Cucumber Mosaic Virus and their associated satellite RNAs infecting banana (*Musa sp. Genomic group AAA)* in Côte d'Ivoire : A molecular characterization.

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Abstract

In Côte d'Ivoire, banana (*Musa sp.*) ranks third among exportation products and represents 3% of the Gross Domestic Product with a national production of up to 500000 tons in 2019. Banana is subject to numerous disease agents among which viruses cause significant losses. To figure out the impact of viruses in Ivorian industrial banana fields, surveys were conducted in the 7 main banana production departments. A total of 260 leaf fragments presenting viral symptoms were collected and analyzed. From the 65 leaf fragments used for biological indexing, 14 showed symptoms related to *Cucumber mosaic virus* (CMV). CMV presence was confirmed by a double-antibody sandwich enzyme-linked immunosorbent assay using CMV polyclonal antibodies. CMV strains we isolated appeared to be highly infectious and to produce various symptoms like mosaic, chlorosis, and necrotic spots on *Cucumis sativus*, *Cucurbita pepo*, and *Nicotiana tabacum*. Satellite RNAs (SatRNAs) associated with CMV isolates were also detected using reverse transcription-polymerase chain reaction (RT-PCR) with a degenerate primer pair. CMV's coat protein and satRNAs were sequenced. Novel Ivorian coat proteins and satRNAs were compared to publicly available CMV sequences from GenBank. We noticed a single amino acid substitution (Serine to Leucine) at position 73 of the novel coat protein that allowed us to divide Ivorian CMV strains into two groups. Molecular and phylogenetic analysis suggested that Ivorian strains might be classified into CMV Subgroup IA. We also discovered that satellite RNA associated with Ivorian CMVs form a separate clade.

Keywords : *Cucumber mosaic virus*, protein coat, micro-satellite RNA, *Musa sp.*, Côte d'Ivoire.

Résumé

***Cucumber Mosaic Virus* et ces ARN satellites associés infectant la banane (Musa sp. groupe génomique AAA) en Côte d'Ivoire : Une caractérisation moléculaire.**

En Côte d'Ivoire, la banane (*Musa sp.*) est le troisième produit d'exportation et représente 3 % du produit intérieur brut avec une production nationale atteignant 500 000 tonnes en 2019. Cependant, la banane est sujette à de nombreuses maladies virales qui provoquent des pertes importantes. A cet effet, une étude à été menée dans les bananeraies industrielles Ivoiriennes des 7 principaux départements de production. Au total, 260 fragments de feuilles présentant des symptômes viraux ont été collectés et analysés. Sur les 65 échantillons utilisés pour l'indexation biologique, 14 présentaient des symptômes liés au virus de la mosaïque du concombre (CMV). La présence du CMV a été confirmée par un test DAS-ELISA utilisant des anticorps polyclonaux du CMV. Les souches de CMV isolées semblaient très infectieuses et produisent divers symptômes tels que la mosaïque, la chlorose et des taches nécrotiques sur *Cucumis sativus*, *Cucurbita pepo* et *Nicotiana tabacum*. Des ARN satellites (satRNA) associés à des isolats de CMV ont également été détectés par RT-PCR. La protéine capsidaire du CMV ainsi que les satRNA ont été séquencés et comparés aux séquences de CMV disponibles publiquement dans GenBank. Une seule substitution d'acide aminé (de la sérine à la leucine) à été identifiée à la position 73 de la nouvelle protéine capsidaire et a permis de diviser les souches de CMV en deux groupes. De plus, l'analyse phylogénétique a suggéré que les souches Ivoiriennes appartiennent au sous-groupe IA des CMV et que les satRNAs qui leur sont associés forment un clade distinct.

Mots-clés : *Cucumber mosaic virus,* protéine capsidaire, ARN microsatellite, *Musa sp.*, Côte d'Ivoire.

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INTRODUCTION

Bananas (Musa spp.) are one of the most important fruit crops in the world both in terms of production volume and trade (FAO, 2014). Nevertheless, only around 13% of bananas produced are globally traded showing their importance in food security and domestic markets (Lescot, 2013). Côte d'Ivoire is the first African exporter of bananas to European countries (FAOSTATS, 2020). Banana represents 3% of the Gross Domestic Product (GDP) of the country. The banana sector represents 9,000 direct jobs and 35,000 indirect jobs in Côte d'Ivoire. With a production of nearly 450,000 tonnes of bananas in 2019, Côte d'Ivoire is at the forefront of African producing countries and records a turnover of 145 billion CFA francs . Banana is therefore of importance for the national economy of Côte d'Ivoire. Nevertheless, several banana pests and diseases are a global threat to global food security (Blomme et al., 2017).

CMV is one of the top ten plant viruses in plant pathology and is reported to infect more than 1200 plant species in over 100 families, both monocots and eudicots: fruit crops, vegetables, and ornamental, including banana (Scholthof et al., 2011). *CMV* belongs to the genus *Cucumovirus* and the family of *Bromoviridae.* CMV is a positive sense RNA plant virus with a tripartite genome (Jacquemond, 2000; Kim et al, 2011; Roossinck et al., 1999). This genome encodes five proteins: the 1a and 2a proteins are encoded by RNA1 and RNA2 respectively and are obligatory for viral replication. The 2b protein is encoded by a subgenomic RNA (RNA4A) from RNA2, and functions as a viral suppressor of RNA silencing (Qiu et al, 2018). RNA3 is bicistronic: the protein

encoded by the 5′ open reading frame (ORF) is the designated movement protein, while the coat protein (CP) encoded by the 3′ ORF is translated from a subgenomic RNA4 synthesized de novo from RNA3 minus-strand progeny (Owen et al., 1990). CMV strains are broadly divided into two subgroups (I and II) based on nucleotide sequence homology. CMV strains in the same subgroup share a high degree of sequence similarity (Lin et al., 2003). Several CMV strains discovered on bananas from Latin America have already been described by proteomics and phylogenetics (Kim et al., 2011, Owen et al., 1990; Lin et al., 2003; Dubey et al., 2010; Hsu et al., 1995; Hord et al., 2001) but very few analyses have been carried out on strains originating from West Africa. Although CMV has been well studied biologically (Aka et al., 2009) and from a molecular point of view in Asian and American countries, molecular and phylogenetic characterization of African CMV strains targeting banana crops is still missing.

