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East African Cassava Mosaic Virus and East African Cassava Mosaic Cameroon Virus: Two Species Emerging in Togo

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Abstract

Cassava mosaic disease (CMD) caused by the whitefly-transmitted *Begomoviruses* (family Geminiviridae) is a major threat to production of cassava (*Manihot esculenta* Crantz) in Togo. Survey was conducted in 2020 in the 5 agroecological zones of Togo to assess the status of East African cassava mosaic virus (EACMV), East African cassava mosaic Cameroon virus (EACMCMV) and its distribution. Polymerase chain reaction (PCR) and Sanger sequencing were used for the detection of cassava mosaic *Begomoviruses* (CMBs) in the sampled leaves. The incidence of EACMV was 47.93% (278/580) and varied between 41.30% (Zone V) and 62.29% (Zone IV) across the agroecological zone but no significant difference was observed. The EACMCMV incidence was 13.67% (38/278) and varied significantly ($p \leq 0.001$) through the agroecological zone. Phylogenetic analysis of the viral isolates showed that they are closely related to those from Nigeria, Ghana, and Ivory coast. Nucleotide sequence analysis of CP revealed an overall genetic diversity (π) of around 3.4%. These results showed that EACMV was the predominant virus and that EACMCMV incidence could be more widespread in Togo.

Keywords

Begomovirus, PCR, EACMV, EACMCMV, Togo, Genetic Diversity

1. Introduction

Cassava (*Manihot esculenta* Crantz) is recognized as one of the most important

tuber crops cultivated in tropical and subtropic regions, providing a major source of food to more than 800 million people worldwide [1]. In Togo, cassava is the important largest food crop with an annual production estimated at 1.15 million tons [2] and its diversity of processing. Despite its many advantages, cassava production is affected by dozens of factors across the producing countries, including [3] especially Cassava Mosaic Disease (CMD). CMD is caused by a group of viruses commonly referred to as Cassava Mosaic Begomoviruses (CMBs), belonging to the genus *Begomovirus* in the family Geminiviridae [4] [5]. It is reported wherever cassava is grown. This disease is spread by a whitefly *Bemisia tabaci* (Gennadius) or by the infected cuttings used for planting a new field [6]. Eleven cassava mosaic *Begomovirus* (CMB) species have been reported in Africa [7]-[11] among which African cassava mosaic virus (ACMV), East African cassava mosaic virus (EACMV), and East African cassava mosaic Cameroon virus (EACMCMV) are known to be widely prevalent in sub-Saharan Africa [12]. The first studies carried out on the geographical distribution of these viral species suggested that EACMV mainly present in East Africa [13] and EACMCMV in Cameroon [7].

East African cassava mosaic virus (EACMV) and East African cassava mosaic Cameroon virus (EACMCMV) were reported in Togo in 2009 and 2017 [14] [15]. EACMV in combination with African cassava mosaic virus (ACMV), is the major cause of cassava mosaic disease in sub-Saharan Africa, with attendant important yield reduction of cassava [16]. Zhou *et al*. [17] provided evidence of inter-specific recombination of DNAs of ACMV and EACMV to give rise to a strain of EACMV in Uganda (EACMV-Ug).

The aim of this study is to determine the status of East African Cassava mosaic virus and East African cassava mosaic Cameroon virus on cassava's farm in Togo. The outcomes could be utilized in the screening of cassava genotypes for resistance to cassava mosaic disease (CMD) on agroecological zones.

2. Material and Methods

2.1. Collection of Cassava Leaves

Surveys were conducted between August-September 2020 throughout Togo. A total of 580 cassava leaves with characteristic CMD symptoms and CMD-free were collected from farmers' fields at 3 - 6 months after planting (MAP) located at intervals of between 10 km in five (05) agroecological zones of Togo [18]. The agroecological zones were Sudanian savannah (Zone I), dense forests and grassy savannah (Zone II), Guinean wooded savannah, clear forests and discontinuous forests along the main rivers (Zone III). Agroecological zone IV corresponds to the humid and semi-deciduous forests zone and Zone V is located at the extreme south of the country with fallow land, thickets, bushes, derived and coastal grassland savannah [19].

