



Molecular evidence for presence of *Manihot esculenta* associated virus 1: CG-Nmb and/or CG-Kah isolates in coast, Tanzania: A threat to cassava production

Herieth R Mero*

University of Dar es Salaam, College of Natural and Applied Sciences, Department of Molecular Biology and Biotechnology,
P.O. Box 35179, Dar es Salaam, Tanzania.

Keywords

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Cassava Brown Streak Disease;
Cassava Brown Streak Virus;
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Abstract

Genetic diversity of *Manihot esculenta* associated virus type 1 (MEaV-1) species suspected to cause the most destructive Brown Streak of Cassava (CBSD) in Tanzania, has remained elusive since the year 2020 when MY-6mb-4 virus isolate was first identified in Chambezi, Coast region. In this study, two additional isolates (CG-Nmb and CG-Kah) of the MEaV-1 species were characterized in the same area after screening leaf samples collected from 292 Kiroba, 12 Albert and 22 Kikombe varieties of cassava using a Reverse Transcription Polymerase Chain Reaction (RT-PCR) assay. One half of the leaf samples collected from each of the three varieties displayed CBSD-like symptoms whilst the other half were CBSD-asymptomatic. The MEaV-1 infection was detected only in the CBSD-symptomatic Kiroba, Albert and Kikombe varieties by 58%, 100% and 82%, respectively. Since the RT-PCR assay could only detect the two MEaV-1 isolates simultaneously rather than discriminating them, it indicates that either one of the isolates or both of them caused the infection in the CBSD-cases. Detection of additional MEaV-1 isolates, has expanded the genetic diversity knowledge on the MEaV-1 species in Tanzania which is essential for thorough comprehension of CBSD epidemiology and guiding invention of more effective CBSD-control methods using recombinant DNA technology.

Introduction

Genetic diversity of causal pathogens of the most destructive Brown Streak Disease of cassava (CBSD) causing a loss of nearly 2 billion US dollars per annum, is still debateable (Mero et al. 2021). Currently, six virus species belonging to two virus families, Potyviridae and Closteroviridae are speculated to cause the disease (Mero et al. 2022b). The two Ipomoviruses, *Cassava Brown Streak Virus* (CBSV) and *Ugandan Cassava Brown Streak Virus* (UCBSV) which are collectively termed as Cassava Brown Streak Viruses (CBSVs) were the first causal viruses of the CBSD to be discovered in the 1930s (Monger et al. 2001 and 2010, Tomlinson et al. 2018). However, two extra *Ipomovirus* species which were predicted to have evolved in the UCBSV clade (Ndunguru et al. 2015), are yet to be characterized (Mero et al. 2022b). Of recent, 7 (MG-Men-241, MG-Mia-403, RE-Ljv, MY-Ren-5, MY-6mb-4, CG-Nmb and CG-Kah) and 6 (MY-6mb-6, MG-Men-9, MG-Mia-10, MG-Mia-362, MG-Mian 2-2 and MY-Ren-8) isolates of the corresponding type 1 and 2 of *Manihot esculenta* associated virus (MEaV-1 and MEaV-2) species belonging to subgroup II of the most heterogenous *Ampelovirus* genus of the *Closteroviride* family, were isolated from diverse cassava genotypes in the Democratic Republic of Congo (DRC), Madagascar, Mayotte and Reunion islands combined (Kwibuka et al. 2021). Among the two Ampeloviruses, the former was

found to be prevalent in the four investigated regions (Mayotte, Reunion Island, Madagascar and DRC) whilst the latter is restricted to only two regions (Mayotte and Madagascar). Even though the MEaV-1 species was reported to be more abundant than the MEaV-2 species in the investigated countries, the case may not necessarily be the same in other un-investigated cassava producer regions in Sub-Saharan Africa.