CMV is not known as a major virus infecting bananas but causes notable losses and has been reported as one of the viruses infecting bananas in Cote d'Ivoire (Hord et al., 2001; Aka et al., 2009). The aim of this study was to investigate the incidence of CMV in industrial banana plantations and to characterize CMV isolates at the sequence levels. By the way, we identified satellite RNA associated with CMV and included the characterization of this satRNA in our study.

In this paper, we report one of the first molecular and phylogenic based characterization of Ivorian CMV strains identified on banana Cavendish, cvs Great Naine, and William, AAA group. These results are based on the analysis of an exhaustive survey that led to the collection of 260 banana leaf fragments from symptomatic banana plants in the 7 main banana production areas of Côte d'Ivoire. After antibody-based virus detection, 14 leaf samples were used for PCR amplification and DNA sequencing, while 65 samples were used for biological indexing. In addition to classical biological comparison (description of induced symptoms on reporting plants) and nucleotide comparison of coat protein, we sequenced partially the detected micro-satellite of 15 CMV isolates.

MATERIALS AND METHODS

1. Sample collection and storage

Banana (variety Cavendish, cvs Great Naine and William, AAA group) leaf samples showing symptoms of possible viral etiology were collected in the seven major production areas from 2010 to 2011. Sampling sites are located in the South and South-East of the country and correspond to the banana production area around the main harbor, Abidjan (Figure 1).

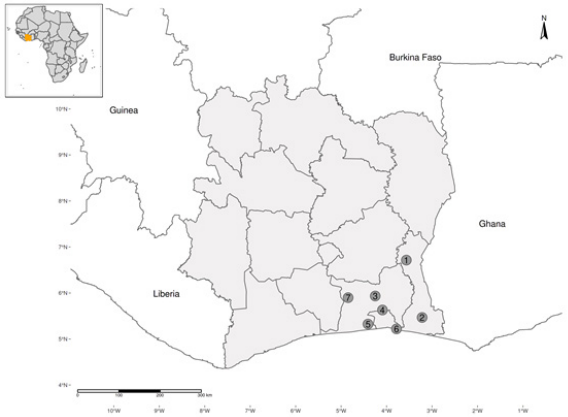

Figure 1. Location of sample collection areas in industrial banana plantations in Côte d'Ivoire. 1. Abengourou (Lat: 6.7146399, Long: -3.5654096), 2. Aboisso (Lat: 5.4675302, Long: -3.2185867), 3. Agboville (Lat: 5.9342509, Long: -4.2500361), 4. Azaguié (Lat: 5.6305067, Long: -4.0907336), 5. Dabou (Lat: 5.3242556, Long: -4.4098582), 6. Grand-Bassam (Lat: 5.2198516, Long: -3.7821731), 7. Tiassalé (Lat: 5.8985662, Long: -4.8458078)

Visited industrial plantations were 80 to 700 ha. Each plantation is divided into blocks and each block into squares. We defined collection spots in the different squares. The number of sampling spots was a function of the initial size of the visited plantation. This number ranged from 3 (for a square of 0,5ha) to 10. For each sampling spot, the number of collected plant fragments was a function of the importance of observed virus-linked symptoms. For each spot, symptomatic and asymptomatic samples were collected and tagged.

A total of 260 bananas leaves were collected in industrial plantations by investigating the symptomatic plants in the previously detected square fields (Table 1). A sample consisted of fragments of a leaf demonstrating one or more symptomatic traits. On the field, samples were placed in a plastic bag, assigned a code, and stored in dried ice. Laboratory storage conditions consisted of a freezer at -20°C. The second part of each sample was kept desiccated on calcium chloride.

Table 1. Summary of sample collections in industrial plantations by location

Sample site	Visited squares	Squares with symptomatic plant	Collected samples
Abengourou	6	3	14
Aboisso	3	3	39
Agboville	7	6	36
Azaguie	8	3	29
Dabou	10	7	55
Grand Bassam	9	5	38
Tiassale	8	6	49
Total	51	33	260

2. Virus detection and sequencing

CMV detection was performed using three complementary approaches: double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA), biological indexing, and reverse transcription-polymerase chain reaction (RT-PCR).

DAS-ELISA was carried out using the Loewe Phytodiagnostica GmbH kit (Sauerlach, Germany). The kit contains two polyclonal antibodies with their related conjugates that are coupled to alkaline phosphatase and also includes positive and negative probes as well as sensitization, extraction, and cleansing buffers. These antibodies (anti-CMV) can detect the two groups of CMV. Leaf samples, stored at -20°C, were defrosted and crushed with a manual ball extractor (AGDIA, France). The grinding was done with a ratio of 0.5 g of sample per 5 ml of the extraction buffer. Settled and clarified juice (kept on ice) was then collected in tubes for antibody detection. Antibody detection was conducted following the manufacturer's recommendation. Optic density was determined using an ELISA plate reader (Titertek Multiskan) after 1 and 2 hours of incubation in darkness. A sample was considered positive when the absorbance (at 405 nm) was greater than 2 times the average absorbance of reference samples (false positive and true negative) (Ayo-John et al., 2008; Sutula et al., 1986).

Molecular detection of CMV using RT-PCR was carried out using specific primers CMV 3': TTTTAGCCGTAAGCTGGATGGACAACCC and CMV 5' : TATGATAAGAAGCTTGTTTCGCGCA (Sharman et al., 2000; Bariana et al., 1994).