The collection of samples was done following the random method based on the presence of viral symptoms. The latitude and longitude of each farm were

recorded using Global Positioning System equipment (Garmin GPSMAP 64s) and the software QGIS 2.4.0 was used to superimpose on the agroecological map of Togo.

Symptom severity of CMD was rated on a 1 to 5 scale as described by ITTA [20] where 1 = asymptomatic, 2 = Mild symptom, 3 = Moderate symptom, 4 = Severe symptom and 5 = Very severe symptom. The samples were kept in the envelopes and dried at 25˚C.

2.2. DNA Extraction

Total DNAs were extracted from 100 mg of leaf tissue of infected plants. Total DNA was extracted from leaf samples using InnuPREP Plant DNA Kit (Endress + hauser Compagny, Germany) according to the manufacturer's instructions. The extracted DNA was stored at −20˚C for analysis. The concentration and purity of extracted DNA samples were determined using a Nano Drop spectrophotometer (Thermo Scientific, Nano drop-2000C, Germany).

2.3. Molecular characterization of Cassava Mosaic *Begomoviruses*

The DNA samples of the cassava were tested for the presence or absence of CMB using primers that could detect EACMV and EACMCMV. Four (04) primers pairs were used (Table 1). The samples positive for EACMV virus were subjected to another round of PCR using specific primers for the detection of EACMCMV (VNF031/VNF032).

The PCR mixture (25 µL) contained 12.5 µL Master Mix (New England Biolab, NEB), 1.25 µL each of forward and reverse primers (10 µM), 8 µL nuclease free PCR water (Inqaba Biotech West Africa Ltd) and 2 µL DNA. The PCR conditions used are those described by the various authors in Table 1. The DNA amplification was carried out in the Applied Bio systems Thermal Cycler. The PCR amplified products were separated by agarose gel electrophoresis on 1% (which was stained with ethidium bromide) for 100 V for 35 min in 1% TBE

Table 1. Primers pairs used for the amplification of CMBs.

Primer Name	Virus	Primer sequence (5' 3')	Tm (˚C)	Amplicon (bp)	References
JSP001	EACMV	ATGTCGAAGCGACCAGGAGAT	55	780	[21]
JSP003		CCTTTATTAATTTGTCACTGC			
CMBRep F		CRTCAATGACGTTGTACCA	56	650	[22]
EACMVRep R		GGTTTGCAGAGAACTACATC			
EAB555/F		TACATCGGCCTTTGAGTCGCATGG	58	550	[7]
EAB555/R		CTTATTAACGCCTATATAAACACC			
VNF031/F	EACMCMV	GGATACAGATAGGGTTCCCAC	52	560	
VNF032/R		GACGAGGACAAGAATTCCAAT			

Buffer. Bands were viewed under UV light using and the images saved using a gel documentation system (MultiDoc-It Digital Imaging System). PCR products of 24 EACMV and EACMCMV positive samples were directly sequenced in both forward and reverse orientations using the Sanger method at Inqaba Biotec company.

Disease incidence per field was calculated using the formula below [23]:

$$\text{Incidence} = \frac{\text{Number of infected plants}}{\text{Total number of plants}} \times 100$$

2.4. Phylogenetic Analysis

The sequence of 24 samples were used phylogenetic analysis. Blastn program was used for preliminary analysis [24] using Consensus sequence obtained from forward and re-verse sequences for each sample. Multiple sequences alignment was performed with Clustalw of the Megalign program included in the Lasergene v10.0 software (DNASTAR). The nucleotide diversity (π) was calculated using a 100-nucleotide (nt) sliding window with 25-nt steps using DNAsp 6.0 software [25]. Phylogenetic trees were constructed using the Maximum Likelihood (ML) method and the Kimura two parameter (K2) nucleotide substitution model [26] The tree was 1000 replicates using MEGA11 [27].

2.5. Statistical Analysis

Data analysis was performed using the R software v. 4.3.2. The normality of the variables was determined using the Shapiro-Wilk test and the generalized linear model was used. Statistical analysis of the incidence by agroecological zone were conducted with the analysis of variance (ANOVA) with one criteria of classification. The difference between the means was compared using the LSD test at 5%.