By the year 2019, there was no any published report which confirmed presence of Ampeloviruses-infecting cassava in the Eastern African zone. However, in the year 2020 a partial genomic sequence of the MEaV-1 species with 100% identity to the MY-6mb-4 isolate (GenBank Accession: MT773585.1) was isolated for the first time at Chambezi village located in Coast region of Tanzania following a metatranscriptomic survey for novel RNA viruses infecting cassava (Mero 2022b). Since then, genetic diversity of the MEaV-1 species circulating in the country has remained elusive. Similarly, no report has confirmed on presence of the MEaV-2 species infecting cassava in the Eastern African zone. With reference to the work by Kwibuka et al. (2021), both MEaV-1 and MEaV-2 species were detected in symptomatic CBSD-cases of cassava however, the influence of each virus on severity of CBSD symptoms on roots is not yet comprehended. Before understanding virulence of the MEaV-1 and MEaV-2, knowing how many isolates of the two-virus species exist in endemic field settings is crucial. Due to

*Corresponding author: mero.herieth@udsm.ac.tz

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resource constraints, investigation conducted in this study was limited to the MEaV-1 species only.

The present study was conducted to determine presence of selected isolates of MEaV-1 species in the study area. Information gathered from this study will guide the ongoing experimental investigations to determine virulence levels of the MEaV-1 species and evaluation of

Materials and Methods

Study area

This study was conducted in Coastal region, Tanzania to characterize selected isolates of the MEaV-1 species at Chambezi village. The village is located nearby Bagamoyo town and positioned 50 kilometers from Dar es Salaam city center (Shirima et al. 2019). Farmers were recruited by a non-randomized (snow-ball) sampling method (Kirchherr and Charles 2018) and a survey of selected MEaV-1 isolates was done in farms whose owners were willing to participate in the study. Global position system (GPS) coordinates (Latitude and Longitude) of the three surveyed farms in the Chambezi village include (-6.560307 and 38.924428), (-6.541372 and 38.923482) and (-6.540478 and 38.923651), correspondingly.

Study design

A blend of case - control (Temfack Deloko et al. 2021) and *in silico* study designs were employed in the current study.

Sample collection

Sample size was estimated with reference to the work by Kaweesi et al. (2014). By a non-randomized purposive sampling technique (Andrade 2021) 12, 22 and 292 leaf samples were collected from the respective Albert, Kikombe and Kiroba varieties. For each variety, one half of the samples were CBSD-symptomatic whilst the other half was apparently healthy (CBSD-control). Unequal number of samples were collected from the three cassava varieties because the visited farms which we had access to, had mixture of distinct varieties with unmatched number. One half of leaf samples collected from each of the three varieties displayed CBSD-like symptoms whilst the other half displayed no CBSD symptoms (apparently healthy). Both the CBSD-symptomatic and asymptomatic cassava plants sampled on 1st December 2022, had age range of 2 - 7 months, regardless the variety. Severity of the CBSD-like symptoms on leaves and stem of suspected CBSD-cases of cassava was graded based on 1 - 5 scoring system described by Shirima et al. (2019). Cassava plants with symptom severity score value of 2 - 5 and 1 were grouped as CBSD-cases and controls, respectively. To maximize the chance of detecting the MEaV-1 species, six leaflets were picked per plant, two from each of the three points (top, middle and bottom) of the plant. The six leaflets were pooled together and put in a tank full of liquid nitrogen immediately after being wrapped-up in sterile, pre-labelled envelope made up of Aluminum foil. Samples were transported in liquid nitrogen from field area to the molecular biology laboratory at the department of Molecular Biology and Biotechnology (MBB) of the University of Dar es Salaam, Tanzania for molecular analyses.

the virus-resistance levels among germplasms of cassava cultivated in Tanzania. The acquired knowledge will also guide appropriate design and implementation of effective control measures of the CBSD caused by the MEaV-1 species for alleviation of poverty and food insecurity in the zone and the rest of Sub-Saharan African countries.

Determination of RT-PCR Amplicon Size of the CP Gene Expressed by MEaV-1: CG-Kah and CG-Nmb Isolates

Forward and reverse primer sequences for simultaneous detection of two isolates (CG-Nmb and CG-Kah) of the MEaV-1 species designed according to the consensus sequence (CP gene) of the reconstructed viral contigs were 5' - AATTTGGGAGGAGTGCGACC - 3' and 5' - AGACCGACTTGTGCTACTCTTG - 3', respectively (Kwibuka et al. 2021). Since information on the expected amplicon (band) size for confirming presence of the two isolates in samples by the RT-PCR assay wasn't provided in Kwibuka et al. (2021), such information was obtained by bioinformatics analysis. By using the known primer sequence, the open access Primer-BLAST tool (Ye et al. 2012) was used to determine the expected amplicon (band) sizes of the analyzed CG-Kah and CG-Nmb isolates.