One milliliter of extraction buffer (137 mM NaCl; 8 mM Na 2 HPO 4; 1.5 mM KH 2 PO 4; 2.7 mM KCl; 80 mM Na 2 SO 3; 3 mM NaN 3; 0.05% Tween 20 – pH 7.2) was added in a grinding bag to 50 mg of dried leaves or 2 ml added to 0.5 g of fresh leaves. After 15 minutes of soaking the dried samples or immediately for fresh leaves, samples were crushed with an electric ball mill (Power Plus X022). Settle clarified juice was diluted 100 times with sterile distilled water. For RT-PCR amplification of nucleic acid sequences, reactions were made in volumes of 25 µl (20 µl of the reaction mixture + 5 µl of the sample corresponding to diluted crude extract) using the Titan RT-PCR kit (Rocher, Mannheim, Germany). A reaction mixture was composed of 5 µl of Buffer (5x Concentrate) Titan RT-PCR, 0.5 µl of dNTP, 0.5 µl CMV 3' (25 µM), 0.5 µl CMV 5' (25 µM), 1.25 µl of DTT (100 mM), 0.5 µl of Titan mixed enzyme, and 11.75 µl of sterile distilled water, i.e. 20 µl by mix. All amplification reactions included both negative and positive controls from banana plants grown in greenhouses as well as Blanco made up of sterile distilled water. Amplification cycles were performed using a My Cycler™ (Biorad, USA) following this program: 50°C for 30min, 94°C for 5min, 40 cycles of 94°C for 30sec, 54°C for 60sec, 72°C for 2min then 72°C for 10min. The last step consisted of the revelation of amplification products on agarose gel 1% (1 g agarose per 100 ml of buffer Tris, Acetate, EDTA (TAE) 1 x) containing 10 µl of Ethidium bromide (BET), in TAE buffer 1 x. Electrophoresis migration was carried out under a constant current of 120 mA for 45 min. The gel was then visualized by UV lighting allowing the observation of amplified bands that were pictured using a digital camera.

CMV's satellite RNAs (satRNAs) identification was carried out by amplification of nucleotide sequences by RT-PCR following the procedure described above but using primers proposed by Gafny et al. (Gafny et al., 1996) and Nouri et al. (Nouri et al., 2014). These primers include header and tail sequences found in all known CMV satRNA and deposited at the National Center for Biotechnology and Information (NCBI) Genbank. A positive control, CMV strain with satellite RNA (DSMZ collection: PV-0029 and PV-0092, Germany), and negative control (CMV strain without satellite RNA) were used. PCR products were purified with the Qiaquick PCR Purification kit (Qiagen, Benelux) and sent for sequencing using the Sanger dideoxy sequencing technology (MACROGEN Inc., Netherland). In all, 16 CMV isolates from the 7 sampling locations were sequenced from amplicons of genes partially coding for the coat protein and part of an untranslated region (UTR) of the RNA3. For the sequencing of satellite RNA, 15 RNA satellite isolates were selected from amplification and purification results.

3. Indicator plant inoculation

The biological indexing was carried out by mechanic infection of *Cucumis sativus cv Poinsett Pepino, Cucurbita pepo Linn. cv Precose Maraîchère,* and *Nicotiana tabacum cv. Samsun.* Three test plants were used per virus isolate. Mechanical inoculation took place at the two-leaf cotyledon stage for Cucurbits (cucumber, courgette) and at the 4-6 leaf stage for tobacco plants. Banana leaf samples were ground in a bag containing phosphate buffer at pH 7.2 (KH 2 PO 4 0.05 M; DIECA 0.01 M) in a ratio of 1:5 (g: ml) for samples stored at -20°C and 1:10 (g: ml) for dried samples. The shred was clarified and collected in a Petri dish, then a pinch of carborundum and a pinch of activated carbon was added. After wearing new gloves for each manipulated plant to avoid any contamination, healthy plant leaves previously sprinkled with carborundum were inoculated by rubbing their top face with a finger moistened in the inoculum. The inoculated leaves were immediately rinsed with distilled water and inoculated plants were shade-stored in a greenhouse for 24 hours: (25°C, 16 hours of auxiliary lighting at the phytopathology laboratory, Gembloux) or in anti-insect cages (ambient temperature average of 30°C at the phytopathology laboratory, INP-HB). Two CMV isolates from the DSMZ collection (DeutscheSammlung Von Mikroorganismen und Zell-kulturen, PV-0029 and PV-0092; Germany) and healthy plants were used as positive and negative controls respectively. All plants (inoculated or not) were regularly watered and monitored during 30-35 days.

4. Sequence analyses

Data issued from sequencing were visualized using multiple sequence alignment obtained from Clustal Omega (Sievers et al., 2014). Similarity searches were realized using the BLASTN program from the BLAST suite (Camacho et al., 2009) and using NCBI GenBank non-redundant nucleotide database as the search database (downloaded and compiled in December 2019). Phylogenetic trees were built using the maximum-likelihood method with 1000 bootstrap replicates under the MEGA 7 software (Kumar et al., 2018). A data set containing 49 sequences from the different CMV subgroups (S1 Table) and 1 sequence used as the outgroup (ER-PSV: U15730) were used to build the CMVs proteins coat tree. A data set of 50 sequences from known CMV's satRNAs (S2 Table) was used for the satRNAs tree. Phylogenetic trees were visualized using iTOL v4 (Letunic and Bork, 2019).

