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Virulence and Molecular Detection of Cassava Mosaic and Brown Streak Virus Genes on Elite Mutant Cassava Lines

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Authors' contributions

This work was carried out in collaboration among all authsors. All authors read and approved the final manuscript.

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ABSTRACT

Unavailability of germplasm with Cassava Brown Streak Disease (CBSD) and Cassava Mosaic Disease (CMD) tolerance genes remains the main bottleneck towards management of the two diseases in tropic and sub-tropic regions in Africa. We assessed the pathogenicity of two viruses and established the presence of genes linked to CBSD and CMD among some elite cassava lines using field techniques and PCR methods. The screen-house experiment was arranged in split plot in completely randomized block design replicated thrice. Severity data was done on a scale of 1 - 5 at an interval of 14 days post inoculation. Symptomatic fresh leaves were used in the extraction of genomic deoxyribonucleic acid and amplification done using markers linked to African cassava mosaic virus (ACMV), separated bands scored as present (+) or absent (-) and visualized under UV trans-illuminator. Virulence and pathogenicity results showed higher tolerance to CBSD and CMD under single inoculation. In contrast, dual inoculation synergistically increased severity expression leading defoliation at early stages of growth. DNA amplification found no CBSD and CMD alleles in CAS1. However, some traces of the alleles were amplified on CAS2, CAS3, KME3 and KME4. Mutant cassava lines exhibited low severity compared to parental lines which were genetically different in terms of viral genes detected by the ACMV linked markers.

Keywords: Virulence; gene detection; cassava mosaic virus; cassava brown streak virus; cassava.

1. INTRODUCTION

Cassava (*Manihot esculenta Crantz*) is ranked second among root crops grown and marketed globally but mostly utilized in Sub-Saharan Africa [1]. In terms of production trends, there has been unpredictable patterns where between 2010 and 2014, yield increased from 323, 389 to 858, 461 metric tons respectively. However, between 2014 and 2016, there was significant reduction to 571,848 metric tons which later increased 970, 587 in 2019 [2]. Nutritionally, cassava roots are rich in magnesium, copper, zinc, manganese while the leaves contain proteins, vitamins and minerals. Additionally, the crop has high energy and carbohydrate content [3].

Despite the existence of approximately 9 million hectares under cassava production in Africa, CBSD and CMD contribute a greater percentage to yield and root quality losses. In previous research documented that a number of cassava varieties expressing tolerance to CMD tend to show high susceptibility to CBSD [4] especially when vertical tolerance is used in breeding other than dual tolerance for the two diseases. These two diseases usually cause leaf necrosis and drying of the roots, significantly reducing their quality and, consequently, their market value. For example, in Tanzania, the CBSD alone has been reported to cause losses of up to 70% [5]. However, dual infections of CBSD and CMD have been reported to cause as high as 100% yield losses in susceptible varieties [6]. Furthermore, CBSD reduces the starch quality of cassava roots hence necessary mitigation measures are needed due to rapid global increase in demand for cassava products and by-products [7,8].

Efforts have been made to reduce severity to CBSD and CMD using a wide range of strategies, but several gaps still exist. For example, open quarantine method [9] and phytosanitary measures are the main strategies known to contain CBSD and CMD. However, these two are hindered by the fact that viruses cannot be eliminated by only selecting asymptomatic materials [10] and the technique is only applicable in emergencies, and when the destination during the transfer of plant materials are near. Additionally, this method proves to be inefficient by its ability to introduce new pathogens and insect pests [11] into new environments.

Furthermore, resistant varieties have been developed for CMD, but for CBSD, little success has been achieved due to ability of the viral pathogen to evolve into more virulent strains. However, fewer varieties have been reported to express tolerance to the two viral diseases but due to increasing demand for the tolerant varieties, there is need to screen newly developed cassava to widen the genetic base for tolerance.