3. Results

3.1. Symptomatology

Cassava samples collection was carried out in 159 farms surveyed. A total of 580 leaf samples including 392 symptomatic samples and 188 asymptomatic samples were collected. Different types of symptom phenotypes occurred in the different locations in all the surveyed fields. Mosaic, leaf distortion, stunting, and leaf reduction were observed on the symptomatic samples collected (**Figure 1**).

3.2. Detection of EACMV and EACMCMV Per Agroecological Zone

Samples analyzed by PCR confirmed the presence of CMD in Togo. PCR amplification with expected sizes of 560 bp and 780 bp were obtained from the cassava samples with VNF031/VNF032, and JSP001/JSP003 respectively (**Figure 2**).

Out of a total of 580 samples, 65.69% (381/580) were positive by PCR for CMD, 44.31% (257/580) reacted positive with ACMV primers (unpublished result) and 47.93% (278/580) also were positive for EACMV primers. The specific

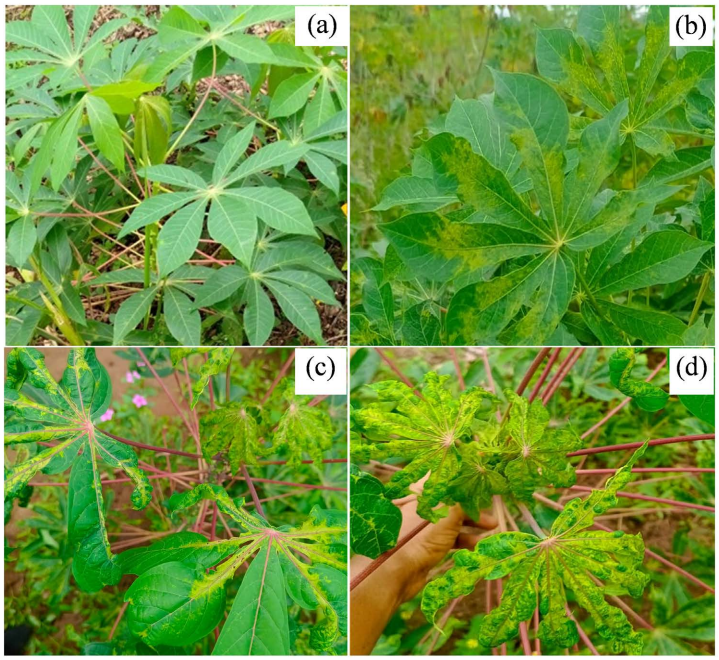

Figure 1. Symptoms of cassava mosaic disease observed on infected cassava plants during the surveys (a) Asymptomatic leaves; (b) Mild mosaic; (c) Moderate mosaic; (d) Severe mosaic.

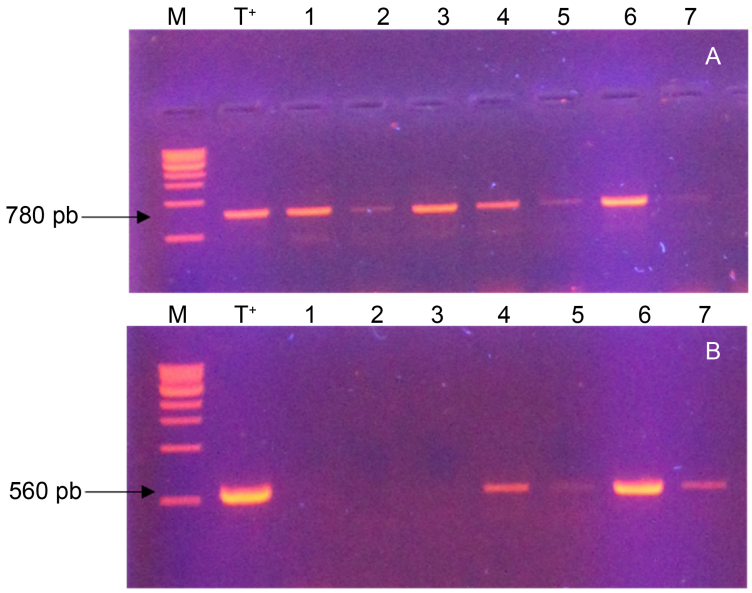

Figure 2. Agarose gel electrophoresis with primers JSP001/JSP003 identifying EACMV, giving fragments of 780 pb (A) and primers VNF031/VNF032 identifying EACMCMV, giving fragments of 560 pb (B). Lanes 1 = T + (Positive sample), 2 to 8 contained separate cassava samples. M = Marker.

