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Zimbabwe is Free from Maize Chlorotic Mottle Virus (MCMY): The Chief Virus for Maize Lethal Necrosis

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ABSTRACT

Zimbabwe is one of the largest exporters of hybrid maize seed to markets in East Africa, Southern Africa and recently into West Africa. This export business is threatened by invasive pests likely to impact market access, food security and nutrition security in Sub-Saharan Africa. Chief among the pests is Maize lethal necrosis (MLN), which vector spreads maize disease caused by the co-infection of Maize Chlorotic Mottle Virus (MCMV) from the genus Machlomovirus in the Tombusviridae family and any Potyviridae virus and it can cause up to 100% yield losses. A surveillance study was carried out from 2016 to 2022 in Zimbabwe, to determine the status of Maize Chlorotic Mottle Virus (MCMV). Maize seeds, maize grain and maize green tissues were collected from the ten provinces of the country (divided into 45 survey sites/districts based on the production status of maize crop). Sampling points were at least 20 km radius apart from each. Green maize tissues were sampled from the fields and Bioreba-agristrips rapid test kits were used to test for the virus. Maize grain and maize seeds were sampled from storage centers from the grain stores, agro-dealer retail and wholesale markets, seed company depots and entry border ports. 1,030 green tissue samples, 1503 seed and maize grain and 45200 maize germplasm were tested for MCMV using Bioreba-agristrips and or Enzyme Linked Immunosorbent Assay (ELISA). All samples tested negative for maize chlorotic mottle virus. A geo-spatial map was drawn. This study also showed that Sugarcane Mosaic Virus, one of the synergistic pathogens for maize lethal necrosis disease development, is positive for Zimbabwe's southern areas. Data from this study revealed that Zimbabwe is currently free from MCMV, the chief Maize Lethal Necrosis causing virus in maize production and productivity.

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Introduction

Maize is a critical food security crop for Zimbabwe [1-3]. It is consumed by the majority of the population with an estimated per capita consumption of 110 kg per year [4]. Production of maize in Zimbabwe covers an area of at least 1.2 million hectares. Mostly, the crop is produced by the small-holder and with the other components coming from large commercial farms. Over 80% of maize in Zimbabwe is produced by smallholder farmers for subsistence and livelihood [5]. Zimbabwe is also currently one of the largest exporters of hybrid maize seed to markets in East Africa, Southern Africa and recently West Africa [6]. Any disruption to maize production including maize seeds due to pests and other production constraints has impacts on the availability and price of maize grain and maize seed nationally and regionally. One of the major duties of quarantine officials and pest risk assessment staff is to institute quarantine control measures as a legal method for pest and disease control. Quarantine is critical in making sure that the introduction of new pests is reduced through border control and the use of phytosanitary measures for imported consignments that could act as pathways for pests' introduction. It is very critical to note that surveillance is one of the key components for the determination of the pest status of a country or production site including the status of pathogens such as Maize Lethal Necrosis (MLN) causing viruses.

MLN is caused by synergistic interaction between the Maize Chlorotic Mottle Virus (MCMV) from the genus Machlomovirus in the family Tombusviridae and any of the poty-viruses infecting cereals such as Sugarcane Mosaic Virus (SCMV), Wheat Streak Mosaic Virus (WSMV) and Maize Dwarf Mosaic Virus (MDMV) [7-9]. The diseases can also be caused by the co-infection of MCMV with other unrelated viruses [10,11].

In Africa, the disease was first reported in Kenya in Sept 2011 and the disease spread to Uganda, Tanzania, Rwanda, D.R. Congo, and Ethiopia by 2016 and currently Mozambique [12-17]. MCMV was first identified in Peru in 1973 and has been subsequently reported in the USA, parts of Latin America, and China, Kenya, Uganda, Tanzania, Democratic Republic of the Congo, South Sudan and Ethiopia [12,16,18-22]. Globally, MLN has also been reported in Ecuador, and China [13,23]. Maize plants are susceptible to MLN at all growth stages.

The diagnostic symptoms of MLN include chlorotic mottling of leaves, necrosis development from the leaf margin to the midrib, and dead heart; later-stage infection could lead to sterile pollen, small cobs with poor seed set, or death of the plants. Possible factors that contribute to the devastating effect of MLN include new and perhaps highly virulent strains of MCMV and SCMV, conducive environment for survival, proliferation and spread of insect-vectors of the viruses as well as continuous maize cropping in certain regions leading to build-up of virus inoculum [24].