The forward and reverse primer sequences were copied and pasted on the respective search boxes namely, use my own forward (5' - >3' on plus strand) and use my own reverse (5' - >3' on negative strand), correspondingly. Distinct primer's parameters were set differently before running the Primer-BLAST especially on the Primer Parameters, Exon/intron selection and Primer Pair Specificity Checking Parameters categories.

Speaking of the primer's parameter, the PCR product size was set at minimum and maximum values of 70 and 1000, respectively. Number of primers to return was set at 10. Minimum (Min), Optimum (Opt), Maximum (Max) and Maximum difference values of Primer melting temperatures (T_m) were set at 57.0, 60.0, 63.0 and 3, correspondingly. The box for Preferring primers at 3' side of the template was unchecked. Parameters for the Exon/Intron Selection were set at default values whereby, no preference was selected for the Exon junction span. For the exon junction match, the minimum 5' match, minimum 3' match and maximum 3' match was set at respective 7, 4 and 8 values. For the Intron inclusion, the box was unchecked for primer pair which must be separated by at least one intron on the corresponding genomic DNA. Intron length range was set at minimum and maximum values of 1000 and 10000, respectively. For the specificity check, the box for enabling search for primer pairs specific to the intended PCR template was checked and the search mode was automatic. Regarding the Exclusion, two boxes were unchecked, one for Exclude predicted Refseq transcripts (accession with XM, XR prefix) and another for Exclude uncultured/environmental sample sequences. Nucleotide (nt) database was selected. *Manihot esculenta* associated ampelovirus 1 (taxid: 2843331) was the selected organism. Entrez query (optional) was set at default. Speaking of primer specificity stringency, the following parameters were set at default values: Primer must have at least 2 total mismatches to unintended targets including

at least 2 mismatches within the last 5 bps at 3' end and ignore target that have 6 or more mismatches to the primer. The maximum target amplicon size was set at 4000 value. Splice variants were not allowed because the box for Allow primer to amplify mRNA splice variants (requires refseq mRNA sequence as PCR template input) was unchecked. Eventually, the Get Primers button was clicked and the results were shown in new windows.

Sample Analysis

Isolation of the Total Plant RNA from Cassava Leaf Samples

Total RNA was extracted from cassava leaves by using a modified Cetyltrimethylammonium (CTAB) protocol (Shirima et al. 2017). Purity and concentration of the isolated RNA was measured by using NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

Molecular Detection of MEaV-1: CG-Nmb and/or CG-Kah Isolates in Cassava

Two randomly selected isolates (CG-Nmb and CG-Kah) of the MEaV-1 species were characterized by a two-steps Reverse Transcription Polymerase Chain Reaction (RT-PCR) assay (Goodwin et al. 2016). Complementary DNA (cDNA) of all total RNA extracts of cassava were synthesized by using ProtoScript II First Strand cDNA Synthesis Kit (New England BioLab, NEB) (Kwibuka et al. 2021). A master mix (20 µl) composed of RNA template (1 µl), 60 µM Random primer mix (2 µl), 2X ProtoScript II reaction mix (10 µl), 10X ProtoScript II enzyme mix (2 µl) and nuclease free water (5 µl); was initially incubated at room temperature for 5 minutes before final incubation at 42°C for 1 hour. Eventually the reaction mix was incubated at 80°C for 5 minutes.

A 25 µl reaction mixture of the PCR assay for typing isolates of the MEaV-1 species, was prepared by mixing 2 µl of cDNA template, 12.5 µl of Q5 High-Fidelity Master Mix (NEB), 0.5 µl of 10 µM of each of the forward and reverse primers and 9.5 µl of nuclease-free water. Denaturation, annealing and elongation steps of the PCR assay were set at respective: 98°C for 3 minutes followed by 35 cycles of 98°C for 10 seconds, 57°C for 10 seconds, 72°C for 30 seconds and then for 2 minutes (Kwibuka et al. 2021).

