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Research Paper

PCR and ELISA detection of cassava mosaic virus in a Congolese cassava landrace

Otono Freddy Bulubulu[1]* • Ndofunsu Aimé Diamuini[1] • Nakweti Ruffin Kikakedimau[1] • Ntumbula Alexandre Mbaya[1] • Hity Mutambel[1] • Kasali Lumande[2] • Ndiku Luyindula[1] • Gladys Rufflard[3] • Philippe Lepoivre[3]

[1]Département de Biotechnologie et Génétique moléculaire, Commissariat Général à l'Energie Atomique (CGEA/CREN-K.), B.P. 868 Kin XI Kinshasa/République Démocratique du Congo (RDC)
[2]Faculté des Sciences, Université de Kinshasa, B.P. 190 KinXI, RDC.
[3]Laboratoire de Phytopathologie, Gembloux Agro-BioTech, Université de Liège, Passage des Déportés 2, Gembloux 5030, Belgique.

*Corresponding author. E-mail: freddy.bulubulu@upn.ac.cd, freddybbl@yahoo.fr.

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Abstract. The aim of this study is to diagnosing the cassava mosaic virus in the cuttings of the Congolese cassava landrace *Manihot esculenta* var. Boma. Symptoms severity was evaluated by visual observation of leaves in field and in greenhouse. The PCR (polymerase chain reaction) and DAS-ELISA tests were used to detect the cassava mosaic virus in this variety. The results showed that the disease index on infected plants were about 3.3 in the field and 2.8 in the greenhouse respectively. The presence of *African Cassava Mosaic Virus* (ACMV) in samples of this variety was detected by DAS-ELISA. The polymerase chain reaction was well performed for the identification of ACMV and not successful for the Uganda variant of *East African Mosaic Virus* (EACMV-UG) identification in the leaves samples with or without symptoms.

Keywords: Cassava, virus identification, severity, molecular and serology detection

INTRODUCTION

Cassava (*Manihot esculenta Crantz, Euphorbiaceae*) is a major food crop for more than half a billion people in Africa, Latin America and Asia. It plays a role of food security for more than 300 million people of sub-Saharan African population. It is one of the sources of income for farmers in several African countries (FAO, 2000; Ewuzie, 2008).

Cassava nourishes more than 60% people of the Democratic Republic of Congo (DRC) population (FAOSTAT, 2009). It is cultivated in all the DRC provinces with a total production of 15,300 tons (FAO, 2008, 2009).

The cassava production is seriously constrained by several pests and diseases, among them: Cassava Mosaic Virus Disease (CMD), whose etiological agent is transmitted through an insect vector, the whitefly (*Bemisia tabaci* Gennadius), as well as through the cuttings harvested from infected plants (IITA, 1990; Thresh et al., 1997; IITA, 2000; Ambang et al., 2006; Legg et al., 2006).

Seven begomovirus species widely distributed in Sub-Saharian Africa are recognized (Bisimwa et al., 2012). A recombining stock exceptionally virulent, the Uganda variant of *East African Cassava Mosaic Virus* (EACMV-UG) was associated the severe pandemia of *African Cassava Mosaic Virus* (ACMV) in Central and East Africa. Both, ACMV and EACMV-UG, were recorded in DRC in the West (Kinshasa and Bas-Congo Provinces) (Neuenschwander et al., 2002), and in the East, in particular in Yangambi region (Monde et al., 2010), the South-Kivu and North-Kivu (Karakacha, 2001; Obonyo et al., 2007; Bisimwa et al., 2012).

Manihot esculenta var. Boma, one of the congolese varieties of cassava appreciated by the population in the

Bas-Congo Province, is sensitive to CMV (Tata-Hangry et al., 2009). In the frame of the program of breeding of this variety, the establishment of a reliable diagnosis represents an essential stage. Although the Uganda variant is associated with severe pandemic of cassava mosaic disease, different studies showed that plants showing severe CMD symptoms are dually infected by both ACMV and the EACMV-Ug (Colvin et al., 2004; Thresh and Cooter, 2005).

The presence of the disease in a field can be detected by a simple visual observation of the virus symptoms on the leaves. These symptoms are variable according to whether the field is moderately or severely infected. The symptoms are however sometimes indistinct, in particular for the periods of dryness, at the time of mineral deficiency of the cassava seedlings or at the time of a serious attack of cassava green acarian or cassava cochineals (Compendium of the CABI, 2007). This indicates the limits of diagnoses based on disease symptoms. Such obviousness should be confirmed by more significant diagnostic tests.