Method

Site Selection and Sampling

The surveillance covered all ten provinces of the country and the exit and entry points (Table 1). The country was divided into three regions by virtue of them being maize-producing regions. Each region was divided into 15 survey sites/districts based on the production status of maize crops (Table 1).

Table 1: Sites where Maize Samples were collected for the Surveillance of MCMV in Zimbabwe from 2016 to 2022

Region	Description of the region	District / site	Border port
Region 1	Mashonaland Central and parts of Mashonaland West Provinces	Guruve, Centenary, Mazowe, Bindura, Shamva, Mt Darwin, Muzarabani, Glendale, Mvurwi, Karoi, Mhangura, Chinhoyi, Banket, Lions Den, Kadoma, Kariba.	Mkumbura; Kariba, Chirundu One stop Border Port.
Region 2	Midlands, parts of Mashonaland West, Mashonaland East and Matabeleland North Provinces	Harare, Mhondoro, Shurugwi, Gokwe North, Gokwe South, Mvuma, Kwekwe, Beatrice, Chivhu, Chegutu, Gweru, Kadoma, Sanyati, Lupane, Bulawayo Umguza.	Harare Airport, Bulawayo Railway station.
Region 3	Mashonaland East, Manicaland, Masvingo and Matabeleland South Provinces	Chiredzi, Zaka, Bikita, Gutu, Mrehwa, Mutoko, Macheke, Marondera, Goromonzi, Arcturus, Nyanga, Mutare, Rusape, Chipinge, Chimanimani, Beitbridge.	Forbes, Nyamapanda, Beit bridge, Plumtree, Maitengwe border port and Mphengs.

Sampling points were at least 20 km radius apart from each. Green tissue for the maize grain and maize seed crops were sampled from within the 45 survey areas from the three regions. Maize grain and seed were drawn from storage centers from the Grain Marketing Board deports, farmers' grain stores, agro-dealer retail and wholesale markets, seed company depots and exit and entry border ports.

Rapid Diagnostic Testing of MCMV

A rapid testing approach was one of the techniques used during the surveillance of MCMV in Zimbabwe. Visual inspection of fields with growing maize crops was done at sites indicated in Table 1. From a field with an estimated area of about 10 hectares, six leaf samples were taken using the 'X' field inspection pattern from the CDFA Phytosanitary Certification Manual, 1985 (Figure 1). The six samples were randomly selected from farmers' fields within every 20km radius making an average total of 90 samples per region. This sample size was relatively maintained for the period of surveillance. For the testing of maize seed crops using the rapid diagnostic testing (RDT) technique, five (5) samples were randomly selected from seed production fields and maize agro dealers making a total of 45 samples per each in all the regions.

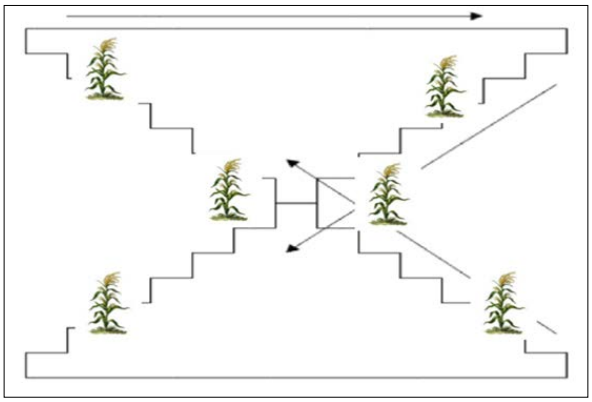

Figure 1: Staggered Sampling

The sampling procedure ensured that all parts of the field were adequately and proportionately represented for the plants inspected within the various usual microclimates of the field. Leaf samples taken were the youngest leaves of the maize plant and mostly, the flag leaf was sampled. Five to six-centimeter lengths segments of fresh leaf tissue were cut using bleach-cleaned scissors and enclosed in a clean sheet of fresh tissue paper. Aseptic techniques were followed to avoid contamination. The six leaf samples were placed singularly into an envelope perforated with air holes at each site. Completed sample labels and a unique quick response (QR) code (DENSO International America, Inc. 1994) were stuck on each sample bag and the unique sample code was also put on each leaf envelope. Each labeled sample bag was placed inside a medium zip-lock plastic bag. The six individually labeled leaf sample bags from the same sampling field were put inside one large zip-lock plastic bag (Bulk sample bag). Bulked six leaf

samples from the sampling fields were immunostrip assayed together. Scissors and other implements were cleaned thoroughly with bleach solution between each sampling of a maize plant and test and between fields and between farms.

