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# Infectivity of *Maize Chlorotic Mottle Virus* from Contaminated Maize Seeds

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**Abstract**

Seeds have been identified as major sources of introduction and spread of pathogens, with viruses being detected in the seed and also on the seed coat. In this study, the infectivity of *Maize chlorotic mottle virus* (MCMV) through seeds was investigated. Maize seeds that had tested positive for MCMV previously using double antibody enzyme-linked immunosorbent assay (DAS-ELISA) and real-time reverse transcription polymerase chain reaction (real time RT-PCR) were obtained from various sources. The seeds were soaked in phosphate buffer overnight and the solution used to inoculate maize seedlings. The whole seed was also ground and mixed with the buffer and used for inoculation of seedlings by hand rubbing. Visible MCMV symptoms were observed on less than 2% of the 547 seedlings inoculated with the seed soak and seed extract from contaminated seed 28 days after inoculation and this was confirmed using DAS-ELISA. Use of real time reverse transcription polymerase chain reaction revealed infectivity of MCMV from one of the seed sources used. The mean cycle threshold (Ct) values of samples that showed infectivity ranged from 28.21 to 29.40 cycles. The means were significantly different ($P<0.001$) from the other samples

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tested, the healthy and negative controls. When compared to seedlings inoculated with MCMV-infected leaf sap, there was visible development of symptoms associated with MCMV infection, with a severity score of three and Ct values as low as 11.53. The results show evidence of infection of MCMV on maize seedlings caused by virus present in seed extract. Despite rare occurrence of infectivity, the presence of viable virus may cause spread of the virus in the field, leading to development of maize lethal necrosis disease where a cereal potyvirus is present.

## Introduction

*Maize chlorotic mottle virus* (MCMV) is the only member of the genus *Machlomovirus*, found in the *Tombusviridae* family. It is a single stranded RNA virus, 4.4 kb with icosahedral shape.[1] The virus has a smooth surface[2] and has a thermal inactivation point of 80-85°C, and can maintain its infectivity at 20°C for 33 days.[3] The virus causes maize lethal necrosis (MLN) disease in the presence of a cereal-infecting potyvirus. Yield losses due to MLN can be up to 100% .[4–6] with symptoms of chlorosis and mottling of leaves; leaf necrosis, "dead heart" symptom and complete death of the plants.[7] The disease is considered a food security threat in the Eastern Africa region.[8]

*Sugarcane mosaic virus* (SCMV) is the most predominant potyvirus identified in MLN-infected plants in eastern Africa,[4,9,10] and has been present in the region for decades,[11] while MCMV was first reported in the eastern Africa region in 2011.[7,12] However, globally the virus has been reported in Peru, Hawaii, Nebraska and Kansas in the period of 1973-1992.[13] Other parts of the world where MCMV has been reported include China,[14] Taiwan,[15] Ecuador[16] and Spain.[17] The presence and increase in incidence of MLN continue to be reported in Kenya, Uganda and Tanzania since the first reports[12] despite the efforts to manage the disease. The presence of MCMV in some of the areas in Kenya was attributed to continuous planting of maize throughout the year and use of non- certified seed by small scale farmers.[18] *Maize chlorotic mottle virus* is transmitted through maize seeds[19–22] and by insect vectors.[23–27] Corn thrips (*Frankliniella williamsi*) are the most common vectors of MCMV found in eastern Africa region, and both the adult and larva transmit the virus.[4,7,13,23] The corn thrips are attracted to maize plants that are infected with MCMV, due to changes in volatile profiles of the plants, thus increasing the transmission of the virus.[28] It is therefore important to avoid introduction of MCMV into the fields as the secondary spread may create a pandemic. Transmission also occurs via contaminated soil[13] and in the presence of disease infected maize residues.[29]

*Maize chlorotic mottle virus* is not only transmitted by seed, but the virus has also been detected on seed parts. The contaminating virus may be transmitted by mechanical means to the seedlings, similar to other viruses where it is located outside the embryos.[30] These viruses are reported to be stable and can survive on the testa and endosperm of the seeds. *Maize chlorotic mottle virus*, unlike other maize-infecting viruses, is readily transmitted mechanically to seedlings and is also stable at 20°C for 30 days.[31] Due to the economic losses that the virus can cause in development of the maize lethal necrosis disease, there is need to gain understanding of the extent of infectivity of the virus that is commonly detected in seed samples in spread of MLN.

