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Occurrence of Cassava Mosaic Begomovirus New Species and *Ageratum Leaf Curl Cameroon Virus* on Pepper (*Capsicum annuum* L.) in Togo

Kodjovi Atassé Dansou-Kodjo[1,2], Assion Sétu Mivedor[1], Kossikouma Djodji Adjata[1*], Jérôme Duclercq[2], Yawovi Mawuena Dieudonné Gumedzoe[1]

[1]Laboratory of Plant Virology and Biotechnology (LVBV), Ecole Supérieure d'Agronomie (ESA), University of Lome, Lomé, Togo
[2]Laboratory of Agroecology, Ecophysiology and Integrative Biology (AEB), Unit EDYSAN FRE 3498 CNRS/University of Picardie Jules Verne, Amiens, France
Email: *dadjata@yahoo.fr

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Abstract

Cassava mosaic disease caused by the whitefly-transmitted begomoviruses (family *Geminiviridae*) is a major threat to cassava (*Manihot esculenta* Crantz) production, which can be intercropped with other plants such as pepper (*Capsicum annuum* L.). The aim of this study is to identify cassava begomoviruses on other crops in cassava intercropping systems. Thus, foliar samples showing typical symptoms of virus diseases in cassava intercropping systems were collected from pepper and submitted to PCR analysis and direct sequencing. Three begomovirus species ACMV, EACMV and ALCCMV were identified and characterized in samples. Isolates of these species shared respectively 90% - 93%, 74% and 80% nucleotide identities with begomoviruses. These findings show that cassava begomoviruses can infect other crops and will help in understanding the epidemiology related to whitefly-transmitted begomoviruses in cassava intercropping systems.

Keywords

ACMV, ALCCMV, Begomovirus, EACMV, Pepper, Sequencing

1. Introduction

The most damaging and economically important diseases of crops, especially in tropical and subtropical regions are caused by the whitefly-transmitted begomoviruses. These viruses are included in the genus *Begomovirus* of the family *Geminiviridae* and are responsible for causing crop losses ranging from 30% to 100% [1]. Begomoviruses are single-stranded circular DNA viruses, which repli-

cate in the nucleus of their host cells and are classified in monopartite or bipartite viruses depending on their genome organization [2]. They have emerged everywhere in the world where environmental conditions support large whitefly (*Bemisia tabaci* Genn.) populations and in Western Africa, emergence of begomoviruses is caused by genetically distinct species that have evolved locally [3] [4].

Cassava mosaic disease (CMD) caused by whitefly-transmitted begomoviruses is the main factor of cassava (*Manihot esculenta* Crantz) decrease yields [5]. Eight cassava mosaic begomovirus species have been reported in Africa [6] [7] [8] of which three species affect cassava in Togo: ACMV (*African cassava mosaic virus*), EACMV (*East African cassava mosaic virus*) and ICMV (*Indian cassava mosaic virus*) [8]. Begomoviruses are also reported in pepper (*Capsicum* sp.) [9] [4] [10] which can be intercropped with cassava. Pepper is an important cash crop for smallholder farmers in developing countries. Among the five cultivated species of the genus *Capsicum*, *Capsicum annuum* (both hot and sweet pepper) is the most widely cultivated. In cassava production, pepper is often intercropped with it in Togo.

This study was initiated to identify cassava begomoviruses on other crops in cassava intercropping systems. Results of this study will help to better understand the disease epidemics, related to whitefly-transmitted begomoviruses as well as *Bemisia tabaci* biotypes behavior within these systems.

2. Material and Methods

2.1. Foliar Sampling and DNA Extraction

Foliar samples from pepper plants intercropped with cassava showing typical symptoms of viral diseases (mosaic, distortion, leaf curling, yellowing and deformation) were collected through the five economic regions of Togo.

Total DNA was extracted from collected leaves using the DNA minipreparation method [11].