Mutation breeding technique has largely focused on increasing genetic variability through combination of natural and engineered virus resistance to combat multiple cassava viral diseases worldwide [12]. Therefore, utilization of

elite genetic resources provides the most recommended and rapid strategy in conferring tolerance to CBSD and CMD [13]. This can be accurately achieved by establishing the genes linked to the previously observed phenotypic responses by assessing resistance in elite cassava lines to cassava mosaic and cassava brown streak viruses using 2wssequence characterized amplified region (SCAR) markers. The use of specific primers would identify specific genes and base-pairs responsible for the observed severities in each of the cassava lines.

2. MATERIALS AND METHODS

2.1 Evaluating Mutant Lines for Tolerance to CBSV and CMV

The planting materials were sourced from University of Eldoret, School of Agriculture and Biotechnology breeding program (International Atomic Energy Agency - IAEA). These included three candidate mutant lines coded as CAS1, CAS2 and CAS3 which were developed through mutation breeding from KMA2,KMA3and KMA4 respectively and two parental lines namely KME3 (Resistant) and KME4 (Susceptible) farmers' varieties that had been released for commercial production.

The experiment was planted in split-plot arrangement in completely randomized design replicated three times. The main plots consisted of inoculum (control, single CMV, single CBSV dual – CMV + CBSV) while the sub-plots were the five cassava mutant' lines. Approximately, 15 cm long stem cuttings were planted inside plastic pots and watered daily until sprouting. The inoculum was prepared from 10 months old infected leaves collected from experimental sites and famers fields. Infected leaves were crushed using pestle and mortar; mixed with 10 ml distilled water. The inoculum was injected into 30 days old leaf petioles of the experimental plants and data collected from 14 days post-inoculation to 42 days after inoculation. The control experiments however were not inoculated as described by [14]

2.2 Disease Severity Assessment

The severity of CBSD was determined following a 1-5 severity rating scale adopted from [14] where: 1 = no symptoms, 2 = slight chlorosis of the leaves and no symptoms on the stem, 3 = visible and progressed leaf chlorosis with wild lesions on the stem but no dieback, 4 = pronounced chlorosis and stem lesions, 5 = large lesions and dieback. Other than foliar severity, at the final phase of field screening, the severity expression on the cassava roots was quantified and recorded using a 1 to 5 scale, where: 1 = zero necrosis, 2 = less than 5% of root necrosis, 3 = 5-10% necrosis, 4 = 11 – 25% necrosis and 5 = more than 25% necrosis and severe root constriction. For CMD, the response of mutant lines were measured using a 1-5 severity rating scale [15].

2.3 Statistical Data Analysis

Data analysis was done using Genstat statistical software 16th Edition. Descriptive statistics was used to test the significant differences between the lines on their response to single and dual-inoculations by the two viruses. The mean difference for the cassava lines and the inoculants was presented using line graphs with error bars. The phenotypic differences in response between cassava lines and the two viruses were presented in descriptive figures showing the degree of virulence and pathogenicity under dual and single inoculation by the mosaic and brown streak viruses.

2.4 Detection of Cassava Mosaic and Brown Streak Virus on the Elite Cassava Lines

2.4.1 Deoxyribonucleic acid (DNA) extraction

Genomic DNA was extracted from cassava leaves expressing symptoms of African cassava mosaic virus (ACMV) from greenhouse plants. The harvested leaves were labeled and immediately put in icebox and taken to the laboratory where they were placed in -80°C for ease of grinding. Established Cetyltrimethyl ammonium bromide (CTAB) and modified protocol as suggested by [16] was used to extract the DNA where 200 mg leaf samples were weighed and crushed to form a homogenous paste in 500 µl CTAB buffer (10% SDS, 0.5 M EDTA, 1 M Tris-HCl with a final pH of 8.0). 10 ul of 100 mg/ml RNase A (Bioneer) was added and the homogenate transferred into 1.5 ml eppendorf tube and incubated at 65 °C water-bath for 30 minutes. The tube was inverted 4-5 times after every five minutes during the incubation period to ensure uniform distribution of the crushed leaf tissues in the buffer. The sample was then cooled down in fridge for 15 minutes and 200 ul of 6 M Ammonium Acetate added and vortexed. The mixture was kept in

