

Maize Lethal Necrosis (MLN): A Technical Manual for Disease Management



Editor
B.M. Prasanna



In collaboration with international and national research
and development partners

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CIMMYT – The International Maize and Wheat Improvement Center – is one of the centers under the Consultative Group on International Agricultural Research (CGIAR) and is the global leader in publicly funded maize and wheat research and related farming systems. Headquartered near Mexico City, CIMMYT works with hundreds of partners throughout the developing world to sustainably increase the productivity of maize and wheat cropping systems, thus improving global food security and reducing poverty. CIMMYT leads the CGIAR Research Programs on Maize and Wheat and the Excellence in Breeding Platform. The Center receives support from national governments, foundations, development banks, and other public and private agencies. For more information, visit www.cimmyt.org.

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Preface

This publication on *Maize Lethal Necrosis (MLN): A Technical Manual for Disease Management* is intended as a comprehensive guide on best practices and protocols for sustainable management of the MLN disease in countries where the disease is already prevalent as well as for technically supporting “high-risk” countries globally for proactive implementation of practices that can possibly prevent the incursion and spread of the disease.

The manual is organized in 10 chapters, as below:

- Chapter 1: Maize Lethal Necrosis (MLN) in Africa: Incidence, Impact, Rapid Response, and Management
- Chapter 2: MLN-causing Viruses in Africa, and their Symptoms
- Chapter 3: Modes of Transmission of MLN-causing Viruses
- Chapter 4: MLN Surveillance, Leaf and Seed Sampling Protocols
- Chapter 5: Diagnostic Protocols for MCMV and SCMV
- Chapter 6: Managing MLN Quarantine Facilities: Phytosanitary Guidelines
- Chapter 7: Maize Germplasm Phenotyping for MLN, MCMV and SCMV under Artificial Inoculation at the MLN Screening Facility, Naivasha, Kenya
- Chapter 8: MLN Pathogen-free Commercial Seed Production: Standard Operating Procedures
- Chapter 9: MLN Early Warning and Emergency Preparedness Plans
- Chapter 10: MLN Management: Conclusions and Future Perspective

This publication is the product of contributions by subject-matter specialists and technical staff from CIMMYT and several partner institutions, to whom I express deep appreciation. While formulating various chapters in this manual, the authors considered relevant lessons from dealing with MLN in sub-Saharan Africa over the last 10 years. We recognize that these lessons could also be of immense value in effectively tackling the disease in countries in Latin America and Asia where the disease has been reported as well as for stakeholders in those maize-growing countries where MCMV/MLN has so far not occurred to take proactive steps.

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CIMMYT is grateful to various national and international partners for their intensive engagement over the years, and support in co-designing and implementing an array of key management practices to effectively tackle MLN in Africa. Special thanks are to:

- **Kenya Agricultural and Livestock Research Organization (KALRO)** for its exemplary partnership in tackling the MLN challenge, especially by enabling CIMMYT to establish and operate the MLN Screening Facility at KALRO-Naivasha, Kenya, and for co-developing strong scientific data to support MLN diagnostics and management practices.
- National partners, especially NARES and National Plant Protection Organizations (NPPOs), in **Ethiopia** (Ethiopian Institute of Agricultural Research/EIAR), **Kenya** (Kenya Plant Health Inspectorate Service/KEPHIS), **Rwanda** (Rwanda Agriculture Board/RAB), **Tanzania** (Plant Quarantine & Phytosanitary Services, Ministry of Agriculture, Food Security & Cooperatives), **Uganda** [National Crop Resources Research Institute (NaCRRRI) & Crop Protection Division, Ministry of Agriculture, Animal Industry and Fisheries], **Malawi** (Department of Agricultural Research Services/DARS), **Zambia** (Plant Quarantine and Phytosanitary Service/PQPS, Ministry of Agriculture), and **Zimbabwe** (Department of Research & Specialist Services/DR&SS) for generating and sharing the MLN surveillance data during 2015-2019.
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- **NARES, NPPOs, and commercial seed companies in Africa** for partnering with CIMMYT, AGRA and AATF on MLN-free commercial seed production and exchange in sub-Saharan Africa.
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- **International Institute of Tropical Agriculture (IITA)** for conducting regional awareness workshops on MLN in West Africa.

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List of Acronyms & Abbreviations

AATF	African Agricultural Technology Foundation
AGRA	Alliance for Green Revolution in Africa
BMGF	Bill and Melinda Gates Foundation
bp	base pair
CIMMYT	International Maize and Wheat Improvement Center
COMESA	Common Market for Eastern and Southern Africa
CoP	Community of Practice
dap	Days after planting
DARS	Department of Agricultural Research Services (Malawi)
DAS-ELISA	Double Antibody Sandwich-Enzyme-linked Immunosorbent Assay
dpi	days post-inoculation
DR&SS	Department of Research & Specialist Services (Zimbabwe)
EAC	East African Community
EIAR	Ethiopian Institute of Agricultural Research
ELISA	Enzyme-linked immunosorbent assay
EPP	Emergency Preparedness Plan
ESA	Eastern and Southern Africa
fg	femtogram (1 fg = 10 ⁻¹⁵ gram)
GPS	Global Positioning System
hectare	ha
<i>icipe</i>	International Centre of Insect Physiology and Ecology
IEC	Information Education and Communication
IITA	International Institute of Tropical Agriculture
IPPC	International Plant Protection Convention
KALRO	Kenya Agricultural and Livestock Research Organization
KEPHIS	Kenya Plant Health Inspectorate Service
M ha	Million hectares
MCMV	Maize chlorotic mottle virus
MDMV	Maize dwarf mosaic virus
MLN	Maize lethal necrosis
MLN-QF	MLN Quarantine Facility
MMT	Million metric tonnes
NaCRRRI	National Crop Resources Research Institute
NARES	National Agricultural Research and Extension System
ng	nanogram (1 ng = 10 ⁻⁹ gram)
NPPO	National Plant Protection Organization
ODK	Open Data Kit
PCR	Polymerase chain reaction
pg	Picogram (1 pg = 10 ⁻¹² gram)
PPE	Personal Protective Equipment
PQI	Plant Quarantine Institute
PQPS	Plant Quarantine and Phytosanitary Service (Zambia)
QR	Quick Response
RAB	Rwanda Agriculture Board
RNA	Ribonucleic acid
RT-PCR	Reverse Transcription-Polymerase Chain Reaction
SADC	Southern Africa Development Community
SCMV	Sugarcane mosaic virus
SFSA	Syngenta Foundation for Sustainable Agriculture
SME	Small- and medium-enterprise
SOPs	Standard Operating Procedures
SrMV	Sorghum mosaic virus
SSA	Sub-Saharan Africa
ssRNA	single-stranded ribonucleic acid
SSS	Seed soak solution
SExT	Seed extract
Sq.m.	Square meter
t/ha	tonnes per hectare
µg	microgram (1 µg = 10 ⁻⁶ gram)
USAID	United States Agency for International Development
WSMV	Wheat streak mosaic virus

Chapter 1

Maize Lethal Necrosis (MLN) in Africa: Incidence, Impact, Rapid Response, and Management

B.M. Prasanna*

1. Background

Maize (*Zea mays* L.) is the most important cereal crop in sub-Saharan Africa (SSA), covering over 40 million ha, largely in smallholder farming systems, with a production of over 70 million metric tonnes (MMT) of grain (FAOSTAT, 2021). The crop is critical for food security, incomes, and livelihoods of several million smallholders across SSA, especially in eastern and southern Africa where nearly 85% of the maize produced is used as food (Shiferaw et al., 2011). However, average maize yield in SSA (~2 t/ha) is far below the global average (~5 t/ha), due to various reasons, including frequent occurrence of drought, poor soil fertility, inadequate use of inputs (both improved seed and fertilizers), and challenges imposed by various pests and diseases (Prasanna et al., 2021).

The spread of transboundary pests and diseases has increased significantly in the recent years, affecting the food security and livelihoods of several million resource-constrained smallholders, especially in SSA, Asia, and Latin America. Globalization, trade, and climate change, as well as reduced resilience in production systems due to decades of agricultural intensification, have all played a part. One such major example is the emergence of maize lethal necrosis (MLN) in sub-Saharan Africa, which was first reported in the southern Rift valley area of Kenya in 2011 (Wangai et al., 2012), and then rapidly spread to several other eastern Africa countries during 2012 to 2014 (Mahuku et al., 2015; Redinbaugh and Stewart, 2019; Prasanna et al., 2020). MLN is a viral disease caused by combined infection of maize plants with Maize chlorotic mottle virus (MCMV) with any one of the members of family *Potyviridae*, such as sugarcane mosaic virus (SCMV), Maize dwarf mosaic virus (MDMV) or Wheat streak mosaic virus (WSMV) or Johnson grass mosaic virus (Stewart et al., 2017). MCMV was a recent introduction into eastern Africa, possibly in 2011, while SCMV has a worldwide distribution, including in SSA, over many decades. Therefore, the outbreak of MLN in Africa was primarily triggered by the introduction of MCMV.

2. Global Occurrence and Impact of MLN

MCMV was first reported in Peru in 1971 (Castillo and Hebertt 1974), and subsequently in the USA in the 1970s (Niblett and Claflin 1978). The disease later emerged in several countries across the Americas, Asia, and Africa, including Argentina (1982), Mexico (1987), Thailand (1983), Brazil (1983), Mexico (1987), China (2010), Kenya (2011), Tanzania (2012), Uganda (2012), Rwanda (2013), D.R. Congo (2014), Ethiopia (2014), Taiwan (2014), South Sudan (2015), Ecuador (2016), and Spain (2016) [reviewed in detail by Redinbaugh and Stewart, 2018; Prasanna et al., 2020].

MLN had a serious impact on maize production and grain yield in eastern Africa. During 2012-2013, the estimated maize yield losses in Kenya due to MLN were reported as 23-100% in the affected counties in the country (Prasanna et al., 2020). De Groote et al. (2016) estimated that the aggregate national loss of maize production due to MLN in Kenya alone was about 0.5 million tons with a value of US\$180 million. An average yield reduction of 1.4 t/ha was reported in Uganda, estimated at US\$ 332 per ha (ASARECA, 2014; Kagoda et al., 2016). In 2016, Isabirye and Rwomushana (2016) indicated that MLN may pose high potential yield losses in several countries in SSA, including Uganda (81.1%), Tanzania (65.9%), Ethiopia (59.8%), Malawi (53.8%) and Madagascar (45.1%). Annual economic impact associated with MLN on smallholder farmers in eastern Africa was estimated as about US\$ 261 million (Marenja et al., 2018).

The adverse impact of MLN was not just limited to the maize crops and the livelihoods of the resource-poor farmers in the affected countries, but also on other actors in the maize seed value chain, especially small- and medium enterprise (SME) seed companies. Although not empirically quantified and published, rejection of MLN-contaminated commercial seed by the regulatory authorities and decreased demand for seed of commercial maize varieties in the years soon after the MLN outbreak in eastern Africa led to significant losses to SME seed companies.

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3. Tackling the MLN Challenge on Multiple Fronts

Effectively countering the incidence, spread and adverse impacts of MLN in Africa requires strong, coordinated, and synergistic efforts from multiple institutions as the challenge is complex and multi-faceted. Since 2012, a team including CIMMYT, Kenya Agricultural and Livestock Research Organization (KALRO), NPPOs and commercial seed companies across sub-Saharan Africa, International Institute of Tropical Agriculture (IITA), International Centre of Insect Physiology and Ecology (icipe), several advanced research institutions in the USA and Europe, and non-government organizations, such as Alliance for Green Revolution in Africa (AGRA), and African Agricultural Technology Foundation (AATF), has been intensively implementing a multi-disciplinary strategy for curbing the spread and impact of MLN in Africa. These initiatives are briefly highlighted in this Chapter, and the practical details were elaborated in subsequent Chapters in this Technical Manual.

3.1. Breeding and Deployment of Elite, MLN-Tolerant/Resistant Maize Hybrids in Africa

Studies during 2012-2013 confirmed that nearly all commercial maize varieties (approximately 98%) in Kenya were susceptible to MLN, both under natural and artificial infection (Marenya et al., 2018; Prasanna et al., 2020). Severe MLN infection in the farmers' fields can cause up to 100% yield loss in susceptible varieties (Mahuku et al., 2015). Development of MLN-tolerant/resistant maize varieties is, therefore, the most economically viable and environmentally sustainable approach. This requires intensive screening of germplasm, identification of resistant genotypes, and then incorporation of MLN resistance in combination with other relevant farmer-preferred traits into suitable genetic backgrounds. All this needs to be done in an accelerated manner so that improved varieties with MLN tolerance/resistance are released in the affected countries, and farmers can access the seed of such varieties.

As an important first step, in partnership with KALRO, CIMMYT established a dedicated and centralized **MLN Screening Facility at KALRO Research Center, Naivasha**, in September 2013. The 20-hectare facility, established with financial support from support from the Bill & Melinda Gates Foundation (BMGF) and Syngenta Foundation for Sustainable Agriculture (SFSA), includes 17ha for field screening under MLN artificial inoculation, an MLN diagnostics laboratory, nearly 2000sq.m. of greenhouses, 3500sq.m. of net houses (for screening separately for MCMV and SCMV under artificial inoculation.), etc. MLN phenotyping is carried out throughout the year (two times a year for field-based MLN screening, and thrice for MLN indexing). The facility provides **MLN phenotyping service** to both public and private sector partners across Africa under artificial inoculation, with uniform disease pressure across field trials and high-quality data on the responses of genotypes to MLN. During 2014 to 2021, CIMMYT has screened over 200,000 germplasm entries with more than 300,000 rows (3 m each) at the MLN Screening Facility in Naivasha under artificial inoculation. Of these, 61% were from CIMMYT, 17% were from NARS institutions, and 22% from private sector.

From less than 5 inbred lines with tolerance/resistance to MLN in 2013, today we have more than 50 elite and diverse CIMMYT lines with MLN resistance. Before the onslaught of Covid19 pandemic, annual field days at the MLN Screening Facility at Naivasha (Figure 1) provided public and private sector partners with the first-hand information on the performance of MLN-resistant inbred lines and hybrids (under artificial inoculation). Since 2015, an array of public and private sector institutions globally accessed the seed of CIMMYT's MLN-resistant inbred lines.



Figure 1. Field Day at the MLN Screening Facility, Naivasha, Kenya (2019).

Breeding for MLN resistance is now an integral component of maize breeding pipelines at CIMMYT, especially in the ESA product profiles. This includes routine screening of breeding materials in various breeding stages under MLN artificial inoculation at the Naivasha facility; identification of resistance sources from diverse germplasm; accelerated breeding using doubled haploids (DH) technology and molecular markers; stage-gate product advancement, and varietal release and deployment of elite MLN resistant hybrids through public and private sector partners.

By 2020, a total of 19 MLN-tolerant/resistant hybrids were released in East Africa (Prasanna et al., 2020). On-farm trials conducted in eastern Africa (Kenya, Uganda, Tanzania, and Rwanda) also confirmed the superior performance of these hybrids for MLN tolerance as well as grain yield under high disease pressure, besides other agronomic traits, as compared to the popular commercial checks in the region. Two of the prominent examples of CIMMYT-derived, MLN-tolerant maize hybrids that are being commercialized in eastern Africa are “Bazooka” by NAECO in Uganda, Burundi, and D.R. Congo, and “H6506” by Kenya Seed Company in Kenya (Figure 2).



Figure 2. Bazooka (A) and H6506 (= H12ML1) (B), two of the CIMMYT-derived, elite, MLN-tolerant maize hybrids being commercialized by seed companies in eastern Africa.

Besides deriving MLN-tolerant/resistant inbred lines and hybrids through conventional breeding, molecular marker-based analyses undertaken by CIMMYT has helped in: a) understanding the genetic architecture of resistance to MLN and its causal pathogens; b) identifying the molecular markers associated with resistance, which could be used to improve the resistance or as potential diagnostic markers for early-generation identification of MLN resistant materials or as a part of forward breeding strategy to select lines for MLN resistance during early generations of breeding.

An **MLN Quarantine Facility** was established by CIMMYT, with funding support from USAID and CGIAR Research Program MAIZE, at the Plant Quarantine Institute (PQI) at Mazowe (near Harare), Zimbabwe. The facility, functional since April 2017, is enabling safe introduction and exchange of maize breeding materials from CIMMYT (including from the Kenya breeding hub) to partners in southern Africa. The committed NPPO in Zimbabwe is undertaking monitoring and surveillance during the cropping cycle at the MLN Quarantine Facility, and seed from MLN-free plants is multiplied under quarantine conditions. Seed is further tested at the Quarantine Laboratory in the PQI before declaring it virus-free and suitable for further distribution to partners across Africa. CIMMYT has also developed detailed operational guidelines for the management of MLN Quarantine Facilities (see Chapter 6).

3.2. An MLN Surveillance and Monitoring System in SSA

MLN is one of the successful examples where a surveillance and diagnostic system was rapidly developed and deployed by a CGIAR center (CIMMYT) together with an array of national and international partners. The complexity of transmission of MLN in the field through insect-vectors, contaminated seed lots, and mechanical means have made surveillance and diagnosis a key activity in tracking the disease and minimizing its spread in the continent. The surveillance and disease tracking for MLN were modelled on another successful initiative i.e., for wheat stem rust (Ug99) (Hodson et al., 2012; Park et al., 2011). The MLN surveillance system was developed by CIMMYT in partnership with National Plant Protection Organizations (NPPOs) and Aarhus University, Denmark, under the USAID-funded MLN Diagnostics and Management Project. After its initial development in 2015, a regional MLN surveillance system was put in place in ESA.

MLN surveillance and diagnostic protocols (<https://mln.cimmyt.org/mln-status/protocols-survey-forms/>), as described in Chapters 4 and 5, was carried in eight sub-Saharan African countries, namely Kenya, Uganda, Tanzania, Rwanda, Malawi, Zambia and Zimbabwe during 2016-2019 (Prasanna et al., 2020). All the surveys conducted since 2016 used MCMV immunostrips to detect the presence of MCMV from a bulk sample of six young leaves per field, collected randomly using a staggered X pattern in each of the surveyed fields. Survey fields were selected at random every 10-20 km in maize growing areas. Fields sprayed with pesticide were not surveyed. All the surveyed fields were geo-referenced using GPS. Field survey data from 2017, 2018 and 2019 are depicted in Figure 3 (the results were explained by Prasanna et al., 2020). The surveys indicated that MCMV continues to prevail in eastern Africa. Kenya, Uganda, Rwanda, northern Tanzania, and Ethiopia all detected the presence of MCMV in farmers' fields. However, no further spread to new countries in SSA has been detected, and the current survey data indicates the continued absence of MCMV/MLN in southern and West Africa.

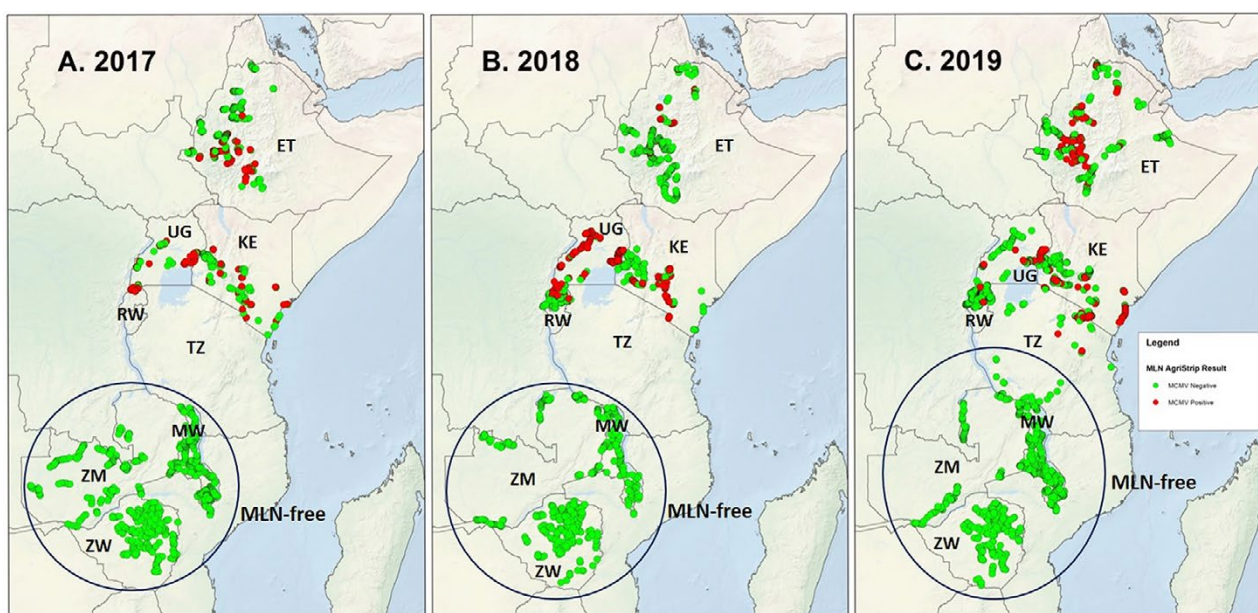


Figure 3. Results of MLN surveys (based on MCMV immunostrip data, coupled with evaluation for MLN symptoms) undertaken by the NPPOs in eight countries in eastern and southern Africa, in partnership with CIMMYT, in (A) 2017; (B) 2018; and (C) 2019. Source: Prasanna et al. (2020).

CIMMYT, in partnership with Aarhus University, Denmark, developed an **MLN Surveillance Data Management Toolbox**, an on-line data management system that supports field surveillance and seed surveys of MLN and other major maize diseases in SSA. The MLN toolbox enables centralized and secure management of standardized data for transboundary diseases at the continental level but with data managed, validated, and published at the country level. Features of the MLN toolbox include controlled access, secure storage in structured databases, data editing, data visualization through interactive maps and charts, and data export of country-specific data. Only when data has been checked and approved by authorized country managers does it enter in public domain data dissemination tools. The MLN toolbox represents an increasingly rich data resource on the status of MLN in SSA, with over 4500 field survey records from eight countries and more than 400 seed survey records currently in the database (Prasanna et al., 2020).

3.3. Production and Exchange of MLN Pathogen-free Commercial Maize Seed

As a rule, planting of healthy, certified, and treated seed is the first step for production of healthy crop that can in turn result in healthy seed. If MLN-causing viruses, especially MCMV, enter a new area through contaminated seed, and the infected plants are not diagnosed and rogued out in a timely manner, the control of the disease could become quite difficult. This is due to the possible presence of insect-vectors in the field that can potentially transmit the viruses within and across the fields.

In a recent study, Kimani et al. (2021) analyzed the seed contamination rates of MCMV in four seed lots; the results ranged from 4.9 to 15.9%. MCMV transmission frequency for 37,617 seedlings, tested in 820 pools of varying seed amounts, by Double Antibody Sandwich-Enzyme-linked Immunosorbent Assay (DAS-ELISA), was 0.17%, whereas a transmission frequency of 0.025% was obtained from 8,322 seedlings tested in 242 pools by real-time RT-PCR (Reverse Transcription-Polymerase Chain Reaction). Seeds from plants mechanically inoculated with MCMV had an overall seed transmission rate of 0.04% in 7,846 seedlings tested in 197 pools. The study showed that even with substantial contamination of maize seed with MCMV, the transmission of the virus from the seed to seedlings was low. Nevertheless, even such low rates of transmission can be significant under field conditions where insect vectors can further spread the disease from infected seedlings, unless diseased plants are detected in time and properly managed.