2.2. PCR Diagnosis

Detection of cassava mosaic begomoviruses (CMBs) in samples was performed by PCR amplification using three sets of specific primers targeting the coat protein (**Table 1**): JSP001/JSP002, JSP001/JSP003 and JSP012/JSP013 allow to identify respectively *African cassava mosaic virus* (ACMV), *East African cassava mosaic virus* (EACMV) and *Indian cassava mosaic virus* (ICMV) [12]. Another PCR was performed using begomoviruses coat protein specific primers AC1048/AV494 [13] to identify other begomoviruses apart from CMBs. PCR tests were done in the thermocycle "Mastercycle gradients Eppendorf (Hamburg, Germany). All PCR reactions were prepared in a 25 µl reaction mixture containing: template DNA 2 µl, dNTPs 0.5 µl (200 µM), primers 1.25 µl (each), Taq polymerase 0.16 µl (0.8 U), reaction buffer 2.5 µl (1X), MgCl2 2.5 µl (2.5 mM), BSA 0.5 µL (0.4 µg/µL), and Sterile water 14.34 µl.

Table 1. Primers used in this study.

Primer	Sequence 5'……3'	Size (bp)	Reference
JSP001	ATGTCGAAGCGACCAGGAGAT	770	[12]
JSP002	TGTTTATTAATTGCCAATACT		
JSP003	CCTTTATTAATTTGTCACTGC		
JSP012	GTCCATATAGGTAARGTNATG		
JSP013	CCTGCTCCTTGCTNGCYTART		
AC 1048	GGRTTDGARGCATGHGTACATG	560	[13]
AV 494	GCCYATRTAYAGRAAGCCMAG		

D = A, G, T; H = A, C, T; K = G, T; M = A, C; N = A, C, G, T; R = A, G; W = A, T; Y = C, T.

The PCR thermal profile for CMBs was as followed: a 94˚C denaturation step of 2 min followed by 30 cycles of denaturing at 94˚C for 30 secs, annealing at (50˚C for ACMV and EACMV and 52˚C for ICMV) for 30 secs and extension at 72˚C for 1 min and then a final extension step at 72˚C for 10 min [12]. The PCR thermal profile with primers AC1048/AV494 was: a 94˚C denaturation step of 2 min followed by 35 cycles of denaturing at 94˚C for 1 min, annealing at 55˚C for 2 min and extension at 72˚C for 1 min and then a final extension step at 72˚C for 10 min [13].

PCR amplified products were resolved by agarose gel electrophoresis and visualized under ultraviolet (UV) light using Gel Doc 2000 (Bio-Rad, Hercules, CA, USA). A 100 bp DNA molecular weight marker (Smart ladder small fragments Eurogentec) was run in each gel as a reference to estimate the size of the virus-specific DNA band in the PCR amplified products.

2.3. DNA Sequencing and Phylogenetic Analysis

Illumina Nextera XT Index kit (Illumina Inc., San Diego, CA, USA) was used according to the manufacturer's instructions to assign a code to each sample before the sequencing. Purity of the PCR products was verified with the Agilent Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA, USA) and the sequencing was performed at the UPJV Molecular Biology platform based on "Sequencing by Synthesis" technology. Illumina Miseq Reagent Kit V2 (Illumina Inc., San Diego, CA, USA) was used to perform this sequencing.

BLASTn program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used for preliminary analysis [14]. Multiple sequence alignments were performed with ClustalX 2.1 [15] and pairwise nucleotide identities were calculated with SDT 1.2 software [16].

Phylogenetic analysis was performed by Neighbor-Joining method using Darwin6. A thousand bootstrap replications, was used to evaluate the robustness of each individual branch. Tree was visualized and edited using Darwin6.