fridge at 4°C for 15 minutes. Using Eppendorf centrifuge, the mixture was centrifuged for 5 minutes at 13000 rpm. The supernatant was transferred to a new eppendorf tube and 50 µl of 10% CTAB preceding addition of 700 ul of chloroform: isoamyl alcohol (24:1) and gently mixed by inversion. The mixture was centrifuged at 13,000g for 5 minutes. The upper phase (approx. 500 ul) was transferred to a new eppendorf tube and DNA precipitated by addition of 350ul ice cold isopropanol, the tubes were gently inverted and kept at -20°C for 15 minutes. The precipitated DNA was pelleted by centrifuging at 14000 rpm for 20 minutes followed by 70% and 90% ethanol washing the pellets. The pellets were air dried on a clean bench and dissolved in 100ul 1x TE (tris EDTA) buffer.

2.4.2 DNA amplification

The extracted DNA was amplified using Eppendorf master cycler gradient PCR. Four primer pairs obtained from INQABA BIOTECH specific to the ACMV were used to amplify DNA from three Cassava lines (CAS1, CAS2 and CAS3) and their parents (KME3 and KME4). The primer codes and base pairs are shown in Table 1. Solis BioDyne Firepol Master mix was used to run the PCR reaction at a volume of 20 µl per reaction and constitutions of 4 µl Firepol Master mix, 1 µl forward, 1 µl reverse primer, 2.5 µl template DNA and 11.5 µl molecular water. The PCR program was 94 °C initial denaturation for 3 min, 94°C denaturation for 30 seconds with varied annealing temperature depending on the primer (between 49-60°C) for 1 minute, 72°C extension for 1 minute and final extension for 7 minutes. The PCR product was run for 1 hour at 110 Volts in a 2.5% gel stained with Green star nucleic acid stain followed by visualization in BioDoc IT gel documentation.

2.4.3 Cassava brown streak virus RNA extraction and amplification

RNA was extracted from fresh cassava leaves with symptoms of cassava brown streak virus (CBSV). Acetyl trimetyl ammonium bromide total nucleic acid extraction protocol optimized for cassava with slight modifications followed. Fifty (50) mg of fresh cassava leaf was placed in -80°C for easy grinding. The leaves were removed from the freezer; ground using a sterile mortar and pestle, the ground leaf powder was transferred into a sterile Eppendorf 1.5 ml tube for each sample separately. The tubes with leaf powder were transferred into a fume hood and 750 µL CTAB buffer was added (2.0%, w/v CTAB, 2.0 M NaCl, 2.0% PVP, 25 mM EDTA, 100 mM Tris-HCl pH 8.0, 0.2% fresh β-mercaptoethanol).The mixture was shaken vigorously on a vortex mixer and incubated at 65°C for 30 min in a water bath.

The RNA was extracted by adding 750 µL Phenol: Chloroform: Isoamyl mixture (25: 24:1), the mixture was then inverted for 10 minutes and centrifuged at 13300 × g for 10 minutes. 500 mL of the aqueous upper phase was transferred to a new sterile 1.5 mL micro centrifuge tube. The RNA was precipitated in 0.6 vol (300 µL) of isopropanol by incubating at −20°C for 1 h and pelleted by centrifuging at 15600 × g. The pellets were washed twice in 700 µL of 70% ethanol, centrifuged at 15600 × g and the ethanol was discarded by decanting. The remaining ethanol was completely removed by pipetting and air drying for 30 min at room temperature. The extracted RNA pellets were dissolved in 100 µL of 1xTris-ethylene diaminetetra acetic acid (TE, Invitrogen). Finally, the RNA quantification was done using a NanoDrop2000 spectrophotometer (Thermo Scientific) and used immediately for one step RT-PCR analysis [17].