The bulked six leaves were cut into small pieces of ≈ 5 cm² and put into an extraction bag. 4 ml of Agri-Strip extraction buffer was added with a disposable pipette. The tissue was homogenized with a handheld homogenizer with a few movements for not more than 2 seconds. 1 drop of extract solution was transferred into a cuvette and diluted with 3 drops of extraction buffer. The end of the strip marked «sample» was inserted into the extract and the formation of colored bends was observed. Results were recorded after 15 minutes. Positive results indicated two bands of color changes whilst negative results indicated one line of colour change. The techniques with agri-strips testing used antibody and antigen reactions.

Testing of Maize and Grain Samples using Enzyme-Linked Immunosorbent Assay (ELISA)

Coating Test-Wells of ELISA Plate

A humid box for keeping test wells during incubation was prepared by lining an airtight container with a wet paper towel to help prevent samples from evaporating. Antibodies and enzyme conjugates were prepared in a glass container that does not readily bind antibodies. The capture antibody was diluted with carbonate coating buffer before use on a ratio of 1:200. 100 µl of carbonate coating buffer was put in each test-well by mixing 10 ml of carbonate coating buffer with 50 µl of the concentrated capture antibody. 100 µl of the prepared capture antibody solution was put into each well. The plates were incubated in a humid box overnight in the refrigerator (4°C). Wells were emptied into a sink before filling them with 1X PBST (wash buffer). PBST was quickly emptied and test wells were washed two more times. The plates were held upside down and tapped firmly on a folded paper towel to remove excess liquid. Seed and grain samples were ground in an IKA tube mill. The ground samples were mixed with Agdia's general extract buffer (GEB) at a 1:10 ratio (grain weight in g: buffer volume in ml. 100 µl of diluted sample extraction per test-well.

Testing Procedure

100 µl of prepared sample was dispensed into sample wells. 100 µl of positive control was dispensed into positive control wells, and 100 µl of sample extraction buffer was dispensed into buffer wells. The plates were incubated inside the humid box overnight in the refrigerator (4°C). 10 ml of the enzyme conjugate was prepared by dispensing 10 ml of ECI buffer. Then, adding 50 µl from bottle with concentrated detection antibody and alkaline phosphatase enzyme conjugate and 50 µl from bottle alkaline phosphatase enzyme conjugate to the ECI buffer. The samples were washed with 1X PBST seven times. 100 µl of prepared enzyme conjugate solution was dispensed per well.

The plates were incubated in the humid box for 2 hours at room temperature. PNP solution was prepared by mixing 5 ml of room temperature 1X PNP buffer with one PNP tablet 15 minutes before the end of incubation time. Plates were washed 8 times with 1X PBST. 100 µl of PNP substrate was dispensed into each test-well. The plate was incubated for 60 minutes. Wells were examined by eyes. The procedure used was developed by the International Maize and Wheat Improvement Center (CIMMYT) for the detection of MLN-causing viruses which are Sugarcane Mosaic Viruses (SCMV), Maize Chlorotic Mottle Virus (MCMV) and Maize Dwarf Mosaic Virus (MDMV) [9]. Carbonate coating buffer, PBST buffer, General extraction buffer and PNP buffer were prepared.

Preparation of Buffers

Carbonate Coating buffer (1X) was prepared by dissolving 1.59 grams of sodium carbonate (anhydrous) in 1000 ml of distilled water. 2.93 grams of sodium bicarbonate and 0.2g grams of sodium azide were added. The solution was adjusted to pH 9.6 and the mixture was stored at 4°C.