The Enzyme-Linked Immunosorbant Assay (ELISA) is a simple and sensitive method for detection and quantification of virus level in the plant (Clark and Adams, 1977). ELISA has been used for the detection of several geminiviruses (Givord et al., 1994). However, detection of geminiviruses through ELISA has certain limitations such as its inability to distinguish different cassava mosaic begomovirus in mixed virus infections (Thottappilly et al., 2003). One major problem is the cross-reaction of the antibodies produced against one geminivirus coat protein with other geminiviruses. Therefore, it becomes difficult to distinguish between two closely related strains/species (Nirbhay et al., 2010). Moreover, the Polymerase Chain Reaction (PCR), the routine assay for plant viral genome detection, is many times more sensitive than ELISA (Ogbe et al., 2003; Nirbhay et al., *op cit*) and successfully detect mixed infections of ACMV and EACMV-Ug (Ogbe et al., 2006). A single diagnostic test or assay may provide adequate information on the identity of a virus, but a combination of methods is generally needed for unequivocal diagnosis (Acheremu, 2011).

The purpose of this study was to assess the symptom severity and to identify the ACMV, alone or in mixed infection with UG-EACMV, in the stem cuttings of *M. esculenta* var. Boma in order to promote CMD-free planting material.

MATERIALS AND METHODS

Plant material

The plants materials analyzed consisted of: (1) cassava plants variety Boma obtained from the National Institute of Studies and Agronomic Research (INERA)/MVUAZI, in the province of Bas-Congo and multiplied in field during the rainy season and in sandy soils at the Regional Center of Nuclear Research Agency of Kinshasa (CREN-K). Cuttings collected from CREN-K field were also multiplied on peat at 25°C in the greenhouse of Phytopathology unit, Gembloux Agro-BioTech (Belgium); (2) Burundian varieties (Cryolinha and R25) collected from *in vitro* young plantlets and the greenhouse of Gembloux.

Virus detection by visual observation

A preliminary study was conducted in the aims to evaluate the severity of symptoms by visual observation of *M. esculenta* var. Boma leaves in field conditions and in the greenhouse. The observation included 32 plants in fields and 9 plants in the greenhouse.

The severity of disease symptoms was assessed using the 1 to 5 scale (Hahn et al., 1980) that indicates the extent of symptom development: 1 - indicating no symptoms, 2 - slight mosaic covering less than 20% of leaf area without leaf distortion or size reduction, 3 - mosaic covering less than 50% of the leaf area without obvious size reduction but with some distortion, 4 - mosaic covering most of the leaf surface with leaf distortion and some leaf reduction, 5 - the most severe symptoms with leaf distortion and stunting of plants.

Index of severity of symptoms based on all plants (ISS_{AP}), and index of severity of symptoms based only on diseased plants (ISS_{DP}) were used (Acheremu, 2011). The data was subjected to angular transformation (Njock et al., 1994) and analysed:

$$\text{Incidence } (\%) = (X/Y)\ 100$$

$$ISS_{AP} = (a + 2b + 3c + 4d + 5e) / (a + b + c + d + e)$$

$$ISS_{DP} = (2b + 3c + 4d + 5e) / (b + c + d + e)$$

where X is the number of diseased plants and Y the total number of plants scored; a, b, c, d and e are the number of plants scored under the respective severity classes 1, 2, 3, 4 and 5.

ACMV detection by enzyme-linked immunosorbent assay

Sample preparation

The youngest and mature symptomatic cassava leaves were chosen from Boma cassava plants in the greenhouse. The samples were also taken from Cryolinha cassava plants whose ACMV was identified in previous work (Bussogoro et al., 2008). Leaf tissues were ground 1:10 (w/v) in sample extraction buffer. This sap dilution was used in the detection of ACMV.

Double antibody sandwich ELISA (DAS-ELISA)

DAS-ELISA was done essentially as described by Clark and Adams (1977) and Givord et al. (1994). It was used

Table 1. Sequences of couples of primers specific to ACMV and EACMV-UG.