2.4.4 Reverse transcriptase PCR using CBSV 10 and CBSV 11 primers

The extracted RNA was then subjected to a one step RT-PCR for virus detection using primer set CBSV 10 (5''ATCAGAATAGTGTGACTGCTGG-3') and CBSV 11 (5''CCACATTATTATCGTCACCAGG-3') (Mouketou et al. 2022) which amplify ~230 bp length nucleotides. The 10 µl PCR reaction mix contained 6.85µl of sterile nuclease free water, 1 µl of 10x MMLV buffer, 0.3 µldNTPs (2mM), 0.08 µl of Taq polymerase (5U/µl), 0.15µl of the primer mix, and 2 µl of RNA template. Thermal cycling conditions comprised of Pre-PCR program for generating the cDNA in 1 cycle at 42°C for 30 min 94°C for 2 min, 52°C for 2 min and 72°C for 3 minutes. The PCR cycle for cDNA multiplication included 30 cycles of 94 °C for 30 min, 52°C for 30 seconds, 72 °C for 1 minute 25 seconds and stored at 4 °C. Gel electrophoresis was done in 1x TBE at 100V for 1hr and the products visualized on a UV transimilluminator (BioDoc.IT) [18].

2.4.5 Visualization of the PCR bands

A 1% Agarose gel was prepared by mixing 1.0 g of Agarose with 100 ml 1x TBE (Tris-HCL Boric

Acid EDTA) buffer. The solution was then heated in a microwave for proper mixing. The gel was left to cool to approximately 60°C then 3 µl of nucleic acid gel stain Ethidium Bromide was added. The gel was then cast in trays and combs carefully placed and solidification allowed occurring. The PCR products obtained were then mixed with 1 µl of the loading dye (orange G) and 5µl of the sample loaded in each well [18]. The data on band separation was scored as present (+) or absent (-) for the genes responsible for mosaic and brown streak diseases in cassava.

3. RESULTS

3.1 Virulence Expression of CMV and CBSV under Single and Dual Inoculation

All the mutant cassava lines (CAS1, CAS2 and CAS3) expressed significant tolerance to CBSD and CMD under both single and dual infection with CBSV and CMV compared to parental lines. Specifically, single inoculation with CBSV revealed that CAS1 and CAS2 were more tolerant to the virus than CAS3 and all checks at 14, 28 and 42 days after inoculation (DAI). However, for single inoculation with CMV, CAS1 and CAS3 did not differ significantly but CAS2 recorded lower severity compared to the two candidates and parental lines (Fig. 1).

Phenotypically, under dual inoculation with a mixture of CBSV and CMV, all three mutant cassava lines (CAS1, CAS2 and CAS3) performed better than the parental lines (checks) across the duration of assessment. For instance, leaf defoliation was observed with KME4 (susceptible check) compared to the candidates and KME3 (resistant check). However, CAS3 was the most resistant to leaf defoliation under dual inoculation with the two viruses (Plate 1).

3.2 Genetic Diversity in Reference to CMV and CBSV Gene Detection

The two primers for CBSV (CBSV 10 and CBSV 11) did not detect any genes for cassava brown streak virus across all the elite cassava lines and their respective parental lines. However, the ACMV primers detected the presence of genes linked to cassava mosaic virus in all the mutant lines screened. The banding pattern for ACMV1, ACMV-ALI, ACMV-ARO primers showed the presence of CMV genes in CAS2 and CAS3 as well as the parental checks (KME3 and KME4). Among the mutant lines, only CAS1 did not express the presence of CMV genes by three ACMV primers and this corresponds with tolerant phenotypic results where it was the most tolerant line among the elite lines under single inoculation with the virus (Table 1).

ACMV2 primer pair was efficient in detecting the African cassava mosaic virus with positive detection of the genes in all the five lines tested. However, the bands present were faint in the mutant lines (CAS1, CAS2 and CAS3) and more intense in two parental lines genotypes (KME3 and KME4) with a 1,200 bp band. CMV-AL1 primer being co-dominant marker gave 3 different bands at 1,800 bp, 1,700 bp and 1,500 bp. However, these three bands were detected in all parental lines and CAS2 and CAS3 while CAS1 recorded false positive for the 1,800 bp band.