Diagnosis of pathogens in seeds needs to be accurate and sensitive, especially because they are used in determining the status of seed lots. Many methods have been described for diagnosis of viruses in seeds, however, enzyme-linked immunosorbent assays have been described as relatively simple to use reliable, sensitive and suitable for large-scale testing as that found in seed-health testing regulation.[32] Diagnosis using polymerase chain reaction (PCR) based methods are much more sensitive than the ELISA methods, however, they are more costly to use.[32] The real time reverse transcription PCR (real-time RT-PCR) method provides quick results in detection of RNA viruses such as MCMV, and especially where low quantities of the virus are available.[33] This study aimed at determining the infectivity of *Maize chlorotic mottle virus* from contaminated seed. The presence of MCMV was confirmed using DAS-ELISA and real time RT-PCR. The research findings are important in

developing management strategies of MLN disease that may be due to spread through seed.

**Materials and Methods**

Maize seed contaminated with MCMV- Kenya isolate was obtained from two sources- commercial seed lots and experimental plots. The seed from the commercial lots was labelled as Lot A, Lot B, Lot K27 and Lot K4, the seed was infected with MCMV naturally in the field; and those from experimental plots were from MCMV-inoculated field plants of varieties H614, DK777 and Duma 43 described earlier.[21]

The experiments were carried out in the screenhouse located at Biotechnology Research Institute, Kabete Center, Kenya Agricultural and Livestock Research Organization (KALRO). The experiments were repeated six times in the period of 2018- 2020. The experiments were laid in a completely randomized design consisting of four replications per treatment (seed source of inoculum) and three plants in each replication. Certified seed of variety PH30G19, which is susceptible to MCMV, were planted in 20cm-diameter pots half-filled with sterile loam soil mixed with five grams of diammonium phosphate fertilizer. The seedlings were watered daily and the screenhouse was sprayed weekly to control for possible MCMV vectors using either 50g/L lufenuron (Match 050EC, Syngenta), 480g/L flubendiamide (Belt 480SC, Bayer Crop Science) and 19g/litre emamectin benzoate (Escort 19EC, Greenlife Crop Protection).

Twenty-seed samples from the different seed sources contaminated with MCMV and from MCMV free-seed were placed in separate 50ml tubes. Potassium phosphate buffer (0.1M) was added to the seeds at a ratio of 1:1 (amount of seeds/volume of buffer). The mixture was hand shaken for one minute and allowed to stand overnight at 4°C. An aliquot of the soak solution (SS) was tested for MCMV presence using double antibody sandwich-enzyme-linked immunosorbent assay (DAS-ELISA) as described in a similar protocol.[21] Seedlings that were inoculated with seed soak from the contaminated seeds that tested negative for MCMV were removed from the experiment, to retain only those where MCMV was detected. Levels of contamination in the seed samples by MCMV was found to vary for the seed lots used.[21] Positive control samples were prepared from leaves from MCMV-infected plants. The leaves were chopped to small pieces and ground in 0.1M phosphate buffer. The mixture was passed through a muslin cloth to remove debris. This was stored overnight at 4°C alongside the soaked seed and used for inoculation the next day.

Two twenty-seed samples from the different seed sources contaminated with MCMV and those from the MCMV-free control were soaked overnight as described above. The seeds were then removed from the solution and ground to fine powder before re-mixing with phosphate buffer that had been used to soak the seed.[32] The seed extract was then left to stand for 20 minutes at room temperature before using the clear solution to inoculate the maize seedlings. Inoculum from MCMV-infected leaf sap was prepared as described above and included as a positive control. In order to account for viability of the virus due to soaking, seeds from MCMV-contaminated H614 seeds were used as fresh seed extract (FSE) and compared with those soaked overnight. To obtain FSE, seeds were first ground to a fine powder, then mixed with the phosphate buffer and allowed to stand for 20 minutes in room temperature before inoculating the seedlings. Similarly, MCMV-free seeds and MCMV-infected leaf sap controls were included in the experiment under similar conditions.

Two weeks after planting, maize seedlings were inoculated by lightly dusting carborundum powder on the leaves and then applying the inoculum by rubbing on the leaf using a piece of muslin cloth. Gloves were changed between different inoculum. Aside from the seedlings inoculated using MCMV contaminated inocula, three controls were included. In each experiment there were seedlings that were not inoculated, those inoculated with SS from MCMV-free seeds (negative controls) and those inoculated with MCMV infected leaf inoculum (positive control).