CMD virus	Primer name	Sequence (5'-3')
ACMV	Afr 1	ACMV-AL1F : GCG GAA TCC CTA ACA TTA TC
	Afr 2	ACMV-ARO/R : GCT CGT ATG TAT CCT CTA AGG CCT G
EACMV-UG	Ug 1	UV-AL1/F1 : TGT CTT CTG GGA CTT GTG TG
	Ug 2	ACMV-CP/R3 : TGC CTC CTG ATG ATT ATA TGT C

Source: Busogoro et al. (2008).

through the Kit *DAS-ELISA-Biotin* (SEDIAG) specific for the ACMV, following the manufacturer's instructions. Microtiter plate (96 wells) was used. Volumes for each reactant were kept at 100 µl/well. Two replications were used to load samples on the plates. Between incubations, intensive washing steps were carried out by repeated soaking of the plates in washing buffer. Microtiter plates were coated with ACMV antibody diluted 1:1000 (v/v) in coating buffer and incubated for 2 h at 37°C.

Sap extracts, negative and positive controls (from Kit SEDIAG) were added following the loading diagram and incubated overnight at 4°C. Antibody-biotin conjugate, diluted 1:1000 (v/v) in conjugate buffer, was added and incubated for 2 h at 37°C. Alcalin phosphatase-streptavidin conjugate, diluted 1:100 (v/v) in conjugate buffer, was incubated 30 min at 37°C. The substrate, p-nitrophenyl phosphate diluted 1 mg/ml in substrate buffer was added and incubated for 1 h at 37°C. The measurement of the p-nitrophenol substrate conversion resulting in a yellow color was made by determining absorbance at 405 nm in a Multiskan RC model spectrophotometer (Labsystems Co.). Samples with absorbance values of more than twice that of the negative controls were considered positive for the virus.

Virus detection by polymerase chain reaction

The Polymerase Chain Reaction (PCR) was used to detect the ACMV and EACMV-Ug in the cassava leaves samples. Leaf samples with symptoms or without symptoms were collected from the plants growing in the greenhouse and from Cryolinha and R25 *in vitro* plantlets. Previous works have detected ACMV in Cryolinha samples and EACMV-UG in R26 samples (Bussogoro et al., 2008). The target genetic materials were obtained from the crude extracts of leaves with symptoms (Bussogoro et al., *op cit*) or leaves without symptoms. 0.4 g of each sample was introduced in a bag of crushing (LINARIS Biologische Produkte GMBH), contains 2 ml of cold (4°C) KAJI extraction buffer (DNAlis sprl). After leaves crushing, a rapid agitation (vortex) was carried out to suspend the leaves tissue fragments generating thus the primary crude extract which had to be diluted (100 times) in distilled water. The diluted extracts were preserved at –20°C before PCR analysis.

PCR with two primer pairs Afr 1/ Afr 2 or Ug 1/ Ug 2 (Table 1) was done in a 50 µl reaction mixtures consisting, 5 µl of 10x concentrated PCR reaction buffer (Roche, Mannheim, Germany), 1 µl of DNTPs (200 µM), 1 µl of each primer (Forward and Reverse) (0.5 µM), 0.2 µl of the Taq Polymerase solution (5 U/µl), 36.8 µl of sterile distilled water and 5 µl of the 100x diluted plant crude extract suspension.

The PCR amplification was achieved with a PTC 200 Doppler (MJ Research BioRad) thermocycler or C 1000 (BioRad) thermocycler. The PCR cycle used for the amplification consisted of an initial denaturation step at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 1 min, primer annealing at 52°C for 1 min and elongation at 72°C for 2 min. This was then followed by a final elongation step at 72°C for 10 min.

20 µl aliquots of PCR products were mixed with 4 µl of loading dye (6x DNA Dye Loading orange) and electrophoresed on 1% agarose gel containing ethidium bromide (1 µg/10 ml) under a constant current of 120 mA, at 120 V for 45 min.

This migration was followed by visualization of the amplified bands under ultraviolet light and photographed with an imagery Gel Doc system.

RESULTS AND DISCUSSION

Virus disease status in the cassava variety Boma

Cassava Mosaic Disease incidence and severity on leaves of variety Boma cultured *in situ* (CREN-K site) and in the greenhouse are presented in Table 2. This variety is sensitive to the *African cassava mosaic virus*. After three months of culture *in situ*, all plants (100 %) were infected and presented an index of severity 3.34. 59.38% of plants infected with cassava mosaic had a severity level 4. In greenhouse, the symptoms were observed in 55.56 % of plants. The index of severity of symptoms was 1.56 on all plants and 2.8 on diseased plants. More than 55% of plants had a degree of severity between 1 and 4.