Table 1. Presence (+) or absence (-) of ACMV genes linked to CMD in 5 cassava lines

No	CASSAVA LINES	ACMV –PRIMERS			
		ACMV1	ACMV2	ACMV-ALI	ACMV-RO
1	CAS1	-	+	-	-
2	CAS2	+	+	+	+
3	CAS3	+	+	+	+
4	KME3	+	+	+	+
5	KME4	+	+	+	+

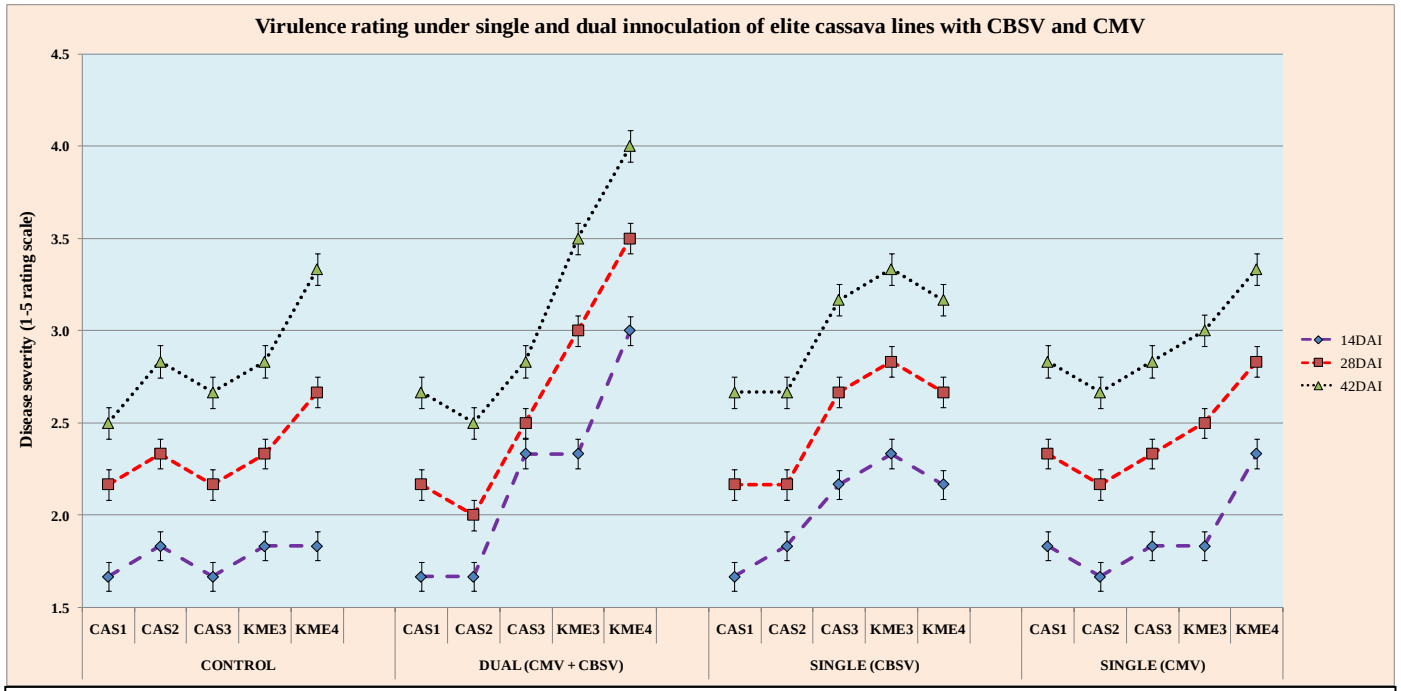

Fig. 1. Severity expression by elite cassava lines and their parental checks under single and dual inoculation with mosaic and brown streak viruses. Assessment were made 14, 28 and 42 days after inoculation