RESULTS

5. Symptoms observation and CMV detection

Virus presence was detected by measuring the absorbance of a solution of clarified leaf juice incubated with antibodies (DAS-ELISA). The serological tests were performed using a negative and positive control for each ELISA plate. The range of optical densities for the negative controls was 0.09 to 0.12 and for the positive controls 2.65 to 3.32. The optical densities of all samples tested ranged from 0.10 to 3.47. The samples reported as positive are those for which the optical density is greater than twice that of the negative controls, i.e. 0.18 to 0.24. DAS-ELISA realized on symptomatic leaves therefore allowed to confirm the presence of CMV: 85.7% of the sample from Abengourou (East) were positive to CMV and 100% for samples from Aboisso (south-East). Table 2 summarizes, for each sample place, percentages of symptomatic banana samples that appeared to be positive to CMV.

Table 2. Percentages of symptomatic banana samples positive to CMV, by sampling sites.

Location	Number of symptomatic samples	Positive to CMV	CMV infected sample percentage
Aboisso	39	39	100
Abengourou	14	12	85.7
Agboville	36	33	91.6
Azaguie	29	28	96.5
Dabou	55	52	94.5
Tiassale	49	48	97.9
Grand-Bassam	38	37	97.3
Total	***260***	***249***	***95.7***

The most common symptoms observed countrywide are chlorosis, mosaics, and leaf necrosis (Figure 2).

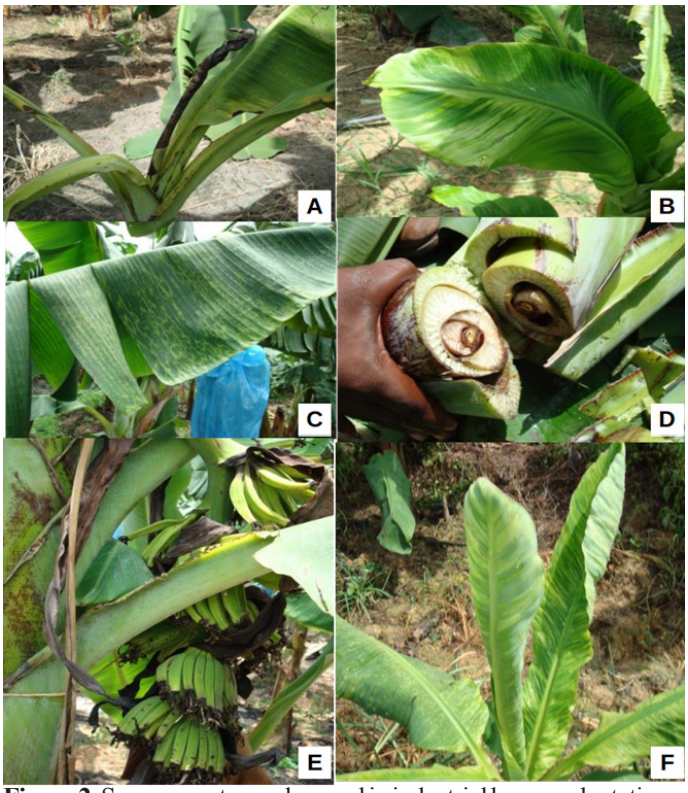

Figure 2. Some symptoms observed in industrial banana plantations.
A: Cigar rot observed on the plant used for the BT2 sample in Tiassalé; B: Mosaic associated with leaf distortion [sample BT 7 taken in Tiassalé; C: Spindle-shaped chlorosis [sample SL16 collected in Dabou]; D: Leaf sheath or pseudostem rot [sample SP23 collected in Dabou]; E: Dwarfing of banana fingers (fruits) (sample BA6 taken in Abengourou); F: Mosaic accompanied by leaf curl [sample CA 13 taken in Aboisso].

Observations concerning the main symptoms associated with banana leaf samples positive to CMV are provided in Table 3.

Table 3. Symptoms associated with samples positive to CMV for each collection place

Location	Number of samples with the corresponding symptom (related percentage)							
	CL	M	FN	CR	LSR	DW	FD	LW
Tiassalé	45 (93.7)	30 (62.5)	32 (66.6)	6 (12.5)	1 (2)	3 (6.2)	3 (6.2)	2 (4.1)
Agboville	23 (69.6)	24 (72.7)	17 (51.5)	1 (3)	0 (0)	0 (0)	2 (6)	1 (3)
Dabou	46 (88.4)	35 (67.3)	38 (73)	9 (17.3)	4 (7.6)	9 (17.3)	6 (11.5)	0 (0)
Aboisso	37 (94.8)	34 (87.1)	28 (71.7)	7 (17.9)	0 (0)	10 (25.6)	3 (7.6)	16 (41)
Abengourou	8 (66.6)	12 (100)	5 (41.6)	5 (41.6)	0 (0)	1 (8.3)	1 (8.3)	0 (0)
Azaguié	27 (96.4)	23 (82.1)	15 (53.5)	2 (7.1)	0 (0)	1 (3.5)	1 (3.5)	0 (0)
Grand-Bassam	33 (89.1)	33 (89.1)	17 (45.9)	0 (0)	0 (0)	0 (0)	1 (2.7)	1 (2.7)

Legend for symptoms: CL: chlorosis, M: mosaic, FN: foliar necrosis, CR: Cigar rot, LSR: Leaf sheath rot, DW: Plant dwarfism, FD: Foliar distortion; LW: Leaf winding. Percentage are indicated in brackets.

Along with DAS-ELISA validation, agarose gel electrophoresis allowed the visualization of RT-PCR products. Fragments of the expected size (950 bp) were identified in infected samples along with positive controls. RT-PCR test confirmed the serologic test for all but for the BT16 sample that finally appeared positive even though the ELISA test was negative. These results indicate that CMV was effectively present in 249 samples collected in the 7 areas known to be the major production sites of banana in the country.

6. Symptomatology on indicator plants

The symptom development on indicator plants was evaluated for CMV isolates. The positive controls caused symptoms of local chlorotic lesions 3-4 days after inoculation and yellow mosaics 8 days after inoculation on tobacco plants.