The nucleotide sequences of the following begomovirus (GenBank accession numbers are given) were included in the analysis: *African cassava mosaic virus*

(AF259894.1, AY562423.1, EU685318.1, FM877473.1, HE979761.1, HG530111.1, KJ887779.1, KR476371.1, KR476372.1); *East African cassava mosaic virus* (AJ717553.1, HG530116.1); *East African cassava mosaic Cameroon virus* (AF112354.1, KJ887944.1); *East African cassava mosaic Kenya virus* (JF909078.1, KJ888087.1); *East African cassava mosaic virus*-Tanzania (Z82356.1); *East African cassava mosaic virus*-Uganda (KT780440.1); *Ageratum leaf curl Cameroon virus* (FR717144.1, FR873228.1, FR873230.1); *Pepper yellow vein Mali virus* (KY271076.1, FM876851.1, AM691555.1, AY502935.1) and *Pepper golden mosaic virus* (EF027236.1).

3. Results

3.1. Incidence Rate of Begomoviruses

A total of nineteen samples were collected (Table 2). Samples number was varied from one region to another, most of them were collected in Maritime and Plateaus regions, which are the main cassava production areas in Togo. In Kara region, during the collection, pepper was not found in association with cassava (Figure 1).

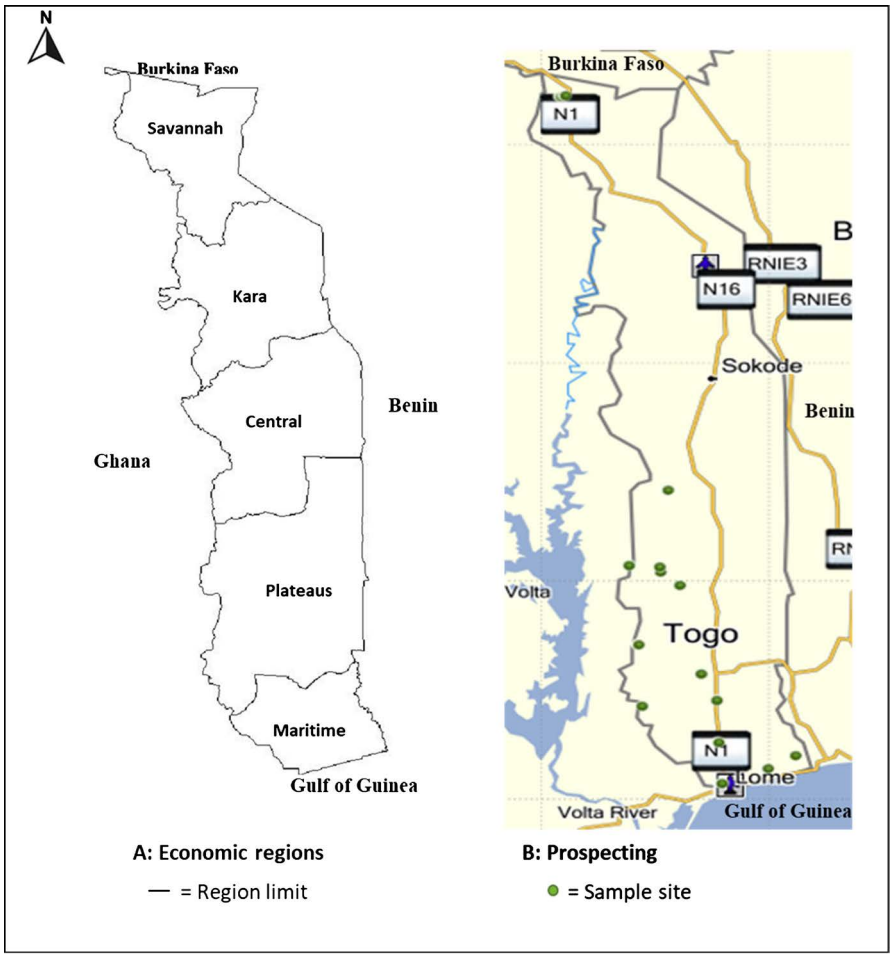

Figure 1. Geographical location of the sample sites.