The PCR amplicons of the CP gene were analyzed using 1% Agarose Gel Electrophoresis (AGE) prepared using 1x Tris Borate Acetate Ethylenediaminetetraacetic acid (TBE) (Kwibuka et al. 2021). The PCR amplicons and a DNA marker, Generuler 1 kb plus ladder (Thermal Scientific, SM1343) were loaded on gel's wells after being mixed with a loading dye, Purple at 5:1 ratio. The AGE was run at 80 volts for 45 minutes (Kwibuka et al. 2021) and the gel was visualized in a LUMINAX gel documentation system.

Detection of Mixed Infection between Distinct RNA Viruses in Cassava

Samples which tested positive for the MEaV-1: CG-Nmb and CG-Kah isolates were screened for mixed

infection by other RNA viruses (CBSV and UCBSV species) belonging to the *Ipomovirus* genus by using the same RT-PCR protocol described above, except that the used primers and PCR conditions were specific for the targeted CBSV and UCBSV species.

A single primer set (CBSDDF2/CBSDDR) was used for simultaneous detection of the CBSV and UCBSV species (Mbanzibwa et al. 2011) in the (MEaV-1) - positive samples. Sequence of the forward (CBSDDF2) and reverse (CBSDDR) primers which targeted the respective partial sequence of the CP gene and 3'-UTR regions were; (5'- GCTMGAAATGCYGGRTAYACAA - 3') and (5'- GGATATGGAGAAAGRKCTCC - 3'), respectively (Mbanzibwa et al. 2011). Denaturation, annealing and elongation steps of the PCR assay were set at respective: 94°C for 2 minutes followed by 35 cycles of 94°C for 30 seconds, 51°C for 10 seconds, 72°C for 30 seconds (Mbanzibwa et al. 2011). Sizes of PCR amplicons for the detected CBSV and UCBSV species were 344 and 430 - 440 base pairs, respectively (Mbanzibwa et al. 2011).

Data Analysis

Percentage infection by the MEaV-1 species was determined as a proportion of both CBSD-cases and controls which tested positive for the CG-Kah and/or CG-Nmb isolates using the two-step RT-PCR assay. Statistical significance of the CBSD infections by the CG-Kah and/or CG-Nmb isolates of MEaV-1 species in Albert, Kikombe and Kiroba varieties was determined by Logistic Regression Analysis using a graphical Jeffreys's Amazing Statistics Program (JASP) version 0.95.4 (Love et al. 2019). Evaluation of the influence of independent variables (sample size and variety type) on occurrence of CBSD caused by MEaV-1 infection (Dependent variable) were done at 95% Confidence Interval.

Results and Discussion

Infection Status of the MEaV-1: CG-Nmb and/or CG-Kah Isolates in varieties of cassava

Regardless the variety type, all (163) of the CBSD - asymptomatic cassava plants (Negative control) tested negative for both the MEaV-1: CG-Nmb and CG-Kah isolates whilst 100 (31%) and 63 (19%) of the 163 CBSD - symptomatic plants tested positive and negative, respectively, for the investigated MEaV-1 isolates detected by the two-step RT-PCR assay (Figure 1).

To be clear, the assay was desirable for molecular screening (detection) of the CG-Nmb and CG-Kah isolates, rather than distinguishing them because a partial conserved sequence of the CP which was targeted by the primer, produced same sized bands (221 bp) for both the former and latter (Figure 2). The forward and reverse primers bind to the corresponding 12488 - 12507 and 12687 - 12708) and (12493 - 12512 and 12692 - 12713) genomic spans of the former and the latter.