PBST buffer (Wash Buffer) (1X) was prepared by dissolving 8 grams of sodium chloride into 1000 ml of distilled water. 1.15 g of sodium phosphate, dibasic (anhydrous); 0.2 grams of potassium phosphate, monobasic (anhydrous); 0.2 g of potassium chloride; and tween-20 were added to the solution mixture. The solution pH was adjusted to 7.4.

ECI Buffer (1X) was prepared by dissolving in 1000 ml of distilled water PBXT (1X). 2 grams of Bovine Serum Albumin (BSA), 20.0 grams of Polyvinylpyrolidone (PVP) MW 24-40,000 and 0.2 grams of Sodium azide were added to the mixture. The pH was adjusted to 7.4 and the solution stored at 4oC.

General Extraction Buffer (GEB 1X) was prepared by adding to 1000 ml distilled the PBST (1X). 1.3 g Sodium sulfite (anhydrous); 20.0 grams of Polyvinylpyrolidone(PVP) MW 24-40,000; 2.0 grams of Sodium azide; 2.o grams of Powdered egg (chicken) albumin, Grade II and 20 grams of Tween-20 were added to the mixture. The mixture was adjusted to pH 7.4.

PNP Buffer (1X) was prepared by dissolving the PNP buffer (1X) into 800 ml of distilled water. 0.1g of Magnesium Chloride hexahydrate, 0.2 g Sodium Azide; 97 ml of Diethanolamine were added to the mixture and the pH was adjusted to 9.8 with hydrochloric acid. The final volume was adjusted to 1000ml with distilled water and solution was stored at 4oC.

Preparation of Capture Antibody

The capture antibody was diluted with carbonate coating buffer using the ratio given on the label before use. 100 µl of carbonate coating buffer was used for each plate with 10 ml required for the full plate of the test. 20 µl volume of concentrated capture antibody was added to the carbonate coating buffer at the dilution of 20µl 20ml and mixed thoroughly. All antibodies and antigens were prepared on polyethylene and glass materials to prevent the container material from readily binding with the antibodies. No polystyrene materials were used.

Coating and Incubation of the Plate

100ul of the prepared capture antibody were pipetted in each well of the micro-titre plate and the plate was incubated in a humid box for six hours at room temperature and or overnight in the refrigerator with a temperature of 4°C. Coated plates were not stored for any period above 24 hrs.

Washing of Plates and Wells

Using a quick flipping motion, the contents of the wells were emptied from the wells into a sink without mixing the contents. The wells were filled completely with 1X PBST and quickly emptied again as a rinsing measure. This washing technique was repeated twice. The plates were held upside down and tapped firmly on a folded paper towel to remove excess liquid.

Sample Preparation
For maize seeds and grains; 4 grams of the kennels were ground in a miller and put in a centrifuge tube. 40ml of the general extraction buffer (GEB) at a ratio of 1:10 (1g of the sample: 10ml of GEB) were added. For maize green leaf tissue; leaf samples showing symptoms were collected and put in a sample bag. General extraction buffer (GEB) at a ratio of 1:10 (tissue weight in g: buffer volume in ml) was added and the sample was ground.

Sample Loading
100ul of the ground sample was dispensed into each well and 100ul of positive control and negative control was added into the positive and negative wells respectively

Plate Incubation
The plate was placed inside the humid box and incubated for 2 hours at room temperature and or overnight in the refrigerator set at 4oC.

Preparation of Enzyme Conjugate
Alkaline phosphatase enzyme conjugate was diluted at 1000 x with ECI buffer in a dedicated container as indicated on the bottle. The enzyme conjugate solution was mixed thoroughly. The enzyme conjugate solution was prepared not more than 10 minutes before use.

Washing of the Plate
Using a quick flipping motion, the plate wells were emptied into a sink without mixing the contents. All the wells were then filled completely with 1X PBST, and then quickly emptied again as a rinsing mechanism. This was repeated 7 times. The frame was held upside down and tapped firmly on a folded paper towel to remove all droplets of wash buffer.

Adding the Enzyme Conjugate
100 µl of prepared enzyme conjugate was dispensed per well after mixing it thoroughly.

Incubation of the Plate
The plate was incubated in the humid box for 2 hours at room temperature.