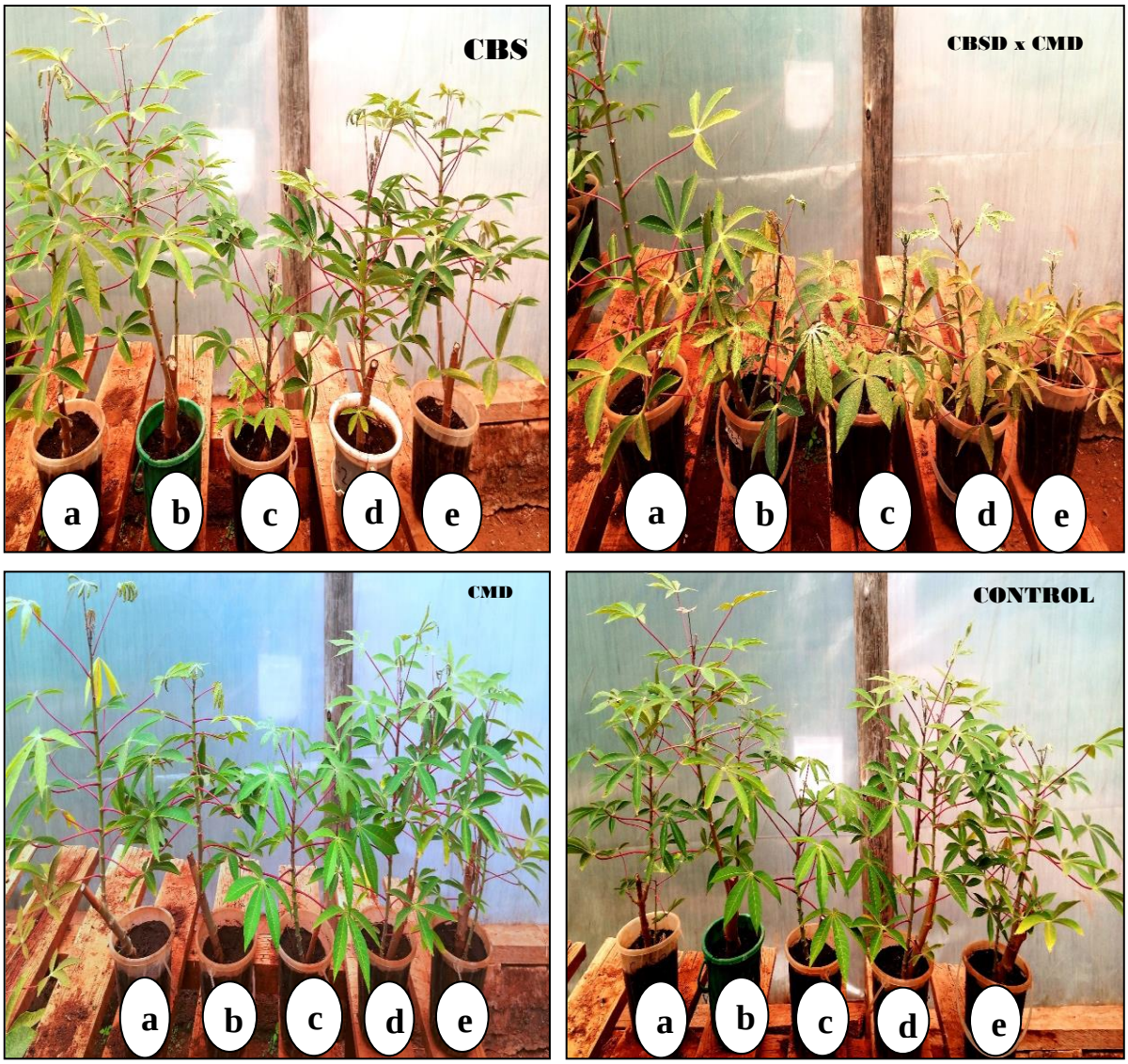

Plate 1. Response of cassava lines to CBSD and CMD under single and dual (CBSD x CMD) viral inoculant combination under greenhouse conditions. The cassava lines identified as 'a', 'b', 'c', 'd', and 'e' were CAS3, KME3, CAS1, CAS2 and KME4 respectively. The single inoculation by either CBSV and CMV were less severe *compared to dual infection by the two viruses*