ELISA detection of ACMV

The double-antibody sandwich enzyme-linked immuno-

Table 2. Scores of African Cassava Mosaic virus disease status on leaves of *M. esculenta* var. Boma cultured field and in the greenhouse.

Site of culture	Severity		CMD Incidence (%)
	ISS_{AP}	ISS_{DP}	
CREN-K	3.34	3.34	100 (0.20)
Greenhouse	1.56	2.8	55.56 (0.11)
Grand mean			0.15
LSD (0,05)			0.28
Cv (%)			104.96%

ISS_{AP} = Index of severity of symptoms on all plants. ISS_{DP} = Index of severity of symptoms on diseased plants. Parenthesis = Transformed data used for analysis.

Table 3. DAS-ELISA detection of African cassava mosaic virus in leaf extracts of *M. esculenta* var. Boma

Accession	Well number	Absorbance (405 nm)	ELISA reaction (+ or -)
Boma	2A	0.042	+
	3A	0.048	+
	2B	0.039	-
	3B	0.040	-
	2C	0.007	-
	3C	0.006	-
	2D	0.419	+
	3D	0.311	+
	2F	0.098	+
	3F	0.092	+
Cryolinha	2E	0.152	+
	3E	0.157	+
Positive control	G2	0.079	+
Negative control	H2	0.0299	-

+ = ACMV positive; - = ACMV negative.

sorbent assay (DAS-ELISA) detected ACMV in leaf extracts of Boma samples (well A, D and F) and Cryolinha (well E). These samples had UV absorbance values twice that of ACMV-free (negative control), indicating that they were infected by the virus. By contrast virus was not detected in Wells B and C (Table 3).

PCR detection of the cassava mosaic virus in the extracts of *M. esculenta* Var. Boma

The amplification profile was obtained for four of the five samples analyzed by using the primer pairs Afr 1/Afr 2 relative to ACMV (Figure 1). The presence of the ACMV results in the amplification of a fragment of approximately 1000 bp corresponding to part of gene coding for protein of the viral capsid.

However, the infection with the Uganda variant of East African cassava mosaic virus (UG-EACMV) was not revealed in Boma samples (Figure 2). Except for the positive control (R25) where the virus fragment amplified is 1600 bp, no amplification profile was obtained for all samples analyzed by using the primer pairs Ug 1/Ug 2 relative to UG-EACMV.

DISCUSSION

In the present study, the visual inspection of Boma cassava plants revealed that cuttings of this Congolese variety were infected by cassava mosaic virus. With respectively an index of symptom severity on all plants and on diseased plants of 3.34 *in situ,* Boma presented severe symptoms of the disease. Tata-Hangy et al. (2009)

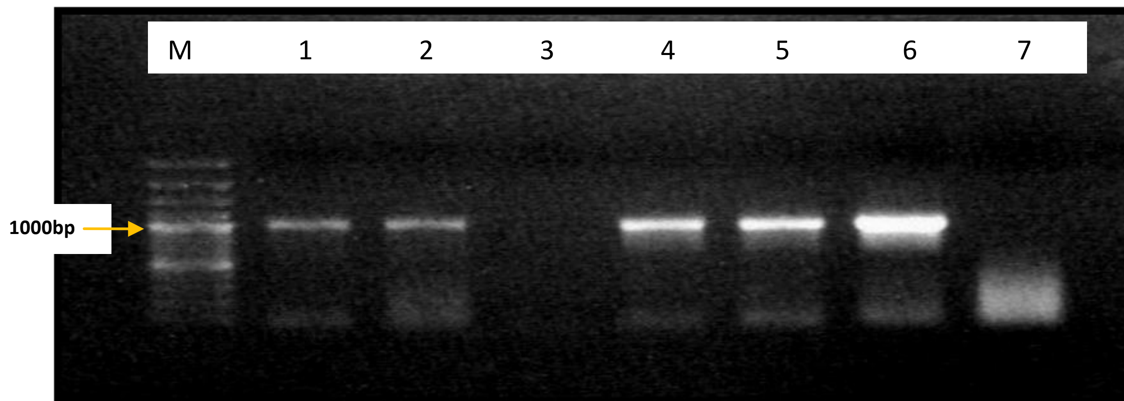

Figure 1. PCR detection of African cassava mosaic virus (ACMV) in the cassava samples. Lane M: 100 bp ladder; lanes 1-4: Boma samples; lane 5: Cryolinha sample; lane 6: positive control (Cryolinha); lane 7: negative control.

