Maize Lethal Necrosis (MLN):

A Technical Manual for Disease Management

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Chapter 7

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

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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^{0}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/0.2m^2 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)
  K_2HPO_4 10.8 g
  KH_2PO_4 4.8 g
  Na_2SO_4 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