The CMV-ARO molecular marker amplified two distinct bands with 1,400 bp and 1,500 bp. The 1400 bp band amplified by ACMV-ARO was detected in all cassava lines while 1,500 bp band was expressed in all parental lines and CAS2 and CAS3 but lacked in CAS1. Molecular band visualization of amplified genes by ACMV1 marker revealed a matched susceptible viral allele with 1,800 bp that was found in the susceptible and resistant checks (KME3 and KME4) as well as a false positive band for CAS2 and CAS3. However, there was no amplification of this susceptible gene in CAS1 using this primer (Fig. 2).

4. DISCUSSION

The low severity expression for CBSD and CMD under single inoculation especially by the elite mutant lines could be due to the existence of resistant genes randomly created through mutagens used in generating the lines. Conversely, the higher severity that resulted in defoliation in some lines may be an indication of synergistic effect on virulence and pathogenicity when the two viruses successfully infect a single host at the same time [19]. Similarly, the severity increase from 14 days post-inoculation to 42 days after inoculation corresponds with the

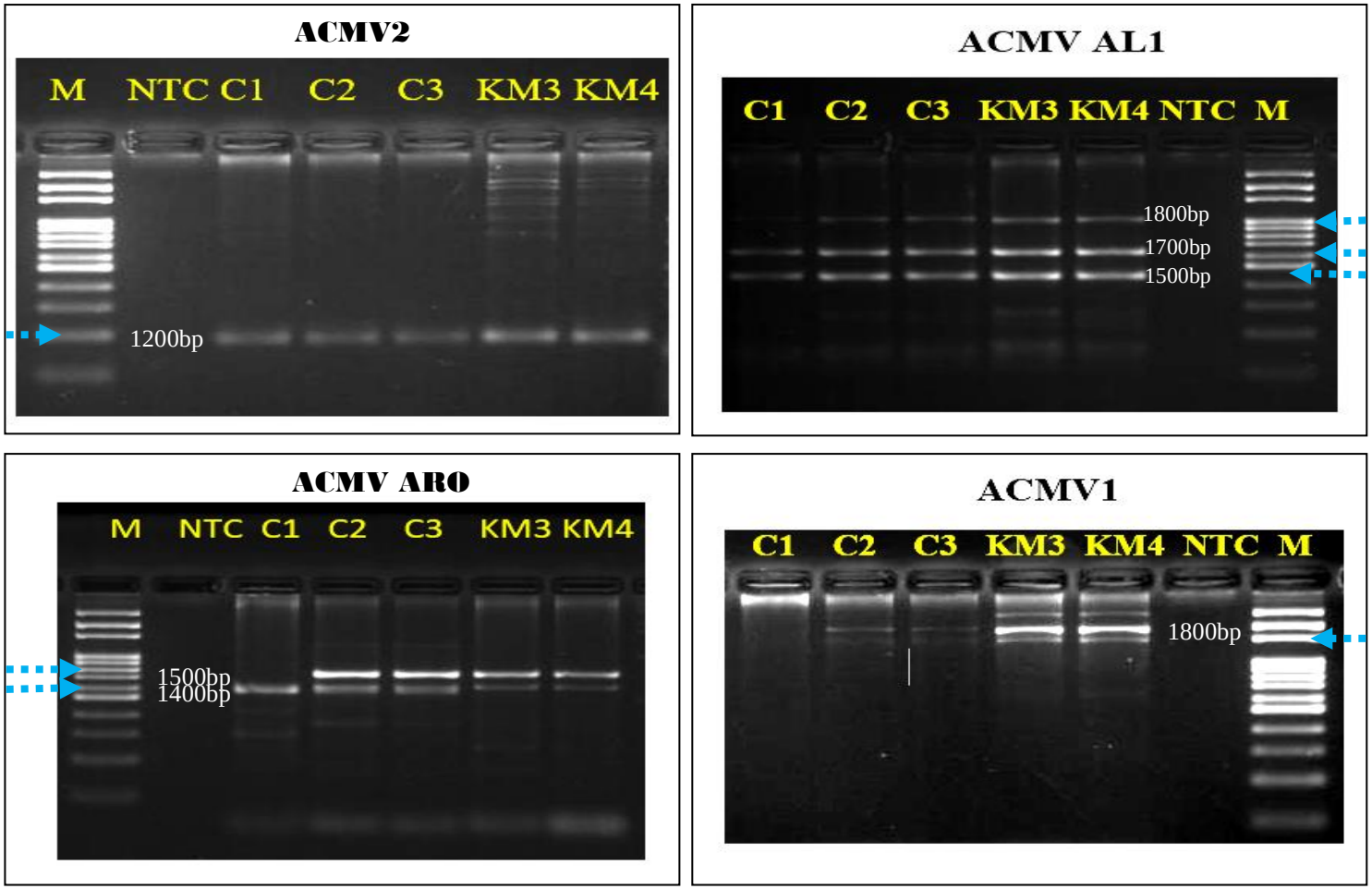

Fig. 2. Genomic DNA diversity and similarity among the elite cassava lines (CAS1-C1, CAS2-C2 and CAS3-C3) alongside resistant (KME-KM3) and susceptible (KME4-KM4) parental lines amplified by ACMV2, ACMV-ALI, ACMV-ARO and ACMV1 markers linked to CMV

findings by other researchers where after 28 days of inoculation, the severity expressions were higher on the susceptible checks than the test lines [20].

The higher severity which affected even the elite mutant lines under the dual infection by the CBSV combined with CMV may imply that when the two viruses combine within a single host, much of the physiological processes are affected due to the mixed signals sent by the two viruses under host-pathogen interactions [21]. This could also mean that the replication signals sent by the two viruses under dual infection caused rapid synthesis of biochemicals that synergistically destroyed plant cells in response to infection hence silencing the expression of resistant genes in CAS1, CAS2 and CAS3 which later expressed some level of susceptibility to the two viruses under dual infection [22]. Lastly, the expression of symptoms of viral infection by the control experiment (non-inoculated) could be as a result of vector transmission that sucked sap from the infected cassava lines as well as from non-inoculated lines and such observation is similar to previous studies where non-inoculated plants showed some level of infection [23].