**Laboratory Confirmation Tests**

**Double Antibody Sandwich Enzyme-linked Immunosorbent Assay (DAS-ELISA)**

Twenty-eight days after inoculation, leaf samples from the inoculated seedlings in each pot were tested for presence of MCMV using DAS-ELISA. In each microplate, three controls were included. These were two negative controls comprising of the ELISA extraction buffer and sap from MCMV-

free leaf; and one sap from MCMV infected leaf (positive DAS-ELISA control).

**Real Time Taqman Reverse Transcription Polymerase Chain Reaction (Real time Taqman RT-PCR)**

Real time Taqman RT-PCR was used to detect MCMV in seedlings in the experiment where plants were inoculated using FSE and SE. RNA was extracted using the Purelink RNA minikit (Ambion 1283018A, ThermoFischer Scientific, USA). The 10µl reaction components for real time RT-PCR and the cycling conditions for amplification of PCR product were as reported previously.[21] The fluorescence probe and primers amplified a 131bp molecular marker, a region located from position 4,000 to 4,437bp of the nucleotide sequence of MCMV. In every microplate, negative and positive controls were included comprising of wells where no sample was added; with RNA from MCMV-free leaf, and third control with RNA from MCMV-infected leaf. The samples and controls were tested in duplicate.

**Data Collection and Analysis**

The severity of MCMV infection was scored once every week for four weeks after inoculation using a scale of 1 to 5,[34] where 1 = no MCMV symptoms, 2 = fine chlorotic streaks on leaves, 3 = chlorotic mottling throughout plant, 4 = excessive chlorotic mottling, necrosis on leaves and in some cases dead heart symptom and 5 = complete plant necrosis. Data collected from the DAS-ELISA consisted of mean absorbance values ($A_{405nm}$). A sample was considered positive for MCMV infection when the mean absorbance reading of duplicate samples at 405nm wavelength ($A_{405nm}$) were above twice the mean of the negative controls included on the same microplate. The number of positive samples were counted and reported. MCMV severity ordinal data was analysed using the Kruskal-Wallis test in the base package in R software, and the scores of the various entries compared against each other using the Dunn's test in the rstatix package.[35]

Data obtained from the real time Taqman RT-PCR was the threshold cycle (Ct) value from the StepOnePlus software (Applied Biosystems, Thermo Fischer Scientific), applying the automatic generated threshold setting and baseline settings. The mean Ct values were subjected to analysis of variance (ANOVA), and the means separated using the Tukey's honestly significant differences (HSD) test in R software[35] since the sample sizes were unbalanced. A positive sample was determined if the mean Ct values of the sample was lower than that of healthy and negative controls and also significantly different at P<0.05.

**Results**

**Effect of MCMV from Soak Solution on Maize Seedlings**

The seed soak (SS) solution tested to confirm the presence of MCMV prior to inoculation showed positive samples absorbance values ($A_{405nm}$) (Table1) with more inoculum from Lot B having positive detection of the virus, thus more seedlings inoculated with this seed source (Table 1). All the seedlings that were inoculated with seed soak from the different sources had average disease severity score of one, where there were no visible MCMV symptoms (Table 1). Similarly, DAS-ELISA did not reveal any MCMV positive samples from the inoculated seedlings (Table 1). There were significant differences of the inoculum entries on the severity of MCMV on seedlings ($\chi^2(9) = 297.39$, $p<0.0001$). The significant differences were due to the seedlings that were inoculated with sap from MCMV-infected leaf. All the seedlings in this category developed symptoms.

**The Effect of MCMV from Seed Extract on Maize Seedlings**

The results showed that when MCMV contaminated seeds were soaked and then ground, or when the seeds were ground and inoculation buffer added prior to inoculation as fresh seed extract (FSE), the resulting solution/inoculum tested positive with absorbance readings twice higher than that of the negative control (Table 2). However, most of the seedlings inoculated with seed extract (SE) and FSE showed no visible MCMV symptoms at 28 days post inoculation, except one seedling (Table 2). This was unlike the progressive development of MCMV symptoms observed on maize seedlings from day seven after inoculation when seedlings were inoculated with MCMV infected leaf sap (Table 2). There was significant differences in the mean severity of MCMV symptoms ($\chi^2(8) = 273.23$, $P<0.0001$mainly due to the higher severity on seedlings inoculated using the MCMV-infected leaf sap.).