From the phytosanitary perspective, it is critical to follow rigorous procedures to produce and exchange MLN pathogen-free commercial maize seed within and between countries to prevent the further spread and negative impact of the disease in Africa or in other continents where the disease is prevalent (see **Chapter 8**). In principle, the level of tolerance should be zero for acceptance or rejection of a seed lot where one of the two viruses causing MLN is detected. Ideally, seed produced from a plot/field that had MLN-infected plants must NOT be transferred to a known MLN-free location in the same country or outside the country. In practice, keeping a commercial seed production field completely free from the MLN-causing viruses in prevalent areas requires significant efforts and resources, but is important for protecting the food security, income, and livelihoods of the resource-poor smallholder farmers. Testing for MLN viruses in the seed is also important for NPPOs to ensure that the seed shipped to other countries, especially those which are free from MLN, is devoid of the pathogens.

During the early years of MLN outbreak in eastern Africa, most of the local/regional seed companies in the MLN-prevalent countries lacked necessary knowledge of the disease and its transmission, as well as access to protocols to produce and exchange MLN pathogen-free clean commercial seed. It was, therefore, critical to develop and implement protocols for MLN-free commercial seed production and exchange, especially from the affected to non-affected areas, and make these protocols widely accessible to the regulatory agencies and commercial seed companies. Several strategies have been put in place to achieve this objective, including development of comprehensive checklists and standard operating procedures (SOPs) for MLN-free seed production at various points along the seed value chain (see **Chapter 8**). Three consultative meetings, one each in Kenya, Tanzania, and Ethiopia, organized jointly by CIMMYT, AATF and AGRA, with active participation of researchers, seed companies and extension agents, helped in harmonizing the MLN-free seed production checklist and SOPs. Several training workshops were organized, targeting seed companies, seed trade associations, contract growers, NARS institutions, regulatory and extension agencies for disseminating SOPs and MLN management checklist. Various communication materials on MLN diagnostics and management were developed and disseminated to relevant seed stakeholders in MLN-prevalent as well as MLN-free countries in Africa and Latin America (see **Chapter 8**).

3.4. MLN Information Portal

As part of the strategy to ensure wider dissemination of information and increase awareness among relevant stakeholders, CIMMYT and partners in Africa established an MLN Information Portal (<https://mln.cimmyt.org/>). This portal is indeed a single source for updated information on MLN in sub-Saharan Africa, and provides access to research information (e.g., the availability of new MLN-tolerant/resistant germplasm), MLN screening facility updates, MLN surveillance status, communication products, and training course materials (Figure 4). The MLN Toolbox (data management system) is connected directly to the MLN Information Portal, enabling database-driven interactive maps and charts of surveillance data to be displayed automatically. User statistics for the MLN Information Portal indicate an increasing number of visitors with a near global distribution.



Figure 4. MLN Information Portal (<https://mln.cimmyt.org>).

3.5. MLN Phytosanitary Community of Practice

MLN mitigation strategies requires well-coordinated institutional efforts that effectively leverage expertise across multiple institutions. To achieve this, the MLN Phytosanitary Community of Practice (CoP) was established by CIMMYT in Africa in 2016, bringing together diverse partners, including phytosanitary and regulatory organizations, seed trade associations, NARES scientists, regional bodies etc. The objectives of the CoP are: a) to identify, gather, and seek agreement on the phytosanitary community requirements, especially for effective control of MLN in SSA; b) to provide a forum/platform for cooperation on activities where the CoP adds value to the existing initiatives; c) to share learning across borders on key aspects, such as standardized MLN diagnostics procedure(s), providing training on MLN diagnostics, expediting adoption of appropriate phytosanitary and diagnostic procedures, identifying/validating and deploying novel and low-cost MLN diagnostic protocols, etc.; d) to identify linkages and opportunities for collaborative strategic and technical projects related to MLN phytosanitation and diagnostics in SSA; e) to report on progress and provide updates of the projects and programs that have phytosanitary and diagnostics components related to MLN; and f) to provide information for the review of maize seed certification and import/export procedures in relation to MLN for formulation of appropriate SOPs (Prasanna et al., 2020).

4. Conclusions

The first outbreak of MLN in Kenya in 2011, followed by its rapid spread to several countries in eastern Africa within a span of 3-4 years, caused huge concern to stakeholders, including maize-dependent smallholder farmers, researchers, national plant protection authorities, commercial seed sector, etc. across the African continent. Rapid response and Intensive multi-disciplinary and multi-institutional efforts by an array of national and international institutions resulted in co-development and deployment of an array of tools/technologies to effectively tackle the MLN challenge (Prasanna et al., 2020). The fact that no maize-growing country in southern Africa or West Africa has reported further outbreak of MLN is a testimony to the huge and successful initiative to collectively manage the deadly disease. Nevertheless, there is no room for complacency! MLN is still prevalent in eastern Africa and has not been eradicated. The threat of the disease spreading to other regions in sub-Saharan Africa still looms. It is, therefore, imperative to sustain MLN disease management, as outlined in various Chapters of this Technical Manual, not only in Africa but also globally through proactive and synergistic efforts.

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Chapter 2

MLN-causing Viruses in Africa, and their Symptoms

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1. Introduction

MLN is a maize viral disease caused by a synergistic interaction of maize chlorotic mottle virus (MCMV) and sugarcane mosaic virus (SCMV) or other potyviruses such as maize dwarf mosaic virus (MDMV) or wheat streak mosaic virus (WSMV) (Mahuku et al., 2015; Redinbaugh and Stewart, 2018; Prasanna et al., 2020). MLN outbreaks in the east African region are closely associated with co-infection of plants with MCMV and SCMV. MCMV and SCMV alone are also capable of causing significant damage.

Disease symptoms are often the outward expression of the effects of viruses and other pathogens on growth and development of plants. “Symptoms” are defined as perceptible changes in the plants or its functions that indicate a disturbance in the normal course of the physiological processes (Bos, 1977). Virus infection in plants can often be difficult to identify visually, as symptoms are often subtle and could be easily confused with nutrient deficiency or herbicide injury. In addition, disease development and its associated symptoms are often influenced by the plant genotype, crop stage, environment, and the crop development stage at which the infection occurs.

2. MLN Symptoms

MLN is initially associated with development of fine chlorotic specks or mottling of young growing leaves (Fig. 1.1) that coalesce to produce chlorotic stripes (Fig. 1.2). Eventually the whole leaf becomes necrotic (Fig. 1.3). After 15 days post infection, the leaves gradually start showing some mottling. However, symptoms severity and progression vary with crop growth stages, plant vigor and nutrient management, local prevailing environment, host susceptibility and local agronomic conditions. Growth is stunted in MLN affected plants (Fig. 1.4). Plant infected at earlier growth stages generally develop more severe (chlorotic/necrotic) symptoms, which can lead to plant death (Fig. 1.5). Death of young leaves in the whorl results in “dead heart” (Fig. 1.6). Plants also develop smaller ears which start drying (Fig. 1.7). Other symptoms include distortion of the male inflorescence (Fig. 1.8), with hard panicles, a short rachis, and few spikelets; reduced numbers and length of malformed and partially filled ears (Fig. 1.9). Complete fields may be killed before tasseling in case of severe infection at the early crop growth stages.

3. Maize Chlorotic Mottle Virus (MCMV)

3.1. Host Range

Host range or those plant species infected by MCMV is restricted to plants within the grass family (Poaceae). These include wild grasses (e.g., *Digitaria abyssinica*, *Cynodon dactylon*, *Pennisetum clandestinum*, *Digitaria velutina*, *Cyperus rotundus*, *Brachiaria brizantha*, teosinte), cereals [*Sorghum bicolor* (sorghum), *Eleusine coracana* (finger millet), *Saccharum officinarum* (sugarcane), *Zea mays* (maize), *Triticum* spp. (wheat), *Pennisetum purpureum* (Pearl millet), *Sorghum halepense* (Johnson grass)], and other grasses (*Andropogon*, *Bromus*, *Digitaria*, *Eragrostis*, *Panicum*, *Setaria*, *Spartina* spp. etc.) (Mahuku et al., 2015, Kusia et al., 2015). The virus is not known to infect dicotyledonous species (Castillo and Hebert, 1974; Niblett and Claffin, 1978). Maize is an important natural host of MCMV (Bockelman et al., 1982), but maize genotypes range from highly susceptible to resistant. Experimental host range and alternative host species reported are also restricted to the Poaceae (Castillo and Hebert, 1974).

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Figure 1.1. Chlorotic specks on young leaves



Figure 1.2. Chlorotic stripes on leaves



Figure 1.3. Severe chlorosis of leaves



Figure 1.4. Shortening of internodes



Figure 1.5. Severe chlorosis and necrosis of leaves



Figure 1.6. Dead heart symptoms of plants



Figure 1.7. Premature drying of husks at grain filling stage



Figure 1.8. Tassel blasting at flowering stage



Figure 1.9. Poor or no grain filling

Figure 1. MLN symptoms and effects at various maize growth stages.

Note: The symptoms in these photos were on MLN-infected maize plants artificially inoculated with MCMV plus SCMV at the MLN Screening Facility, Naivasha, Kenya.

3.2. Symptoms

Maize plants infected with MCMV alone develop symptoms (Fig. 2.1-2.3) that are milder than when infected along with a potyvirus, causing MLN. Early symptoms begin as chlorotic specs and form chlorotic stripes developing from the base of youngest leaves, progressing upward towards the leaf tips. The stripes later coalesce to form uneven patches with chlorotic mottle that finally turn necrotic. In severe infections of particularly susceptible lines, leaf necrosis can result in plant death (Castillo and Hebert, 1974). Male inflorescences have hard panicles, short rachis and few spikelets. Fewer ears and ear malformation can also occur in severe infections (Castillo, 1976).

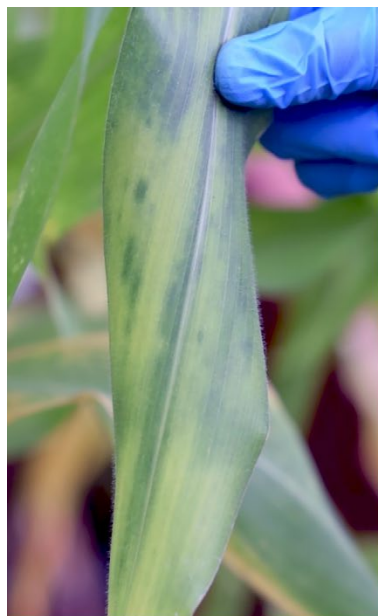


Figure 2.1. Mottle / mosaic symptom due to MCMV infection of maize plants



Figure 2.2. Chlorotic stripes on leaves due to MCMV infection



Figure 2.3. Chlorotic spots, coalescing to stripes, on a MCMV-infected leaf

Figure 2. MCMV symptoms on maize leaves. **Note:** The symptoms were on maize plants that were artificially inoculated with MCMV in nethouses under controlled conditions at the MLN Screening Facility, Naivasha, Kenya (as per the inoculation protocol described in [Chapter 7](#)).

3.3. Conditions for Disease Development

MCMV is efficiently transmitted mechanically and by chrysomelid beetles and thrips. It is also transmitted through contaminated seed at very low rates (see [Chapter 3](#)). MCMV has been detected serologically in all parts of an infected maize plant, including leaf, stem, roots, cob, husk, silk, kernel, seed, anther, and sheath tissues. When MCMV co-infects maize with any potyviruses, a synergistic interaction occurs causing MLN. The MLN symptoms are much more severe than the additive symptoms of either MCMV or the potyvirus alone. The virus complex causes a severe systemic necrosis which may even culminate in death of a plant. If maize plants exhibit a rapid onset of necrosis followed by rapid plant death, it is likely that they are infected with both MCMV and a maize-infecting potyvirus. From an epidemiological perspective, MLN can occur wherever both MCMV and a maize infecting potyvirus are prevalent.

It has been reported that leaves of maize plants inoculated early (3-7 leaf stage) are more adversely affected and become stunted, die prematurely, and bear small, deformed and partially filled or no ears while the plants inoculated later (14-leaf stage) do not often develop symptoms as severe as those of the earlier-infected plants although the kernel weight may be greatly reduced. The virus has been associated with some late-infected plants that showed normal green foliage but bore prematurely yellowed and necrotic ears with shriveled kernels (Uyemoto, 1983).

4. Sugarcane Mosaic Virus (SCMV)

The SCMV complex (Family *Potyviridae*) is known to consist of four distinct potyviruses and includes strains of Johnsongrass mosaic virus (JGMV), maize dwarf mosaic virus (MDMV), sorghum mosaic virus (SrMV), and sugarcane mosaic virus (SCMV) (Shulka et al., 1994). SCMV is the most widespread virus disease affecting sugarcane production. As many as 21 different strains were found in the United States (Yang and Mirkov, 1997). This disease occurs in sugarcane-growing countries worldwide and has significant economic impacts. Yield losses due to SCMV complex were reported to be as high as 21% in the United States (Grisham, 2000) and up to 42% in South Africa (Balarabe et al., 2014).

4.1. Host Range

SCMV causes mosaic diseases in sugarcane (Koike and Gillaspie, 1989) but different strains of SCMV usually infects various members of the crop and wild species of the *Poaceae* family. Some hosts that have been identified are *Sorghum bicolor*, *Zea mays*, *Brachiaria piligera* (Sabi grass), *Sorghum verticilliflorum* (wild sorghum), *Urochloa mosambicensis*, *Dinebra retroflexa*, *Eragrostis ciliaris*, *Pennisetum glaucum* (pearl millet) and *Digitaria didactyla* (Teakle and Grylls, 1973; Persley and Greber, 1977). The SCMV strain formerly known as maize dwarf mosaic virus (MDMV) strain B infects maize and may infect other wild *Poaceae* grasses.

4.2. Symptoms

The classical symptoms of SCMV consist of contrasting shades of green on a background of paler green to yellow chlorotic areas. Sometimes yellow stripes and/or necrosis also occur. The symptoms expression and intensity vary depending on the virus strain, the host cultivar and the environmental conditions, particularly temperature.

Infected plants develop a distinct mosaic (Fig. 3.1), and irregularities in the distribution of normal green color (Fig. 3.2), on the youngest leaf bases. Sometimes the mosaic appearance is enhanced by narrow chlorotic streaks (Fig. 3.3) extending parallel to the veins. Later, the youngest leaves show a general chlorosis, and streaks are larger and more abundant. As plants approach maturity, the foliage can turn purple or purplish red. Depending on the time of infection, there may be severe stunting of the plant. Plants infected early may become totally barren.



Figure 3.1. Mosaic symptoms on maize leaves



Figure 3.2. Irregular mosaic symptoms on maize leaves



Figure 3.3. Narrow chlorotic streaks on maize leaves

Figure 3. SCMV symptoms on maize leaves. **Note:** The symptoms were on maize plants that were artificially inoculated with SCMV in net houses under controlled conditions at the MLN Screening Facility, Naivasha, Kenya (as per the inoculation protocol described in **Chapter 7**).

4.3. Conditions for Disease Development

SCMV infection occurs at the seedling or other vegetative growing stages, but maximum concentration of the viral particles is found in young leaves and minimum in the roots of older infected plants. Seed transmission has also been reported (Ford et al., 1989). The main source of primary infection is the vegetative parts used for propagation of sugarcane. The virus overwinters in infected sugarcane or in appropriate perennial hosts of the specific strain. The virus is transmitted in a non-persistent manner by several species of aphids including *Rhopalosiphum maidis*, *R. padi*, *Myzus persicae*, *Schizaphis graminum*, and *Aphis craccivora* (Noone et al., 1994). The virus is easily sap-transmissible. Infected plants begin to show symptoms at about 4-6 weeks after planting. Crops of maize and sorghum are good hosts of SCMV vectors such as *R. maidis*, and should not be grown near infected sugarcane crops. Altering the times of planting and harvesting so that they do not coincide with high aphid vector populations can also reduce losses (Bailey and Fox, 1980).

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Chapter 3

Modes of Transmission of MLN-causing Viruses

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1. Introduction

Plant viruses have devised several mechanisms for infection, transmission and spread among the hosts. Insect vectors play a key role in the survival and propagation of the plant viruses. The epidemiology of plant viruses is indeed largely dependent on the insect vector population dynamics including their long- and short-range dispersal, host selection and feeding behaviours (Eigenbrode and Bosque-Perez, 2016). In the case of transmission and spread of MLN-causing viruses, the following appear to play a prominent role:

- Insect vectors
- Seed contamination and transmission
- Transmission through soil
- Mechanical transmission

2. Insect Vectors of MLN-causing Viruses

Transmission of Insect vectored plant viruses can be classified into four types namely, non-persistent; semi-persistent; persistent-circulative and persistent-propagative (Ng and Falk, 2006). MLN is caused by co-infection of maize plants with *Maize chlorotic mottle virus* (MCMV) belonging to the genus *Machlomovirus* in the family *Tombusviridae*, and one of the potyviruses, especially *Sugarcane mosaic virus* (SCMV) belonging to the Family *Potyviridae*. MCMV is transmitted in a semi-persistent manner (Cabanas et al., 2013) by thrips, especially corn (maize) thrips, *Frankliniella williamsi* Hood (Nault et al., 1978, 1981; Cabanas et al., 2013), and Chrysomelid leaf beetles, belonging to the genera *Diabrotica*, *Chaetocnema*, *Systema* and *Oulema* (Nault et al., 1978). SCMV and other potyviruses are transmitted in a non-persistent manner by various aphid species infesting cereals, especially belonging to genus, *Aphis*, *Rhopalosiphum*, *Sitobion* and *Macrosiphum* (Adams et al., 2014, Brault et al., 2010; CABI, 2019).

The range of vectors for MCMV and SCMV in Africa are not fully understood. However, several insect pests belonging the families Thripidae, Chrysomelidae, Curculionidae, Nitidulidae and Aphididae that can transmit MCMV and SCMV infests maize in East Africa. Some of these insects have proven to be efficient in vectoring the two component viruses of MLN (Table 1). However, further research is needed to determine the ability of these vectors in virus transmission, their ecology, impacts and their role in the epidemiology of MLN in Africa (Mahuku et al., 2015).

2.1. Thrips

2.1.1. Maize thrips (*Frankliniella williamsi*) [Order: Thysanoptera; Family: Thripidae]






The maize thrips, widely distributed in East Africa (Moritz et al., 2013), are very slender, cigar-shaped insect (2mm long). The insect is yellowish orange to greyish black in color, with narrow wings fringed with hairs. The nymphs are smaller, paler, and wingless. Adults and nymphs infest the underside of young leaves, within the leaf sheaths and especially in growing points, during the first four weeks in the seedling stage and during the tasseling stage. Direct damage due to their feeding on the plant sap in maize is very minimal, but as a vector of MCMV it causes significant damage to the crop. Host range of *F. williamsi* is largely restricted to cereal crops, such as maize, wheat, finger millet, sorghum, and other grasses. Maize thrips do not transmit SCMV even when exposed to plants with mixed infections (Nyasani et al., 2015). Transmission of MCMV by maize thrips is estimated at 78% (Nyasani et al., 2015).

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Table 1. Insect vectors of MLN-causing viruses in East Africa.

Common Name	Scientific Name	Order & Family	Transmits	Photo	Reference(s)
Maize thrips	<i>Frankliniella williamsi</i>	Thysanoptera: Thripidae	MCMV		Nault et al. (1978, 1981); Cabanas et al. (2013); Nyasani et al. (2015); Mwando et al. (2018)
Onion thrips	<i>Thrips tabaci</i>	Thysanoptera: Thripidae	MCMV		Nyasani et al. (2015); Mwando et al. (2018)
Common blossom thrips – pale form	<i>Frankliniella schultzei</i>	Thysanoptera: Thripidae	MCMV		Nyasani et al. (2015)
Western Flower thrips	<i>Frankliniella occidentalis</i>	Thysanoptera: Thripidae	Reported to transmit MCMV in China, but not in Kenya.		Zhao et al. (2014); Nyasani et al. (2015); Kinyungu et al. (2018)
Flea beetle	<i>Chaetocnema pulicaria</i>	Coleoptera: Chrysomelidae	MCMV		Nault et al. (1978, 1981)
Nitidulid beetle	<i>Carpophilus</i> sp.	Coleoptera: Nitidulidae	MCMV		Nyasani et al. (2014)
Maize weevil	<i>Sitophilus zeamais</i>	Coleoptera: Curculionidae	MCMV		Nyasani et al. (2014)
Maize coccinellid leaf beetle	<i>Epilachna</i> sp.	Coleoptera: Coccinellidae	MCMV		Nyasani et al. (2014)
Green maize aphid	<i>Rhopalosiphum maidis</i>	Hemiptera: Aphididae	SCMV		Nyasani et al. (2014); Kinyungu et al. (2018); Klein and Smith (2020)
Wheat Aphid	<i>Sitobion avenae</i>	Hemiptera: Aphididae	SCMV		Yasmin et al. (2011)

2.1.2. Common blossom thrips (*Frankliniella schultzei*) [Order: Thysanoptera; Family: Thripidae]

Common blossom thrips have a very wide distribution and a broad host range. It is mainly found in tropical and subtropical areas throughout the world. *Frankliniella schultzei* manifests in two different colour morphs, a dark and a pale form. The two colour morphs have distinct differences in morphology, molecular profiles, and biology (Gikonyo et al., 2017; Moritz et al., 2013). Mixed colonies of both colour forms are reported in Kenya, Sudan and Uganda in Africa. Only the pale form of *F. schultzei* has been found as a vector of MCMV (Nyasani et al., 2015) with more than 70% transmission. Common blossom thrips can be differentiated from the maize thrips by characters such as placement of the third ocellar setae inside and lower in the ocellar triangle, lack of companiform sensilla in the metanotum, and differences in the metanotal markings (Moritz et al., 2013).

2.1.3. Western flower thrips (*Frankliniella occidentalis*) [Order: Thysanoptera; Family: Thripidae]

Western flower thrips, *Frankliniella occidentalis*, have a broad host range and are normally prevalent in the mid- to high altitude regions. Adult females are very variable in colour, ranging from yellowish orange to dark brown. It is a major pest of ornamentals, French bean, tomato and other solanaceous crops. It is occasionally found on maize (Moritz et al., 2013). Female of these thrips can be differentiated from *F. williamsi* by darker body colour, absence of discal setae in the abdomen, and type of coloration of the antennal segment (Moritz et al., 2013).

2.1.4. Onion thrips (*Thrips tabaci*) [Order: Thysanoptera; Family: Thripidae]

The onion thrips, *Thrips tabaci*, prefers to feed on plants belonging to Alliaceae (onion, garlic), Brassicaceae (cabbage, kale) and Solanaceae (tomato, potato, eggplant). It is occasionally observed on cereal crops such as maize. The adult, which is about 1mm in length, is pale yellow to brownish. It can be differentiated from thrips belonging to *Frankliniella* with seven segmented antennae, incomplete row of setae on the forewing, and presence of rows of microtrichia in the pleurotergites (Moritz et al., 2013). Onion thrips can cause up to 70% transmission of MCMV in maize.

2.2. Beetles

2.2.1. Flea beetle (*Chaetocnema* sp.) [Order: Coleoptera; Family: Chrysomelidae]

The adults are small to moderately sized with enlarged hind femora that allow for the springing action of these insects when disturbed. Many flea beetles are attractively coloured; dark, shiny, and often metallic colours predominate. Adult flea beetles cause the most damage by feeding on the leaves and stems. They create shallow pits and small rounded, irregular holes (usually less than 1/8th inch) in the leaves. Flea beetle, *Chaetocnema pulicaria* identified as a vector of MCMV in the US (Nault et al., 1978, 1981) is also occasionally observed on maize in East Africa. However, its ability to vector MCMV in Africa needs to be confirmed.

2.2.2. Maize weevil (*Sitophilus zeamais*) [Order: Coleoptera; Family: Curculionidae]

The adults are small brownish-black weevils with elongated snout and geniculate antennae. The elytra have fine microsculpture and is shinier with four pale reddish- or orange-brown markings on elytra. However, this is largely a stored product pest, which also infests maize occasionally. Laboratory assays indicate its ability to transmit MCMV (Nyasani et al., 2014).