The absence of genes linked to CBSV when amplified by the CBSV 10 and CBSV 11 primers despite the symptomatic expression both in the field and under virulence and pathogenicity studies could imply the virus have mutated to form a different allele with different base-pairs as previously known. This should be further studied and tested. However, the diverse expression of CMV genes by the mutant lines as well as their parental lines proves that there is genetic diversity among the elite cassava lines screened against CMD [24]. Further, the variation in base-pairs for the detected alleles by the different ACMV primers depict the possibility of existence of about 5 viral alleles with 1,200 bp, 1,400 bp, 1,500 bp, 1,700 bp and 1,800 bp that are responsible for symptom expression for CMV infection in cassava.

The consistency in tolerance to the two viruses by CAS1 mutant line and the subsequent lack of a number of genes linked to susceptibility to CMV indicate that mutation breeding has a great potential to alter allelic genes with possible beneficial impact on plant tolerance to viruses [25]. This was also phenotypically expressed by other two mutant lines (CAS2 and CAS3) compared to parental lines which expressed all viral genes for all the primers used as markers [24].

5. CONCLUSION

Mutant cassava lines (CAS1, CAS2 and CAS3) expressed low virulence and pathogenicity to CBSV and CMV under single and dual inoculation compared to unimproved parental lines. Also, fewer CMV alleles were detected in elite mutant lines compared to the parental resistant and susceptible checks. The study recommends the use of CAS1, CAS2 and CAS3 mutant lines for variety improvement and screening against CMV and CBSV as resistant checks and the resistant traits in CAS1 should be exploited at genetic level to confer tolerance to these two viruses in commercially grown cassava varieties.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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