PCR products with the expected size were amplified from collected samples with primers JSP001/JSP003 and AC1048/AV494 confirming the association of begomovirus with symptoms. This indicates in addition, that pepper is natural infected by *East African cassava mosaic virus* (EACMV). Based on PCR results with primers JSP001/JSP003 and AC1048/AV494, 10.53% and 26.31% of samples were respectively infected (Table 2). *African cassava mosaic virus* and *Indian cassava mosaic virus* were not identified in none sample respectively with primers sets JSP001/JSP002 and JSP012/JSP013.

3.2. Sequence Analysis and Comparison

PCR products were submitted to direct sequencing. Three sequences obtained from these products were selected for phylogenetic and pairwise nucleotide analysis

Table 2. Incidence rate of begomoviruses.

Region	Locality	Sample number	Samples reaction to PCR			
			JSP001/ JSP002	JSP001/ JSP003	JSP012/ JSP013	AC1048/ AV494
Maritime	Lome	1	-	-	-	+
Maritime	Alokoegbe	1	-	+	-	-
Maritime	Gblainvie	1	-	-	-	-
Maritime	Alokoegbe	1	-	-	-	-
Maritime	Blama Kondji	1	-	+ (Isolate B51)	-	+
Maritime	Blama Kondji	1	-	-	-	-
Maritime	Game	1	-	-	-	-
Plateaus	Tikoe/Agou Agotime	1	-	-	-	-
Plateaus	Tikoe/Agou Agotime	1	-	-	-	-
Plateaus	Koudravi	1	-	-	-	-
Plateaus	Tokudjahoui	1	-	-	-	-
Plateaus	Tokudjahoui	1	-	-	-	+
Plateaus	Houdje	1	-	-	-	-
Plateaus	Kougnonhou	1	-	-	-	+
Plateaus	Amouka	1	-	-	-	-
Plateaus	Kessibo/Dandjodji	1	-	-	-	-
Savannah	Nabou (Biangou)	1	-	-	-	-
Savannah	Kambere	1	-	-	-	+ (Isolates G44a and G44b)
Central	Bowou Kope	1	-	-	-	-
		Incidence (%)	**0**	**10.53**	**0**	**26.31**

- = Sample reacted negatively to PCR; + = Samples reacted positively to PCR.

(Table 2). The criteria used to distinguish between different viruses are: 90% - 100% for isolates, 80% - 90% for strains and less than 80% for species demarcation [17].

Sequence comparisons revealed that isolate B51 from primers JSP001/JSP003 specifics to *East African cassava mosaic virus* (EACMV), shared the highest sequence identity (74%) with EACMV AJ717553.1; but also, with *African cassava mosaic virus* (ACMV) KJ887779.1 and *Pepper yellow vein Mali virus* (PepYVMV) isolates AM691555.1, AY502935.1 and KY271076.1 (Figure 2). Thus, the new strain of EACMV found in pepper seemed to be related to ACMV and PepYVMV species. This finding is supported by the phylogenetic analysis which showed isolate B51 forming a distinguish group with isolate G44a. Both isolates were closely related to group of *Ageratum leaf curl Cameroon virus* (ALCCMV) (Figure 3).

Isolates G44a and G44b from primers AC1048/AV494 shared high level of nucleotides identity respectively with *Ageratum leaf curl Cameroon virus* (ALCCMV) isolate FR873230.1 (80%) and ACMV AF259894.1 (93%). Isolate