Apart from flea beetles and maize weevils, maize in east Africa is also infested by other occasional beetles such as *Epilachna* sp. (Coccinellidae: Coleoptera) and Nitidulid corn-sap beetle, *Carpophilus* sp. which can also transmit MCMV (Nyasani et al., 2014).

2.3. Aphids

Aphids are small, pear shaped, soft-bodied insects that suck the plant sap and vector some plant viruses. Aphids' development rate, lifespan, and quantity of offspring are essentially determined by temperature and host plant quality. They reproduce both parthenogenetically and sexually. Under optimal conditions, more than 40 nymphs per female and up to 50 generations per year are laid. An early infestation is often discovered when aphids are found on leaves or on the unopened tassels. Symptoms of infestation include yellow-brown spots on the leaves, leaf wilting and curling, and development of sooty molds on honey dew produced by aphids. Direct damage due to aphids is only significant with high infestation levels, while as vector of viruses such as SCMV and other potyviruses they can significantly damage maize.

2.3.1. Green corn aphid (*Rhopalosiphum maidis*) [Order: Hemiptera; Family: Aphididae]

The head, antennae, legs, cornicles, tail, and transverse bands on the abdomen are blackish brown, with the rest of the body green in colour. The body has sparse short hairs. The length of the antennae is less than half the length of the body. Cornicles are not longer than the finger-like tail. In winged females, the head and thoracic section are black-brown and the cornicles are shorter than in the wingless females. The corn aphid is primarily a species of warm and humid areas and mostly reproduces parthenogenetically. A closely related species, *Rhopalosiphum padi* is also infrequently observed in maize. It can be differentiated from *R. maidis* due to a darker body colour.

2.3.2. Wheat aphid (*Sitobion avenae*) [Order: Hemiptera; Family: Aphididae]

The adults are medium-sized, spindle shaped and can be observed in green and brown colour forms. The antennae are black, shorter than the body length, legs are yellow with the tips of the segments black. The cornicles are longer.

2.4. Management of Insect Vectors of MLN-causing Viruses

Management of cropping systems and crop habitats is critical for effective management of key insect vectors such as thrips and aphids. For instance, avoiding intercropping or mixed cropping of maize with cruciferous vegetables (cabbage, kale) and Alliaceae crops (onion, garlic) is critical to reduce infestation of thrips such as *Thrips tabaci*. Since most of the cereal aphids and corn thrips prefer graminaceous hosts, effective management of grass weeds in the maize farms can reduce early onset of thrips and aphid population. High population of thrips and aphids, especially maize thrips and green corn aphids, at the early stages of crop growth can be detrimental. Coating maize seeds with systemic insecticides can ensure early-stage protection of seedlings against thrips and aphids, and thereby MCMV and SCMV.

Both thrips and aphids can be controlled naturally by a wide array of natural enemies, such as ladybird beetles, lacewing bugs, pirate bugs, syrphid flies, braconid and eulophid parasitoids, and predatory mites. Outbreaks of thrips and aphids often occurs with extensive use of organophosphates and synthetic pyrethroids for the control of other major pests, such as stemborers and fall armyworm. These pesticides kill the natural enemies of aphids and thrips, resulting in their resurgence. Hence it is critical to effectively monitor aphids and thrips population with yellow sticky traps for timely and need-based management interventions, preferably with biorational pesticides.

Application of biopesticides based on entomopathogenic fungi, *Metarhizium anisopliae* can provide early season protection against thrips and aphids. For sustainable management of MLN, the control strategies for insect vectors should be well integrated with other MLN management efforts, such as clean seeds, resistant cultivars, closed season planting, and maize-legume crop rotation.

3. Seed Contamination versus Seed Transmission

“Seed contamination” refers to the presence of a pathogen within a seed or on the seed surface. “Seed transmission” refers to the passage of a pathogen from the seed to the seedling, and further to the whole plant (Sastry, 2013). Any pathogen that may be either inside or attached to the outside surface of a seed that can affect the plant germination or affect an emerging seedling causing the disease symptoms may, in broad sense, be referred to as “seed-borne”. It is well established that plant viruses are effectively introduced into new countries and continents through contaminated or infected seed.

3.1. Seed Transmission of MCMV

There is a serious concern about the transmission of plant pathogens through seed, including MLN-causing viruses like MCMV. Jensen et al. (1991) indicated that seed transmission rates of MCMV in maize seed from MCMV-infected plants range from 0 to 0.33%. To gain a better understanding of the mechanisms underlying MCMV transmission through seed, Bernardo et al. (2018) investigated the MCMV distribution and infectivity in infected seeds from Kenya and Hawaii. The virus was detected at high levels in the pericarp and pedicel in hand-dissected seeds using ELISA. Significantly lower levels of virus were detected in the endosperm and embryo, and no virus was detected in embryos that were washed after dissection. Subsequent immunofluorescence microscopy of seed sections indicated MCMV was localized to the pericarp and pedicel. These results indicated that MCMV virions are limited to maternal tissues in the seed, and seed treatments may reduce seed contamination and transmission of MCMV by seed.

Kimani et al. (2021) analyzed the seed contamination rates of MCMV in four commercial seed lots; the results ranged from 4.9 to 15.9%. MCMV transmission frequency for 37,617 seedlings, tested in 820 pools of varying seed amounts, by Double Antibody Sandwich-Enzyme-linked Immunosorbent Assay (DAS-ELISA), was 0.17%, whereas a transmission frequency of 0.025% was obtained from 8,322 seedlings tested in 242 pools by real-time RT-PCR (Reverse Transcription-Polymerase Chain Reaction). Seeds from plants mechanically inoculated with MCMV had an overall seed transmission rate of 0.04% in 7,846 seedlings tested in 197 pools. The study showed that even with substantial contamination of maize seed with MCMV, the transmission of the virus from the seed to seedlings was low. However, even a low rate of seed transmission could be epidemiologically significant because viruses may be introduced into new areas/countries through infected seed (Mahuku et al., 2015). In conjunction with secondary spread by insect vectors, low rates of seed transmission can translate into high numbers of infected plants, resulting in epiphytotic (Maule and Wang, 1996). Recent grow-out tests with maize seeds obtained from plants with varied levels of MLN infection, revealed high levels of MLN incidence in the seedlings in both the laboratory (55–100%) and field (10.9–36.5%). MLN transmission was not observed with certified seeds obtained from plants with no incidence of MLN (Kinyungu et al., 2021).

3.2. Seed Transmission of SCMV

SCMV has not been reported to be transmitted by seed in sugarcane. However, seed cane (stalk pieces or setts), used to propagate sugarcane vegetatively commonly transmits SCMV and other viruses from one crop to the next. In the case of maize, SCMV-MB (Maize dwarf mosaic virus strain B) has been detected in the pericarp, but rarely in the endosperm or embryo of seeds 21 days after pollination. In mature seeds, it was occasionally detected in the pericarp and endosperm, but not in the embryo (Mikel et al., 1984). Experimental studies by Li et al. (2011) reported seed transmission rate of SCMV between 2.3% and 3.9% in two groups of maize seed tested. SCMV was reported to be mechanically and seed transmitted but not pollen transmitted (Brunt et al., 1996).

4. Transmission through Soil

By definition, a virus is soil-borne if it holds the capacity to survive in the soil debris or other living organisms and infect the plants growing in that soil. To be soil-borne, a virus should have an existence in soil outside of its natural host (Hiruki and Teakle, 1987). The majority of plant viruses are transmitted into the aerial plant parts by a variety of arthropods, mainly sap-sucking insects, such as thrips, aphids and whiteflies, while some soil-inhabiting zoosporic organisms and root-feeding nematodes can transmit a number of plant viruses into roots (Hull, 2013). Several plant viruses with single-stranded RNA (ssRNA), belonging to at least 17 genera in eight virus families, were reported to be transmitted by soil-inhabiting organisms (Andika et al., 2016).

So far, there are no published reports with conclusive evidence on the specific mode(s) of soil transmission of MLN-causing viruses like MCMV. However, soil-based vectors that have been associated with transmission of viruses in the family *Tombusviridae* (to which MCMV belongs) are fungi in the genus *Olpidium*, and at least five genera of nematodes (*Longidorus* spp., *Paralongidorus maximus*, *Xiphinema* spp., *Trichodorus* spp., and *Paratrichodorus* spp. (Andika et al., 2016).

5. Mechanical Transmission

All members of the virus family *Tombusviridae* (to which MCMV belongs) are readily transmitted by mechanical means. Experimentally, tombusviruses are readily sap transmissible and infected leaf extracts may retain infectivity after freezing for several years (Rochon, 1999). Thus, farm tools and machineries used in the maize fields infected with MCMV/MLN can serve as a source of inoculum. Hence, vehicles, and farm machinery/equipment entering the farmers' maize fields or seed production fields should be properly cleaned using disinfectants both before and after use.

The maize plant is also an important source of fodder for animals in the smallholders' agri-food systems. When MLN outbreak occurred in eastern Africa, farmers in some countries were advised to scout and rogue out plants showing MLN symptoms at the early stages of crop growth, and feed the rogued maize plants (with stalk, leaves and husks) to animals. The dung of the farm animals is often used as an organic manure in the fields, either alone or in combination with inorganic fertilizers. It is, thus, conceivable that animals including cattle grazing on the MLN-infected plants or fed with MLN-infected plants could potentially transmit the MLN-causing viruses to either mechanically or through organic manure. There is no evidence, however, that MLN-causing viruses can transmit to maize plants through this specific mode. However, as a precautionary measure, it is advised not to feed the farm animals with MLN-infected maize plants or other plants infected by MLN-causing viruses.

6. Conclusions

One of the major challenges regarding MLN management is the existence of multiple modes of transmission. The theory behind the diversity and evolution of plant virus transmission patterns observed in nature were explained by Hamelin et al. (2016). Although there is a lot still to understand about the pathways of transmissions of MLN-causing viruses, especially from the perspective of seed and soil, the existing knowledge can aid in devising and implementing appropriate management practices that can mitigate the threat.

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Chapter 4

MLN Surveillance, Leaf and Seed Sampling Protocols

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1. Introduction

Continuous surveillance for MLN causing viruses is required to monitor the disease incidence in farmers' maize fields and seed production fields. Surveillance informs decisions on deployment of management practices to limit the effect of the disease at the farm-, country- and regional levels. Effective diagnostics and surveillance of the possible incidence of MLN in the seed production fields is essential for producing and exchanging MLN-free seed (see Chapter 8).

2. Field Survey Protocol

For a successful MLN field survey, the following elaborate strategy should be adopted.

2.1. Checklist for Field Surveys

Prior to starting field survey work, field survey teams should have the following:

- Pre-printed field survey forms (sufficient number plus spares)
- Sample collection envelopes and labels (sufficient number plus spares)
- Pens/Pencils (at least 3 per team member; pencils should be used for writing if there is a lot of moisture in the environment)
- GPS Unit (1 per team) – with standard settings for units
- Spare AA batteries (at least 4 per team)
- Tablet (or smartphone) and charger (if using electronic survey)
- Pocket knife/scissors
- Tape
- Sampling plastic bags
- Diagnostic tools (e.g., MCMV immunostrips kit)
- Sample storage equipment (e.g., cool box for temporary storage of samples, if needed)
- Hand counter
- MLN diagnostic illustrations

2.2. Field Inspection/Sampling Pattern

Make a preliminary survey of the field to identify and collect the survey information using survey forms in **Annex 1A and/or 1B**. Give particular attention to:

- a. Possible micro-climates in the field that appear different enough to warrant special attention when inspected. This may include locations in which high moisture levels may be retained due to proximity to rivers and streams, drainage areas, low spots, etc.
- b. Weedy areas
- c. Areas of the field affected by borders, such as field edges, tree lines in the field, adjacent fields of a similar crop, presence of buildings etc.
- d. Drought-stressed areas in the field

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The field inspection pattern must ensure that all parts of the field are adequately and proportionately represented in the plants inspected within the various possible microclimates of the field. For maize field inspections and surveys, the staggered “X” pattern is recommended (CDFA, 1985). It requires:

- i. Examination of plants along one side of the field.
- ii. Then diagonally in a staggered pattern across rows to the far corner, and across the far side of the field.
- iii. Diagonally back to starting corner (Fig. 1).

Additional examinations may be necessary for field environments not covered by the inspection pattern. Counting the number of plants between a sample and another with the hand counter maybe useful in case positives are detected. Counting the number of plants between a sample and another with the hand counter maybe useful in case positives are detected.

2.3. Sampling the Plants for Virus Testing

1. Operators should wear laboratory gloves while sampling. Sample should be collected before pesticide application, if any, is done.
2. Samples should be collected from the youngest rapidly growing leaves of plants.
 - 2.1. Invert the plastic sample bag over one hand, grasp the leaf to be sampled through the bag and revert (Fig. 2a-c)
 - 2.2. Using your other hand, grasp the leaf below the bag and cut it into the plastic bag (Fig. 2d). Always use separate bags for different plots.
 - 2.3. Important caution: Do not touch the interior of the plastic bag with fingers, implements or any other leaves; remove any leaf exudate (sap) from hand and cutting implements immediately after sampling to prevent cross-contamination of samples.
 - 2.4. Place a completed sample label on the side of the sample bag and record the unique sample code with details, as shown in Fig. 3A and B. Place the labelled sample bag inside another plastic bag to protect the label from any possible damage.
 - 2.5. Store the labelled samples in a styrofoam or thermos-cool box with ice packs. Individual, labeled samples from the same plot maybe placed inside one large plastic bag to keep them altogether (Fig. 2e-f).
 - 2.6. Send the styrofoam with samples secured in a cardboard box to the recommended laboratory for ELISA testing for confirmation., Keep these in paper layers and put it in a labelled polythene bag with a few holes, and place the polythene bag in a carton.
 - 2.7. Please mention on the box “Plant sample“ “RUSH IMMEDIATELY”.

If samples cannot be mailed immediately, keep them refrigerated (preferred) or in a cool dark place.
3. Label each individual sample bag with a computer-generated adhesive label with all the relevant information (date, site, plot number, crop stage etc.) (Fig. 3A). A computer generated unique QR code label must also be attached to each individual sample bag (Fig. 3B) and the unique code recorded on the corresponding field survey form.
4. The labeled leaf samples must be put immediately in a cooler containing freezer blocks.
5. If the samples are not processed immediately, they must be refrigerated at 4°C and no longer than 48h. After that time, leaf samples can deteriorate, and the results will not be reliable.

Note: Although symptomatic plants must be tested, it is critical to analyze asymptomatic plants as well. In general, it is preferable to undertake systematic sampling across the field (including both symptomatic and symptom-free/asymptomatic plants) for analysis.
6. For every symptomatic plant sampled, it is required to sample at least three neighboring symptom-free plants into separate sampling bags and with the same procedure as described above.
7. Complete the Sample Collection Form (Annex 2) and include it with the sample.

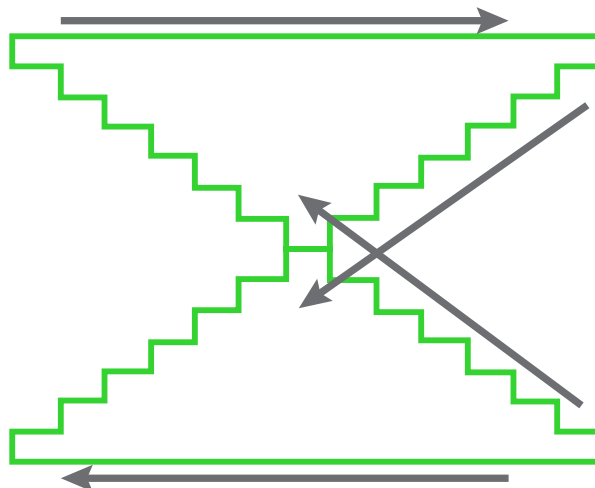


Figure 1. Staggered sampling in the field.



Figure 2. Leaf sampling in the field (a-d) and storage (e-i) for testing of the MLN viruses.

Field location:
Farmer's or Seed Producer's Name:
Trial / Plot Number (if any)
Sample type (leaf / Seed):
Sample number:
Virus symptoms present: Yes / No
Date of sampling (d/m/y):
Name of the person who sampled:
Remarks, if any:

A



1SQsaFOR

B

Figure 3. Example of the sample label (A) and the unique QR code (B).

2.4. Seed Sampling

This procedure describes how to obtain a suitable sample size in which probability of a constituent (MLN-causing virus) being present is determined only by its level of occurrence in the seed lot (**Annex 1C**). The seed sample to be tested must represent as homogeneously as possible the composition of the whole seed lot. The International Seed Testing Association (ISTA, 2004) has provided procedures and tables with number of seed to be sampled according to the size of the seed lot under evaluation. Composite samples can be used to help overcome issues with possible irregular distribution of virus across a seed lot.

2.4.1. Sampling threshold for MLN testing

Generally, if the threshold for tolerance of a pathogen transmitted through seed is 1%, then the sample size taken for laboratory analysis after the procedure for reduction of the sample withdrawn from the whole seed lot should be 400 seeds per seed lot according to ISTA (ISTA, 2004). If the threshold is much lower than 1%, the sample size is scaled up accordingly. The sensitivity of test used (ELISA or PCR) and the incidence of infected seed in a lot are the factors that play a role in the equation for the calculation of the sample size to withdraw from a seed lot (Morrison, 1999).

In case of MLN, different strategies may be required depending on where the seed is being distributed to: (a) MLN-free countries, the tolerance (column in Table 1 named “proportion of MCMV infection”) level should be zero; and (b) for MLN prevalent areas, a higher level of tolerance can be used depending on the guidelines from specific phytosanitary agencies.

2.4.2. Sampling procedure for primary samples

- Generally, if the threshold for tolerance of a pathogen transmitted through seed is 1%, then the sample size should be 300-400 seeds per seed lot according to ISTA (ISTA, 2004). If the threshold is much lower than 1%, the sample size is scaled up accordingly.

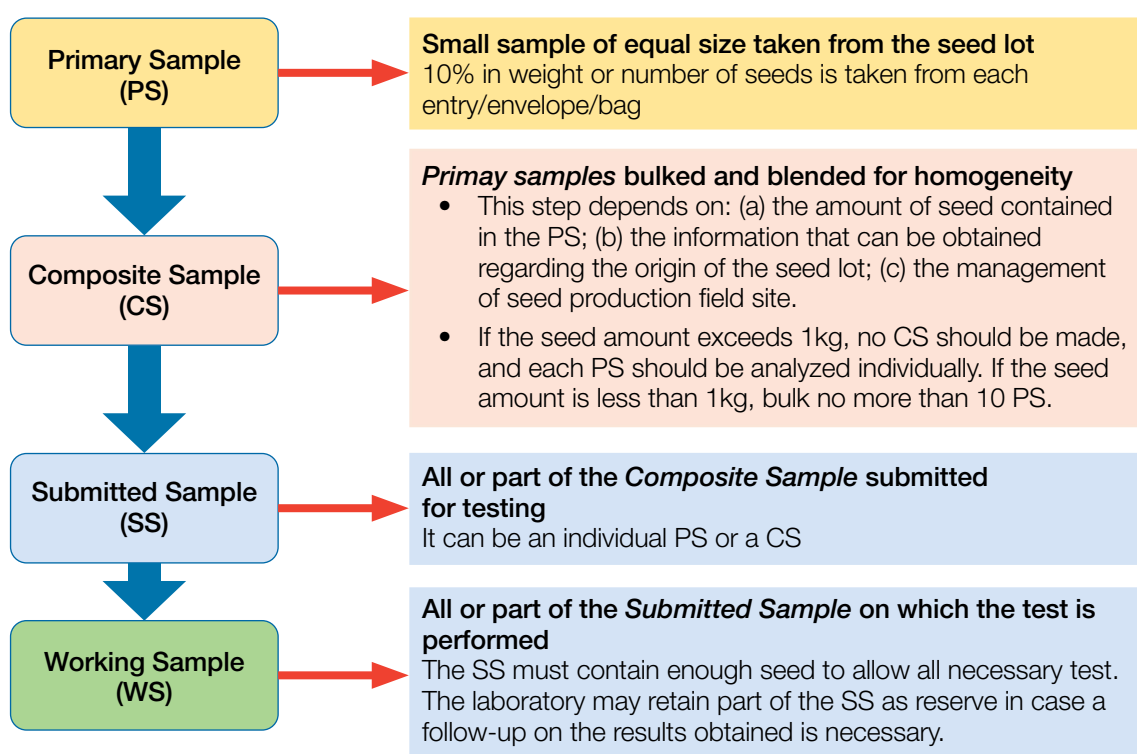


Figure 4. Definition of a primary sample, composite sample, and submitted sample. Source: Mezzalama et al. (2015).

- For experimental seed lots for hybrids, inbred lines and OPVs:
 - i. Sample between 100 g to 1 kg, 10% of the total seed quantity in weight or number of kernels. However, for seeds quantities less than this, 5 % is recommended under the condition that the seed has been produced in an area where the plants were inspected and found free of MLN causing viruses.
 - ii. Collect between 1-14 kg, 10% from each entry
 - iii. Quantities between 15-100 kg.

Table 1. Minimum sampling intensity for seed lots in containers of up to 15-100 kg capacity (inclusively).

Number of containers in the lot	Minimum number of primary samples to be taken
1-4	3 primary samples from each container
5-8	2 primary samples from each container
9-15	1 primary samples from each container
16-30	15 primary samples from each seed lot
31-59	20 primary samples from each seed lot
60 or more	30 primary samples from each seed lot

- iv. Draw small amounts of seed from 100 kg and above (usually commercial seed lots) from as many points in the seed lot as possible.

Table 2. Number of primary samples to be taken from seed lots of more than 100 kg or from the seed stream.

Lot size	Number of primary samples to be taken
Up to 500 kg	At least five primary samples
501 to 3,000 kg	One primary sample for each 300 kg but not less than five
3,001 to 20,000 kg	One primary sample for each 500 kg but not less than 10
20,001 kg and above	One primary sample for each 700 kg, but not less than 40

2.4.3. Seed sampling in the laboratory

In the laboratory there are two categories of samples:

(a) Single samples: For seed quantity greater than or equal to 500 g, a single sample of 10% of the seed quantity either by weight or number of kernels is collected after thoroughly mixing the seed in the bag to homogenize it.

(b) Composite samples: Depending on the number of entries in the list submitted and the quantity of seed (less than 500g in weight or number) per entry, take 10% from each entry and homogenize to constitute a composite sample.

In both cases, take 50% of the total amount of seed and use it for analysis and the remaining 50% should be kept as a reserve.

Sample preparation in the laboratory

Once a representative seed sample is obtained, identify and store it in plastic or paper bags until you reach the laboratory.

3. MLN Electronic Survey Forms

Electronic versions of the MLN Field Survey Forms (**Annex 1A & 1B**) and the MLN Seed Survey Form (**Annex 1C**) have been developed using ODK software. The survey tools may be downloaded without cost and will work on any Android device that has GPS (smartphone or tablet). No internet connection is required to collect data in the field, as data and data forms will be stored on the device and sent when a connection becomes available.

The survey data collected using these tools will be stored by CIMMYT on a secure server in the MLN Toolbox Data Management System developed in partnership with Aarhus University, Denmark. Survey data will not be released to the public domain prior to approval of a country's authorized official (i.e., the country's designated national plant protection officer).

3.1. Download and Installation

Download ODK Collect using the link below; <https://play.google.com/store/apps/details?id=org.odk.collect.android&hl=en>

Download ZXing barcode scanner using the link below; <https://play.google.com/store/apps/details?id=com.google.zxing.client.android&hl=en>

Note: Device GPS MUST be switched on in order to complete the survey forms.