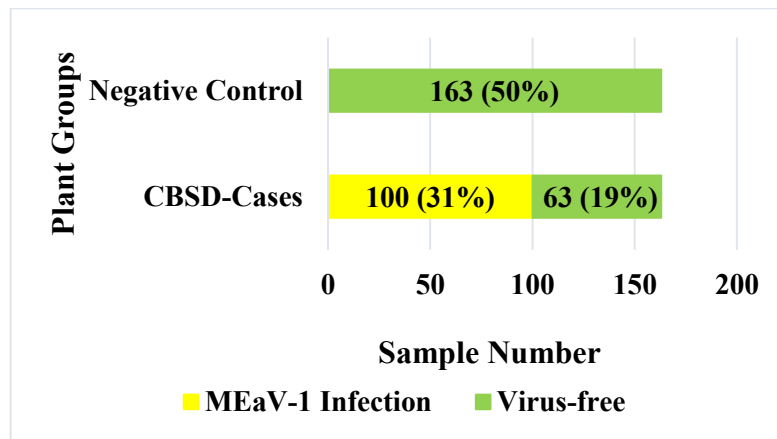


Figure 1: Infection status of the MEaV-1 species only between CBSD-cases and control group of Cassava

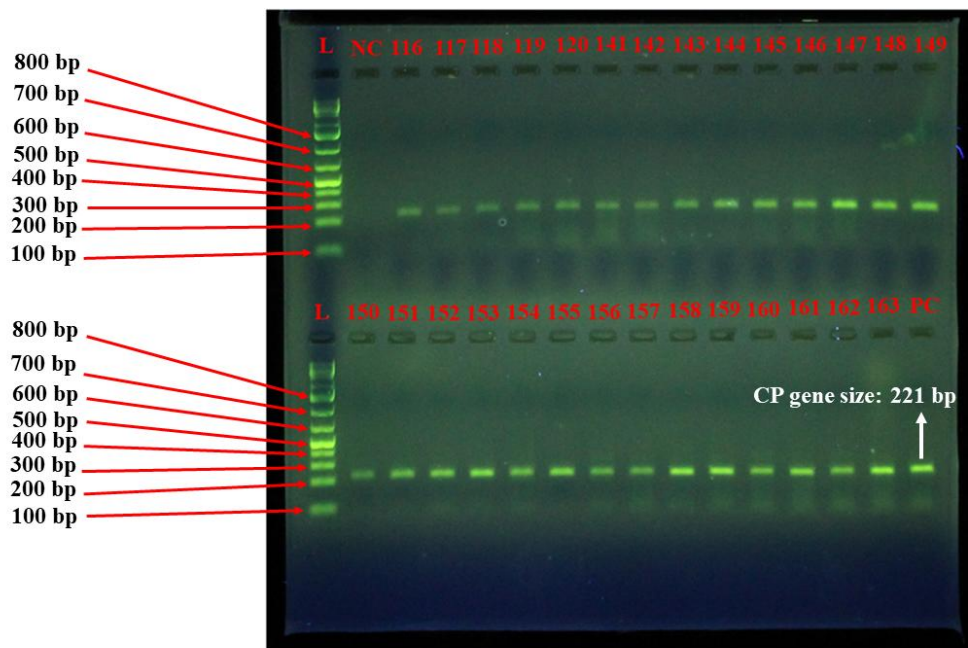


Figure 2: Gel Electrophoresis for detection of the MEaV-1: CG-Nmb and CG-Kah isolates in Cassava Leaf Samples. L, PC and NC Represented the Generuler 1Kb Plus Ladder (Thermal Scientific, SM1343), Positive and Negative Controls for the MEaV-1 species, correspondingly.

The fact that, a single primer produced amplicons with same size (221 bp) for each of the two (CG-Kah and CG-Nmb) isolates implies that, the infections detected in the CBSD cases were possibly caused by: (i) one of the two isolates (two distinct, single infection existing in separate plants) and/or (ii) two isolates mixed in the same host plant (dual or co-infection). Despite the said MEaV-1 infection scenarios, findings of this study confirm that, more isolate(s) of the virus species are circulating in cassava fields in addition to the MY-6mb-4 isolate (GenBank Accession: MT773585.1) characterized in the 2020 year. With regard to future prospects of cassava production in Tanzania, these findings call for further omic-based investigations in order to understand in detail about viral diversity and molecular epidemiology of the CBSD.