Prepare of the PNP Solution
About 15 minutes before the end of the above incubation step, 5 ml of 1X PNP buffer was measured at room temperature for each tablet. Then, without touching the tablets, PNP tablets were added to the buffer. Each PNP tablet (ACC 00404) made 5 ml of PNP solution, at a concentration of 1 mg/ml, which was enough for five 8-well strips.

Washing of the Plate
Wash the plate as indicated before and this rinsing was repeat 8 times. No air bubbles were allowed in each well after rinsing. The plate was tapped firmly on the paper towel to remove remaining wash buffer and any air bubbles. Remaining air bubbles were broken with a clean pipette tip.

Adding PNP Substrate
100 µl of PNP substrate were added into each test well.

Incubation of the Plate
The plate was pit inside the humid box and incubated for 60 minutes away from direct or intense light. After 60 minutes, 60µl of the NaOH 3M were added to stop the reaction.

Evaluation of the Results
The wells were examined by eye for color change and also the plate was read using micro-titre plate ELISA-reader at 405 nm wavelength. All air bubbles present at the time if reading were eliminated to avoid wrong readings due to interference of the reader-light path.

Interpretation of the Results
Wells in which color changes to yellow indicated positive results while wells in which there was no significant color development indicated negative result. Test results are valid only if positive control wells give a positive result while negative control and buffer wells remain colorless or give negative results.

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 or above 2 x healthy control= positive result.
- If 1.5-2 x healthy control = tentative positive.
- If equal or less than healthy control= negative results.

Note: Buffer control was a must to be less than or equal to the healthy control. The general ELISA process is given diagrammatically in Plate 1.

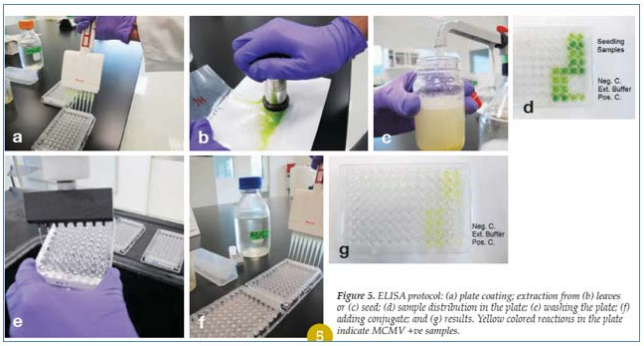

Figure 5. ELISA protocol: (a) plate coating; extraction from (b) leaves or (c) seed; (d) sample distribution in the plate; (e) washing the plate; (f) adding conjugate; and (g) results. Yellow colored reactions in the plate indicate MCMV +ve samples.

Plate 1: Diagrammatic Protocol for testing using ELISA

Construction of Geospatial Data of the Sampling Sites
GPS coordinate were collected from sampling points and these were subjected to spatial data maps using free GPS tools.

Results
All the samples tested negative for MCMV using either RDT or ELISA (Table 3 and Table 4). The tests carried out for the leaf samples, produced a control band that showed the absence of the MCMV (Figure 2). Where ELISA protocol was used for the testing of the MCM, the positive result appeared as a band which is yellow for the control (Figure 3). Figure 4 shows the geospatial maps for the data collections points excluding ports of entries used during this study.