3.2. Device Settings (First time Use Only)

Click on menu icon on main ODK page (see Fig. 5). Select "General Settings"; Click on "Configure platform settings" and enter the following

- i. URL: <https://kc.kobotoolbox.org>
- ii. Username: mlnsurvey_yourcountryname (e.g., mlnsurvey_malawi)
- iii. Password: mlnsurvey_yourcountryname (e.g., mlnsurvey_malawi)

The password can be changed by going to kc.kobotoolbox.org, then login using old password. Click on the three lines at the top left corner of the page and choose settings among the list in the left column. Click on change password and submit the new password and click ok.

In "AUTO SEND". It is recommended to select "Auto send with Wi-Fi" and "Auto send with network" [This will ensure automatic sending of data forms when connected to internet].

Load the MLN FIELD and SEED survey forms (**Note:** For first time use only, or if an updated version of the forms is available).

- On main ODK page. Click on "Get Blank Form" (Fig. 5).
- Enter username and password if needed. Click OK for server authentication.
- Select MLN FIELD survey form v1.0 and MLN SEED survey form v1.0.
- Click on "Get Selected".

Open the MLN FIELD Survey Form v1.0

- On main ODK page. Click on "Fill Blank Form" (Fig. 5).
- Select MLN FIELD Survey Form v1.0.
- Swipe screen and fill in form. Enter text or select options from lists.
- To collect GPS coordinates. Click on "Record Location" button. Once the GPS signal has been received the latitude, longitude and elevation will be automatically recorded.
- If MLN symptoms are observed in a plot, you have the option to take a photo of a symptomatic leaf/plant. It is recommended to take a photo of a leaf that has clear symptoms.
- For ALL leaf samples collected the unique QR code for the sample MUST be recorded. Stick a unique QR code label on each sample bag and also on the bulk sample bag. Click on the "Get Barcode" button in the survey form. The camera will open. Align the camera directly over the QR code. Once the QR code is scanned the code will automatically appear in the survey form. Check that this matches the code on the sample bag (If it does not match – click on "Replace barcode" and repeat).

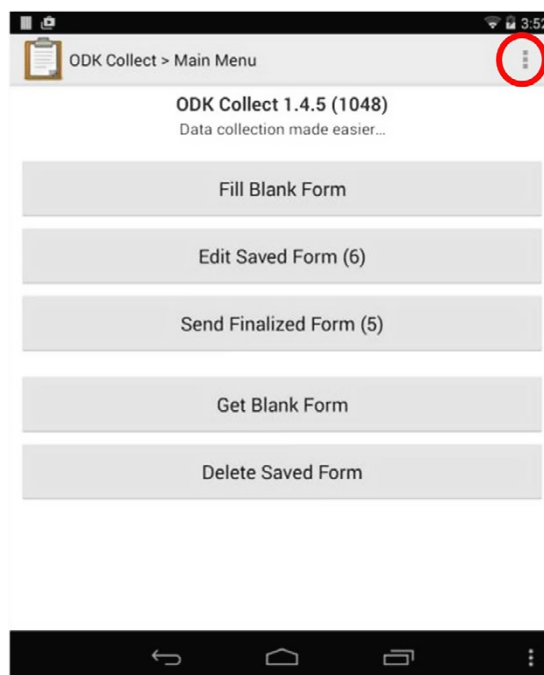


Figure 5. ODK Collect Main Menu screen. Settings menu icon highlighted in red circle.

- Please note some of the questions on the survey form are conditional – selection of a response will lead to additional relevant questions.
Note: If a number (e.g., number of other diseases etc.) needs to be changed. It is possible to scroll back, edit the number and add the additional data without losing any previously entered data.

Once the survey is completed click on “Save Form and Exit”. The completed forms will also be sent when a WI-FI connection is available. Open ODK. Saved forms will either be sent automatically or click on “Send Finalized Form” button on the main ODK menu screen (Fig. 5).

3.3. Using the MLN Seed Survey Form

Open the MLN SEED Survey Form v1.0 or the newest version on main ODK page. Click on “Fill Blank Form” (Fig. 5). Select MLN SEED Survey Form v1.0. Swipe screen and fill in form. Enter text or select options from lists. To collect GPS coordinates. Click on “Record Location” button. Once the GPS signal has been received the latitude, longitude and elevation will be automatically recorded.

It is essential that you enter the number of seed samples collected. For ALL seed samples collected the unique QR code for the sample MUST be recorded. Stick a unique QR code label on each sample bag. Click on the “Get Barcode” button in the survey form. The camera will open. Align the camera directly over the QR code. Once the QR code is scanned the code will automatically appear in the survey form. Check that this matches the code on the sample bag (If it does not match – click on “Replace barcode” and repeat).

Please note some of the questions on the survey form are conditional – selection of a response will lead to additional relevant questions.

3.4. MLN Field Survey using a GPS

If surveys are conducted using paper forms, a handheld GPS should be used to record field location (latitude and longitude). Display features vary depending on the GPS model being used. General operating procedure for handheld GPS units is as follows:

- Turn on the GPS and get a satellite signal. Once in the field, switch on GPS unit by pressing the power button.
- View the main satellite display page. Wait for 2-3 minutes for the GPS to get a location fix using the satellites overhead. Once a fix has been obtained, satellite symbols and signal strength bars will turn black. Once signals from at least 4 satellites have been received, Latitude and longitude data (and GPS accuracy) will be displayed.
- Once latitude and longitude are displayed and accuracy is 10m or less, you can now record the location on the survey form.
- Turn off the GPS after recording the location, to switch off GPS – press and hold Power key. Complete the MLN Survey Form and sampling and move to next survey location.

Note:

The first time you use a GPS in a completely new region it can take up to 5+ minutes to receive satellite signals. After initial use, signal reception will be much faster – about a minute or less. The more satellites you receive signals from the more accurate will be the location. However, the maximum accuracy possible with handheld units is +/- 4 or 5 meters. Anything less than 10 meters is good enough.

4. References

- CDFA (1985) California Department of Field and Agriculture (CDFA) Phytosanitary Field Inspection Manual.
- ISTA (2004) International Seed Testing Association (ISTA) Handbook on Seed Sampling, 2nd Edition. ISTA, Zurich, Switzerland.
- Mezzalama M, Das B, Prasanna BM (2015) MLN Pathogen Diagnosis, MLN-free Seed Production and Safe Exchange to Non-endemic Countries. CIMMYT, Mexico DF. <https://repository.cimmyt.org/xmlui/bitstream/handle/10883/4284/56880.pdf?sequence=3&isAllowed=y>
- Morrison RH (1999) Sampling in seed health testing. *Phytopathology* 89: 1084-1087.
- Redinbaugh MG, Stewart LR (2018) Maize lethal necrosis: an emerging, synergistic viral disease. *Annual Review of Virology* 5: 301-322. <https://doi.org/10.1146/annurev-virology-092917-043413>

Annex 1A. Field Survey Form for MLN Detection

Country/Institution: _____

Date of Survey (d/m/y): _____ / _____ / _____

Location: _____

Latitude (decimal degrees): N S

		.						
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Longitude (decimal degrees): E W

		.						
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Elevation: _____ (meters above sea level)

Survey Site: Farmer's field Seed Production Field Maize trial

Growth Stage: Vegetative (VE-VT) _____ Reproductive: R1. Silk R2. Blister R3. Milk
R4. Dough R5. Dent R6. Maturity

Plot Seed Source: 1. Farmer saved seed 2. Neighbour/Friend/Relative 3. Purchased from Agro Dealer
4. Donation (Gov/Project/NGO) 5. Other _____

Date of Planting (d/m/y): _____ / _____ / _____

Field area size: _____ ha Variety: _____

Disease	Present (Y/N)	Plot Incidence (% of plot infected)	Plot Severity (Avg % severity on plants)
1. MLN (visible symptoms)		L M H	L M H
2.		L M H	L M H
3.		L M H	L M H
4.		L M H	L M H
5.		L M H	L M H

L (Low) = 1-20%

M (Moderate) = 20-40%

H (High) = more than 40%

[Note: If other diseases / symptoms observed – record in disease column. If no diseases observed leave table blank]

Insects present: Thrips Whitefly Aphids Leaf beetles Others _____

Visible Insect Damage: Leaf: L M H Stem: L M H

MLN Control Measures: None Insecticide Removal of Infected Plants

Insecticide used: _____ Dose (l/ha): _____ Date of Last Application: _____ / _____ / _____

MLN-infected Leaf samples collected: Y N Number of Leaf samples collected: _____

Leaf Sample ID	Source* (Variety)	MLN Visible Symptoms (Y / N)	Bulk Sample ID (6 leaves)	Sent for ELISA Assay (Y / N)	Immunostrip used (Y / N)	Bulk Immunostrip Result (+ / -)
1						Test 1.
2						
3						
4						Test 2. (if test1 +ve)
5						
6						
7						Test 1.
8						
9						
10						Test 2. (if test1 +ve)
11						
12						
13						Test 1.
14						
15						
16						Test 2. (if test1 +ve)
17						
18						
19						Test 1.
20						
21						
22						Test 2. (if test1 +ve)
23						
24						

*Indicate specific source from where the leaf sample was collected (e.g., Variety X if several varieties are grown in the same field)
 [Record exactly same sample ID's as QR label used on sample bags/bulk bag. NB: Codes are case sensitive]

Annex 1B. Survey Form for MLN Detection in Farmer's Field

Farmer's Name:

Farmer's Tel Number (if available):

Is maize cultivated continuously? Y N Previous Crop:

Is maize planting synchronized in the locality? Y N

Has the farmer seen MLN symptoms before? Y N

Does the farmer have access to extension agent? Y N

No. of extension visits / season?

Additional Comments / Observations:

Notes on filling MLN Field Survey Form:

- **Disease Table:** Primary focus of survey is MLN, but if other diseases are observed and can be identified, record them in the disease column and score the plot incidence and severity. If unknown viral symptoms are observed, use the following 6 symptom categories for recording – unknown virus symptoms – Mosaic; Chlorotic stripes; Yellowing; Necrotic leaf margin; Dead heart; Dead plant.
- **Sample Table:** Take 6 leaf samples, create a bulk and test bulk sample with Immunostrip. Record sample IDs and bulk ID and the Immunostrip result. If the Immunostrip result is positive, take another aliquot from the same bulk and re-test using another Immunostrip. If both tests are positive, re-sample and re-test infected plants. If positive tests are obtained, sample 3-4 surrounding / neighboring plots.
- Only send the samples that tested +ve with Immunostrips for follow-up ELISA analysis.

Annex 1C: Survey Form for MLN Detection in Commercial Seed

Name of the Surveyor:

Country/Institution:

Date of Survey (dd/mm/year):

Latitude (decimal degrees): N S

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Longitude (decimal degrees): E W

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Elevation: _____ (meters above sea level)

Name of the Agro Dealer:

Location:

Tel:

If commercial seed samples are collected, provide the following details:

Seed Sample ID (QR code)	Name of the Variety	Company	Seed Lot Number	Seed Source (Country /Location as per label)	Weight of seed lot / bag sampled (kg)	Approx. weight of sample (kg)	Sent for ELISA Assay (Y/N)
1							
2							
3							
4							
5							
6							
7							
8							
9							
10							

Additional Comments / Observations:

Annex 2. Maize Sample Collection and Submission Form for MCMV/MLN Diagnosis in the Laboratory

Maize Sample Collection for MCMV/MLN Diagnosis in the Laboratory	
To be completed by field staff collecting samples	
1. Collection Number:	2. Date of Collection:
3. Submitting Organization:	
4. Name and address of the sample collector:	
5. Place of collection (Name / Station / GPS coordinates etc.):	
6. Type of sample (leaf/seed):	
7. Packaging:	
8. Number of samples submitted:	
9. Name and signature of the sample collector/sender:	

To be completed by laboratory staff analyzing samples	
10. Name and address of the Laboratory:	
11. Remarks by the Laboratory Analyst:	
12. Lab testing method:	
13. Pathogen identified (Common name, abbreviation):	
14. Description notes, if any:	
15. Name and Signature of Laboratory Analyst	
Place:	
Date:	

Chapter 5

Diagnostic Protocols for MCMV and SCMV

Monica Mezzalama^{1*}, Margaret Redinbaugh², Anne Wangai³, and L.M. Suresh⁴

1. Introduction

Early and accurate detection of plant viruses is important in surveillance, seed multiplication, and seed exchange. Maize chlorotic mottle virus (MCMV) infection may be difficult to diagnose based on symptoms alone as some of them (stunting, chlorosis) resemble those caused by nutrient deficiencies, moisture stress or other maize-infecting viruses like maize mosaic virus, maize streak virus and maize stripe virus. There are several diagnostic tools that are available for detection of MCMV and sugarcane mosaic virus (SCMV). The most reliable methods for detecting MCMV in host tissues include ELISA (enzyme-linked immunosorbent assay), immunostrips, and polymerase chain reaction (PCR). In this chapter, we present several validated MCMV and SCMV detection methods and suggest their point of use depending on the objective.

2. MCMV and SCMV Detection in Leaf Samples using Immunostrips in the Field

2.1. Requirements

1. MCMV and SCMV detection immunostrip kits
2. Gloves
3. Scissors
4. Tweezers
5. 1 lt of 10% bleach water solution
6. Cotton wads or gauze
7. Containers (big plastic boxes for carrying out the test under clean conditions (to protect the material and the reagents from dust, dirt etc.)



8. Tissue homogenizer/sap extractor



2.2. Procedure

- Collect the leaf tissue using the same procedure and precautions as described under “Leaf sampling” and according to the company providing the immunostrips.
- Try to find a site in the field with shade, with little or no wind, and with no movement around you.
- Open the plastic box in no. 7 of 2.1 and use it as a bench.
- Wear clean gloves.
- Open the Immunostrips kit and dispense all the reagents inside the plastic box.
- Holding the collected tissue with the tweezers, cut with the scissors a piece of leaf tissue of the size of a coin (approximately 0.1–0.15g) or what is recommended by the provider and place it in the plastic extraction bag provided with the kit.
- Disinfect carefully the scissors and the tweezers with a cotton wad soaked in the bleach solution.
- Carry out the test following the procedure described with the kit.
- Record the results on the survey form.

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In case of a positive result for MCMV (Figure 1), follow-up by sending the sample to the recommended laboratory for ELISA or PCR testing for further confirmation.

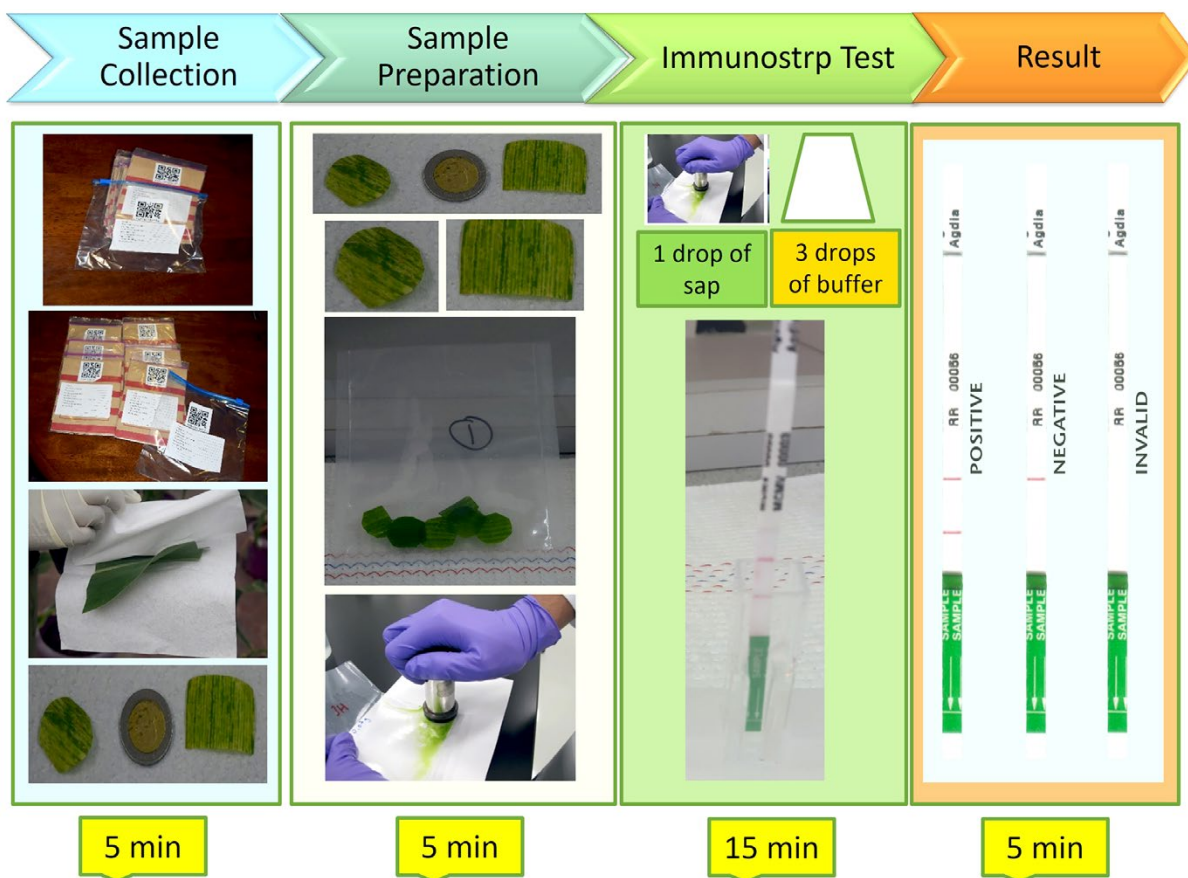


Figure 1. MCMV immunostrip assay using maize leaf samples.

3. MCMV and SCMV Detection using ELISA

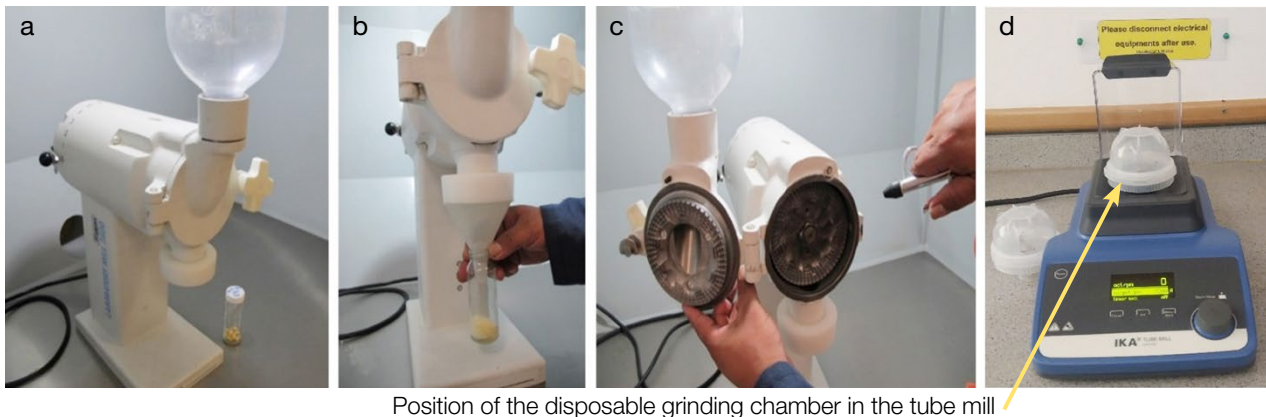
3.1. Antisera, Protocols and Recipes

There are several companies that provide kits (antisera) for ELISA testing for the MLN-causing viruses. Usually, the protocol for the testing is provided along with the kits. It is important to follow the company instructions to perform the test always. The antisera must be (a) stored as per instructions, (b) used at the indicated dilution, (c) not mixed with antisera sourced from different companies, and (d) not to use expired antisera.

3.2 Sample Preparation

The leaf samples obtained must be extracted with a tissue homogenizer or sap extractor. The amount of leaf material needed for the test must be calculated based on the instructions provided by the kit company. The extraction of the virus from the sample usually requires a dilution of the leaf sample at a ratio of 1:10 weight/volume (w/v) in extraction buffer, unless the protocol given with the kit gives different instructions.

The seed samples must be ground with a seed grinder (two examples of seed grinder are given in Figure 2; any other type is suitable so long as it allows a scrupulous cleaning between samples). Proper caution must be taken in cleaning thoroughly between samples, using a brush or by blowing compressed air, followed by cleaning of all surfaces that were in contact with the test material (seed) with 70% ethanol. The amount of ground material needed for the test must be calculated based on the instructions provided by the kit company. The extraction of the virus from the sample usually requires a dilution of the ground sample at a ratio of 1:10 w/v in extraction buffer, unless the protocol given with the kit gives different instructions.



Position of the disposable grinding chamber in the tube mill

Figure 2. (a) Seed grinder; (b) collection of flour after grinding through the funnel; (c) cleaning of the grinder between samples with a shot of high-pressure air; (d) seed grinder with disposable grinding chamber.

3.3. Materials and Equipment

- ELISA reagents
 - ◊ Buffers: Carbonate coating buffer 1X; General extraction buffer 1X; PBST wash buffer 1X; Conjugate buffer 1X; Substrate buffer 1X
 - ◊ Antisera [Capture antibody and alkaline phosphatase enzyme conjugate (enzyme- labelled antibody)]
 - ◊ PNP tablets
- Both positive and negative controls purchased from the company.
- Local healthy and infected controls (leaf tissue or seed) should also be added in the test.
- 96-well microtiter plates (usually these are provided with the kit)
- Plate covers/parafilm
- Distilled or purified water
- Paper towels
- Micropipettes 0-10 µl and 20-200 µl and Micropipette tips
- Tissue homogenizer and seed grinding devices
- Plastic bags, tube mill or disposable grinding chambers
- Plate washer bottle
- Airtight container for incubations
- ELISA reader
- Refrigerator (2-8°C)

3.4. DAS–ELISA Procedure

A general DAS-ELISA procedure is described here (Figure 3), but the protocol provided with the kit must be strictly followed to make sure that the antisera will perform at their best sensitivity and specificity levels.

Buffer preparation: Prepare in advance (at least one day before carrying out the test) carbonate coating buffer, PBST buffer, general extraction buffer, conjugate buffer and PNP buffer, as described in Annex 1.

The day of the experiment:

Prepare the list of the samples to be tested with proper identification and then prepare the layout of the experiment on a log sheet (Fig. 2) that will help to fill the microtiter plate and record the results. Remember that in the 96-wells plate you must use:

- 2 wells per each sample
- 2 wells for the positive control
- 2 wells for the negative control
- 2 wells for extraction buffer only (this will determine whether there are background reactions)
- 2 wells may be left empty (“blank) to standardize the reading of the ELISA reader; this is optional; it depends on the instructions of the equipment.

Therefore, in each plate you will have room for testing 44 or 45 different samples.

- It is not necessary to fill a complete plate in every experiment; if you do not have enough samples, you can use only the wells that you need, preparing the quantity of each reagents according to the number of wells needed. Unused wells can be left empty.
- Remember that in each well you will dispense 100µl of each reagent; therefore, for 96 wells you will need 9.6ml of coating, conjugate and PNP buffers. Usually, 10ml of each reagent is prepared for convenience in the calculation of the dilutions of the antisera and to cope with any pipetting error.

STEP 1: Coating: binding specific antibodies

- Dilute the coating antibody 1:1000 or as recommended by the kit provider in coating buffer (e.g., 10µl of antibody in 10ml of coating buffer)
- Pipette 100µl of the diluted antibody to each well of the microtiter plate.
- Cover the plate with a rubber plate cover or a layer of parafilm.
- Incubate the plate at room temperature (between 20-30°C) for 4h or as recommended by the kit provider in an incubator shaker or overnight in the refrigerator at 4°C.

Note:

- All antibodies and enzyme conjugates should be prepared in a container made of either polyethylene or glass that does not readily bind antibodies. Do not use polystyrene.
- Do not store coated plates longer than 24 hours.

Leaf and seed samples preparation

During the incubation time of STEP 1 prepare the samples to test.