**Table 1: Number of seedlings inoculated using the seed soak solution; seedlings that had MCMV symptoms, the mean severity at 28 days post inoculation and detection using ELISA of the inoculum and seedlings**

| Seed Source | ELISA Absorbance readings for Inoculum | No. of tested seedlings | Plants with symptoms | Mean severity | † ELISA Absorbance readings for seedlings |
|---|---|---|---|---|---|
| K4 | - | 44 | 1 | 1.04 | - |
| K27 | 1.77 | 73 | 2 | 1.02 | 0.09 ± 0.01 |
| Lot A | 2.12 | 78 | 2 | 1.01 | 0.09±0.00 |
| Lot B | 1.84 | 100 | 3 | 1.03 | 0.09±0.00 |
| DK777 | 1.56 | 12 | 0 | 1 | 0.11 ± 0.03 |
| Duma43 | 2.39 | 12 | 0 | 1 | 0.09±0.00 |
| H614 | 3.24 | 12 | 0 | 1 | 0.09±0.00 |
| Plants not inoculated | - | 37 | 0 | 1 | 0.09±0.00 |
| MCMV-free seed | 0.10 | 20 | 0 | 1 | 0.09±0.00 |
| Positive control | 1.18* | 18 | 18 | 3**** | 0.56± 0.12 |

"-" samples were not tested using DAS-ELISA, * positive control was obtained from MCMV infected plant. **** P<0.0001
†Sample were replicated in two wells ±SD absorbance readings at 60 minutes

**Table 2: Number of seedlings inoculated with seed extract, those that had MCMV symptoms at 28 days post inoculation, the mean severity and detection of MCMV by ELISA of the inoculum and seedlings**

| Seed Source | ELISA Absor bance readings for Inoculum | No. of tested seedlings | No. of plants with symptoms | Mean severity | † ELISA Absor bance readings for seedlings |
|---|---|---|---|---|---|
| K4 | - | - | - | - | |
| K27 | 1.53 | 12 | 0 | 1 | 0.09±0.00 |
| Lot A | 1.79 | 36 | 1 | 1.02 | 0.09±0.00 |
| Lot B | 1.57 | 60 | 0 | 1 | 0.09 ± 0.01 |
| DK777 | 3.33 | 12 | 0 | 1 | 0.09±0.00 |
| Duma43 | 3.29 | 12 | 0 | 1 | 0.08 ± 0.01 |
| H614 | 3.46 | 84 | 0 | 1 | 0.09 ± 0.01 |
| Plants not inoculated | - | 24 | 0 | 1 | 0.09±0.01 |
| MCMV-free seed | 0.11 | 36 | 0 | 1 | 0.10 ± 0.01 |
| Positive control | 4.01* | 15 | 15 | 3**** | 0.42±0.01 |

'-' stands for seeds not available * positive sample obtained from seed infected with MCMV. †DAS-ELISA samples were replicated in two wells; absorbance readings ±standard deviation at 60 minutes. P<0.0001

**Real Time Taqman Reverse Transcription Polymerase Chain Reaction**

The use of real time Taqman reverse transcription polymerase chain reaction (real time Taqman RT-PCR), a more sensitive method in the detection of viruses, amplified the targeted molecular marker in some of the seedlings that were tested (Table 3). Two seed sources of MCMV-contaminated H614 samples were used with a total of 72 samples. One seed source had seven positive samples of the 36 tested (19.4%). Ct values from seedlings inoculated using the first H614 seed source had Ct values ranging from 31.43 to 36.24 cycles. These Ct values were not significantly different ($P>0.05$) from those of the negative control.

However, the second H614 seed source had Ct values that were significantly different ($P<0.001$) from those of the negative and healthy controls. The samples that tested positive had Ct values ranging from 27.04 to 30.22 cycles. These values were lower than that of the negative and healthy controls, and the result indicated presence of MCMV. The Ct values were subjected to statistical analysis and means separation by Tukey's HSD test, these samples showed significant differences from the negative and healthy controls (Table 3). The seedlings that were not inoculated and those inoculated using healthy seed source had Ct values ranging from 30.90 to 35.21 cycles. There were significant differences between the positive controls (samples inoculated with MCMV-infected leaf- sap) and all the other samples including the negative and healthy controls with low Ct values ranging from 11.31 to 14.93 cycles.

