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Identification of *Cucumber mosaic virus* (CMV) Isolates Infecting *Musa* spp. and Vegetable Crops in Southern Nigeria

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ABSTRACT

This study was carried out to identify 25 CMV isolates from *Musa* spp. and associated vegetable crops using biological, serological and molecular techniques. The CMV isolates were from 20 *Musa* spp., 2 *Dioscorea* spp., 2 *Capsicum* sp. and 1 *Occimum* sp., crops indexed from a total of 85 leaf samples collected from 31 locations within 5 states in Southern Nigeria in 2004. Seven of the isolates transmitted to indicator plants all belonged to CMV subgroup 1b by inducing necrotic local lesions on *Vigna unguiculata* (TVu 76) and mosaic symptoms on *Nicotiana glutinosa*. None of the isolates reacted with monoclonal antibody 489 with affinity for CMV I while monoclonal antibody 490 with affinity for CMV II isolates reacted with 5 CMV isolates from *Musa* spp. and *Occimum* sp. The monoclonal antibodies used showed a narrow specificity for the CMV isolates as such could not be used to differentiate all the isolates. Thirteen cDNA corresponding to the coat protein gene of CMV were amplified in IC-RT-PCR of which 11 of the isolates showed restriction pattern typical of CMV 1 after digestion with Msp1 and EcoR1 restriction endonucleases. Two isolates were distinct in that they did not react with either of the enzymes. Restriction enzyme digest patterns showed that the CMV isolates all belong to subgroup 1.

Key words: CMV, *Musa*, identification, vegetable crops

INTRODUCTION

Banana chlorosis or heart rot disease has been reported to be caused by *Cucumber mosaic virus* (CMV) (Magee, 1940; Singh *et al.*, 1995; Chatzivassiliou *et al.*, 2004). The virus also causes diseases and economic losses in cereals, fruits vegetables and ornamentals (Kaper and Waterworth, 1981). The virus is one of the most important viruses world-wide for the economic impact of diseases. It induces in many different crops, particularly ornamentals and vegetables (Alonso-Prados *et al.*, 1998). This may be as a result of the transmission by more than 80 aphid species in a non-persistent manner and with different efficiencies, via seed of more than 20 host species in over 1,200 species in more than 100 plant families (Palukaitis and Garcia-Arenal, 2003a, b).

Plantain and banana are among the most important staple food crops in humid forest zone of West and Central Africa and they are important sources of rural income and thus boost food security among many Nigerians. Plantains and bananas are perennial and popularly grown in home backyard farms and gardens in Southern Nigeria where they are often intercropped with crops such as peppers, eggplant, pumpkin, yam, bitter leaf and other vegetables. Vegetables provide essential proteins, minerals and vitamins in the diets of people in the developing countries

and serves as a complement to starchy staple foods. They are high value crops that provide income generating opportunities to small farmers (Selleck and Opena, 1985).

Vegetable crops however are susceptible to a host of diseases and production is limited by pests and pathogens, among which viruses disease are a major constraint (Kostova *et al.*, 2003). Most farmers lack the knowledge of virus symptoms as such do not recognize virus symptoms as been due to disease thereby resulting in low or absence of control measures. CMV has been detected in staple crops in Nigeria such as yam, cowpea and plant *Musa* spp. (Hughes *et al.*, 1997; Shoyinka *et al.*, 1997; Ayo-John *et al.*, 2008). Information on the identity of CMV strains causing diseases of crops in southern Nigeria is scanty, this survey work was carried out to identify CMV isolates infecting *Musa* spp. and vegetable intercrops in southern Nigeria. An understanding of the viruses involved is a pre-requisite for formulating effective control measures and the information would be important for national breeding programs.

MATERIALS AND METHODS

Survey for CMV in *Musa* spp. and vegetable intercrops: Survey for CMV was done in 5 states in Southern Nigeria in 2004 where *Musa* spp. are majorly grown in (1) Backyard home orchards, (2) Farms and orchards along the roads between villages were selected at an interval of 2-5 km. These were areas where virus symptoms had previously been reported. At each site leaf samples of the various virus symptoms such as interveinal chlorosis, chlorotic streaks, chlorosis, crisp malformed leaves, leaf crinkling and puckering and unusually thick lateral veins were collected from *Musa* plant stands showing virus-like symptoms. Also, where found leaf samples from crop plants in association with *Musa* spp., such as *Capsicum* sp., *Dioscorea* sp and *Occimum* sp., with virus-like symptoms were also collected for detection of CMV to determine their role as virus reservoirs. The leaf samples were preserved while in transit by placing them individually in labeled polyethylene sample bags before the bags were placed over ice packs in coolers while in transit. All the samples were later taken to the Virology laboratory, International Institute of Tropical Agriculture (IITA) Ibadan, Nigeria for virus indexing using Enzyme-linked Immunosorbent Assay (ELISA) with polyclonal and monoclonal produced at Deutsche Sammlung von Microorganismen und Zellkulturen GmbH (DSMZ), Germany and Immuno-Capture Reverse Transcription Polymerase Chain Reaction (IC-RT-PCR) technique.