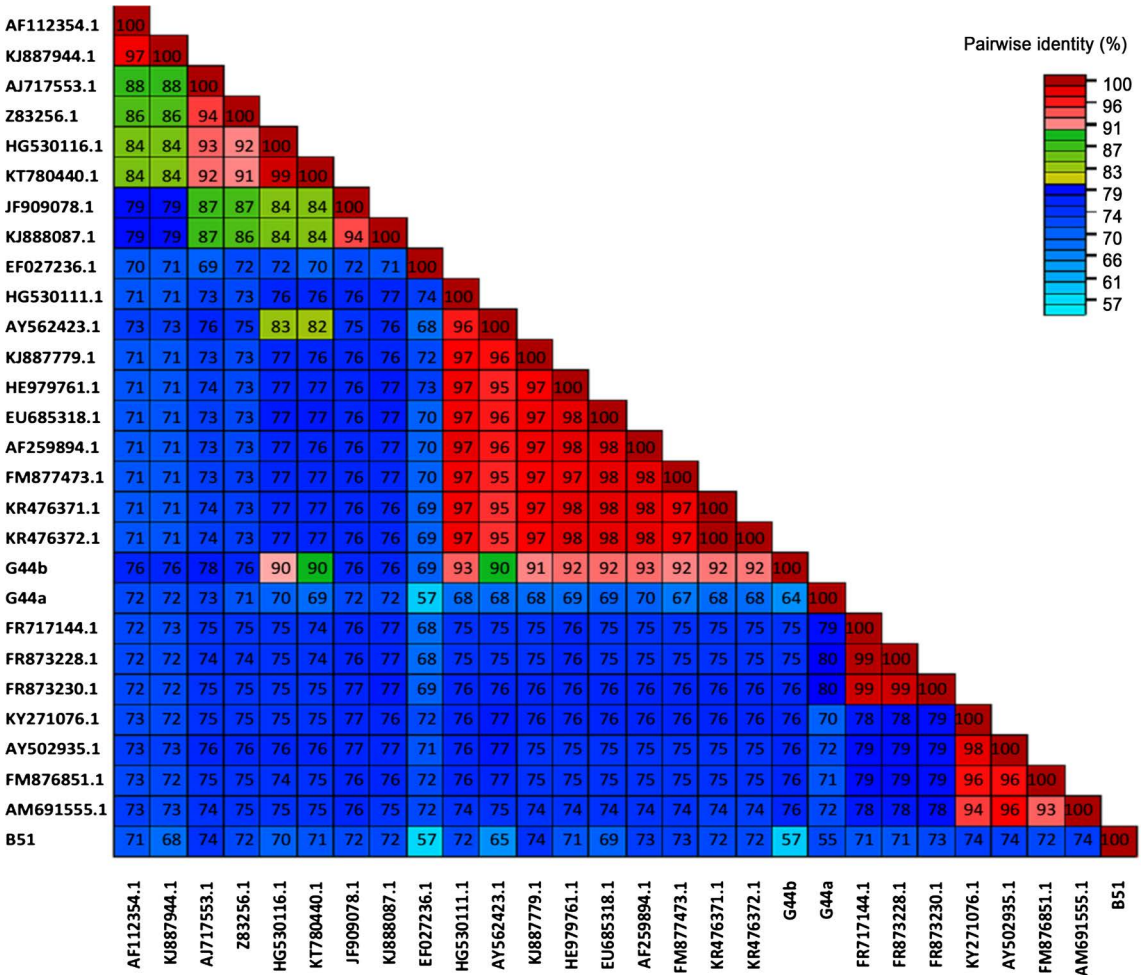

Figure 2. Pairwise nucleotide identities scores between sequences of isolates B51, G44a, G44b and selected begomovirus from GenBank.

