et al*., 2014; Kusia *et al*., 2015; Mahuku *et al*., 2015b). Hence, any management practice aimed at MLN control should take into consideration the presence several potential alternate hosts.

MLN symptomatic leaf samples collected from most of the surveyed areas were not tested against SCMV due to short supply of antiserum. To confirm the presence of MLN disease in maize growing areas of Ethiopia, detection of MCMV alone would be sufficient as MCMV is the newly introduced virus to the country. SCMV and other potyviruses that in combination with MCMV causes MLN are already prevalent in different agro-ecologies of the country including in maize producing areas. Wider distribution of MLN disease in Ethiopia was more evident from the fact that MCMV was frequently detected in most leaf samples obtained from different areas surveyed. This suggests an increasing disease pressure in major maize growing areas in the country and calls for application of appropriate control measures. As indicated in Tables 5 and 6, all leaf samples collected from Oromia, SNNP and Tigray regions during the off-seasons of 2015 and 2016 were positive for MCMV, indicating that MLN disease significantly effects

maize during the off season than during the main season. The plausible reasons might be maize grown under irrigation could be the only green vegetation in the area that attracts massive insect vectors and the dry and hotter conditions during the off-season could be a conducive environment for reproduction and movement of the insect vectors to fast spread MLN causing viruses. The presence of reservoir hosts and other cereal crops in the area serve as green bridge for survival of both the viruses and insect vectors, high activity and population of insect vectors, continuous maize cultivation without break period, as well as lack of resistant/tolerant maize varieties at the hands of growers might have contributed to the disease spread.

The presence of susceptible maize varieties, ample reservoir grass hosts, insect vectors and favorable environmental conditions for both the virus and vectors coupled with lack of strong quarantine restriction for the movement of infected seeds within the country and poor phytosanitary systems might have contributed for fast spread of MLN disease in Ethiopia. Furthermore, a study to understand the spatiotemporal distribution of MCMV and MLN risk in Africa involving an ecological niche models that use a genetic algorithm (GARP) was undertaken (Isabirye and Rwomushana, 2016). According to this study; Ethiopia, Tanzania, and Democratic Republic of Congo have the potential to lose 662,974, 625,690 and 615,940 km2 potential maize landmass, respectively (Isabirye and Rwomushana, 2016). In terms of proportional loss of national maize production area, Rwanda, Burundi, and Swaziland have the potential to lose each 100%, and Uganda 88.1% (Isabirye and Rwomushana, 2016). The model predicted that MLN risk in Africa is high, and suggests "the need for better allocation of resources in management of MLN, with special emphasis on eastern and central Africa, which are and will remain hotspots for these problems in the future". The model prediction seems in agreement and consistent with the current wide spread occurrence of MLN in Ethiopia. This is justified based on the fact that MLN was first noticed in pocket areas of the Upper Awash in the eastern part of the Central Rift Valley in 2013/14, and has since reported from almost all the major maize growing areas in the country over the period of three years (2014 -2016).

During the field assessments conducted in different areas, several insects such as aphids, thrips, beetles and others were found associated with the diseased maize plants in the field. However, these insects are not taxonomically identified to species level and tested for their ability to efficiently transmit SCMV and MCMV in Ethiopia, although reports are available elsewhere in the world that indicate *Chrysomelid* beetles (Nault *et al.*, 1978) and Thrips

(*Frankliniella williamsi*) (Cabanas *et al*., 2013) are vectors of MCMV, and aphids are vectors of SCMV (Brault *et al*., 2010).

Results of seed health testing have generated variable results depending on the type of crop and crop varieties tested. In seed lots sampled and tested during the 2015, MCMV was detected in 0.25% of the samples at maximum. This level of transmission is close to what has been reported by Hill *et al*. (1974) and Mikel *et al.* (1984), respectively, for maize dwarf mosaic and wheat streak mosaic viruses in maize, and maize dwarf mosaic virus in sweet corn, but higher than what has been reported by Jensen *et al.* (1991) for MCMV (0.04%). On the contrary, tests carried out during the 2016 on seeds harvested during the 2015 main and off-seasons revealed that seed infection rate of imported popcorn ranged from 3.3% (for leaf samples raised from seeds) to 25% (when popcorn seeds were directly tested). MCMV was not observed on any of the imported sorghum seeds and popcorn samples collected from local market. When maize seed samples from Hora Koma and Uke maize farms (east Wellega) were tested by DAS-ELISA and LFA, MCMV was detected in none of the seed samples from Hora Koma, while the virus was detected in 6.5% of 46 composite samples (each sample being a composite of 10 plant samples raised from seeds) collected from Uke farm. This later result may be attributed to the fact that maize plants from Uke farm were infected at seedling stage (based on visual symptoms and DAS-ELISA testing), while plants from Hora Koma was free of the virus at seedling stage based on visual symptoms assessment in the field. Seed-borne nature of MCMV was ascertained using DAS-ELISA and LFA tests and further verified by RT-PCR (Figure 5), although the rate of seed-transmission is highly variable within and among crop varieties tested. Factors contributing for this variations are not well understood, and left for further investigation using sensitive diagnostic methods.

Conclusion and Recommendations

The study revealed that MLN disease was distributed in almost all major maize growing areas of Ethiopia. Incidences as high as 100% were recorded in commercial maize and seed farms as well as in smallholder farmers' fields. The disease also recorded in both main and off-season irrigated maize fields and all available local and commercial maize varieties were affected by the disease. Considering the current heavy MLN epiphytotic, all stakeholders working actively in maize research and development including policy makers should come on board and devise short-and long-term strategies and tactical approaches to avert and/or lessen the likely heavy losses that might occur due to the disease.

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