Detection of CMV leaf samples: DAS-ELISA was done as described by Clark and Adams (1977). Mean absorbance values at 405 nm that were twice or more than the mean of the healthy control samples were regarded as positive to CMV. The absorbance of the contents of the wells was read at 405 nm using Dynex-MRX microplate reader after 1 h at room temperature (23-28°C) and overnight at 4°C.

Monoclonal antibody serotyping of CMV isolates: Saps from infected leaf tissues were used in Triple Antibody Sandwich TAS-ELISA for serogroups identification. TAS-ELISA kits containing CMV polyclonal antibodies (ASP 475), monoclonal antibody 489 specific for CMV serogroup 1, monoclonal antibody 490 specific for CMV serogroup II, monoclonal antibody 488 that does not distinguish between serogroups and rabbit anti-mouse alkaline phosphatase were used in TAS-ELISA procedure performed according to the supplier's specifications.

Mechanical inoculation of test plants with CMV isolates: Each CMV infected leaf tissues were ground in cold ($\approx 4^{\circ}\text{C}$) phosphate buffer containing 0.5% sodium sulphite and the saps were rubbed on carborundum-dusted healthy leaves of the following test plants; *Chenopodium quinoa* Willd, *Cucumis sativus* L., *Vigna unguiculata* (L. Walp) TVu 76, *N. tabacum* L., *N. benthamiana* Domin., *N. glutinosa* L. and *Capsicum annuum* L. Identification of CMV subgroup 1a and 1b was done as reported by Daniels and Campbell (1992).

Detection of CMV using immuno-capture RT-PCR technique: A pair of CMV primers (Wylie *et al.*, 1993) was used in IC-RT-PCR to amplify the coat protein gene of the CMV isolates. A fragment length of 482-487 base pairs for CMV subgroup I isolate and 501 base pairs for subgroup II isolates was expected after IC-RT-PCR. The CMV Primer 1 (downstream) had a sequence of 5'GCCGTAAGCTGGATGGACAA3' and Primer 2 (upstream) had a sequence of 5'TATGATAAGAAGCTTGTTCGCG3', respectively. PCR tubes were pre-coated with CMV IgG and incubated for 4 h at 37°C . The tubes were washed with PBS-T before leaf Saps (100 μL) ground in extraction buffer (PBS-T+2% PVP) in ratio 1 to 10 (wt/vol) were added and incubated for 4 h at 37°C . A 50 μL RT-PCR mix (Qiagen Ltd UK) was made into each 500- μL PCR as follows: 5x Qiagen one step RT buffer 10 μL , DNTP mix 2.0 μL , 5x Q solution 10 μL , primer 1 forward 1.0 μL , primer 2 reverse 1.0 μL , Qiagen one step enzyme mix 2.0 μL and sterile distilled water 24 μL . Tubes were left in ice till the Peltier Thermal Cycler (PTC 200) reached a temperature of 50°C before the PCR tubes and contents were arranged in the thermal cycle block. The reverse transcription step was completed after 30 min at 50°C , the amplification process began with a hotstar 95°C for 15 min to activate the Taq polymerase, reverse transcriptases are inactivated and the cDNA template is denatured. This was followed by 40 cycles of 94°C for 1 min, 60°C for 1 min, 72°C for 1 min and a final extension of 72°C for 10 min.

Gel electrophoresis: The amplified PCR products (10 μL) were subjected to electrophoresis in 2% agarose gel stained with ethidium bromide (0.01%). The cDNA bands were visualised under U.V transilluminator (Vilber Lourmat, Serial number 6532). The photograph of the gel was taken using gel documentation and analysis system computer software (model UVP GDS 8000, Ultra-violet product Ltd., UK).

Restriction digests of PCR products: Restriction enzymes digests of the amplified CMV coat protein gene were performed with EcoR1 and MspI restriction endonucleases (Madison, USA). The reaction was made up of 10 μL of the amplified products 2 μL of the reaction buffer (10X Promega), 0.2 μL of bovine serum albumin and 0.5 μL of enzyme were added to a final volume of 20 μL . All digests were carried out at 37°C for 2 h and the digests were subjected to gel electrophoresis and described earlier. Identification of serogroups was done as described by Singh *et al.* (1995).