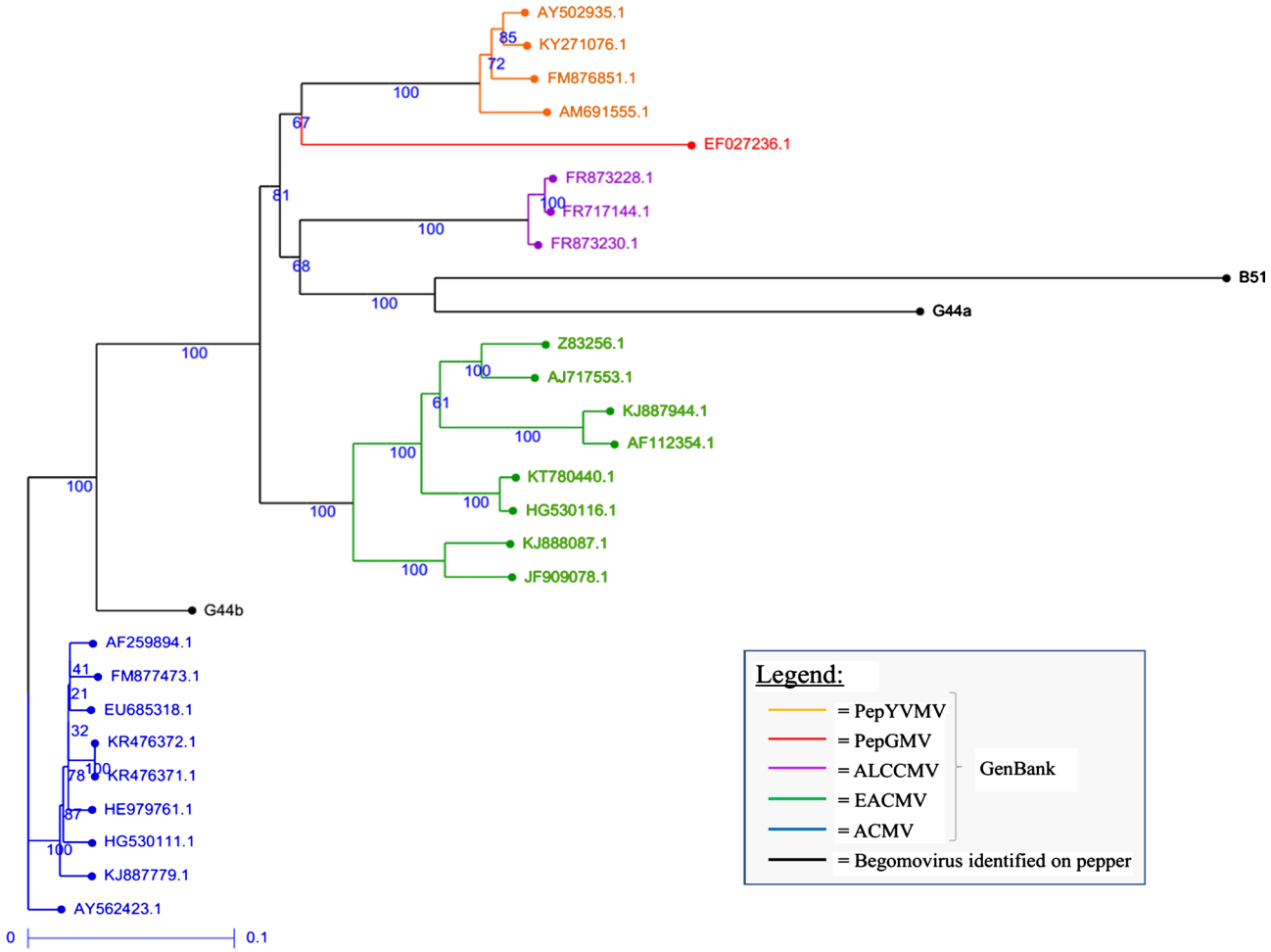

Figure 3. Phylogenetic tree based upon an alignment of isolates B51, G44a, G44b and selected begomovirus sequences from GenBank.

G44b also shared 90% identity with EAMCV references (HG530116.1 and KT780440.1) (**Figure 2**). These results show that isolate G44a is an *Ageratum leaf curl Cameroon virus* and isolate G44b would be a recombinant of *African cassava mosaic virus* and *East African cassava mosaic virus*. In the phylogenetic tree, isolate G44b formed a distinguish group (**Figure 3**).

4. Discussion

This study has demonstrated three unknown infections of begomovirus in pepper foliar samples including *Ageratum leaf curl Cameroon virus* (ALCCMV), a new strain of *East African cassava mosaic virus* (EACMV) and a recombinant of *African cassava mosaic virus* (ACMV) and EACMV.