Among the symptomatic CBSD-cases of 146 Kiroba, 6 Albert and 11 Kikombe varieties 85 (58%), 6 (100%) and 9 (82%) tested positive for the MEaV-1: CG-Nmb and CG-Kah isolates despite some being co-infected with Ipomoviruses. Only 38 (26%), 4 (67%) and 4 (36%) of

the CBSD - symptomatic Kiroba, Albert and Kikombe varieties had a single infection by the MEaV-1: CG-Nmb and/or CG-Kah isolates, respectively. Results of statistical analysis (Logistic regression) using JASP software revealed that infection percentage in each of the three (Albert, Kiroba and Kikombe) varieties (all with p-value of 1) were statistically insignificant (p-value > 0.05) and this implies that the tested null hypothesis was rejected (i.e CG-Nmb and CG-Kah isolates of MEaV-1 species do not cause significant CBSD – infection in cassava farms at Chambezi, Tanzania). Even though the infection by the MEaV-1: CG-Nmb and CG-Kah isolates was found to be statistically insignificant in the investigated varieties, these findings shouldn't be ignored because the RNA virus evolves rapidly and even a single virus infection in fields can result into a huge CBSD outbreaks in the near future.

Some of the CBSD-cases of cassava infected with MEaV-1 species were also co-infected with Ipomoviruses(CBSV and UCBSV species). For varieties which had MEaV-1 infection, 41 (48%) and 6 (7%) of

Kiroba samples were co-infected with (CBSV and UCBSV) species respectively. On the other hand, 2 (33%) and 5 (45%) of the respective Albert and Kikombe

varieties were co-infected with CBSV and CBSV species (Figure 3).

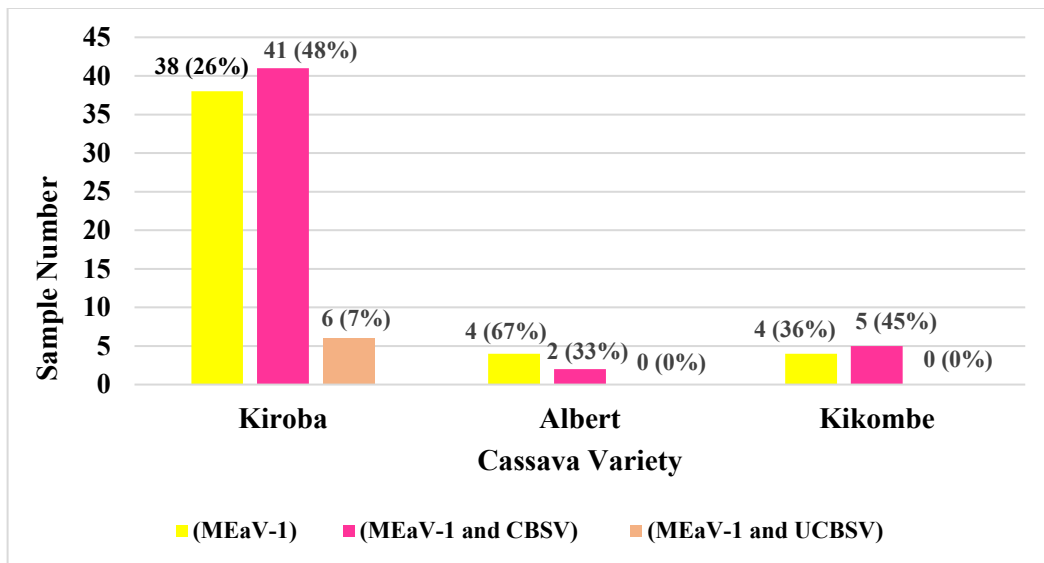


Figure 3: Distribution of the RNA Viruses (MEaV-1, CBSV and UCBSV) Infections in the Kiroba, Albert and Kikombe Varieties of Cassava.

Only CBSV-cases of the Kiroba variety scrutinized in this study, had triple infections by the MEaV-1, CBSV and UCBSV species whilst Albert and Kikombe varieties had dual infection by the MEaV-1 and CBSV species (Figures 4 and 5).