- Weigh the amount of leaf tissue or the ground seed necessary to satisfy the ratio with the general extraction buffer as indicated on the kit protocol: for example, if it is 1:10 w/v it will be 1g of leaf tissue or 1g of ground seed in 10ml of extraction buffer and so on.
- Transfer the amount of leaf in a small plastic bag and the amount of ground seed into a 50ml centrifuge tube.
- Add the required amount of the general extraction buffer (GEB) (1g of the sample: 10ml of GEB).
- Extract the sap from the leaf tissue with a sap extractor; mix the ground seed and the GEB 1X thoroughly.
- While you wait for the incubation of STEP 1 to be over, store the extracted samples (leaf and seed) in the refrigerator at 4°C.

WASH THE PLATE: Use a quick flipping motion to empty the wells into a sink without mixing the contents. Fill the wells completely with 1X PBST using the wash bottle and quickly empty them again. Repeat the washing 3 times. Hold the plate upside down and tap firmly on a folded paper towel to remove excess wash buffer.

STEP 2: Adding samples

- Dispense 100µl of the mixed ground seed sample or leaf extract into each well with a micropipette, changing the tips between samples.
- Dispense 100µl each of positive control and negative control into the positive and negative wells, respectively.
- Cover the plate and incubate at 4°C overnight.
- Wash the plate 6-8 times and blot dry on paper towel. Inspect the wells for presence of sample/plant residue; if present, repeat the wash step and dry the plate.

STEP 3: Prepare enzyme conjugate

- Dilute the conjugate 1:1000 in conjugate buffer or at a ratio indicated by the provider.
- Mix the enzyme conjugate solution thoroughly.

Note: Always prepare the enzyme conjugate within 10min before use.

- Pipette 100µl of the diluted conjugate to each well.
- Cover the plate and incubate at room temperature for 5h.
- Wash the plate thoroughly and blot dry on a paper towel.

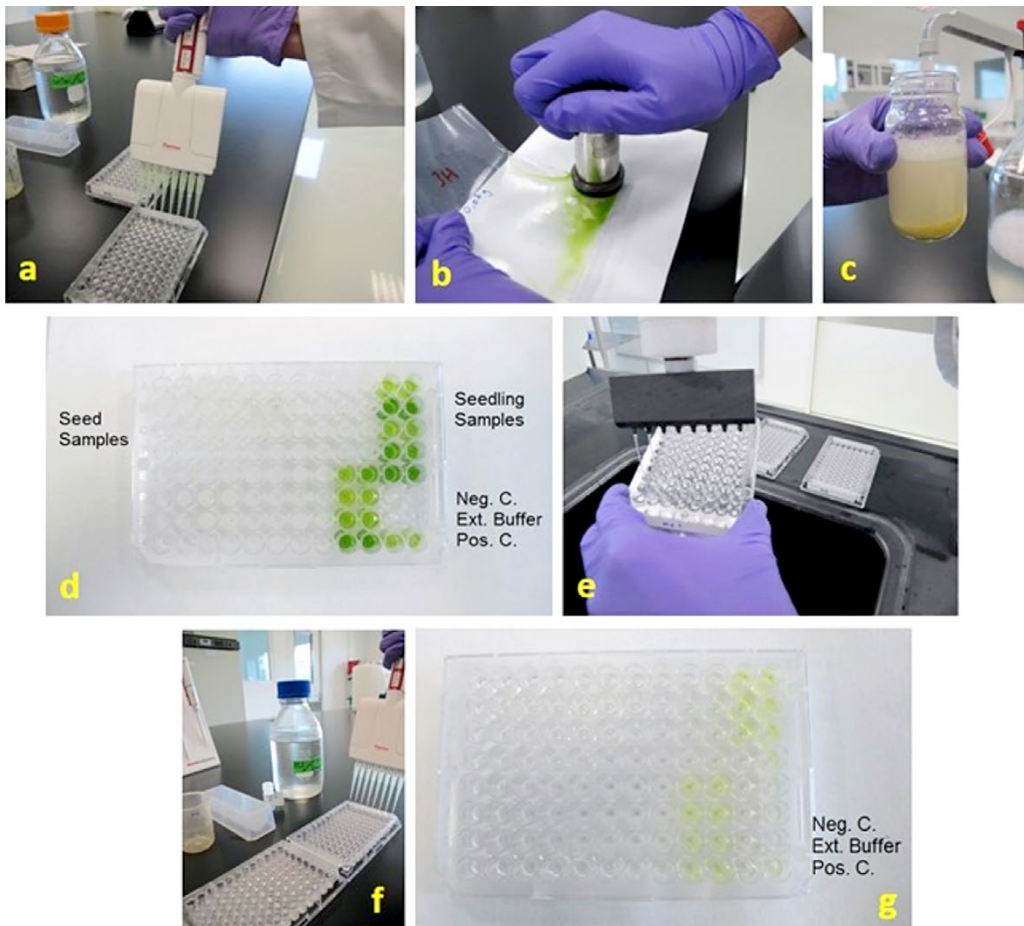


Figure 3. ELISA protocol: (a) plate coating; extraction from (b) leaves or (c) seed; (d) sample distribution in the plate; (e) washing of the plate; (f) adding conjugate; and (g) results. Yellow colored reactions in the plate indicate here MCMV +ve samples.

STEP 4: Substrate preparation

Prepare the PNP solution about 15min before the end of the above incubation step.

- Dissolve the pNPP tablet in substrate buffer to give a final concentration of 1mg/1ml.
- Add 100µl of the substrate solution to each well.
- Cover the plate and incubate at room temperature in the dark.
- Monitor for color change from transparent to yellow both visually and/or photometrically at 405nm (for ELISA readers with individual filters) or at 405/492 nm (for ELISA readers with dual filters).
- Measure optical density values after 60min or when indicated by the kit protocol.

Note: Eliminate all air bubbles present at the time of reading the plate because they can alter results, if in the light path. Add 60ul of 3M NaOH to stop the reaction.

Quality Control

How to assess if ELISA has worked:

1. Wells in which color changes to yellow indicate positive results while wells in which there is no significant color development indicates negative result. Test results are valid only if the positive control wells give a positive result while the negative control and buffer wells remain colorless.
2. Get the average value of the negative controls and multiply by two. Get the average of each sample and compare with the healthy (negative) control.
 - If the average value of the sample is equal to or above 2x healthy control = **positive result**.
 - If 1.5-2x healthy control = **tentatively positive**. In this case the recommendation is to: **REPEAT THE TEST or retest using an RT-PCR assay**.
 - If equal or less than healthy control = **negative result**.

Note: Buffer control must be less than or equal to the healthy control.

Interpretation of ELISA Results

The ELISA results can be interpreted visually based on the color development in the wells of the ELISA plates or with the help of a spectrophotometer, which is more accurate. The wavelength at which the reading should be taken is recommended on the ELISA kit protocols, but not the threshold for establishing whether a sample is positive or negative. There are no easy ways to establish positive-negative thresholds in ELISA, although the threshold to establish positive or negative sample is often 2x the value of the healthy control used in the experiment. Sutula et al. (1986) and Fenlop and Sopp (1991) provided useful guidelines for interpreting ELISA data and determining positive-negative thresholds. In case of ELISA test for MLN viruses, one needs to specifically watch out for “false negatives” (i.e., a test result improperly indicates that there is no pathogen) as these could be more problematic than “false positives” (i.e., a test result improperly indicates presence of the pathogen) because samples can generally be retested to eliminate false positives.

4. Sensitivity and Utility of Diagnostic Methods for MCMV Detection in Commercial Maize Seed Lots

SCMV and other potyviruses are prevalent in sub-Saharan Africa. Their aphid vectors are also prevalent in the region. While control of overall aphid populations might have minor effects on potyvirus transmission, spraying insecticides on the fields infested with aphids does not always reduce potyvirus spread, and is not recommended. Because the viruses are not transmitted through seed, there is no need to test seed for their presence. Therefore, we focus on testing for MCMV.

MCMV is known to contaminate seed of infected plants and could be transmitted through seed (although at very low rates), thus making identification of MCMV-infested seed lots important for preventing long-distance virus spread. We have adapted two commonly used diagnostic approaches for the detection of diverse MCMV isolates in maize seed: ELISA and RT-PCR. Although quantitative RT-PCR (RT-qPCR) has been used for diagnosis of MCMV in seed, experiments in the US indicated that this diagnostic assay does not have increased sensitivity over RT-PCR and the cost is about almost twice higher than RT-PCR (Bernardo et al., 2021; Table 1). The selection of positive and negative controls for all diagnostic assays is quite important. For ELISA especially, the seed extract can produce relatively high background.

Bernardo et al. (2021), based on an analysis of three different diagnostic assays for MCMV detection in seed, indicated that:

1. RT-PCR and RT-qPCR were several orders of magnitude more sensitive than ELISA on a “per virion” basis; however, all the three assays detect viral components, and do not distinguish between infectious and non-infectious virus.
2. ELISA is sufficiently sensitive for most seed diagnostic applications, and has the advantage of the lowest cost per sample (Table 1), with less specialized equipment or training required. Although ELISA is much less sensitive than RT-PCR, the assay is sufficiently sensitive to detect a single infested seed in a sample of 500 seeds.
3. Other diagnostic platforms for MCMV detection are available, including reverse transcription loop-mediated isothermal application (Chen et al., 2017) and Immunostrips, but these diagnostics have relatively lower throughput and require adaptation for use with seed.

Table 1. Assay detection thresholds and costs for MCMV detection. (Source: Bernard et al., 2021).

Assay	Material Detected	Cost/sample ^a	Detection Limit	
			ng ^b	Virions ^c
ELISA	Coat Protein	US\$ 0.94	1.00E-04	13,300,000
RT-PCR	RNA	US\$ 4.97	1.00E-08	4,100
RT-qPCR	RNA	US\$ 10.72	1.00E-08	4,100

^a Approximate cost of reagents per sample.

Costs were calculated based on US market costs, as of February 2020 (details provided by Bernard et al., 2020).

^b Lowest amount of coat protein or RNA always detected across isolates in each assay.

^c Theoretical number of virions detected based on virion RNA and coat protein contents of 2.4E-12 and 7.5E-12 µg/virion, respectively.

In case of Kenya, the calculated cost per sample for ELISA tests ranges between US\$ 5.98 and US\$ 6.31 per sample depending on the source of the MCMV antibody kits. This is relatively higher compared to the cost in the USA (US\$ 0.94) as indicated by Bernardo et al. (2021). In Kenya, the cost of the reagents is the most expensive category (50%) followed by overheads (a third of the total costs). Overheads comprises importation costs (taxes and duties, clearing and forwarding and agents' costs). Molecular-based tests are more expensive (3-4-fold) than ELISA, in general, in Kenya.

4.1. Sample Preparation

There are three options for seed preparation. The seed soak solution (SSS) and seed extract (SExt) produce essentially identical results (Table 2). However, SSS has very low background and does not require grinding. Because many labs test for multiple pathogens or traits in dry ground seed, extracts of ground seed were also tested for MCMV detection. For all the three methods, care must be taken to prevent contamination with MCMV. Air-borne contamination with dust or aerosols from infected seed must be avoided; this is more difficult for dry ground samples.

Table 2. MCMV detection in soaked and dry ground maize seed.

Grind Type	SSS	Sext
Soaked seed	54/54	54/54
Dry seed	-	23/24

4.2. SSS and SExt Preparation

Materials

- 1 quart mason jars (standard mouth) with metal disk and open-center screw lid (or screw-top plastic lids) ([link](#))
- 44 jars = 1 ELISA plate
- 1,000g unknown seed, divided into 100g samples
- 100-200g of healthy background seed, divided into 100g samples
- 5 Liters (lt) 1X GEB ([link](#))
- Agdia ELISA reagent set for MCMV ([link](#))
- 1.5ml tubes
- 200µl and 1000µl pipette tips
- Lab tape
- Weigh boats
- Scale
- Vedco D-256 (viricide for clean-up; [link](#))

Preparation

- Prepare 5 lt 1X GEB ([Annex 1](#)).
- Label jars with tape.
- Weigh healthy control seed (100g) and place in corresponding jar.
- Weight unknown seed (100g) and place in jar.
- Shake each jar by hand for 10 seconds vertically and for 10 seconds horizontally (this is an attempt to mimic mixing that may occur in a seed lot bag).
- Starting with the healthy control jars, pour 1X GEB into each jar. The GEB should cover the seed and be about 2cm above the level of seed. For 100g, this is about 150ml, depending on the size of seed. Use the same amount of GEB for all samples.
- Cover the jars. To minimize laboratory contamination, lids may be sealed with strips of parafilm.
- Jars can be left on bench overnight at room temperature (~23°C).
- Prepare for ELISA next day (Section 3.4).

ELISA using SSS

- Start sampling with healthy control samples, then move to the test samples.
- Cover the jar lid with paper towel; to prevent leaking, shake the jar by hand, first vertically (up and down) for 10sec, then side to side (with the jar horizontal) for 10sec.
- Collect 600µl of the soaking solution from each jar and put into sample tubes.
- Store samples at 4°C until you are ready to load ELISA plate.
- Sample jars should be stored at 4°C until after ELISAs are run, in case resampling is required.

4.3. MCMV Detection using RT-PCR

Reverse-Transcription Polymerase Chain Reaction (RT-PCR) is a sensitive technique that amplifies the virus' genomic nucleic acid. For MCMV and SCMV, the viruses RNA genome is copied into DNA using reverse transcriptase. Then, short nucleic acid sequences (referred as "primers"), in the presence of Taq DNA polymerase, hybridize to opposite strands of the target sequence, and amplify segments during repeated cycles of thermal cycling (warming and cooling). Each amplified segment serves as a new template for amplification in the subsequent thermal cycle. As a result, very small quantities of nucleic acids may be amplified relatively quickly. The results of the amplification may be visualized on an agarose gel, following electrophoresis and staining.

Seed samples (100g) containing a single MCMV-infested seed were either soaked overnight in buffer or dry ground. For soaked seed, the seed soak solution (SSS) and seed extract (SExt) were tested for the presence of MCMV by ELISA. Extracts of dry ground seed were similarly tested. Three independent experiments with a total of 54 soaked seed and 24 dry seed samples were tested. Data presented are the number of positive samples/total number of samples.

To eliminate the need for isolating RNA, samples may consist of seed soak solutions (SSS) prepared as outlined above. If seed extracts (SExt) or ground seed extracts are used, then total RNA should be isolated using a suitable commercial kit (e.g., Direct-zol RNA MiniPrep Plus kit, Zymo Research Corporation). New primers for amplification of a portion of the viral replicase gene that are highly conserved across MCMV isolates were developed that eliminate spurious amplification in RNA from healthy maize. Commercial kits are used for RT-PCR reactions; the instructions below are based on a protocol developed by Peg Redinbaugh (Ohio State University, USA).

Primers

- Specific primers are required for each specific pathogen. Primer specificity and validation are the most difficult steps for obtaining reliable PCR results. The following primers for MCMV and SCMV, as reported by Wangai et al. (2012), can be used to identify MCMV and SCMV:
MCMV Forward Primer: 5'-ATGAGAGCAGTTGGGGAATGCG-3'
MCMV Reverse Primer: 5'-CGAATCTACACACACACTCCAGC-3'
Size of amplicon: 550bp

Primer pair MCMV-2452F (5'-AGTGGAGGTAGGCAGAGTCA-3') and MCMV-3111R (5'-TCCAACAGCAATGTTTCCA-3') produces 660 bp amplicons using one-step RT-PCR was shown to be highly sensitive and specific for MCMV detection in SSS (Bernardo et al., 2021).

SCMV Forward Primer: 5'-GCAATGTCTGAAGAAAATGCG-3'
SCMV Reverse Primer: 5'-GTCTCTACCAAGAGACTCGCAGC-3'
Size of amplicon: 900bp

- Make a master stock solution of each lyophilized primer by adding $x \mu\text{l}$ of Molecular Biology Grade (MBG) to separate primer tubes, according to the concentration declared on the primer manufacturer sheet, to reach a concentration of 100 pmol/ μl (μM).
- Mix thoroughly, and centrifuge briefly (2-3sec) in a microfuge.
- Make from the stock solution 100µl of working solutions 1:10 (10µl of stock solution in 90µl of MBG water).
- Store both the residual primer stock solution and the working primer solutions at -20°C for further use.

Sample and Control Preparation

- **Samples:** The seed soak solution (SSS) may be used directly in RT-PCR. If soaked seed extracts (SExt) are used, then RNA should be extracted from 500µl extract using a commercial kit. Similarly, RNA can be extracted from a thoroughly mixed 10mg sample of dry ground seed and used as samples. Laboratory contamination from grinding should be avoided.
- **Negative controls:** No matter what type of sample is selected, a similarly treated sample from known MCMV-free maize seed should be used as a negative control. A 'water' control should also be included to monitor environmental contamination of reagents with MCMV.
- **Positive control:** RNA isolated from MCMV infected leaf tissue.

RT-PCR amplification

The master-mix for the detection of MCMV by RT-PCR is prepared with reagents as indicated in Table 3. The steps below are followed to make the master-mix.

- Reaction mix: RT-PCR reactions (25µl) are carried out according to the manufacturer's instructions. Add 4 to the number of samples that will be tested to determine how much master mix to make.

Table 3. Master-mix preparation for RT-PCR detection of MCMV.

Component	Initial Conc.	Volume (µl)	Final Conc.	n Rx (µl)
GoTaqBuffer ^a	5X	10	1X	
DTT	100 mM	1.25	5 mM	
Forward Primer	20 µM	1	800 nM	
Reverse Primer	20 µM	1	800 nM	
dNTPs	10 mM	0.5	200 µM	
RNAseOUT ^a	40 U/µl	0.1	4 Units/Rx	
GoTaq DNA Polymerase ^b	5 U/µl	0.25	1.25 Units/Rx	
Superscript III ^c	200 U/µl	0.035	7 Units/Rx	

^a Promega Madison, WI, USA

^b Invitrogen Carlsbad, CA, USA

^c Invitrogen

- Label PCR tubes for each reaction: one for each sample, plus one each for the negative control, water control and positive control.
- Pipet out 23µl of reaction mix into each tube and close the lids.
- Open the tube lids one at a time, add 2µl sample or control into tube. Mix by gently pipetting up and down. Change tips between tubes and take care not to generate aerosols.
- Place tubes into a thermal cycler programmed for 55°C for 40 min, 94°C for 2 min, followed by 33 cycles of 94°C for 15 secs, 55°C for 20 sec and 72°C for 40sec, and then a final elongation step of 72°C for 7 min.
- Amplicons are analyzed in a 1% agarose gel containing ethidium bromide. Use a 100 bp ladder to verify amplicon size of 660 bp.

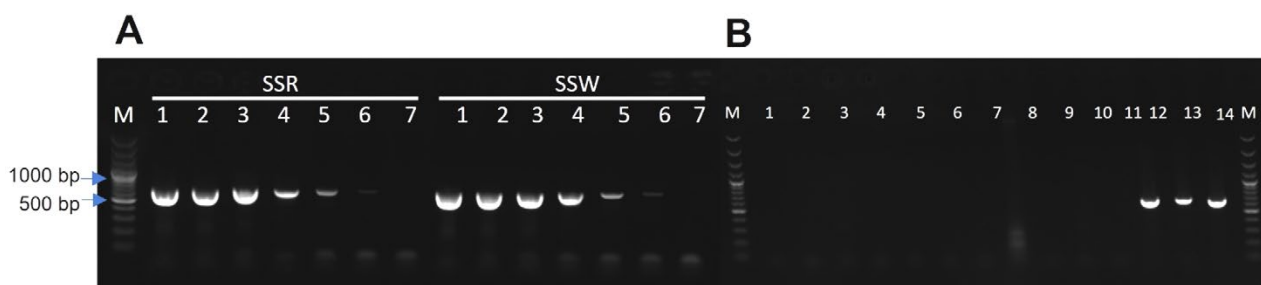


Figure 4. Sensitivity and specificity of RT-PCR for detection of MCMV. **A:** RT-PCR was carried out using serial dilution of RNA purified from MCMV-Kansas virions into Seed Soak RNA (SSR) and Seed Soak Water (SSW) using the MCMV-2452F and MCMV-3111R primers. M, 100bp DNA ladder; lane 1, 100pg MCMV RNA; lane 2, 10pg; lane 3, 1pg; lane 4, 100fg; lane 5, 10fg; lane 6, 1fg; lane 7, water control. **B:** RT-PCR was carried out using total RNA isolated from leaves of maize plants infected with: lane 1, wheat mosaic virus; lane 2, Johnsongrass mosaic virus; lane 3, maize chlorotic dwarf virus; lane 4, maize dwarf mosaic virus; lane 5, maize fine streak virus; lane 6, maize necrotic streak virus; lane 7, maize rough dwarf virus; lane 8, maize rayado fino virus; lane 9, sugarcane mosaic virus; lane 10, wheat streak mosaic virus; lane 11, water control; lane 12, MCMV-Kenya; lane 13, MCMV-Kansas; lane 14, MCMV-Mexico; M, 100bp DNA ladder (ThermoScientific, O'GeneRuler 100bp Plus DNA ladder used above).

5. References

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- Sutula CL, Gillett JM, Morrissey SM, Ramsdell DC (1986) Interpreting ELISA data and establishing the positive-negative threshold. *Plant Disease* 70: 722.
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Annex 1. Preparation of Buffers for MLN Diagnostic Protocols

1. Carbonate Coating Buffer (1X):

Dissolve in distilled water	1000ml
Sodium carbonate (anhydrous)	1.59 g
Sodium bicarbonate	2.93g
Sodium azide	0.2g
Adjust pH to 9.6 and store at 4°C	

2. PBST* (Wash Buffer) (1X):

Dissolve in distilled water	1000ml
Sodium chloride	8 g
Sodium phosphate, dibasic (anhydrous)	1.15g
Potassium phosphate, monobasic (anhydrous)	0.2g
Potassium chloride	0.2g
Tween™-20	0.5g

Adjust pH to 7.4.

* PBST is phosphate-buffered saline solution with a low-concentration detergent solution, such as Tween™ 20.

3. ECI (Enzyme Conjugated Immunoglobulin) Buffer (1X):

Add to PBST (1X)	1000ml
Bovine Serum Albumin (BSA)	2.0g
Poly vinyl pyrolidone (PVP) MW 24-40,000	20.0g
Sodium azide	0.2g
Adjust pH to 7.4 and store at 4°C.	

4. General Extraction Buffer (GEB; 1X)

Add to PBST (1X)	1000ml
Sodium sulfite (anhydrous)	1.3g
Poly vinyl pyrolidone (PVP) MW 24-40,000	20.0g
Sodium azide	0.2g
Powdered egg (chicken) albumin, Grade II	2.0g
Tween-20	20g
Adjust pH to 7.4 and store at 4°C.	

5. PNP (Substrate) Buffer (1X)

Dissolve in distilled water	800ml
Magnesium chloride hexahydrate	0.1g
Sodium azide	0.2g
Diethanolamine	97.0ml

Adjust PH to 9.8 with hydrochloric acid.

Adjust the final volume to 1000ml with distilled water and store at 4°C.

Chapter 6

Managing MLN Quarantine Facilities: Phytosanitary Guidelines

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1. Background

A quarantine site/facility enables prevention of unintentional introduction of pathogens like MLN-causing viruses in MLN-free countries. The MLN Quarantine Facility serves as a platform for safe and thorough evaluation of materials originating at breeding stations or seed production fields typically from outside the country before its use in the breeding programs or deployment efforts in the country or in a subregion.

The Quarantine Facility should be able to contain both the plant and any quarantine pathogen/pest potentially associated with it to prevent the risk of its spread or escape from the facility before the required inspection, testing, treatment, and verification activities are completed and the consignment is released. The facility may consist of a field site, screenhouse/glasshouse, and a laboratory.