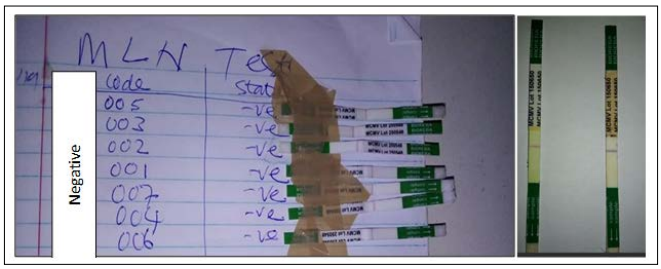

Figure 2: Negative Immunostrip Tests with One Red Band

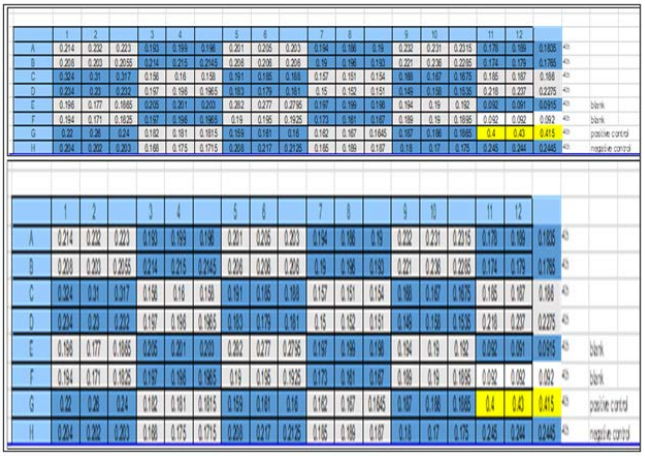

Figure 3: Optical Density from ELISA Reader obtained during the MCMV Testing

It is important to note that during the field sampling of green maize crops, symptoms of nutrient deficiency were observed on leaves of maize crops growing in the fields of small-scale farmers who did not added various artificial fertilizer inputs. Virus symptoms of the Maize Streak Virus (MSV) were the major virus observed during the survey. Grey leaf spot, Curvularia and leaf blight were some of the fungal symptoms detected during the survey. For these specified diseases, the disease incidence ranged from 2- 15% and averaged 3%. Insects observed during the filed inspections include aphids, leaf beetles, whiteflies, fall armyworms and maize stalk borers.

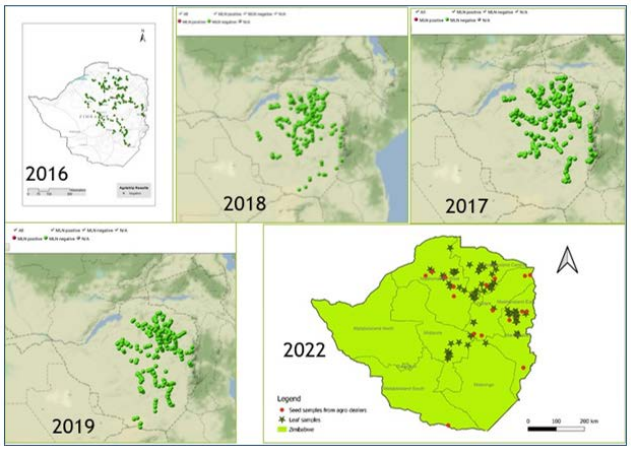

Figure 4: Sampling sites for field leaf samples for the MCMV in Zimbabwe (Maps Drawn by CIMMYT)

Table 4: Number of samples collected and tested for MCMV of the imported maize germplasm, seed and grain samples collected at all entry points from 2016 to 2024

Year	Number Samples Tested	RDT Result	ELISA Test Result
2018	301	Negative	Negative
2019	33	Negative	Negative
2020	174	Negative	Negative
2021	505	Negative	Negative
2022	490	Negative	Negative
Total	1503		

Table 5: Number of samples collected and tested for MCMV of the imported maize germplasm screened at Mazowe PQSI Facility by CIMMYT and PQSI from 2015 to 2022

Year	Number of germplasm lines introduced	MLN RDT test result during vegetative growth	ELISA test Results of the harvested seeds
2015	3800	Negative	Negative
2016	8400	Negative	Negative
2017	6400	Negative	Negative
2018	4800	Negative	Negative
2019	4800	Negative	Negative
2020	4900	Negative	Negative
2021	5800	Negative	Negative
2022	6300	Negative	Negative
Total	45200		

Table 6: Green leaf tissue tested for MCMV from growing maize crops from 2016 to 2022 in Zimbabwe

Year	Number of Samples Tested	MCMV RDT Result	ELISA Test Results
2016	222	Negative	Negative
2017	272	Negative	Negative
2018	198	Negative	Negative
2019	233	Negative	Negative
2022	100	Negative	Negative
Total	1025		

Other Results Collected during the Surveillance Period

Crop stages of the maize crop that were considered were: vegetative (VE-VT) and reproductive (Silk, Blister, Milk, Dough, Dent, and Maturity). The surveys sampled more crops in VE-VT stage as compared to the other stages (Figure 5). The time of planting ranged from October to January. Commercial seed farmers supplemented irrigation as shown by some planting in October. Some small-scale farmers continued planting their crops as they rely on rainfed. Seeds were sourced from seed houses and or retained seeds and the varieties included both hybrids and open-pollinated varieties.