primer (VNF031/VNF032) passed on the EACMV positive samples showed that 13.67% (38/278) of positive EACMV were tested positive to EACMCMV. PCR results showed that EACMV and EACMCMV were present in all the 5 agroeco-

logical zones. The incidence of EACMV across the agroecological zone varied between 41.30% (Zone V) and 62.29% (Zone IV) but no a significant difference was observed. The EACMCMV incidence varied significantly ($p \leq 0.001$) through the agroecological zone. The high incidence was 13% (Zone II) and the lowest 04.92% (Zone IV) (Table 2).

Table 2. Incidence (%) from samples tested by agroecological zones.

Agroecological Zone	Tested samples	EACMV		EACMCMV	
		Positives samples	%	Positives samples	%
Zone I	54	24	44.44 a	3	12.5 b
Zone II	100	35	35 a	13	37.14 a
Zone III	120	67	55.83 a	11	16.42 b
Zone IV	122	76	62.29 a	6	7.89 b
Zone V	184	76	41.30 a	5	6.54 b
Total	580	278	47.93	38	13.67

Note: Percentages followed by the same letters are not significantly different.

EACMCMV occurred in the 5 agroecological zones, Figure 3 shows its distribution. A near-equitable distribution of EACMCMV was observed in agroecological zones IV and V. In other agroecological zone, the distribution of EACMCMV remains concentrated only in certain prefectures (Zone III: Amou, Tchaoudjo, Tchamba, Sotouboua; Zone II: Kozah, Assoli, Bassar, Tchaoudjo and Zone I: Oti, Binah, Dankpen).

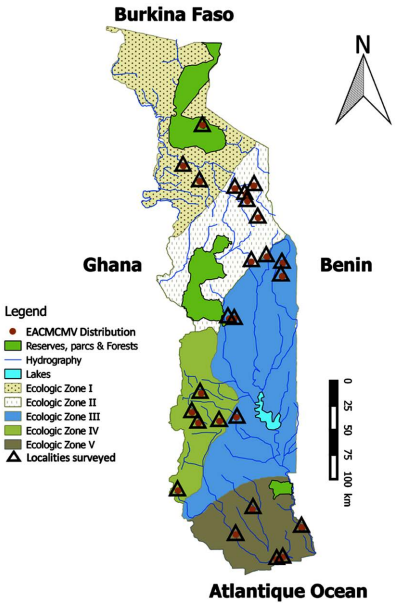

Figure 3. Geographical location of EACMCMV on cassava's farm in Togo.

3.3. Phylogenetic Analysis of EACMV and EACMCMV Isolates

The result of Blastn of sequences in the GenBank database (NCBI) showed that the 24 sequences of EACMV and EACMCMV isolates correspond to those already identified in the sub-region. They shared the highest nucleotide identity (97% - 99%) with EACMV isolates from Nigeria (OM386996.1, OM387001.1, OM387002.1, OM307000.1) and Togo (EU155148.1). The sequences of EACMCMV shared too the highest nucleotide sequence (97% - 99%) with EACMCMV isolates from Nigeria (EU685326.1, EU685321.1, EU685319.1, EU685327.1), Ivory Coast (LC722231.1, AF259896.1, LC722234.1), Ghana (JN165089.1), Madagascar (KJ887944.1, KJ888078.1).