Diagnosis of cassava mosaic begomoviruses (ACMV and EACMV) in pepper showed that these begomoviruses could infect other crop species than cassava. This is the first identification and characterization of begomoviruses responsible of cassava mosaic disease in another cultivated plant in Togo. The ability of begomoviruses to infect plants belonging to different taxonomic families has been

studied. Indeed, *Tomato yellow spot virus* (ToYSV) can infect tomato (*Solanaceae*) and soybean (*Fabaceae*); likewise, *Tomato leaf curl New Delhi virus* (ToLCNDV) infects *Solanaceae* and *Cucurbitaceae* [18].

It is known that genomic plasticity of begomoviruses allows them to adapt to new environments and hosts [19] and results found here suggest the presence of polyphagous biotypes in populations of *Bemisia tabaci* vector of these viruses which can colonize with efficiency cassava and pepper plants. In addition, climate changes directly affect insect behavior, fertility, development and survival or indirectly affect insect by changes in trophic relationships [20] [21]. Pepper samples were all collected in cassava intercropping systems, which means that in fields where cassava and pepper plants were both present. Hence, the polyphagous biotype could easily visit infected cassava plants and transmit cassava virus to pepper plants by feeding on them. [22] found that the emergence of new begomoviruses might be occurring in relationship with some factors like evolution of new virus variants, the appearance of efficient vectors, changes in intercropping systems, introduction of susceptible plant varieties, and changes in climatic conditions. Isolates G44a and G44b found here were both recombinants.

A general consensus exists that *Bemisia tabaci* is a complex of morphologically indistinguishable populations with different biological biotypes. The cassava biotype of *Bemisia tabaci* is known to colonize only cassava and wild eggplant whereas the sweet potato biotype colonizes various plant and weed species but not cassava [23]. But recent work showed that, the sweet potato biotype of *Bemisia tabaci* can colonize cassava. In fact, different species of *Bemisia tabaci* were reported to have cassava and other crops together as host plants [24]. In particular, SSA3 species was found on cassava, cotton, eggplant, groundnut and tomato [25]. In the same way, MED-Q1 adults, larvae, and nymphs were collected on cassava [26]. The capacity of biotype B to adapt gradually to cassava was studied by [27]. These authors found that the biotype B of the insect adaptation started on *Phaseolus vulgaris*, followed on *Euphorbiaceae* plants until finally reaching a commercial cassava variety.

In cassava intercropping systems, these intermediate hosts or plants belonging to the same family are used as secondary crops associated with cassava and weeds can belong to *Fabaceae* and *Euphorbiaceae* families. [28] showed that ACMV and EACMV infect naturally other plant species than cassava namely *Senna occidentalis* (L.), *Leucaena leucocephala* (Lam.), *Combretum confertum* (Benth.) and *Manihot glaziovii* (Müll.Arg). Apart from wild cassava (*M. glaziovii*), [29] also identified EACMV on *Albizia zygia* (DC.), *Senna obtusifolia* (L.), *Pupalia lappacea* (L) and *Strophanthus hispidus* (DC.). Furthermore, Uganda variant EACMV-UG has been detected in a *Jatropha curcas* (L.) [30]. However, it is important to note that none of these authors had identified EACMV (*East African cassava mosaic virus*), in another crop outside cassava.

Ageratum leaf curl Cameroon virus was first identified on *Ageratum conyzoïdes* (L.) in Cameroon [31] and reported in Togo on tomato [32]. The identi-

fication of this begomovirus on pepper now approved that this begomovirus can infect more than one host including weeds as well as cultivated plants.

Occurrence of new begomovirus species on pepper could lead in case of mixed infections with already known begomoviruses infecting this crop to recombinant actions [1] [12] [33]. This situation might result in increasing begomovirus emergence in cassava intercropping systems.

5. Conclusion

This study showed that cassava mosaic begomoviruses could infect other cultivated plants than cassava and suggested a change in *Bemisia tabaci* population or in its feed habit. Further investigations will bring more information about cassava mosaic begomoviruses and pepper relationships.

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Conflicts of Interest

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

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