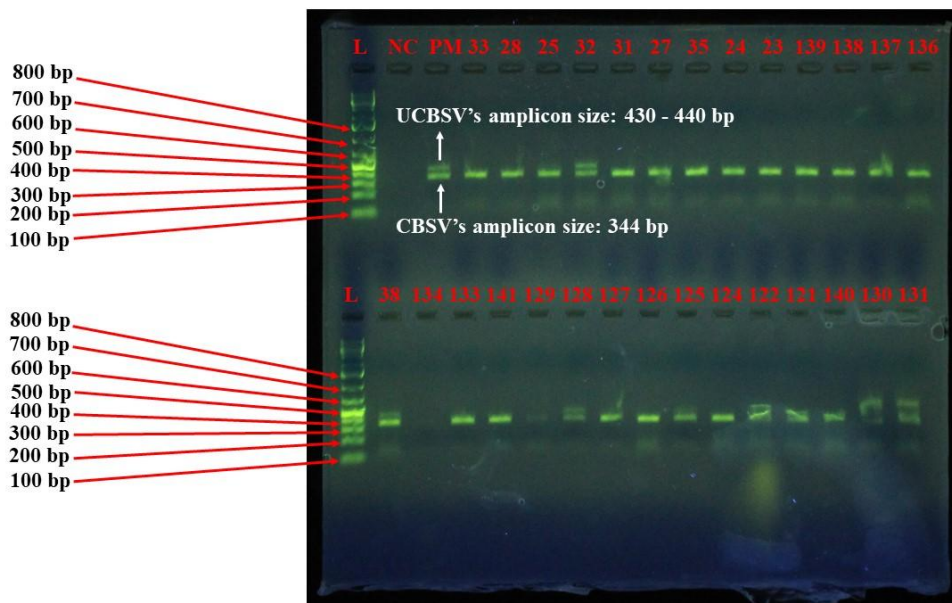


Figure 4: Gel Electrophoresis showing the (MEaV-1)-positive samples of the Kiroba variety (IDs: 32, 38, 128, 125, 122, 130 and 131) with mixed infections by *CBSV* and *UCBSV* species. L, NC and PM represented the Generuler 1 kb plus ladder (Thermal Scientific, SM1343), Negative Control (virus-negative sample), Positive Control with mixed infected by the two ipomoviruses (*UCBSV* and *CBSV* species).

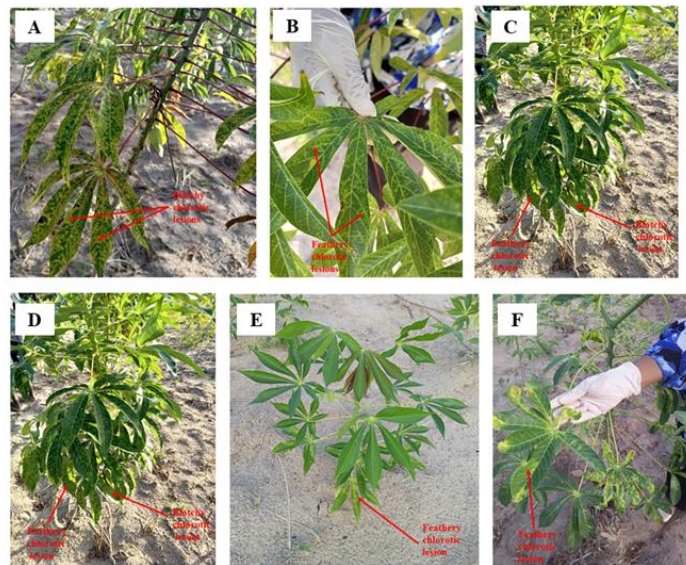


Figure 5: Foliar CBD-like Symptoms (Feathery and/or Blotchy Chlorotic Lesions) Observed on Albert (A - B), Kikombe (C - D) and Kiroba (E - F) Varieties of Cassava.

The mixed virus infection occurred at the inter-genera level, involving a combination of the one *Ampelovirus* (MEaV-1) and two *Ipomovirus* (CBSV and UCBSV) species. Cases of plant diseases caused by multiple virus species are not uncommon (Moreno and López-Moya, 2020, Tugume et al. 2016, Wintermantel et al. 2008). For instance, a cases of triple virus infections involving combinations of (MEaV-2, *East Africa Cassava Mosaic Virus* (EACMV) and UCBSV) and (MEaV-1, EACMV and CBSV) species, belonging to three genera (*Ampelovirus*, *Begomovirus* and *Ipomovirus*) were reported by Kwibuka et al. (2021). Knowledge on how the three viruses belonging to distinct genera co-exist in the same host plants, is not fully comprehended. It is imperative to understand whether the following combinations of dual (MEaV-1 and CBSV and MEaV-1 and UCBSV) and triple (MEaV-1, CBSV and UCBSV) infections occur concurrently or in sequential order within a single host. Thorough understanding on how mixed infection (dual or triple) of the said species belonging to distinct genera (*Ampelovirus* and *Ipomovirus*) is established, could serve as a gateway for discovery of multi-species virus control methods which are based on poly-gene target approach.