The obtained sequences were used to study the phylogenetic relationships between viral populations. The construction of the phylogenetic tree by the maximum likelihood (ML) method, revealed that EACMV and EACMCMV isolates are distinguished into 3 groups (G1, G2, G3) (**Figure 4**). Groups 1 contains

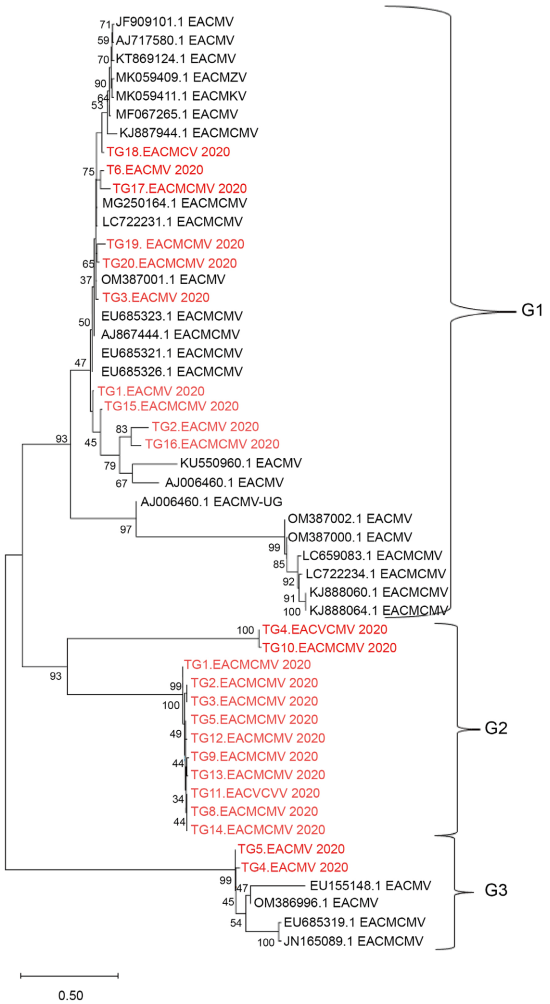

Figure 4. Maximum-likelihood phylogenetic tree obtained from alignment of nucleotide sequences of East African cassava mosaic virus and East African cassava mosaic Cameroon virus. The names of the sequences characterized in this study are in red.

EACMV, EACMCMV isolates from Togo and isolates of Genbank. Groups 2 contains only EACMCMV isolates from Togo and Group 3 EACMV isolates from Togo and Genbank.

The analysis of the structuring of viral populations according to their geographical origin shows that there is no relationship between the geographical origins and the genetic diversity of the isolates.

The analysis of the near-total nucleotide sequences of the capsid protein (CP) gene of EACMV and EACMCMV viruses revealed a genetic diversity of 3.4 % with a peak of 4.6% at the terminus. C-terminal (Figure 5). The alignment of the CP nucleotide showed that they have 768 nucleotides including 577 variable sites corresponding to 75% of the total sequence.

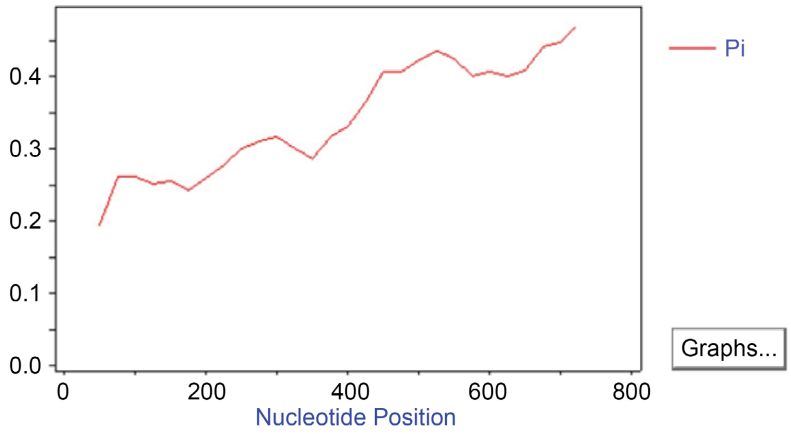

Figure 5. Nucleotide diversity (π) along the EACMV et EACMV capsid protein (CP) gene.