In this Chapter, we elaborate on the general and specific phytosanitary guidelines for managing MLN Quarantine Facilities in both MLN-prevalent and MLN-free countries.

2. Requirements for a Quarantine Facility

The following are the minimum requirements for a Quarantine Facility, based on the guidelines from the International Plant Protection Convention (IPPC; ISPM 34, 2010).

2.1. Location

The facility should be in an isolated area i.e., away from areas where related plant species are abundant. In case of maize, the minimum isolation distance should be at least 500m.

2.2. Physical requirements

The physical design of the facility should consider the biology of the pathogen/pest, the insect-vectors, operational procedures, the workflow in the facility, and specific emergency requirements. These requirements relate to delimitation (in case a plant infected with a quarantine pest/pathogen is detected) within the facility, design of treatment systems and equipment, etc.

2.3. Operational requirements

Any quarantine facility should be authorized by the NPPO of the country. There should be appropriate policies and procedures relating to management, personnel, general operation of the facility, record keeping, contingency planning, occupational health and safety, and other aspects of the facility, as well as audit and review of the management system. The operating procedures for a quarantine facility should consider the biology of the pest, how the pest is spread and its requirements for establishment in the environment. The facility must have an incinerator for disposal of quarantined materials.

2.4. Release from Containment

Consignments should be released from a quarantine facility ONLY on completion of one cropping cycle, periodic field inspections, testing, treatment, and verification. NPPOs should implement necessary system to monitor or trace the consignments once they have left the quarantine facility.

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2.5. Example of a Quarantine Facility Operation: MLN Screening Facility at Naivasha, Kenya.

CIMMYT together with the Kenya Agriculture and Livestock Research Organization (KALRO) established the MLN Screening Facility at Naivasha, Kenya, in September 2013 to enable screening and identification of maize germplasm with tolerance/resistance to MLN under artificial inoculation. This facility operates fully like a Quarantine Facility and is now central to screening maize germplasm from both public and private sector institutions in Africa. This facility provides a quarantined and regulated environment to screen the responses of the maize germplasm against MLN/MCMV/SCMV, but no seed multiplication or breeding activities are permitted at the facility. All the plant and seed materials tested in the facility are incinerated after the experiment, and no seed is allowed to go outside the facility.



Figure 1. KALRO-CIMMYT MLN Screening Facility at Naivasha, Kenya.

2.6. Restricted Access

The quarantine site should be a Restricted Access Facility. Strict adherence to the rules and regulations is a fundamental aspect of disease risk reduction. The phytosanitary guidelines should be applicable to personnel, vehicles, equipment, and plant material.

2.6.1. Personnel

- All the persons working at an MLN Quarantine Facility (MLN QF) are required to register and sign a Log Book at the beginning and at the end of the visit to the facility.
- All the persons accessing the MLN QF must also disinfect their hands, wear necessary apron, and shoes, if necessary, prior to entering and leaving the site. Protective clothing should be provided at the site. Prior to exit from the site, field clothes/protective wear and shoes will be contained within sealed plastic bags and promptly laundered or cleaned at the quarantine site.
- Food items or tobacco products must not be allowed into the facility and must be disposed of before entering the facility.
- No person entering the MLN QF will enter any other maize field on the same day after leaving the quarantine facility. A 24-hour restriction period should be exercised.
- Visitors must read and sign the leaflet (see below) that is distributed at the facility entrance.
- Visitors must inform the person in-charge of the facility of their planned visit to the facility. Access to the site must be authorised by the authorities responsible for the site and has to be documented on the visitor's pass.
- Regulatory authorities within where the site is located must communicate and give a list of authorized personnel that can access the facility (e.g., Government Plant inspectors, scientists etc.). These persons should register themselves at the entrance. The list will be provided in paper to the facility manager to attach to the logbook.

<p>MLN-QF Visitor's Gate Pass Please read this carefully.</p> <p>Maize Lethal Necrosis</p> <ul style="list-style-type: none"> • Maize Lethal Necrosis (MLN) is a potentially damaging disease cause by the interaction of two viruses (typically Maize Chlorotic Mosaic Virus (MCMV) and Sugar Cane Mosaic Virus (SCMV) or Wheat Streak Mosaic Virus (WSMV)) • MLN component viruses are actively spread by insect vectors including thrips, aphids and beetles • Caution must be exercised when entering maize nurseries in MLN-endemic countries or designated Quarantine Facilities since insect vectors carrying the MLN viruses can be unintentionally introduced on clothes or in vehicles <p>Clothing and Vehicle Policy</p> <ul style="list-style-type: none"> • Clothing worn in other nurseries or experimental fields within the previous three days must have been laundered prior to entering the MLN quarantine facilities. • Alternatively, clothing can be sprayed with 70% isopropyl alcohol to remove insects prior to entering the MLN nurseries or experimental sites • Vehicles driven to other nurseries and experimental stations must be cleaned and vacuumed before accessing the MLN facilities <p>Safety Precaution</p> <p>Maize field at this station may be sprayed with insecticides and herbicides to control MLN insect vectors as well as alternate host plant species, respectively. Visitors are instructed to check the central notification board postings regarding recent chemical applications and to clearly understand which fields have restricted access. Visitors must observe and adhere to all posted warnings regarding restricted access intervals. Any questions regarding this policy can be addressed directly to the site manager.</p>	<p style="text-align: right; color: red;">ZW0001</p> <p style="text-align: center;">MLN Clothing and Vehicle Policy</p> <p>I have read and understood the clothing and vehicle policy for entering the quarantine facility. Additionally, I will abide by the site notification procedures regarding chemical applications and I will not enter any field that is designated as restricted access.</p> <p>Name:.....</p> <p>Institution.....</p> <p>Signature..... Date: __/__/20__</p> <p>This individual has been authorised by:.....</p> <p>To access the MLN-Quarantine Facility.</p> <p>Signed:..... Date: __/__/20__</p> <p style="text-align: center; color: green;">Please detach and submit at the entrance</p>
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Figure 2. A sample of a visitor's gate pass for controlled and documented access to the MLN Quarantine Facility.

2.6.2. Vehicles and Equipment

- Vehicles will not be usually permitted within the site, unless such vehicles are required to enter the facility for specific field operations.
- In case any vehicle is authorized to enter the facility, the vehicle must be disinfected before and after its use, as per the quarantine guidelines and washed with a clean, jet/soap water for disinfection. For tractors, the driver should wear appropriate personal protective equipment (PPE) during the disinfection process.
- Field equipment used during the cropping season must be cleaned and disinfected before leaving the quarantine site.
- At the end of daily operations, field equipment should be cleaned and inspected prior to storage within the quarantine site.
- Besides cleaning and disinfecting the equipment, there should be a time delay of at least 12 hours before the equipment is re-used at the MLN QF or used in an outside maize field.

2.6.3. Plant Material

- Plant material (e.g., seeds) from outside must be tested for risky pathogens before planting them at the facility. It should be considered as a quarantine material until its phytosanitary status is tested.
- Employees and visitors who are carrying plant and plant materials away from field and green houses for disease diagnosis are required to collect/submit the samples in a covered bag with necessary details.
- No plant materials, seeds or other plant parts or soil from the field or storehouses should be taken from the facility before, during or after the experiments.
- All the plant materials used during the experiment must be incinerated after the experiment is completed.

3. Guidelines for Operation of a MLN Quarantine Facility in a MLN-free Country

- An MLN QF in an MLN-free country should observe at least a 3-month annual maize-free period.
- Each plot in an MLN QF must be rigorously monitored for MLN symptomatic plants and sampled/analyzed for possible occurrence of MLN-causing viruses. Also, non-symptomatic plants must be routinely analyzed using MCMV immunostrips to ensure no possibility of any escape.
- If an MLN symptomatic plant is found in any of the maize entries grown in an MLN QF, this must be immediately reported to the National Plant Health Regulatory Agency according to relevant national regulations, and samples from such symptomatic plants must be re-tested immediately for MLN-causing viruses.
- If any maize sample tested in an MLN QF was found positive for MLN-causing viruses, there should be “zero tolerance” i.e., the entire field must be destroyed following due procedures and the seed lot must be duly discarded, including the following actions:
 - a) Stover must be sprayed with insecticides and burnt.
 - b) The site must be kept free of maize and weeds for at least six months before any new maize materials are grown.

3.1. MLN Quarantine Facility at Mazowe, Zimbabwe: An Example

Through a joint initiative between the Government of Zimbabwe and CIMMYT, an MLN QF was established by CIMMYT and officially opened on 22nd April 2016 at the Plant Quarantine Institute (PQI) at Mazowe (near Harare) in the Mashonaland Central Province. PQI is one of Zimbabwe’s important research facilities run by the Department of Research and Specialist Services (DR&SS). The QF serves as a platform to safely import maize breeding materials to southern Africa, and enables local institutions to proactively implement breeding for resistance to MLN. The MLN QF the first of its kind in southern Africa, has so far managed to successfully screen and further distribute more than 10,000 maize germplasm entries under strict quarantine conditions.



Figure 3. An aerial view of the MLN Quarantine Facility at Mazowe, Zimbabwe.

3.2. Field Management Practices at a MLN QF in a MLN-free Country

- **Agronomic practices:** These include all the standard crop management practices, including seed treatment, fertilizer application, irrigation, weed control, tillage, etc., necessary for obtaining a good crop.
- **Seed treatment:** For production of MLN-free seeds, all the seeds prior to planting must be treated with an appropriate systemic insecticide (e.g., imidacloprid or imidacloprid + thiodicarb), which provide early-stage protection against thrips, aphids and other potential vectors of the MLN pathogens, including beetles (Elbert et al., 1990).
- **Weed control:** Agronomic practices to control weed populations must be implemented to control other grass species that can potentially host MLN-causing viruses. All the grass roadways and walkways around the MLN QF must be regularly managed and sprayed with appropriate insecticides to prevent incidence of MLN-transmitting insect vectors.
- **Planting plan:** Planting plan should be coordinated to account for the prevailing winds with the first plantings placed as far downwind as possible, and subsequent plantings progressing upwind. This planting plan minimizes the “Green-Bridge” effect, because insect vectors generally move from the older to the younger maize plants and wind direction plays a primary role in vector movement.
- **Crop rotation** between maize production cycles with a leguminous non-host species is important. The known hosts of SCMV and MCMV include cereal crops (e.g., sorghum, oats, millets, sugarcane, etc.), common weeds (e.g., Johnson grass), and wild grasses. MCMV incidence is exacerbated in continuous maize production fields (Nelson et al., 2011).
- **Maize-free period** of at least 2-3 months during each calendar year should be rigorously pursued as a policy in agreement with local authorities. Post-harvest monitoring should be performed weekly during that period, and any maize volunteers should be destroyed within the farm.
- The seed produced at the MLN QF can be released ONLY if it has tested negative to the presence of MLN causing viruses after ELISA or PCR analysis.
- All the maize residues from a season’s harvest should be collected and incinerated within the site.

3.3. Field Inspections and IPC Certification

- Planting dates must be communicated to the regulatory authority for necessary permission.
- Field inspection calendars should be coordinated with national plant health inspectors at the time of planting to ensure that all requisite growth stage monitoring can be accomplished in a timely manner.
- Field inspections must be carried out by trained personnel on a weekly basis from germination to maturity to assess the presence of insect vectors and symptomatic plants
- Guidelines from the National Seed Health System, Phytosanitary Field Inspection Procedures (version 1.3-04.04.2019.pdf) (<https://seedhealth.org/wp-content/uploads/2019/04/Field-Inspection-Procedures-Version-1.3-04.04.2019.pdf>) or similar standardized guidelines must be used for field inspections.
- Field visits by national plant health officials to the MLN QF should be coordinated in compliance with the national and state regulations to obtain an International Phytosanitary Certificate (IPC) for each growing season. Number of minimum official visits required will depend on the national plant regulatory authority regulations.
- If any suspicious MLN symptomatic plants are observed during the inspection:
 - ◇ they must be immediately labelled with a clear identification; and
 - ◇ leaf tissue samples must be collected by trained personnel following a well-outlined procedure and submitted to a competent laboratory for immediate testing.
- If any of the sampled plants tests positive to one or both MLN-causing viruses, the following should be done:
 - ◇ An immediate field inspection must be carried out.
 - ◇ The same plant must be re-sampled and taken to the laboratory for confirmation.
 - ◇ All plants around the suspicious plant (even if non-symptomatic) must be sampled and tested.
 - ◇ If only a particular plot is affected and none of the neighbouring or surrounding plants are, ALL the plants from the affected plot must be removed and incinerated.
 - ◇ If the incidence is more than 10% in the neighbouring plots too, plants from the whole field must be immediately removed and incinerated.

3.4. Insect Vector Control Measures

- Weekly surveys should be conducted by trained and internally certified vector scouts under the supervision of the Seed Health Specialist. Scouting for potential vectors should be carried out, but insect identification will be at the level of saying whether it is a potential vector or not.
- Blue and yellow insect sticky traps previously identified (number, plot, position) should be placed in the field according to the pattern suggested by the manufacturer throughout the nurseries, to effectively monitor the air-borne arrival/presence of potential insect vectors.
- Records of vector monitoring activity should include trap identification, date, operator, presence, and quantity of insects.
- Vector monitoring results will be recorded and communicated weekly to Seed Health Specialist.
- Vector monitoring must be carried out also in the 100m surrounding the facility.

3.5. Pest Management

- Most of the pest management practices at an MLN QF are mainly aimed at minimizing the risk of vector-based transmission of the viruses from the site to other areas and vice-versa. An Integrated Pest Management (IPM) approach should be used, comprising:
 - **Biological practices:** based on weeding and removal of plant material which are host to MLN-vectors in the vicinity of the field; monitoring of insect-vectors through yellow and blue sticky traps. Based on the assessment of the presence or absence of insect-vectors on the sticky traps, the timing of interventions, especially insecticide treatments, should be determined.
 - **Chemical practices:** Appropriate environmentally safer insecticides should be sprayed according to manufacturer's specifications to control the insect vectors. Broad-spectrum insecticides and target specific insecticides should be rotated during the spray schedule to prevent building of resistance to any active ingredient in the target insects.
 - ◇ The MLN-QF Manager must record all insecticide applications during the cropping cycle, including date of application, name of the product, doses applied, name of the operator, observations (if any are necessary).
 - ◇ Insecticides to be used vary with target pest or insect-vector, active ingredient, dosage, and spectrum of application.

Field signs (Figure 4) should be posted at the corners of the applied field blocks to prevent unintentional entry of personnel during the Restricted Entry Interval (REI) periods. The posted signs should indicate the pesticide applied, the time and date of application, the expiration time, date of the REI, and a contact name. Field signs must also contain a weather-proof holder with three copies of the specific MSDS sheet of the chemical applied. Field signs must be promptly removed or flipped down at the end of each REI.



Figure 4. An example of a warning sign for Restricted Entry.

3.6. MLN Diagnostics and Monitoring in the Field

- All the plots must be rigorously monitored by trained staff for detection of any symptomatic plant.
- MLN/MCMV occurrence must be rigorously assessed through periodic sampling and analysis of leaf samples from ALL the plots using immunostrips/ELISA (following the protocols described in Chapter 5).
- Internal field inspection records should be maintained, with all the relevant data (date, operator, samples collected, results of testing, number of plants rogued).
- After harvesting, seed lots intended for distribution and exchange to other locations within or outside the country must also be sampled and analyzed using ELISA for MLN- causing viruses.
- If any sample (leaf or seed) of any plot tests positive, seed produced from that specific plot must NOT be transferred to a known MLN-free location either in the same country or outside.

3.7. Seed Harvesting and Testing for MLN-causing Viruses

- Seed should be harvested according to standard procedures. An inventory of the harvested entries should be prepared. This inventory should include identification of the entry, date of planting, date of harvest, weight of the harvested seed among other information needed for further seed distribution purposes.
- After harvest, crop residues should be collected and incinerated at the facility.
- The plots should be monitored for the presence of volunteers; any such volunteers must be eliminated either manually or by using an appropriate herbicide.
- Seed collected from MLN-free plots (stored in the bags) should be sampled using recommended sampling procedures (as described in Chapter 4) and tested for MLN-causing viruses.
- Dry seed of each harvested entry will be tested with ELISA for the detection of MCMV and SCMV, as per the protocol described in Chapter 5.
- Optional seed germination test will be carried out to confirm ELISA results.
- All entries with negative results to ELISA test for both viruses will be released from the MLN-QF and prepared for further distribution.
- Any entry with positive results to ELISA test for one or both viruses must be incinerated at the MLN-QF.
- All the MLN-free seed produced in the QF should be fumigated for insect control by authorized personnel, and a certificate must be issued by an appropriate authority before leaving the QF.

4. Guidelines for MLN-free Maize Seed Production in an MLN-prevalent Country

In an MLN-prevalent country (e.g., Kenya), all the maize nurseries and seed production fields must be rigorously monitored and sampled/analyzed for possible presence of MLN-causing viruses, as below.

- Besides visual inspection, collection of leaf samples and analysis using MCMV immunostrips or ELISA for detection of any infected plant must be undertaken.
- If any leaf sample from a plot in a nursery/seed production block tests positive, the plants in that plot should be removed and incinerated, and the neighboring or surrounding plots should be rechecked.
- Seed for international shipment from an MLN-prevalent country can only be produced in an MLN-free location or seed production field and following due procedures. Even after internal testing, seed lots from that site must be checked for any evidence of MLN-causing viruses, and duly authorized by the appropriate regulatory agency (e.g., KEPHIS) before export to an MLN-free country
- Seed produced from an MLN-free location in an MLN-prevalent country (e.g., Kenya) can be shared with other MLN-prevalent countries in eastern Africa (e.g., Tanzania and Uganda), but NOT to any partner institution in an MLN-free country where a MLN quarantine site has not been established.

NOTE: To the maximum extent possible, seed produced from locations in MLN-free countries must be exchanged with partner institutions in countries where MLN-causing viruses or MLN has not been reported yet. If considered inevitable, international shipment from a location in an MLN-prevalent country (e.g., Kenya) to a partner institution in an MLN-free country can be done **ONLY** through a location that has an MLN Quarantine Facility/Site established. In such cases, the seed material must be first tested in an MLN Quarantine Site before its multiplication and/or further use. The partner institution **MUST** implement rigorously and without fail due procedures either equivalent or as outlined in this document at the MLN QF.

5. Exchange of MLN-free Maize Seed from an MLN-prevalent Country to an MLN-free Country

For seeds to be safely exchanged from an MLN-prevalent country (e.g., Kenya) to an MLN-free country (e.g., Zimbabwe), a rigorous multi-stage testing process is followed by CIMMYT to ensure that there is no escape of any MLN-infected seed. In addition, guidelines stipulated by the the NPPOs of both countries are rigorously followed. We urge every institution (public/private) to follow this protocol for safe exchange of MLN-free maize seed.

- During the seed production, the concerned NPPO must inspect the crop during active growth to rule out the presence of any MLN infection.

- For exchange of maize breeding materials from CIMMYT-Kenya, seeds produced in the nurseries and fields free from MLN-causing viruses at the Maize Research Center at Kiboko, Kenya, are further tested in the CIMMYT laboratory by authorized personnel using ELISA (following the protocol described in Chapter 5).
- The seed lots that tested negative are then subjected to phytosanitary testing by the regulatory authority (KEPHIS). Only when the seed is tested negative for MLN viruses and other pests/pathogens of phytosanitary concern, and a phytosanitary permit is issued, the export process will be initiated.
- When the CIMMYT seed materials are to be exported from Kenya to any MLN-free country (e.g., Zimbabwe), they must invariably pass through the MLN QF (e.g., at Mazowe, as described above). A complete set of documents (list of entries, copy of the international phytosanitary certificate and copy of the seed testing carried out at source) must be sent with the seed and electronically to the concerned official overseeing the MLN QF at the Plant Quarantine Institute (PQI) at Mazowe, Zimbabwe.
- On receipt of the seed by the PQI in Zimbabwe, storage, packing and any other activity related to the seed shall be carried out only at the MLN-QF.
- Storage of the seed will occur in a locked room. Only the MLN QF Manager, the SHS and the Principal Scientist of PQI can have the access key to the seed.
 - ◇ Unpacking: This activity must be carried out under the supervision of the SHS and by personnel authorized by the Principal Scientist.
 - ◇ Before planting, record must be kept of the condition of the seed: condition of the packing on arrival, complete documentation, chemical treatment present or not, number of entries, number of seeds per entry, etc. All this information should match with the information sent from the origin. In case of any discrepancies the Principal Scientist and the SHS must contact the sender to clarify before planting.
 - ◇ Seed preparation for planting and the planting process will follow instructions from the Principal Scientist.
 - ◇ Seed dressing: the seed should be appropriately treated at origin. If it arrives untreated then a suitable insecticide should be applied prior to planting. Thiamethoxam (Cruiser) or imidacloprid (Gaucho) will be used as stipulated in P-5 and WI-4.
 - ◇ Any remnant seed after planting must be kept in its original envelope, counted, and returned to the storage room and eventually sent for incineration.
 - ◇ Record of incineration must be kept at the MLN-QF. The information recorded includes type of material, quantity, identification of the seed, date, and operator.

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Chapter 7

Maize Germplasm Phenotyping for MLN, MCMV and SCMV under Artificial Inoculation at the MLN Screening Facility, Naivasha, Kenya

L.M. Suresh^{1*} and B.M. Prasanna¹

1. Introduction

We describe here the protocols followed by CIMMYT at the MLN Screening Facility, Naivasha, Kenya, for culturing and increase of inoculum for MLN-causing viruses (MCMV and SCMV) in eastern Africa, followed by phenotyping of maize germplasm against MLN (under artificial inoculation with MCMV + SCMV) or for individual viruses (MCMV or SCMV) under controlled (nethouse) conditions.

2. Germplasm Screening against MLN

2.1. Storage of Isolates

Purified MCMV and SCMV isolates (from Kenya) are stored at -80°C, and are also maintained in separate greenhouses, with monthly checks on virus purity.

2.2. Inoculum Increase

Mother cultures of MCMV and SCMV isolates are maintained separately, and the inoculum is increased under controlled conditions, as described below:

- The two viruses (MCMV and SCMV) are maintained separately on susceptible maize hybrids in different greenhouses where strict quarantine measures are observed to avoid cross contamination.
 - Fill at least 10 pots with sterile soil, add diammonium phosphate fertilizer (DAP) and sow each with 5 seeds from a susceptible commercial maize hybrid in each of the greenhouses. These plants will be ready for inoculation two weeks after planting when the plants are at 2-3 leaf stage.
 - From the stock inoculum source (previously tested to confirm virus purity), harvest a few leaves infected with of each of the two viruses.
 - Grind leaves infected with the single virus (confirmed through ELISA) in a mortar and pestle separately in cold, freshly prepared 0.1M phosphate buffer (pH 7.0) in the ratio of 1:10 (1g leaf material: 10ml buffer) and sieve the sap using cheese cloth. Carborundum dust (600 mesh) is added to the extracted sap to create microscopic injuries to the plant leaves for the virus to effectively infiltrate.
 - The young seedlings are inoculated mechanically at the 4th leaf stage by gently rubbing the sap on all the leaves using fingers. A piece of cheese cloth can be wrapped on the inoculating fingers to increase the friction while rubbing.
 - The excess Carborundum is rinsed with distilled water immediately after inoculation.
- Note:** It is advisable to have each of the MLN-causing viruses (MCMV/SCMV) inoculated on different days or by a different person to avoid any cross-contamination.
- Symptom development should be visible about 6 days post-inoculation (dpi) for SCMV, and within 10 dpi in case of MCMV/MLN, starting from the inoculated leaves, but with symptoms more intense on newly emerging leaves. Symptom expression is most prominent within two weeks after inoculation.
 - The presence of the viruses can be confirmed serologically by ELISA two weeks after inoculation. Routine testing for quality control is conducted every two weeks to ensure no possibility of cross-contamination.
 - A weekly spray regime in the greenhouse with systemic insecticides at the recommended rates is maintained to reduce the presence of insect-vectors.