In the context of dual virus infections, CBSV species was more prevalent in the (MEaV-1) - positive samples than the UCBSV species. These findings imply that the CBSV species co-existed with the MEaV-1 species in the same host more easily than the UCBSV species during dual infection. The fact that *CBSV* species is more aggressive (hyper-virulent) than the UCBSV species (Alicai et al. 2016), could be the reason why it better competes with the MEaV-1 species and outshine it in colonizing the same host plant unlike the UCBSV species. Intra-species genetic recombination was reported to be common in MEaV-1 species, but the inter-species genetic recombination between the MEaV-1 and CBSV species was confirmed to be non-evident by Kwibuka et al. (2021). It is suggested herein that, sequencing genomic RNA of more strain populations of the MEaV-1 species and *Ipomoviruses* (CBSV and UCBSV species) plus

subsequent bioinformatics analysis of the generated datasets, may provide better insight(s) on the same. Besides this report, no other study has described CBD cases of cassava with dual infection caused by the MEaV-1 and UCBSV species. In other words, there's neither genomic data nor base-line knowledge justifying whether inter-species recombination is possible between the MEaV-1 and UCBSV species. Furthermore, the cassava research community is not fully informed whether topotypes of the MEaV-1 species circulating in cassava producer regions of Tanzania are more or less genetically diverse than those circulating in surveyed regions (DRC, Madagascar, Mayotte and Reunion islands) reported by Kwibuka et al. (2021) or other non-surveyed cassava producer regions in Sub-Saharan Africa.

Sixty-three (39%) suspected CBD cases of cassava tested negative for the *MEaV-1* species, despite displaying evident chlorotic lesions on leaves. It was suspected that, presence of other un-identified novel viruses could be the reason why the 39% cases with evident foliar CBD symptoms, tested negative for both *Ampelovirus* (MEaV-1) and *Ipomovirus* (CBSV and UCBSV) species investigated herein. Another possible reason could be associated with presence of very low, virus titres in the sample leaf tissues which couldn't be detected by the RT-PCR assay.

It was mentioned in Kwibuka et al. (2021) that, mealybugs and soft-scale insects transmit virus species of the *Ampelovirus* genus. Nonetheless, it is not yet elucidated, which among the said vectors are responsible for disseminating the MEaV-1 between cassava plants in Tanzania and similarly for the unscrutinised MEaV-2 species. In other words, the question on whether, the two species (MEaV-1 and MEaV-2) are transmitted by the same or different vector species remain unanswered. Furthermore, the possibility for mechanical transmission of the two *Ampeloviruses* by mechanical means mainly the use sharp farming equipment (axes, machetes, knives, daggers etc.) by farmers also remain underexplored.

Conclusion

The present study reports molecular evidence for presence of CG-Kah and/or CG-Nmb isolates of the MEaV-1 species in Albert, Kikombe and Kiroba varieties by 100%, 82% and 58% respectively at Chambezi, Tanzania, where they are suggested to exist either as a single or mixed infection. Even though preliminary results reported herein are inconclusive with regards to mixed infection these findings provide guidance for ongoing experimental studies to understand virulence levels of distinct MEaV-1 isolates. The following research questions need to be addressed: (i) Which novel isolates of the MEaV-1 species are prevalent in all cassava-producer regions in Tanzania? (ii) Which mechanisms underly virus-virus interactions during dual and triple virus infection cutting across members of the *Ampelovirus*, *Ipomovirus* and *Begamovirus* genera and its consequence on disease symptomatology and root yield? (iii) What is the economic impact of infection by MEaV-1 species in cassava? (iv) What is the resistance level of Eastern African germplasm of cassava against infection by the MEaV-1 species?

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