A total of 14 samples out of the 65 tested induced symptoms on the indicator plants. These symptoms included chlorosis and/or mosaic associated with leaf puffiness (S3 Table). Symptoms appeared 4 to 5 days after inoculation on Cucurbits and 14 days after inoculation for tobacco plants. The following isolates produced symptoms: AZ1, AZ2, AZ3, AZ5, AZ13 from Azaguié; BM32 and BM40 from Grand-Bassam; AG6, AG10, AG20, AG24 from Agboville; BT18 from Tiassalé; SP24 from Dabou and CA26 from Aboisso. Figure 3 illustrates some of the observed symptoms on reporting plants (zucchini, cucumber, and tobacco).

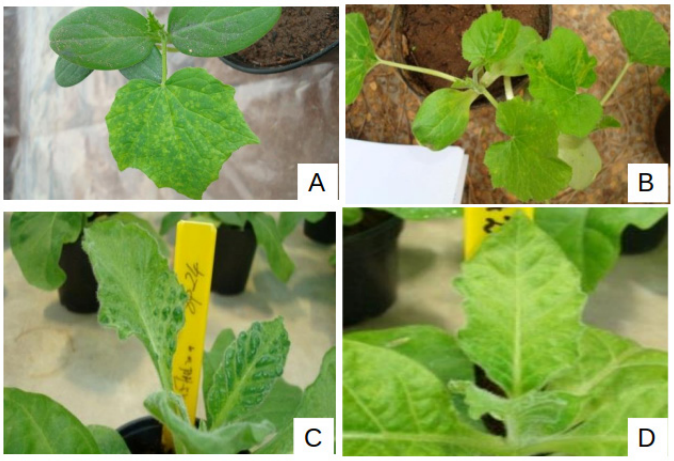

Figure 3. Symptoms observed on reporting plants after CMV's biological indexing.
Top-right: Mosaic on a cucumber leaf inoculated with AZ1 isolate, 7 days after inoculation; Top-left: Chlorosis on Zucchini leaves inoculated with AZ2 isolate, observed 12 days after inoculation; Bottom-right: Chlorosis including blisters on leaves of *Nicotiana tabacum* inoculated with isolate SP24, observed 20 days after inoculation; Bottom-left: Mosaic on leaves of *Nicotiana tabacum* inoculated by isolate CA26, 20 days after inoculation.

7. Analysis of nucleotide sequence of CMV protein coats

Amplicons including a portion of the coat protein and the RNA3 3'-UTR of 16 samples were sequenced and resulted in 11 unique sequences that were further analyzed. Similarity scores are reported in Table 4.

Table 4: Similarity matrix between sequences of RNA3 3'-UTR of Ivorian CMV strains

	BM26	AZ6	AB15	ST17	AG6	ST18	AB12	BM32	AZ2	AG10	BT18
BM26	100	98,9	97,3	98,1	97,5	98,1	97,3	96,1	97,9	98,1	96,9
AZ6		100	98,3	98,1	98,1	99,1	96,9	96,9	98,3	98,7	97,7
AB15			100	96,9	97,1	97,9	96,7	97,3	97,3	97,7	97,1
ST17				100	97,7	98,3	96,7	96,7	96,7	97,1	96,3
AG6					100	98,1	96,5	96,5	97,5	97,1	98,9
ST18						100	97,1	96,5	97,7	97,7	98,1
AB12							100	97,3	96,1	96,7	96,3
BM32								100	96,1	96,3	95,9
AZ2									100	98,1	97,5
AG10										100	97,1
BT18											100

It appeared that Ivorian strains shares a similarity of at least 94,3%. The Basic Local Alignment (BLAST) analysis indicated that the sequence identified in this study share a similarity of 94% to 98% with CMV belonging to the CMV Subgroup IA. The tree resulting from the multiple sequence alignment is provided in Figure 4.

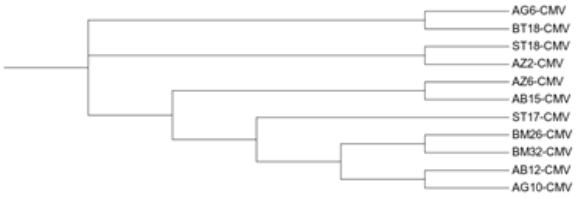

Figure 4. CMV's strains phylogenetic tree using Maximum Likelihood method and JTT matrix-based model.

The tree with the highest log likelihood is shown. A uniform rate is used among sites. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa we analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed.

The 11 distinct coat protein sequences were deposited in GenBank with accession numbers ranging from KC189911 to KC189921 (Table 5).

Table 5. List of identifiers of coat protein and satRNA sequence submitted to the NCBI GenBank Database

Strains reference	Collection place	GenBank identifier of Coat Protein	GenBank identifier of Satellite RNA
BM26	Grand-Bassam	KC189911	KC713593
AZ6	Azaguié	KC189912	MT968978
AZ15	Azaguié	KC189912	MT968984
AB15	Abengourou	KC189913	MT968985
ST17	Tiassalé	KC189914	MT968977
AG6 / AG16	Agboville	KC189915	MT968975
ST18	Tiassalé	KC189916	Not sequenced
AB12	Abengourou	KC189917	Not sequenced
BM13/ BM32	Grand-Bassam	KC189918	MT968976
BM15	Grand-Bassam	KC189918	MT968983
AZ2 / AZ36	Azaguié	KC189919	MT968979
AG10/ AG33	Agboville	KC189920	MT968980
BT18	Tiassalé	KC189921	Not sequenced
SP16	Dabou	KC189919	MT968986
SP24	Dabou	KC189919	MT968988
SP30	Dabou	KC189919	MT968982
SP37	Dabou	KC189919	MT968981
SP39	Dabou	KC189919	MT968987

Multiple sequence alignment of Ivorian CMV coat protein sequences revealed a sequence similarity of at least 99%. The only difference between our sequences concerns position 73. We noticed an amino acid substitution: Serine to Leucine at position 73. The alignment of identified coat protein sequences is provided in Figure 5.