Diseases symptoms observed included the grey leaf spot, leaf blight, maize streak virus, common rust, corn smut, and Phaesphaeria leaf spot, Northern corn leaf blight. Maize streak virus and common rust were the major diseases observed. Insects observed in farmer's fields, were aphids, leaf beetles, whiteflies, fall armyworms, maize stalk borers, webworms and weevils [20,25].

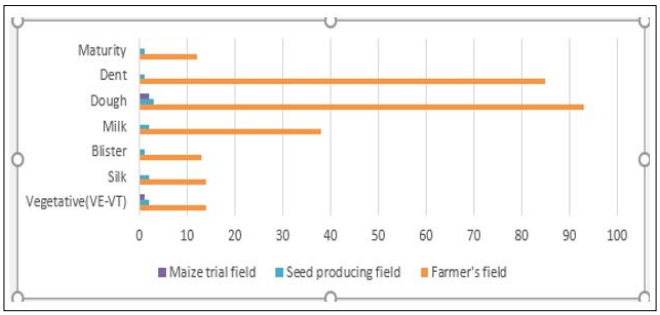

Figure 5: Percentage distribution of the growth stages sampled and tested for MCMV using green tissue maize matter in 2016 to 2022

Discussion

The investigation concludes the absence of MCMV in Zimbabwe as observed from other countries using the same MCMV protocol adopted from CIMMYT [6]. The absence of MCMV is positive result for the country's maize value chain. It is also important for Zimbabwe to maintain this status for food security as well as being a hub of seed as evidenced by the processing of seed maize phytosanitary certificates to regional and COMESA countries.

Research in US and Kenya shows that insect vector control, crop rotation, and crop diversification are among the agronomic practices that play an important role in preventing or reducing the risk of diseases [26]. During the survey, the practice of monoculture was observed. Mono-culturing maize crop can lead to high disease build-up as in tobacco production [27]. Whilst no a MCMV pathogen was detected in the sampled maize products, the fear for this virus remains high as many people tend to be overhead and feel carrying few seeds across border for home experimentations. The proximity of the detection of the MLN causing virus at the neighboring Mozambique with a relatively free movement of people across the porous borders threatens the country biosecurity.

Even though studies have shown low rates of seed transmission, these low rates of seed transmission are significant to cause the introduction with vectors coming in to play a larger role in disease dissemination [28,29]. Zimbabwe has aphids, thrips, and beetles which are vectors of MLN, and their presence if not controlled. Epidemics can occur very quickly through infected seed [8,30]. In Tanzania and DR Congo, the disease seems to be in the northern parts of the two countries, a bit distant from the Zimbabwe territories [6]. Given the increased trade amongst these states with Zimbabwe, plant biosecurity mechanism at ports of entry requires strengthening. Probably by strengthening and instituting all the preventive and control measures, the disease will be kept at bay for some time. Though Zimbabwe if free from MCMV as of the time of writing this script, continued institution of quarantine measures for MCMV is important. The presence of SCMV puts Zimbabwe at risk for a serious epidemic once the MCMV is accidental introduced into the country.

Research in US and Kenya showed that insect vector control, crop rotation, and Crop diversification are among the agronomic practices play an important role in preventing or reducing the risk of diseases [27]. Further, in Kenya, effective monitoring, rigorous implementation of maize-free periods and rotation with non-cereal crops have helped in minimizing MLN incidence [27]. Whilst these can be as good to mitigate the challenge of the disease, pest freedom remains a critical answer to biological threat and plant biosecurity [31].

Recommendations

Yearly surveillance of MLN causing viruses is critical for quick and early detection. The country stands to benefit through putting in management and control measures to combat the disease given the early warning systems in cases of inversions. Awareness of MLN to all stakeholders should be intensified. Where possible, adapted maize varieties under commercialization in the country requires to be considered for screening for MCMV in advance of time especially by sending samples in endemic areas to check for their reaction to the viruses [32,33]. Breeding for resistance put Zimbabwe ahead in terms of accessing maize seeds market where MLN is endemic [34-41].

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- Department of Agricultural Extension and Technical Services,

Ministry of Lands and Agriculture, Zimbabwe
- Ministry of Local Government of Zimbabwe

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