**Table 3: Seedlings inoculated and the reaction of MCMV testing using real time reverse transcription polymerase chain reaction**

| Seed source | seedlings tested | No. samples with Ct values lower than negative control | Ct Mean (cycles) |
|---|---|---|---|
| H614-1 | 12 | 0 | 34.83a |
| H614-1B | 12 | 0 | 34.48a |
| H614-1A | 12 | 0 | 34.14a |
| not inoculated | 12 | 0 | 35.22a |
| healthy_B | 12 | 0 | 33.00a |
| healthy_A | 12 | 0 | 32.77a |
| H614-2 | 12 | 2 | 29.40b |
| H614-2A | 12 | 2 | 28.73b |
| H614-2B | 12 | 3 | 28.21b |
| MCMV_leaf-A | 12 | 12 | 13.70c |
| MCMV_leaf | 12 | 12 | 12.36cd |
| MCMV_leaf-B | 12 | 12 | 11.53cd |
| PCR Negative control | | | 33.53a |
| Positive control | | | 9.79d |
| Tukey's HSD | | | 2.44 |

Ct- mean cycle threshold obtained. The greenhouse data is included in the previous tables

Seed sources with a letter A or B were inoculated with inoculum of freshly ground seed extract while those without the letters were ground and soaked overnight in buffer at 4°C before the inoculation process the following day. Healthy seed source was from MCMV-free seed. 'MCMV-leaf' labelled samples were seedlings inoculated with sap from MCMV-infected leaf, while the 'PCR positive' was obtained from RNA of fresh MCMV-infected leaves. Negative control represents non template control included

in the real-time RT-PCR. Ct means followed by the same letter are not significantly different

## Discussion

*Maize chlorotic mottle virus* causes the devastating maize lethal necrosis disease when in combination with a cereal potyvvirus.[7] *Maize chlorotic mottle virus* has been detected in seed obtained from MLN or MCMV infected maize plants.[33,36] In this study we determined the infectivity of the detectable virus, by infecting young maize seedlings with inoculum from the solution used to soak MCMV contaminated seed and also the seed extract.

There was low severity of MCMV symptoms observed at 28 days after inoculation in 1.93% of the 547 seedlings tested, with the highest score of 2 recorded. The use of DAS-ELISA method to confirm for virus presence in the seedlings also did not detect virus in all samples tested. However, using real time RT-PCR, MCMV was detected from seedlings that were inocoulated with seed extract inoculum. Real time RT-PCR method is significantly more sensitive than DAS-ELISA, and lower viral loads are detectable.[33,36] However, only a few samples were tested using this method due to availability of the assay's reagents. Despite the DAS-ELISA method being easier to employ for diagnosis and more cost effective, it is a less sensitive technique where there is low virus concentration[36]

The lack of infectivity from most of the samples may be attributed to lack of viable virus in the seeds. This may be due to inactivation of the virus during maturation and drying or lack of survival of the virus outside the embryo. Loss of infectivity during maturation has been demonstrated for *Rice yellow mottle virus,* where the infectivity decreased significantly when the seeds were tested just after harvest and after drying for one month 37. Similarly, inactivation of virus has been shown for Sugarcane mosaic virus, where decrease in infectivity is suggested to occur due to the lowering of moisture in the seeds, thereby altering the environment for survival of the virus[38,39]

Viruses obtained from seed samples that have been reported to be infectious include *Cucumber green mottle mosaic virus*[40] and *Pepino mosaic virus,* that was found to be infective from mature and dry *Nicotiana benthamiana* and *Solanum lycopersicum*[41] seeds. Viable virus is detected by analytical methods alongside the inactivated virus in seed extracts and soak solution.[42] The infection revealed in this study may have been due to viable virus available in the seed. Availability of viable MCMV in maize seed is possible and has been confirmed from the transmission of MCMV via seed to seedlings.[20,21] However, there is need to quantify the amount of virus found in seed and the effect to infectivity. Future studies should also include analysis of the compounds found in maize seed and other factors that may inhibit infection of the seedlings by virus found in or on seeds.

*Maize chlorotic mottle virus* is a quarantine pathogen in Kenya and other maize growing countries globally. The study results have shown that MCMV in seed extract may cause infection on young leaf of maize seedlings, implying that the virus is viable, though infrequent. This information is important in implementation of phytosanitary protocols in ensuring management of the spread of MCMV in the fields.

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## Conflict of Interest

The authors declare no conflict of interest.

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