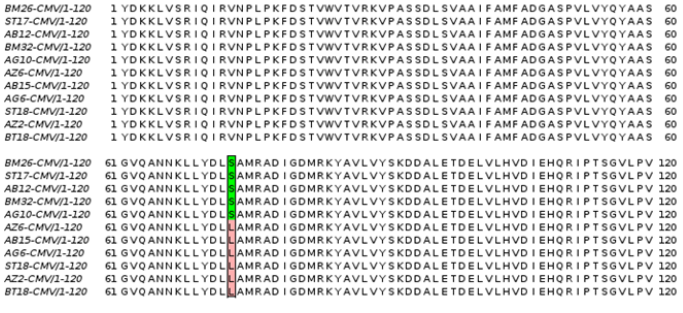

Figure 5. Multiple sequences alignment of Ivorian CMV strains. Amino acids at position 73 are colored following the Zappo color scheme.

Phylogenetic analysis combining Ivorian and worldwide CMV's strains showed KC189912 (AZ6) and KC189911 (BM26) sequences to be closely related to FC-CMV and BAN-CMV respectively. FC-CMV is originated in Israel and BAN-CMV was identified in the United States (Figure 6).

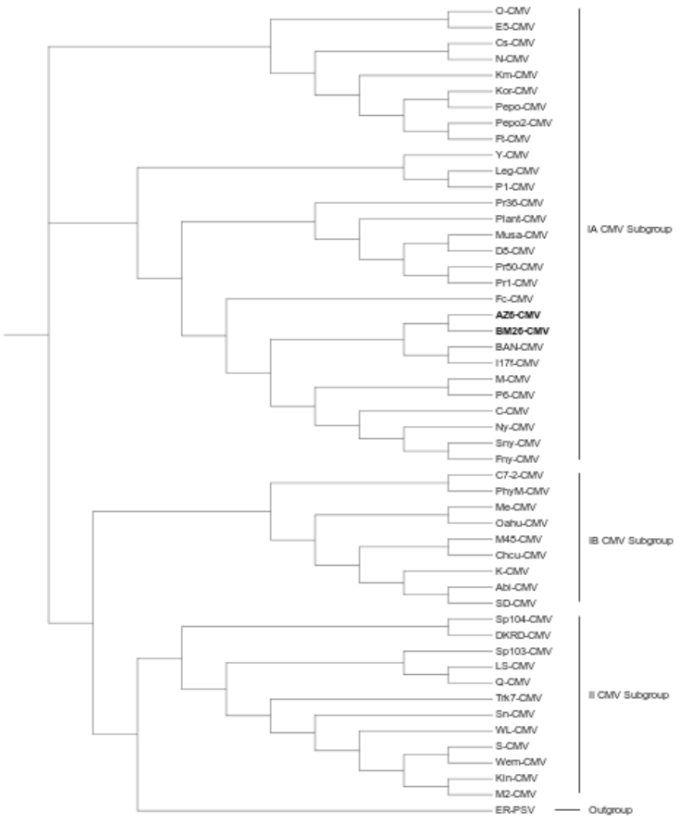

Figure 6. Estimation of Ivorian CMV's phylogenetic relationship with known CMVs using the Maximum Likelihood method and JTT matrix-based model.

Ivorian strains are in bold. The tree with the highest log-likelihood is shown. A uniform rate is used among sites. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed.

8. Analysis of satellite RNA associated with Ivorian CMVs

RT-PCR tests revealed the presence of satellite RNA (satRNAs) in 35 strains among the 249 isolates confirmed to present CMV. SatRNA occurrence in this study is about 14%. At least one strain with satRNAs was detected in each sampling place except the locality of Aboisso (Table 5).

Table 6. Proportions of CMV satellite RNAs detected in banana plantations

Location	Number of symptomatic samples	Positive to CMV	Rate of infected	Number of isolates with RNA satellite
Aboisso	39	39	100	0
Abengourou	14	12	85.7	2
Agboville	36	33	91.6	3
Azaguie	29	28	96.5	10
Dabou	55	52	94.5	12
Tiassale	49	48	97.9	6
Grand-Bassam	38	37	97.3	2
Total	***260***	***249***	***95.7***	***35***

Five samples (AZ1, AZ13, BM40, BT18, SP24) out of the fourteen that induced symptoms on reporting plants appeared to possess a satRNA.

The sequencing of satRNA amplicons resulted in 15 unique sequences of 314 to 321 nucleotides long (Figure 7). Ivorian satRNA sequences were deposited in Genbank and are available under accession numbers ranging from MT968975 to MT968988 and KC713593 (Table 5). Fragment of satRNA identified in this study share 93.04% to 96.52% similarity.