4. Discussion

In the present study, the status of East African Cassava Mosaic Virus (EACMV) and East African Cassava Mosaic Cameroon Virus (EACMCMV) on cassava's farm in Togo has been reported. PCR analysis with the 4 pairs of primers specific to EACMV and EACMCMV confirmed the presence of these viruses in the leaf samples, respectively at proportions of 47.93% and 13,67%. EACMV was detected in the majority of CMD-affected cassava leaves, and was the predominant Cassava mosaic *Begomovirus* species in Togo, which is not consistent with earlier findings in Togo [14] [15] [28], Burkina Faso [29], Nigeria [30] in which they reported ACMV as the frequently detected CMD. These results show that the situation has evolved. Indeed, most samples (47.93%) tested positive for EACMV im-plying that EACMV has displaced ACMV. The use of a large number of primers (04) gives better results, hence a more specific real idea of these diseases in Togo. This finding is similar to an earlier observation by Legg and Thresh [31] and Were *et al*., [32]. These authors showed that in post epidemic areas ACMV had been largely displaced by the more virulent EACMV-UG2. This increase could be explained by the exchange of cuttings from diseased

plants from East Africa via Cameroon and Nigeria given that phylogenetic analyzes showed that the majority of isolates from Togo would be closer to those from Nigeria. East African cassava mosaic Cameroon virus (EACMCMV) was found in 13.67 % samples that positive for EACMV.

The expansion of CMD is mainly favored by biological vectors of the disease and by the use of infected cuttings from previous seasons. Similar observations are made by [33] and [34], who assimilated the increase in incidence to the recycling of infected cuttings by farmers. The importance of the incidence could also be justified by the high susceptibility to cassava mosaic disease of the sampled cultivars. The high incidence rates observed in various fields suggests that stem cuttings are the likely origin of the virus due of the use of planting materials stems which are often infected by viruses. Sources of inoculum are naturally infected plants when used as planting materials in successive years and also other herbaceous hosts of *Begomovirus* [22] [35] [36].

The majority of EACMV isolates characterized belong to the East African cassava mosaic Cameroon virus (EACMCMV) species first described by Fondong *et al.,* [7]. EACMCMV species of cassava mosaic *Begomovirus* result from two recombination events at ORFs AC2/AC3 [7]. East African cassava mosaic Cameroon virus acts in synergy with the African cassava mosaic virus species and is responsible for the expression of severe symptoms on cassava.

The results from the phylogenetic analysis showed that 3 groups were obtained. Group 2 is made up only of EACMCMV. It should be noted that, the most isolates of EACMV and EACMCMV are specific to Togo. The connection between the sequences of the characterized isolates and those of neighboring countries (Nigeria, Ghana, Ivory Coast, Madagascar) could be explained by the exchanges of plant material between Togo and these countries. It is possible that EACMV was introduced from coastal areas of East Africa, including Madagascar, where the virus is predominant [16] [37]. Among these countries, Nigeria appears to be the country whose sequences share the most nucleotide identity with the isolates in this study.

The nucleotide sequences analysis (π) of EACMV and EACMCMV revealed overall low diversity compared to low diversity RNA viruses (3% - 10%) [38] [39]. The analysis of the distribution of isolates from Togo according to their geographical origins indicates an absence of correlation between the geographical structure and the genetic diversity of EACMV and EACMCMV viruses. This absence of correlation represents ideal conditions for the emergence of interspecific variants which would represent a major epidemiological risk for cassava cultivation in Togo. Previous perennial crop studies investigating Tobacco Mild Green Mosaic Virus (TMGMV) and Citrus Treza Virus (CTV) with results reported similar results [40] [41].

The current work confirms that the situation has rather evolved through the emergence of EACMV viruses, unlike previous work [14] [42] in Togo between 2010 and 2020. These results show that the presence of EACMCMV and even

ACMV could be more widespread in Togo than previously thought. The coexistence of species native to Togo and those of foreign strains of cassava mosaic *Begomovirus* could cause mixed infections and therefore new recombinations [15].

5. Conclusion

East African cassava mosaic virus (EACMV) and East African cassava mosaic Cameroon virus were characterized in this study. The analysis of the geographical distribution of the isolates obtained suggests that the two species are distributed in the five agroecological zones of Togo. The use of virus-free cuttings of cassava genotypes with enhanced resistance and the varietal improvement remains the most important control option.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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