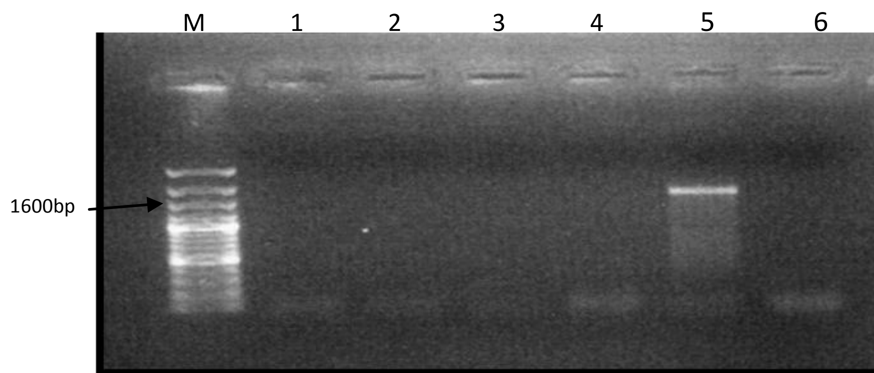

Figure 2. PCR detection of Uganda variant of East African cassava mosaic virus (UG-EACMV) in the cassava samples. Lane M: 100 bp ladder; lanes 1 - 4: samples of the Boma variety; lane 5: Positive control (R25); lane 6: negative control (Mix PCR without sample extract).

reported that the Congolese cassava variety “Boma” is sensitive to *Cassava mosaic virus* and the disease develops quickly. The principal mode of infection in central Africa being through cuttings, a higher percentage of cassava mosaic disease, 100% *in situ* and 55.56 in controlled conditions, was due to the use of infecting planting material.

ACMV was not observed in samples containing in wells B and C by ELISA because leaves were mature. Fargette et al. (1987) reported that virus content of the leaves increased with increasing symptom intensity, but decreased with leaf age and ACMV was not detected by ELISA technique in mature leaves.

The use of the techniques of DAS-ELISA and PCR in this study made it possible to confirm the infection with ACMV of the congolese variety of cassava. According to the literature, the amplification of a fragment of approximately 1000 bp, corresponding to part of gene coding for protein of the viral capside, indicates the presence of the ACMV and a fragment of 1600 bp indicates the presence of the alternative Uganda strain of EACMV (Karakacha, 2001; Busogoro et al., 2008). ACMV has been known to occur in most of the cassava producing areas of Africa (Sseruwagi et al., 2004). By contrast EACMV-UG has a more limited known distribution. It has been reported in western (Kinshasa and Bas-Congo provinces) of the Democratic Republic of Congo (Neuenschwander et al., 2002). In the present study, it was noted in Boma leaf extracts, amplification using the pair of primers specific to the ACMV and the absence of amplification when the primers specific to the EACMV-UG were used. A number of factors play a role in disease severity; these include cultivar, virus strain/species, rainfall and the quality of soil. Another factor in disease severity is occurrence of mixed infections (Zinga et al., 2008; Gaza, 2007). It is generally observed that mixed infections occur in areas with high CMD incidence where both ACMV and EACMV-UG are prevalent (Sseruwagi et al., 2005). Mixed infection ACMV-EACMV-UG was not observed in Boma samples multiplied in Gembloux greenhouse. The severity observed could be attributed to the use of sensitive

cultivars by the peasants. It would be however desirable that the diagnosis is carried out on Boma samples taken from Kinshasa and Bas-Congo fields.

Conclusion

The results of this study demonstrate that stem cuttings of cassava variety Boma are infected. ACMV was detected by DAS-ELISA and PCR techniques while EACMV-UG was not detected. There is need to clean cassava cuttings against cassava mosaic virus using *in vitro* meristem culture coupled to the thermotherapy and/or chemotherapy.

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