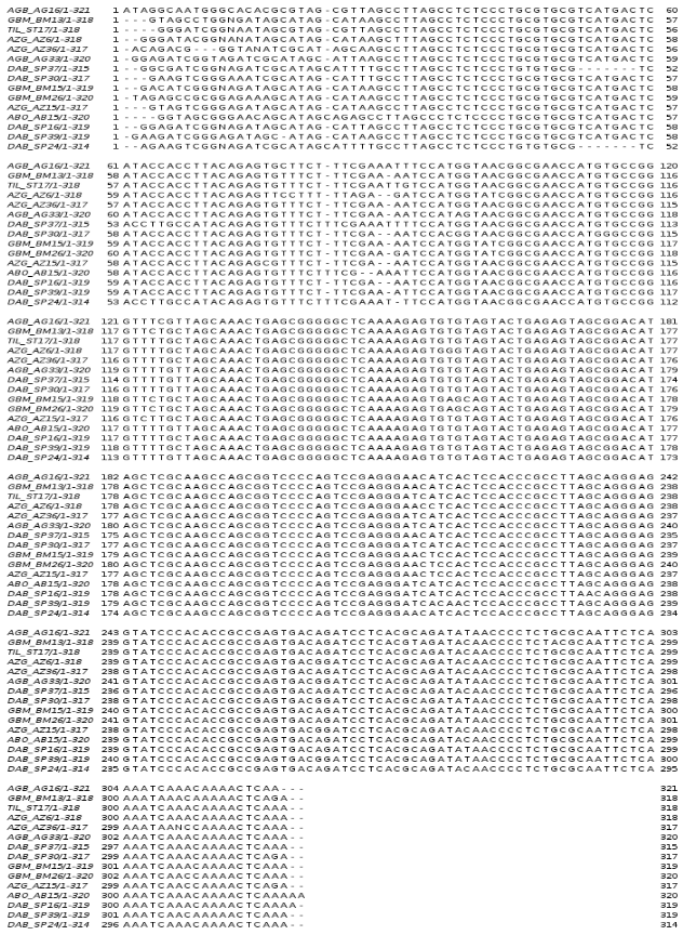

Figure 7. Multiple sequences alignment of satRNAs from Cote d'Ivoire.

We compute a tree with satRNA associated with Ivorian CMV and previously deposited satRNA sequences downloaded from Genbank. It appears that Ivorian satRNAs sequences clearly form a distinct clade (Figure 8).

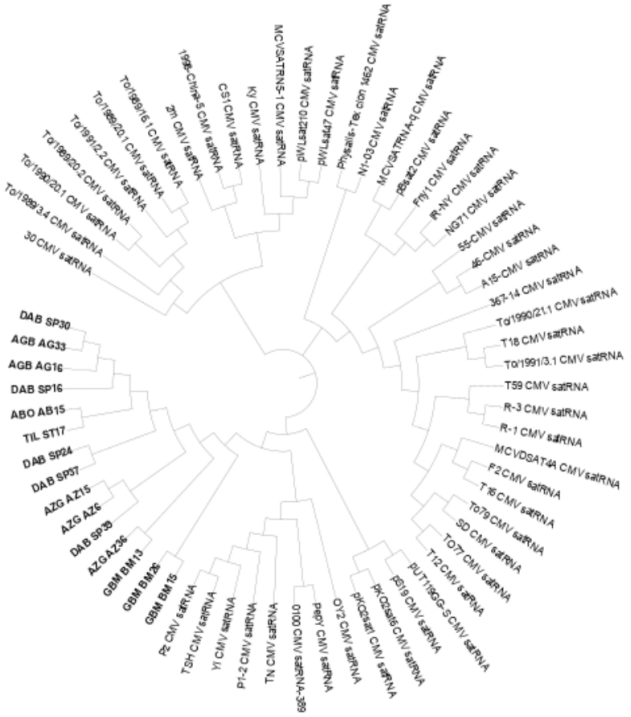

Figure 8. Estimation of Ivorian CMV-related satRNAs phylogenetic relationship with known satRNAs using the Maximum Likelihood method and Jukes-Cantor model.

The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. A Gamma distribution was used to model the substitution rate with 5 discrete Gamma categories.

DISCUSSION

Côte d'Ivoire is the first African exporter of desert bananas (FAO, 2020). Our survey aimed to measure the real level of CMV occurrence in industrial banana plantations. A second goal was to achieve a proper molecular characterization of the concerned strains of the virus since symptoms observed on the field are sometimes very confusing. Our study focused on banana varieties including mainly Cavendish, cvs Great Naine, and William, AAA group.

On the basis of the symptoms observed on these banana plants, 260 leaf samples were collected. In the Ivorian industrial plantations visited, no symptomatic banana plants showed marginal chlorosis or tufted tops characteristic of the Banana bunchy top virus (BBTV). BBTV is a banana virus for which symptomatology is a reliable basis for diagnosis (Lokossou et al., 2012; Adegbola et al., 2013). BBTV is expanding rapidly in Africa (Kumar et al., 2011) and most recently it has been reported in Togo, Benin, and Nigeria (Kolombia et al., 2021; Lokossou et al., 2012; Adegbola et al., 2013). Although we have not observed these symptoms, we will have to be alert to the spread of BBTV in Africa.

Aka et al (2009) had already mentioned the occurrence of CMV in industrial banana plantations located in 3 departments (taken into account in our study) of Côte d'Ivoire. The identification of CMV in our collected samples shows that this virus is present in the 7 departments visited. The DAS-ELISA and RT-PCR tests carried out to confirm CMV infection in banana plantations have already been used by other authors (Aglave et al., 2007; Aka et al., 2009; Chou et al., 2009). Except for one sample, the results of the serological tests were confirmed by RT-PCR. RT-PCR is indeed more sensitive than DAS-ELISA (Hu et al., 1995). Symptoms observed such as chlorosis, mosaic, cigar rot, dwarfing and leaf deformations are similar to those caused by CMV on bananas already reported in Côte d'Ivoire (Aka et al., 2009), Nigeria (Ayo-John et al, 2008), India (Aglave et al., 2007) and Costa Rica (Hord et al., 2001). As biological indexing is often used to diagnose CMV, we inoculated 65 isolates showing symptoms of banana CMV on tobacco (*Nicotiana tabacum*) and Cucurbitaceae (*Cucumis sativus*, *Cucurbita pepo*). While these samples were found to be infected on the basis of serological and molecular tests, our results show that the use of mechanical inoculation requires optimization of the protocol. In fact, only 14% of the samples used caused chlorosis or mosaics on the test plants one month after inoculation. The absence of symptoms following inoculation on these indicator plants could be due to inhibiting substances such as phenolic compounds from banana leaf extracts, the presence of asymptomatic leaves in the samples, or the combination of CMVs with other factors like abiotic stress and/or other viruses. The mechanical transmission of banana CMV isolates on indicator plants has often been difficult (Dheepa and Paranjothi, 2010) although some authors have done so more easily (Aglave et al., 2007; Chou et al., 2009). Compared to studies where only the biological indexing was carried out, our results demonstrate the relative difficulty to directly correlate results from biological indexing, serological data from DAS-ELISA, and genomic information from Sequencing. On the 65 infected samples (confirmed by both DAS-ELISA and amplicon sequencing) used to perform biological indexing, only 14 caused symptoms. This demonstrates, once again, the low reliability of biological

indexing compared to both DAS-ELISA and amplicon sequencing (Dubey et al., 2010; Gafny et al., 1996).