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Notes:

- Plant a susceptible maize hybrid in two separate greenhouses (one for MCMV, and another for SCMV) at a density of 50 seeds/0.2m² in potting trays. If possible, maintain SCMV culture on a variety resistant to MCMV, and MCMV culture on a variety resistant to SCMV to avoid cross-contamination. Maintain cultures on moderately susceptible materials to maintain virulence of the culture.
- Harvest SCMV-infected and MCMV-infected leaves separately after 3rd week of 1st post inoculation (10th leaf stage), cut into 2-inch (5 cm) pieces, and grind in a mortar and pestle in buffer (1g of leaf tissue: 5ml of buffer). Obtain the extract (infectious sap) directly from mortar or by centrifuging for 2 min at 12,000 rpm.
- Add 0.1g of Carborundum per 5ml of SCMV or MCMV extract (infectious sap) and inoculate the susceptible host plants at 1- or 2-leaf stage by mixing and rubbing infectious sap onto the leaves between fingers.

2.3. Inoculum Preparation for Artificial Inoculation of Test Entries

- The material in the greenhouses should be ready for harvesting six weeks after inoculation.
- Harvest leaves from symptomatic plants (1x MCMV: 4x SCMV infected plants)
- Prepare grinding buffer (10mM potassium-phosphate at pH 7.0)
K₂HPO₄ 10.8 g
KH₂PO₄ 4.8 g
Na₂SO₄ 1.26 g
- Dissolve in 1 lt distilled water
- For field inoculation, 6 kg of infected leaf material is required inoculating one hectare of maize plants, following the protocol described in the Section 2.4.
- The inoculum with MCMV + SCMV (1:4) is transferred into mist blowers (motorized power sprayers) that dispense the inoculum at high pressure.

2.4. Artificial Inoculation of Test Entries with MLN (MCMV + SCMV)

- Planting of each of the test entries is done on 3m rows, with a spacing of 75 cm x 25 cm (13 hills per row). Two seeds are sown per hill, but later thinned to one plant per hill.
- Along with test entries, plant appropriate resistant and susceptible checks.
- The first inoculation is done at 28 days after planting or when the crop is at 4-6 leaf stage.
- While inoculating, the person with proper personal protective equipment (PPE) walks along inter-row alleys and spraying seedlings by quickly moving the spray nozzle back and forth, perpendicular to the row to get a 'whipping' action (as if the plants are under a strong wind).
- While the motorized power sprayer is operated by one person, another person guides the action (to avoid skipping any row or plant).
- A second inoculation is conducted seven days after the first, to ensure there are absolutely no escapes from inoculation.
- Standard agronomic practices are followed to encourage good plant growth; however, no insecticides are sprayed during incubation and post-incubation so as to encourage sufficient disease spread in the field through vector transmission.



Figure 1. Inoculation with a motorized sprayer.

2.5. Rating of Germplasm Responses against MLN (MCMV + SCMV)

- Beginning two weeks after the second inoculation, plants are scored for the MLN severity on a weekly (inbred lines) or bi-weekly (hybrids) basis.
- Disease Incidence: Number of plants out of total number of plants in each plot displaying MLN symptoms.

Note: The score is given on a plot basis; however, for some high-precision experiments like fine-mapping or marker validation trials, similar scale is followed but on an individual plant basis.

- MLN disease severity scoring (Figures 2 & 3): Plot level visual scores are recorded on a 1 to 9 scale, as below:
 - 1 = Completely clean plants with no visible MLN disease symptoms
 - 2 = Fine or no chlorotic specks, but no loss of plant vigor
 - 3 = Mild chlorotic streaks on emerging leaves
 - 4 = Moderate chlorotic streaks on emerging leaves
 - 5 = Chlorotic streaks and mottling throughout the plant
 - 6 = Intense chlorotic mottling throughout the plant, with necrosis of leaf margins
 - 7 = Severe chlorotic mottling, mosaic, and leaf necrosis all through the plant
 - 8 = Severe chlorotic mottling, leaf necrosis, dead heart, and premature death of plants.
 - 9 = Complete plant necrosis, and dead plants

3. Germplasm Screening against MCMV in Dedicated Nethouse(s)

The protocols for maintaining MCMV greenhouse-based inoculum increase, and preparation of inoculum are described in Section 2.2. Here we describe methods for screening germplasm responses ONLY for MCMV under artificial inoculation in dedicated nethouse(s) to prevent any possible infection by SCMV (or other possible viral diseases like MSV) through insect vectors.

3.1. Artificial Inoculation of Test Entries with MCMV

- Plant the test entries in 3m rows, with a spacing of 75 cm x 25 cm (13 hills per row). Two seeds are sown per hill, and later thinned to one plant per hill.
- Along with test entries, plant appropriate resistant and susceptible checks.
- The first inoculation is done 28 days after planting or when the crop is at 4-leaf stage as outlined.
- The young seedlings are inoculated mechanically by gently rubbing the sap (with MCMV inoculum) on the leaves using fingers. A piece of cheese cloth can be wrapped on the inoculating fingers to increase the friction while rubbing.
- Second inoculation is conducted seven days after the first, to ensure there are absolutely no escapes from inoculation.
- Normal agronomic practices are followed to encourage good plant growth; however, no insecticides are sprayed during incubation and post-incubation.

3.2. Rating of Germplasm Responses against MCMV

- Evaluate plants weekly for MCMV symptoms after the second inoculation, and repeat this weekly for inbred lines and bi-weekly for hybrids.
- Disease incidence: Number of plants out of total number of plants in each plot displaying MCMV symptoms.

Note: The score is given on a row basis; however, for specific high precision experiments like fine-mapping or marker validation trials, a similar scale is followed but on an individual plant basis.
- MCMV disease severity scoring: Plot level visual scores are recorded on a 1 to 9 scale, as below:
 - 1 = No visible MCMV symptoms
 - 2 = Fine or no chlorotic specks, but no loss of plant vigor
 - 3 = Mild chlorotic streaks on emerging leaves
 - 4 = Moderate chlorotic streaks on emerging new leaves
 - 5 = Chlorotic streaks and mottling throughout the plant
 - 6 = Intense chlorotic mottling throughout the plant, with necrosis of leaf margins
 - 7 = Severe chlorotic mottling, mosaic, and leaf necrosis all through the plant
 - 8 = Severe chlorotic mottling, leaf necrosis, dead heart, and sometimes premature death of plants.
 - 9 = Plant death



Figure 2. MLN disease scoring of maize inbred lines on a 1-9 scale.



Figure 3. MLN disease scoring of maize hybrids on a 1-9 scale.

4. Germplasm Screening against SCMV in Dedicated Nethouse(s)

The protocols for maintaining SCMV increasing viral inoculum in greenhouse-based inoculum increase, and preparation of inoculum are described above in Section 2. Here we describe methods for screening germplasm responses to SCMV under artificial inoculation in dedicated nethouse(s) to prevent any possible infection by MCMV (or other possible viral diseases like MSV) through insect vectors.

4.1. Artificial Inoculation of Test Entries with SCMV

- Plant the test entries in 3m rows, with a spacing of 75 cm x 25 cm (13 hills per row). Two seeds are sown per hill, and later thinned to one plant per hill.
- Along with test entries, plant appropriate resistant and susceptible checks.
- Inoculate plants at 28 dap or when the crop is at 4-leaf stage as outlined.
- The young seedlings are inoculated mechanically by gently rubbing the sap (with SCMV inoculum) on the leaves using fingers. A piece of cheese cloth can be wrapped on the inoculating fingers to increase the friction while rubbing.
- Second inoculation is conducted seven days after the first, to ensure there are absolutely no escapes from inoculation.
- Agronomic practices are followed to encourage good plant growth; however, no insecticides are sprayed during incubation and post-incubation.

4.2. Rating of Germplasm Responses against SCMV

- Evaluate plants weekly for SCMV symptoms after the second inoculation, and repeated weekly for inbred lines and bi-weekly for hybrids.
- Disease Incidence: Number of plants out of total number of plants in each plot displaying SCMV symptoms.
Note: The score is given on a row basis; however, for specific high precision experiments like fine-mapping or marker validation trials, similar scale is followed but on an individual plant basis.
- SCMV disease severity scoring: Plot level visual scores are recorded on a 1 to 9 scale, as below:
 - 1 = No visible SCMV symptoms
 - 2 = Fine or no chlorotic specks, but no loss of plant vigor
 - 3 = Mild chlorotic streaks or mosaic on emerging leaves
 - 4 = Moderate chlorotic streaks or mosaic on emerging new leaves
 - 5 = Chlorotic streaks and mottling throughout the plant
 - 6 = Intense chlorotic mottling throughout the plant, with necrosis of leaf margins
 - 7 = Severe chlorotic mottling, mosaic, and leaf necrosis all through the plant
 - 8 = Severe chlorotic mottling, leaf necrosis, dead heart, and sometimes premature death of plants.
 - 9 = Plant death
- SCMV scores are recorded, starting two weeks after the second inoculation, and repeated weekly for inbred lines and bi-weekly for hybrids.
- Disease Incidence: Number of plants out of total number of plants in each plot displaying SCMV symptoms.
Note: The score is given on a row basis; however, for specific high precision experiments like fine-mapping or marker validation trials, similar scale is followed but on an individual plant basis.
- SCMV disease severity scoring: Plot level visual scores are recorded on a 1 to 9 scale, as below:
 - 1 = Completely clean plants with no visible SCMV symptoms
 - 2 = Fine or no mosaics, but no loss of plant vigor
 - 3 = Mild mosaic symptoms on emerging leaves
 - 4 = Moderate mosaic symptoms on emerging new leaves
 - 5 = Mosaic symptoms throughout the plant
 - 6 = Intense mosaic symptoms throughout the plant, with necrosis of leaf margins
 - 7 = Excessive mosaic symptoms, and leaf necrosis all through the plant
 - 8 = Excessive mosaic symptoms, and sometimes premature death of plants.
 - 9 = Complete plant necrosis, and sometimes even dead plants

Chapter 8

MLN Pathogen-free Commercial Seed Production: Standard Operating Procedures

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1. Need for MLN/MCMV-free Commercial Seed Production and Deployment

The preceding chapters have shown the extent to which viruses that cause MLN can be transmitted through contaminated maize seeds, thereby contributing to the spread of MLN disease within and across countries. Formal and informal seed movement locally and across borders plays a significant role in the spread of transboundary diseases like MLN. Therefore, diagnostic tests for MLN viruses at various stages during seed production and before shipment of seeds is crucial.

Quality seed production leading to regulator's approval as certified seed is key for effective management of MCMV/MLN. Even if a 0.01% infection in a seed field of about 55,000 plants per hectare is missed, this would mean about 5-6 infected plants, which then become sources for further transmission by insect-vectors. If this infected seed production field is harvested, and the seeds find their way to the farmers' fields, in the presence of vectors like thrips and aphids, not only the entire field but also the village itself will be at the risk of devastation by MLN (especially when the farmers plant MLN-susceptible varieties). Therefore, MLN threshold levels in commercial maize seed production fields during field inspections should be close to zero ($\leq 1\%$ infection) from the perspective of infected plants. Fields with higher infection levels should NOT be certified for sale of seed to the farmers. At the seed level, the stringency level should be even higher: zero tolerance for any contaminated seed.

Seed producers/growers, therefore, need to maintain high levels of quality crop management during commercial maize seed production, beginning from field practices to processing. This also calls for the importance of harmonization of seed laws across borders, riding on regional platforms such as EAC, COMESA, SADC, etc. to ensure that the threshold/tolerance levels for MLN in seed fields are the same for ensuring free movement of seed without the risks of MLN spread.

During the early years of MLN outbreak in eastern Africa, most of the local/regional seed companies in the MLN-prevalent countries lacked necessary knowledge of the disease and its transmission, as well as protocols to produce MLN pathogen-free clean seed. Recognizing this critical gap, the African Agricultural Technology Foundation (AATF), the Alliance for a Green Revolution in Africa (AGRA) and CIMMYT, under the USAID-funded MLN Diagnostics and Management Project (2015-2019), worked intensively to: (i) support the commercial seed sector in Kenya, Uganda, Tanzania, Rwanda and Ethiopia in the production of MCMV/MLN-free seed throughout the breeder-foundation-certified seed value chain, and (ii) promote the use of certified clean seeds by farmers in respective countries.

2. A Harmonized MLN-free Commercial Seed Production Checklist

To ensure MCMV/MLN-free commercial seed production and access to the certified seeds by end users, there was a need of developing country-specific harmonized checklists for MLN control and management (**Annex 1**). We, therefore, focused on the development of harmonized MLN management checklists, with proven standard operating procedures (SOPs) for production of MCMV/MLN-free commercial maize seed by the seed sector in MLN-prevalent countries. The SOPs were adapted from the protocols successfully used by major maize seed companies in the USA which produce large quantities of maize seed in MCMV-prevalent areas, especially Hawaii. These protocols were appropriately refined and customized to suit respective eastern Africa countries', including targeted agro-ecologies, existing seed laws, governance and local agricultural pest management practices. The SOPs are expected to guide breeders and seed producers/growers for production and exchange of MCMV/MLN-free seed.

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Representatives of key seed stakeholders in respective countries in MLN-prevalent countries in eastern Africa were systematically involved in the formulation of the SOPs. The stakeholders included representatives of seed companies, contract seed producers/growers, breeders, plant pathologists, national seed trade associations, NPPOs and the Ministry of Agriculture comprising Crop Protection, Inspectorate and Plant Health departments. Discussions revolved around assessing the practicability of the SOPs with a view of identifying key practices that were adoptable according to the country seed laws and common practices, how best to popularize the SOPs, and how to mitigate possible challenges that could arise during the implementation of the SOPs. After these consultative meetings, official communiques were prepared as agreed by the participants. Harmonised checklist with SOPs (Annex 1) along with an ODK-based survey questionnaire (Annex 2) were formulated and used in five MLN-affected countries (Ethiopia, Kenya, Rwanda, Tanzania and Uganda).

3. Fostering Adoption of Harmonized Checklist and SOPs for MLN-free Commercial Seed Production

A major strategy that was used to foster adoption of the SOPs was the involvement of key seed stakeholders in respective countries during the development stage. This instilled ownership of the document by the stakeholders especially seed companies and contract growers, and hence quick adoption and use. Buy-in of the National Plant Protection Organizations (NPPOs) in respective countries was also key to include MLN tolerance thresholds in seed certification procedures. Other strategies for the adoption and use of SOPs are listed below.

3.1. Training of Seed Companies and their Contract Growers on SOPs

A total of 574 participants from NPPOs and NARS institutions, 544 participants from commercial seed companies, and 2313 small-scale contract seed growers in eastern Africa were trained during 2016-2019 on the SOPs for MLN-free seed production. The course content included on-farm MLN diagnostics, disease scouting, leaf and seed sampling, and testing using immunostrips and ELISA.

Most seed companies conduct their seed production using contract growers. It was therefore important to conduct training of not only seed companies but also their contract growers to assist them in understanding and agreeing on the contents of the harmonized checklists for proper implementation. Special focus was given to practices which required hands-on attention; stakeholders were therefore taken to the maize fields for trainings. Some of these trainings included MLN symptoms identification, field scouting and detection using rapid diagnostic kits among others. Rapid MLN diagnostic kits (MCMV immunostrips) were procured and distributed in small quantities as trial kits to the seed companies following individual trainings on their use to facilitate internal quality assurance and early detection of MLN. Seed companies were also encouraged to make private procurement of the kits for their continued MLN surveillance programs.

3.2. Data Collection Tools

Constant follow-up missions with seed companies and their contract growers are key to ascertain the status of MLN in seed production fields, level of adoption and use of the SOPs, and further gauge the effectiveness of the SOPs to seed production. These follow-ups aid in identifying challenges towards adoption and use of the SOPs as well as for devising mitigative actions to ease the use of these SOPs.

Hence, detailed data collection tools were developed and encrypted into the Open Data Kit (ODK) tool (ODK) for real-time monitoring and data collection. From the analysed data, most effective practices as perceived by stakeholders were: a) observing cropping and disease history before planting; b) timely planting (at the onset of rains); c) crop rotation with legume/non-cereal crops; and d) having maize-free windows (2-3 months) in a year. The most challenging practices, on the other hand, were: a) cleaning vehicles and farm equipment before and after use; b) soil testing (sited as expensive); and c) ensuring that the source of basic seed is MLN-free.

3.3. Engagement with Farming Communities

The expected output from the MLN-free commercial seed production initiative is to ensure the end-users i.e., farmers have access to clean MLN-free seed for their planting. Farmer education on MLN management practices is important to prevent MLN viruses' re-infection in farmer fields despite having obtained clean seeds. This is also important because informal seed systems are highly prevalent in SSA, even in a crop like maize (McGuire and Sperling, 2016), including includes farmer-saved seeds. Farmer field schools and trainings were held on MLN management practices, with particular emphasis on obtaining and using clean seed, besides

adoption of MLN-tolerant varieties. Where MLN-tolerant varieties were available, small seed packs of these varieties were distributed in farmer forums in partnership with seed companies to achieve rapid adoption through farmer-led demos.

Farmer sensitization was further conducted by the development and dissemination of relevant Information, Education and Communication materials (IECs) on MLN Management (**Annex 3**). The IEC materials were also translated to Swahili (**Annex 4**) and Amharic (**Annex 5**) for better comprehension by stakeholders, including farmers and maize seed growers. Farmer engagements also included visits to extract first-hand testimonies from the farmers on awareness of MLN disease presence, ability to identify MLN symptoms, routine on-farm agronomic management practices, seed sources, etc.

4. Feedback on Relevance, Efficacy and Practicability of the Harmonized SOPs towards MLN-free Commercial Seed Production

- Some seed growers had a challenge differentiating MLN symptoms from symptoms of nutrient deficiency and moisture stress. This caused a delay in rogueing suspected plants at the early stages of crop growth.
- There were some challenges with the use of MCMV immunostrip kits as they required to be stored under refrigeration for long term use; this limited the use of the kits by some stakeholders who did not have refrigeration facilities.
- SOPs recommend testing of harvested maize seed samples. This was not practiced by many small seed companies (except in Kenya) as the requirement is not enforced and there are no facilities available for its implementation in some countries.
- Adherence to different practices differed; for instance, most seed companies observed timely planting (95%) and management of weeds in seed fields, scouting, rogueing, and destroying of infected plants (90%); while practices such as establishing field history based on last MLN disease records (35%) were less implemented. Some SOPs were not much practiced, such as ensuring healthy seed from the source, seed dressing with systemic insecticides specific for MLN-transmitting vectors, and MLN testing of the harvested seed (with a few exemptions).

5. Summary of Steps towards MLN/MCMV-free Commercial Seed Production and Lessons Learnt

- A questionnaire for data collection was developed (**Annex 2**), especially targeting seed company personnel and breeders.
- The data collection tool was customized to the ODK app.
- In-country consultants were engaged to individually visit seed companies and/or contract growers to collect information related to MLN disease incidence, severity, production site history, scouting and vector management as well as adoption and use of the MLN management SOPs. The consultants were identified on the basis of understanding the MLN terrain and geographical locations of the seed companies and were engaged in the consultative meetings.
- The field officers were instrumental in identifying the MLN-affected regions in each country, identifying the seed companies/contract growers/breeders etc. operating in each of the targeted countries, and in determining the capacity gaps.
- Rapid diagnostic kits (MCMV immunostrips) were piloted and promoted with selected seed companies and NARS institutions for MLN field testing in order to strengthen internal quality control systems for MLN management. This activity was augmented by on-site training and distribution of 50 MCMV immunostrip testing kits to each of targeted beneficiary institutions.

6. Conclusions

While detection of MLN-causing viruses on contaminated seed may not necessarily lead to transmission of the disease to the next generation, from the phytosanitary perspective, it is important to evaluate the presence of MCMV and SCMV in commercial seed lots, especially those meant for exportation to countries where MLN/MCMV is not reported. In practice, keeping a commercial seed production field completely free from the MLN-causing viruses in areas where the disease is widely prevalent requires significant efforts and resources,

but is important for protecting the food security, income, and livelihoods of the resource-poor smallholder farmers (Prasanna et al., 2020). More than 30 seed companies are presently implementing the MLN-free seed production checklist on a voluntary basis in Kenya, Uganda, Rwanda, Tanzania and Ethiopia, and there is scope to further scaling up this to ensure that MLN does not spread to other countries in Africa through commercial seed trade.

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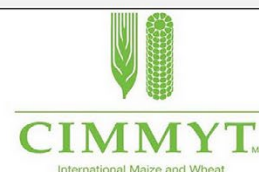
Annex 1. Harmonized MLN Management Checklist & SOPs (Kenya, as an example).



HARMONIZED MLN MANAGEMENT CHECKLIST & SOPs (KENYA)					
No.	TASK	START DATE	DUE DATE	% COMPLETE	NOTES
1.	Monitor crop disease history of seed production fields to enable adequate control plans				
2.	Maintain adequate levels of soil fertility based on soil tests to ensure healthy crops				
3.	Timely planting to facilitate disease escape and eliminate disease incidence due to late planted crop				
4.	Use of disease free seed stocks in subsequent seed production				
5.	Clean farming equipment to remove contaminated soil debris and minimize spread of disease from one field to another				
6.	Eliminate grasses and other weeds from fields and plot borders to remove vector hosting plants				
7.	Monitor and control insect vector population through tested spraying regimes				
8.	Scout for viral symptoms to ensure early detection and control				
9.	Rogue symptomatic plants and burn/bury to minimize spread of disease				
10.	Sample suspect plants for diagnostic testing within the internal quality control to confirm disease presence				
11.	Post-harvest cob selection and seed testing for MLN causing viruses				
12.	Seed treatment using systemic insecticides to ensure early control of the disease				
13.	Crop rotations with non-cereal crops for 1- 3 seasons depending on disease history				
14.	Closed maize season where appropriate				

Name: Position:

Organization: Signature:



Ethiopia: [Harmonized MLN Management Checklist & SOPs](#)
Tanzania: [Harmonized MLN Management Checklist & SOPs](#)
Uganda & Rwanda: [Harmonized MLN Management Checklist & SOPs](#)

Annex 2. ODK Survey Questionnaire for Recording MLN Status, Creating Awareness, and Ascertaining Uptake of MLN Management SOPs and Use of Rapid Diagnostic Kit

1. Name and Location of the Seed Company/ Breeder:

Name of respondent:

Country:

2. Have you experienced MLN infection in your production/ breeding fields? (Y/N) *(For those who answer NO here please let them proceed with Q5 to Q7 for those who answer YES they go on with 3 and 4 then proceed from 8)*

3. If [Yes], when was the last time you experienced the MLN pandemic?

Season (Short/Long)	Year

4. Do you have exposure to MLN SOP? (Y/N)

List of SOPs for verification

5. If Q2 is [No], what are your thoughts with regards to the relevance of SOPs in management of MLN *(Alex here please least at SOPs against a relevant and irrelevant checklist)*

6. Which of these SOPs have been included in your quality management system as seed producers? *(Here list all the SOPs against Y/N)*

7. From your knowledge could you pick the top 4 most effective sops in the control of MLN? *(Alex, only allow for 4 responses here for all the SOPs listed)*

8. If Q4 is [Yes], where was your source of SOP?

AATF/AGRA

Self-generated

Ministry of Agriculture

Other source? Specify

9. How severe was the disease (Scale of 1-5)?

*1 – Not Severe; *5 – Extremely Severe

10. Did you experience any yield loss caused by the disease? (Y/N)

11. How severe was the yield reduction? (100%, 50%, 30%, 20%, 10%, <10%)

12. Specify the location of the disease (country, County, region, district, location/village)

13. Did the disease cause abandonment of any breeding, testing or seed production sites? [Yes/No]; If [Yes], where _____?