Molecular characterization by partial sequencing of the protein coat gene followed by phylogenetic analysis showed that 16 banana CMV isolates from 7 departments in Côte d'Ivoire belong to subgroup IA. In our work, Ivorian isolates with different biological properties on tobacco and Cucurbits (cucumber, courgette) belong to the same subgroup. The presence of only subgroup IA despite the variability between the Ivorian isolates could help in the long term in varietal resistance programs (Akhtar et al., 2010; Lu et al., 2011). Also included in this subgroup IA are CMV strains isolated from banana in Cameroon and Israel as well as CMV strains isolated from yam in Benin (Gafny et al., 1996; Eni et al., 2008). However, Hord et al. (2001) showed that in Costa Rica, bananas were infected by both CMV I and CMV II isolates. Furthermore, a high similarity score between coat protein sequences of Ivorian CMVs was recorded. In addition to considering this result as good conservation of the concerned strains, we also hypothesize that the observed CMV strains may have the same origin. Indeed, in Côte d'Ivoire, for new banana field installation, farmers actually take clones from their neighborhood, causing the spread of the viral infection from area to area. We, therefore, recommend farmers pay much attention to banana seeds. Systematic virus detection should be carried out prior to the introduction of seeds on farms. In this context, the DAS-ELISA protocol could be proposed since it is economically accessible to fruit companies and will avoid important production losses. Currently, symptomatic plants are detected one month after planting and replaced by healthy ones. However, in some cases, one month is already too late to avoid large contamination. Systematic detection before planting remains, to our opinion, the best-preventing strategy.

Based on a single amino acid difference in the protein coat sequences, we distinguished two groups in Ivorian strains. As previously established, it is worth mentioning that a single amino acid variation can induce different symptoms on plants: shifting from light green/dark mosaic to bright yellow/white chlorosis in tobacco (Shintaku et al., 1992) or causing white mosaic, pale green mosaic, veinal chlorosis, veinal necrosis, systemic necrosis, and necrotic local lesions and affect thylakoid structure (Mochizuki and Ohki, 2011). It is also worth indicating that we only partially sequenced the coat protein. The diversity and somehow inconsistency in symptoms observed during the field survey may reside in additional differences that would appear in the full-length coat protein sequence. This point should be considered for further studies on Ivorian CMV strains infecting *Musa spp.* trees. As indicated, we were not able to attribute particular symptoms to specific amino acid variation in Ivorian CMV based on biological indexing. Phylogenetic analysis showed that AZ6 (KC189912) and BM26 (KC189911) sequences are closed to E5-CMV and D8-CMV respectively, both coming from Japan. This result can be explained by the convergent evolution of CMV strains worldwide.

The search for satellite RNA by RT-PCR in the banana samples collected revealed the presence of these subviral RNAs in some of the CMV strains isolated from the 7 departments, except for one. The present study is, to the best of our knowledge, the first report of micro-satellite RNA sequences associated with CMV naturally occurring on *Musa sp.* in Côte d'Ivoire. In other countries, such as the USA and China, some authors have not found CMV satRNAs in banana samples (Kearney et al., 1990; Chou et al., 2009) although Gafny et al. (1996) revealed that in banana plantations in Jordan Valley, Israel satRNAs was associated with CMV strains. Our study also showed that the presence of satRNAs had no effect on the nature of CMV symptoms in Ivorian banana plantations. Similarly, we did not find any link between the observation of symptoms and the presence of satRNAs in the CMV strain on the indicator plants used. However, molecular characterization of 15 satRNAs associated with the Ivorian CMV strains showed that the sequences contained oligonucleotides identical to those responsible for necrosis on tomatoes in Serbia, Croatia, Greece, and Italy (Stanković et al., 2021; Sleat et al., 1994; Skoric et al., 1996; Grieco et al., 1997; Valeri and Boutsika, 1999). Other work had revealed that necrogenic satellite RNAs on tomatoes could induce only chlorosis on tobacco or have no effect on melon (Garcia-Arenal and Palukaitis, 1999; Betancourt et al., 2011). However, biological indexing of satellite CMV-RNA strains on tomato plants would be timely to assess this necrogenic ability. Further molecular characterization by constructing and analyzing infectious clones will be required for a complete characterization of the impact of these satellites on pathogenicity. This point needs a deeper analysis along with the presence or absence of micro-satellite. Indeed, the presence of satRNA does not seem to influence pathogenicity: observed symptoms are almost the same in all collecting places including Aboisso where no satRNA was detected.

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SUPPORTING INFORMATION

S1 Table. Accession number and origin of selected CMV coat protein sequences used for sequence comparison with Ivorian CMVs. *: IA CMV subgroup, **: IB CMV subgroup, ***: II CMV subgroup

S2 Table. List of satRNAs sequences used for sequence comparison with Ivorian satRNAs.

S3 Table. Symptoms observed on reporting plants after CMV's biological indexing. C: Chlorosis, M: Mosaic, FN: Foliar Necrosis, CR: Cigar Rot, LD: Leaf Distortion, LC: Leaf Curling, D: Dwarfing of the banana tree, TS: Thin Sheet