14. If Q10 is [Yes], how was the infected material destroyed? [1=Burning, 2=Burying,3=Fed to livestock, 4=used as grain]

15. Do you have any management practice for the disease? (Y/N)

If [Yes]: List them *(Tick against SOPs documented at number 4).*

16. What are your top 4 most effective MLN Management practices?

If [No] in Q15: Why?

17. How often do you monitor your seed production field for MLN symptoms and vectors? *The frequency should be per season (Short and Long seasons)*

18. Have you in the past sampled any suspected MLN plants in your fields for diagnosis? (Y/N)

19. If [Yes], what technique was used to confirm samples for MLN viruses? (PCR, Rapid kits, LAMP, sent to external Lab, I do not know)

20. If Q19 is [sent to external lab] please give the name of the lab?.....
21. Have you used the MLN Rapid Diagnostic Kits? (Y/N)
If [No] in Q21: Provide a reason:
22. Do you routinely use MLN Rapid Diagnostic Kits? (Y/N)
If [Yes] in Q20 what is the frequency? (*Number of times per season*)
23. Provide a reason for this rate of frequency:
24. How would you rate efficiency of the RPD kits? (Not effective, Effective, Very effective)
(Explain)
25. Have you attended a training on MLN diagnostic Kits (Y/N)?
If [Yes], when and where?
How many times in the last 5 years?
26. Number of Kits provided by AGRA/AATF
27. How long has your company participated in seed production and commercialization?
28. How long has your company been involved in MLN disease management?
29. Approximately how much is spent on chemicals for controlling MLN insect vector population?
30. What is the total area of land that you use for maize seed production? (in ha)
31. Out of this area what proportion has been affected by MLN disease? (in %)
32. Are your neighbor fields (farmers' fields) infected by MLN disease (Y/N)?
If [Yes], what measures are used by farmers in controlling it?
33. Do you import any seeds (Y/N)?
34. If q.33 is Yes, where do you import the seeds (maize) from?
35. If q.33 is Yes, what measure do you put in place to ensure you import disease free seed?

Annex 3. MLN Disease Management in Farmers' Fields.

IEC Materials - Fliers

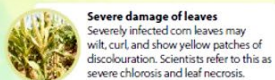
12 steps for control of MLN disease in farmers' fields

What is MLN disease?

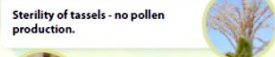
- Maize Lethal Necrosis (MLN) disease is a viral disease that affects maize
- MLN disease is caused by the co-infection of Maize Chlorotic Mottle Virus (MCMV) and any of the following viruses: Sugarcane Mosaic Virus (SCMV), Maize Dwarf Mosaic Virus (MDMV) and Wheat streak mosaic virus (WSMV).
- In Africa, the disease was first reported in Kenya in September 2011, and later in Tanzania, Uganda, Rwanda, Democratic Republic of Congo, South Sudan and Ethiopia by 2014.
- MLN disease is a major challenge in maize production and is considered a threat to food security in Africa because it causes yield losses of up to 100 per cent.

Symptoms of MLN disease and Transmission Modes

MLN disease symptoms include:



Severe damage of leaves
Severely infected corn leaves may wilt, curl, and show yellow patches of discoloration. Scientists refer to this as severe chlorosis and leaf necrosis.



Sterility of tassels - no pollen production.



Poor or no grain filling on cobs.



Premature drying of husks



Short internodes (part of a plant where leaves emerge)



Dead heart symptoms - withering and drying of central shoot.

How is MLN disease transmitted?

Insects can host and transmit MLN virus (Insect Vectors). Some of these insects include thrips, aphids, leaf beetles and rootworms.



Thrips



Aphids

MLN disease is also seed-borne (i.e. seed produced from an infected plant may carry the virus).



Animal, people, farm machinery or farm tools can also spread MLN viruses through movements in infected fields.



How can farmers prevent spread of MLN disease? Farmers can avoid the transmission by following 12-steps

- Find out the cropping or disease history of the field before planting. Practice crop rotation where necessary.
- Monitor the hold every week for presence of insect vector population. A high insect vector population increases chances of attack.
- Make sure the soil in the farm is fertile at all times. This ensures crops are healthy thus can fight disease infections.
- Remember chemical control of insect vectors can be done using recommended insecticides (Once every 1-2 weeks).
- Use certified seed that is free of MLN disease.
- Try to search weekly for MLN viral symptoms for early detection and control of insect vector.
- Avoid using grains as seed.
- Uproot MLN symptomatic plants and destroy them through burning and burying outside the farm to reduce spread of disease. Do not feed infected plants to livestock - animals such as cattle grazing in MLN infected plants can transmit the viruses from infected MLN plots to healthy maize fields.
- Clean farm equipment or tools using disinfectants before and after use to eliminate MLN virus contamination.
- Plant early at the onset of rains.
- Maintain a clean farm by removing grasses, weeds and other alternative hosts from fields.
- Practice crop rotation for at least one season by growing non-cereal crops preferably legumes (beans, soya and peas). Avoid continuous cropping of maize ensuring a closed maize season of at least 2 months where possible.



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Annex 4. MLN Disease Management in Maize Fields (in Swahili)



Hatua 12 za kuzingatia ili kupata mbegu bora ya mahindi isiyokuwa na Ugonjwa wa Mnyauko (Maize Lethal Necrosis-MLN)

Ugonjwa wa Mnyauko (Maize Lethal Necrosis-MLN) ni nini?

Ni Ugonjwa wa mahindi unaosababishwa na virusi. Ugonjwa huu husababishwa na muunganiko wa virusi vya aina mbili: Virusi vya Mabaka na Madoo ya Mahindi-Maize Chlorotic

Mottle Virus (MCMV) na mojawapo kati ya virusi viuatavyo: Virusi vya Batobato ya Miwa-Sugarcane Mosaic Virus (SCMV), Virusi Vidumaza vya Batobato ya Mahindi-Maize Dwarf Mosaic

Virus (MDMV) na Virusi Michirizi vya Batobato ya Ngano-Wheat Streak Mosaic Virus (WSMV)

	<p>1 Pata historia ya shamba pamoja na ugonjwa katika shamba la kuzalisha mbegu kabla ya kupanda</p>		<p>2 Hakikisha udongo una rutuba ya kutosha ili kuweza kupata mimea iliyo bora. Inashauriwa kupima rutuba ya udongo kila baada ya kipindi kisichozidi miaka mitatu ili kuweza kutambua aina ya mbolea itakayotumika</p>		<p>3 Tumia mbegu iliyothibitishwa kutokwa na virusi vya ugonjwa wa Mnyauko katika kuzalisha mbegu.</p>
	<p>4 Safisha zana za kilimo kabla na baada ya kulima ili kuondoa uchafu unaoweza kuwa na virusi vya ugonjwa</p>		<p>5 Panda mvua zinapozna kunyeshwa ili kuepukana na ugonjwa</p>		<p>6 Kagua shamba kila wakati ili kuweza kutambua kama kuna dalili za ugonjwa. Ondoa nyasi, magugu na takataka zingine shambani zinazoweza kuhifadhi au kuficha virusi vya ugonjwa wa Mnyauko</p>
	<p>7 Thibiti wadudu wanaoenea virusi vinavyosababisha Ugonjwa wa Mnyauko kwa kupuliza viuatilifu kwa kutuata utaratibu unaokubalika.</p>		<p>8 Chunguza dalili za ugonjwa shambani, ng'oa, choma au tukia mimea yote itakayoonekana kuwa na dalili za ugonjwa nje ya shamba.</p>		<p>9 Chukua sampuli za mimea itakayohisiwa kuwa na virusi, chunguza sampuli hizo kwenye baabara (au kwa kutumia kifaa maalum) ili kuthibitisha kama mimea hiyo ina ugonjwa.</p>
	<p>10 Baada ya kuvuna chagua magunzi pamoja na punje zenye ugonjwa, viteketeze kwa kuvichoma moto.</p>		<p>11 Pulizia mbegu viuatilifu vinavyoshauriwa ili kuweza kuepukana na Ugonjwa wa Mnyauko</p>		<p>12 Tumia kilimo cha mzunguko wa mazao (kwa kupanda mimea aina ya mikundekunde) angalau kila baada ya msimu mmoja</p>

Dalili za Ugonjwa wa Mnyauko wa Mahindi

- Madoadoa machache au mengi kwenye jani ambayo huanzia chini ya shina la jani changa na kusambaa kuelekea kwenye ncha yake.
- Majani machanga hukauka kabla ya mmea kuchanua, hali hiyo husababisha dalili inayoitwa 'kifo cha moyo', halimaye kifo cha mmea mzima.
- Jani hukauka kuanzia kwenye kicho zake na kusambaa kuelekea katikali, halimaye jani lote.
- Mmea hudumaa na kukomaa kabla ya wakati wake.
- Wakati mwingine mimea huwa tasa (hakuna utengenezaji wa chumvua).
- Gunzi huwa na mbegu chache zilizo hafu au kutokuwa na mbegu kabisa.
- Maganda ya mhindi hukauka kabla hayajakomaa wakati sehemu zingine za mmea zikiwa bado ni mbichi (zikiwa na rangi ya kijani).
- Gunzi huozwa
- Mashambulizi ya kuvu wenye rangi ya kijivu kwenye magunzi yaliyoza hujitokeza kama maambukizi nyemelezi



Namna Ugonjwa wa Mnyauko unavyoeneza

Ugonjwa wa Mnyauko hueneza kwa njia zifuatazo:

- Wadudu wa aina mbalimbali, wakiwemo: Vidukari na Vithiripi-hula kwa kufyonza utomvu wa mahindi; minyoo ya mizizi; panzi na mbawa kavu-hushambulia majani.

- Wanyama wa aina mbalimbali, binadamu pamoja na zana za kilimo-katika kuzihamisha kutoka shamba moja hadi jingine (kunaweza kukawa na shamba lenye ugonjwa)
- Huenezwa pia na mbegu ila ni kwa kiasi kidogo



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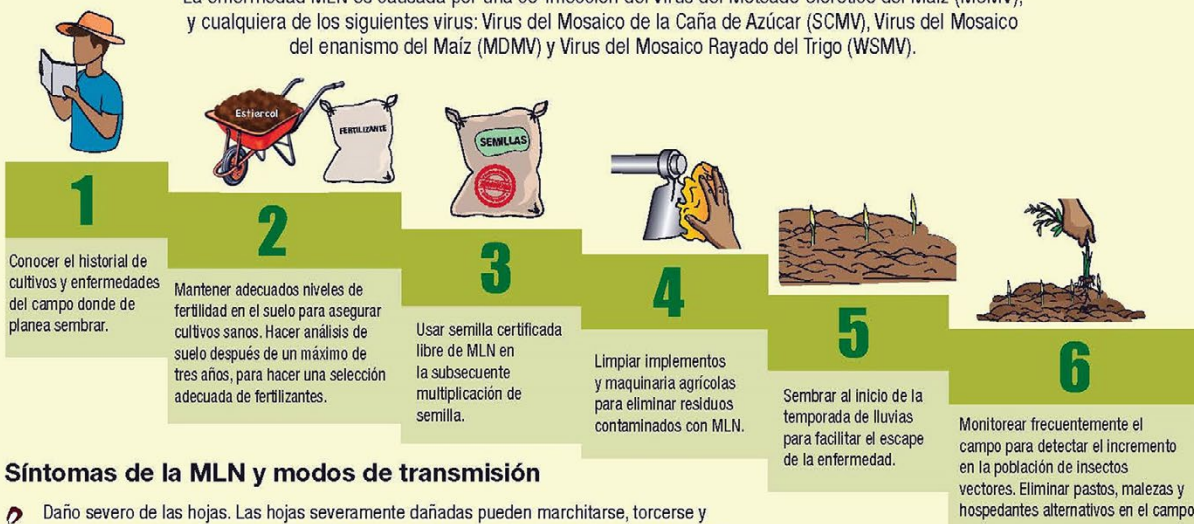
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Annex 6. Twelve Steps for MLN-free Seed Production (in Spanish).

Doce pasos para tener semilla libre de MLN

La Necrosis Letal del Maíz o MLN (por sus siglas en inglés, Maize Lethal Necrosis) es una enfermedad viral que afecta al maíz.

La enfermedad MLN es causada por una co-infección del Virus del Moteado Clorótico del Maíz (MCMV), y cualquiera de los siguientes virus: Virus del Mosaico de la Caña de Azúcar (SCMV), Virus del Mosaico del enanismo del Maíz (MDMV) y Virus del Mosaico Rayado del Trigo (WSMV).



Síntomas de la MLN y modos de transmisión

- Daño severo de las hojas. Las hojas severamente dañadas pueden marchitarse, torcerse y presentar manchas amarillas y un secado prematuro. Los científicos se refieren a esto como clorosis severa y necrosis foliar.
- Espigas estériles. No hay producción de polen.
- Pobre llenado de grano, o no desarrollo de grano en las mazorcas.
- Secado prematuro de las hojas que cubren las mazorcas.
- Entrenudos cortos (los nudos son donde crecen las hojas).
- Síntomas de corazón muerto (marchitez y secado del brote central o cogollo).



¿Cómo se transmite la MLN?

- Los insectos son vectores y transmiten el virus de la MLN; estos vectores incluyen trips, áfidos, escarabajos foliares y gusanos de la raíz.
- La MLN también es transmitida a través de la semilla, es decir, la semilla producida en una planta enferma puede llevar el virus.
- La gente, los animales, la maquinaria e implementos agrícolas, también pueden acarrear el virus a través del movimiento en los campos infectados.

Chapter 9

MLN Early Warning and Emergency Preparedness Plans

Francis Mwatuni¹ and B.M. Prasanna^{2*}

1. Introduction

According to Article IV of the FAO-International Plant Protection Convention (IPPC), the NPPOs are responsible for conducting surveillance of cultivated and wild plants to determine the status of pathogens/pests in the country (IPPC 2015-2016 Procedure Manual) including reporting of the outbreak (ISPM 17) occurrence, and spread of such pathogens/pests and their control. Article VIII of the IPPC describes the importance of the obligation to notify pathogen/pest occurrences and the status of such pathogens/pests to trading partners. However, it is upon the country-specific NPPOs and regional plant health authorities in collaboration with other partners to monitor for any new occurrence of a pathogen/pest, effectively control the threat, and inform the country authorities and regional neighbors on its occurrence.

2. MLN Early Warning System

An early warning and rapid alert system on the emergence of MLN or other transboundary plant health threats is of utmost importance. This includes guidelines for effective and rapid response after detection of a new pathogen/pest, proper identification, and mitigation using an emergency preparedness plan (EPP). A strong understanding of proper diagnosis and management of a devastating transboundary threat, such as MLN, is critical for the NPPOs and regional plant health authorities for preventing further incursion. In case an incursion of MLN could not be prevented in a presently MLN-free country for any reason, putting together comprehensive rapid response measures to prevent the establishment and further spread of the disease is important to protect the food security, income, and livelihoods of maize-dependent smallholder farmers.

3. Emergency Preparedness Plan (EPP) against MLN

An EPP against MLN should include the following elements:

1. Steps for quickly monitoring the location(s) of outbreak of MLN, if an incursion happens in a presently MLN-free country or a specific area within a country, along with appropriate mitigation measures
2. Identification of emergency response actions, and institutions responsible for implementation of these measures, if MCMV/MLN is detected in a new area.
3. Outlining a concrete MLN surveillance system, including “delimiting surveys”.
4. Description of requirements for quarantining the pathogen(s) to contain the disease to a specific area, and to prevent the pathogen(s) from spreading to other areas.
5. Designing an MLN mitigation program in consultation with relevant agencies.
6. Advocacy for sound legislative measures to enforce emergency response, including containment and eradication actions.
7. Strengthening institutional arrangements to provide funding to execute the MLN early warning system and mitigation plan.

4. Plant Health Decision Framework

A well-formulated decision-making process is vital for timely implementation of activities in an emergency preparedness plan. The following may be considered for the plant health decision framework:

- Prevention, preparedness, response, and recovery are broad terms within a framework of an emergency, such as MLN outbreak.
- Plant health emergencies typically unfold in a series of steps, beginning with the initial detection, and ending with eradication, if possible.

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- Parallel tracks may also be established with necessary operational aspects. These could include scientific, technical support, and regulatory development. Others are enforcement, data collection, and communication with relevant stakeholders, including partner agencies.
- Effective communication and cooperation with the industry and the public is paramount for transboundary pest/disease management.

5. MLN Surveillance and Detection Procedures

Prevention is always better than cure! NPPOs and other plant health authorities in the presently MLN-free but high-risk countries in sub-Saharan Africa are advised to proactively implement MLN surveillance (based on the protocols described in **Chapter 4**) on a routine basis during the major maize crop seasons. MLN survey procedures are designed to assist with the detection, delimitation and monitoring of disease incidence. These protocols need to go hand-in-hand with diagnostics (especially using immunostrips or ELISA, as described in **Chapter 5**), so as to ensure that even non-symptomatic plants do not have MLN-causing viruses.

Various entities may make the first identification or diagnosis, if MLN incidence is suspected. These include NPPO staff or MLN experts from relevant research institutions. State departments, university, or private/regional laboratories can also be engaged, if already trained on MLN diagnostics and surveillance protocols. If required, an overseas laboratory may also be utilized for proper preliminary diagnosis, especially if the local diagnostics capacity is inadequate. Final confirmation by internationally recognized entities could be helpful.

A delimiting survey should be used to establish the magnitude of pathogen incidence in a specific area ([ISPM No.6, Updated 2018](#)). The results from the delimiting survey could be used to determine the type and extent of control measures to apply. The response may be immediate or may require further deliberation and consultation within the NPPO and with necessary Government agencies.

6. Emergency Action Notification

Emergency Action Notification can be issued pending positive identification or further instruction from the Ministry of Agriculture or the official NPPO in that country. This is based on the information from plant health officers from the affected areas. If necessary, the NPPO Administrator will issue a letter directing the field offices to initiate a specific emergency action under the Plant Protection Act of the country. This will then facilitate Emergency Quarantine Action.

7. Initiating an Emergency Quarantine Action and Response Program

The Program consists of detection and delimitation, and may be followed by programs in regulation containment, eradication, and control. It may be advisable to form a country or regional New Pest Advisory Team to evaluate the outbreak of a new pathogen/pest, assess the risk to country's/regional plant health, and the potential economic impact. The team may also consult relevant experts and regulatory personnel and make recommendations to the regional plant health authorities and NPPO management for a specific course of action.

The multi-disciplinary response program for a transboundary threat like MLN may include various activities, such as:

- Undertaking rapid detection and delimiting surveys
- Formation of a technical working group
- Strengthening capacity for diagnosis and management of MLN-causing viruses, especially MCMV
- Emergency funding for the response program
- Emergency response coordination
- Mobilization of relevant institutions, including public and private sectors.

8. MLN Monitoring and Evaluation

- Use monitoring surveys to gather relevant information that assists in planning and implementing a strategy for MLN containment, management and eradication.
- Monitoring surveys are also used to evaluate the effectiveness of actions taken to contain/manage/eradicate MLN.
- Continue management and eradication measures for as long as considered necessary.
- After the termination of suppression or eradication measures, keep monitoring the success of the program with periodic (half-yearly) reports on MLN incidence status.

Chapter 10

MLN Management: Conclusions and Future Perspective

B.M. Prasanna*

1. Introduction

MLN management must be effectively addressed through several simultaneously implemented strategies, including development and deployment of elite MLN resistant varieties, agronomic mitigation practices, crop rotations (especially with legumes), etc. (Prasanna et al., 2020). Prophylactic measures are very important to prevent further spread of MCMV/MLN from the presently prevalent to the non-endemic areas either within a country or across countries. While significant success has been achieved so far in preventing the spread and impact of MLN from the eastern African countries to the MLN-free southern Africa or West Africa (which are also major maize-growing regions), there is no scope for complacency. The best management practices outlined in this Manual need to be rigorously implemented by stakeholders at various levels.

2. Key Responsibilities of Different Stakeholders for Effective Management of MLN

MLN management requires coordinated and synergistic efforts of various institutions engaged in maize R&D, support from the policy makers and the Governments, and greater commitment from all the players involved in the maize seed value chain in Africa.

Governments

- Mandate and enforce synchronized maize planting, maize-free time windows (at least 2-3 months each year), and maize crop rotation with legumes in MLN-affected areas to break the MLN virus cycle.
- Strengthen national phytosanitary capacities for effective surveillance and monitoring of major crop pathogens and pests in the region by linking the NPPOs.
- Implement harmonized MLN surveillance and diagnostic protocols.
- Mobilize a dynamic extension system to create adequate awareness among the farming communities on appropriate MLN diagnosis and management measures.

NPPOs

- Ensure proper phytosanitary certification to ensure that MLN-contaminated commercial seed is not exchanged even involuntarily between MLN-prevalent and MLN-free countries.
- Establish MLN quarantine sites in countries where the disease is not prevalent for safe exchange of maize germplasm for research-for-development.
- Use accredited laboratories with harmonized MLN virus diagnostic protocols to test for MLN viruses and issue appropriate certification.

Researchers

- Identify, validate and deploy effective measures to curb the spread and impact of MLN, and to eliminate the possibility of MLN-contaminated commercial seed.
- Proactively develop and deploy elite varieties with MLN resistance and other farmer-preferred traits.
- Analyze and recommend economically viable options in terms of agronomic management, including crop diversification, crop rotations etc. in MLN-affected areas/countries.

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Commercial Seed Companies

- Rigorously Implement the checklist with SOPs, as described in this Manual, to produce MLN-free seed all along the seed value chain.
- Commercialize ONLY MLN-free seed to farming communities within or across countries.

Extension Service Providers

- Provide well-informed, science-based, and clear instructions to the farmers as to the actions to be taken for proper diagnosis and management of MLN.
- Create awareness among farming communities and encourage use of integrated MLN management practices, including use of certified seed of elite MLN-resistant varieties, synchronized plantings, proper agronomic management of maize fields, maize-free time windows (for at least 2-3 months in a year), crop rotations with non-cereals (especially legumes) etc.

Farmers

- Adopt and use proven practices for integrated MLN management, especially in the prevalent areas/countries.
- Share indigenous knowledge and technologies related to MLN management to enhance generation of sustainable solutions in terms of MLN disease management.

3. Conclusions

MLN management in sub-Saharan Africa (SSA) is a complex challenge. Nevertheless, through extensive partnerships, research and development institutions have been able to respond rapidly to this serious threat to the food security, income and livelihoods of millions of smallholder farmers and their families in SSA. MLN management has been effectively addressed through several simultaneously-implemented strategies, including a) development and deployment of elite MLN tolerant/resistant varieties adapted to Africa; b) strong engagement of the NARES and NPPOs on MLN surveillance; c) synergistic multi-disciplinary efforts of various national and international institutions; d) intensive awareness creation among stakeholders, and capacity building of relevant public and private sector institutions on MLN diagnostics and management; e) codeveloping with national partners, and implementing harmonized checklists and SOPs for MLN-free commercial seed production and exchange, etc. (Prasanna et al., 2020).

While significant progress has been made on curbing the spread and impact of MLN in Africa (Prasanna et al., 2020), it is important to continue implementing an integrated disease management approach for sustainable management of the disease in the MLN-prevalent countries whether in Africa, Americas or Asia, and continued efforts on MLN disease monitoring and surveillance globally. Elite maize hybrids with climate resilience and tolerance/resistance to major diseases and insect-pests must be deployed at scale. Good agronomic practices (e.g., maize-free window for at least 2-3 months in areas where monocropping is being practiced; crop rotation with legumes, etc.) are critical to break the cycle of MLN-causing viruses like MCMV. Intercropping of maize with suitable leguminous crops is also key for effective management of diseases like MLN and insect-pests like fall armyworm (*Spodoptera frugiperda*) (Prasanna et al., 2018, 2021).

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