RESULTS

Survey and detection of CMV: Eight-five leaf samples comprising 71 *Musa* spp., 5 *Dioscorea* sp., 6 *Capsicum* sp., 1 *Occimum* sp and 2 *Solanum melogena* were collected from 31 locations within Abia, Akwa Ibom, Cross River, Imo and Rivers States in 2004. CMV polyclonal antibodies in DAS-ELISA reacted positively with 25 leaf tissues comprising of 20 *Musa* leaf samples,

Table 1: Serological detection of CMV in *Musa* spp. and vegetable crops in home gardens in Southern Nigeria in 2004

Crops	No. collected	DAS-ELISA		TAS-ELISA Mabs (488)		TAS-ELISA Mabs (489)		TAS-ELISA Mabs (490)	
		No. positive	(%)	No. positive	(%)	No. positive	(%)	No. positive	(%)
<i>Musa</i> spp.	71	20	28.2	3	4.2	0	0	4	5.6
<i>Dioscorea</i> sp.	5	2	40.0	0	0.0	0	0	0	0.0
<i>Capsicum</i> sp.	6	2	33.3	1	16.6	0	0	0	0.0
<i>Occimum</i> sp.	1	1	100.0	1	100.0	0	0	1	100.0
<i>Solanum melogena</i>	2	0	0.0	0	0.0	0	0	0	0.0
Total	85	25	29.4	5	5.9	0	0	5	5.9

Table 2: Reactions of test plants to mechanical inoculations with CMV isolates from *Musa* spp. and vegetable crops in Southern Nigeria

Test plants	<i>Dioscorea</i>	<i>Capsicum</i>	<i>Musa</i> spp., I	<i>Capsicum</i> 16	<i>Capsicum</i> 20	<i>Occimum</i> 73	<i>Musa</i> spp. II
<i>N. tabacum</i>	M	M	M	M	M	M	M
<i>C. frutescence</i>	M	M	M	LD, M	LD, M	M, LD	NT
<i>C. sativus</i>	M	M	M	NT	NT	NT	NT
<i>N. benthamiana</i>	M	M	M	M	M	M	M
<i>V. unguiculata</i>	NS	M	M	NS	NT	NT	NT
<i>N. glutinosa</i>	M	M	M	M	M	M	VC, M
<i>C. quinoa</i>	CLS	CLS	CLS	NT	LNS	LNS	LNS

*NT: Not transmitted, M: Mosaic, LD: Lead distortion, VC: Vein clearing, LNS: Local necrotic spots

3 *Capsicum* leaf samples, 1 *Dioscorea* leaf sample and 1 *Occimum* sp., leaf sample. The 2 *S. melogena* leaf samples did not react with any of CMV antibodies (Table 1).

Monoclonal antibody (Mab) serogrouping of CMV isolates: MAb 488 and 490 each reacted positively with 5 out of 25 leaf samples, respectively. The leaf samples that were positive in the TAS-ELISA were also positive in the DAS-ELISA. None of the leaf samples reacted with monoclonal antibody 489 Table 1.

Mechanical inoculations of CMV isolates to test plants: Seven CMV isolates from *Capsicum annum* (3) *Dioscorea* sp., (1) *Musa* spp., (2) and *Occimum gratusimum* (1) were successfully mechanically transmitted to the various test plants used. Symptoms of mosaic and leaf distortion observed on the original *Capsicum* hosts were reproduced on *C. frutisense* in the greenhouse. On *N. tabacum*, *N. benthamiana*, *N. glutinosa* and *C. sativus* mosaic symptoms were observed while local chlorotic spots were induced on *C. quinoa* and fine necrotic spots on *V. unguiculata* TVu 76 (Table 2). The 7 CMV isolates all belonged to CMV subgroup 1b by inducing necrotic local lesions on *Vigna unguiculata* (Tvu 76) and mosaic symptoms on *Nicotiana glutinosa*.

IC-RT-PCR and restriction enzyme digests of CMV isolates: The coat protein genes of 13 of the CMV isolates were successfully amplified with IC-RT-PCR technique (Fig. 1). The expected band size of approximately 500 base pairs was observed. Following enzyme digests with *Msp1*; two DNA bands of approximately 350 and 150 base pairs typical of CMV 1 isolates were obtained (Fig. 2) from 11 isolates. All the CMV isolates except 2 showed CMV 1 restriction pattern after enzyme digest analysis. Two isolates were not digested by *Msp1* and *EcoR1* restriction enzymes as shown in Fig. 2 (lanes a and c).

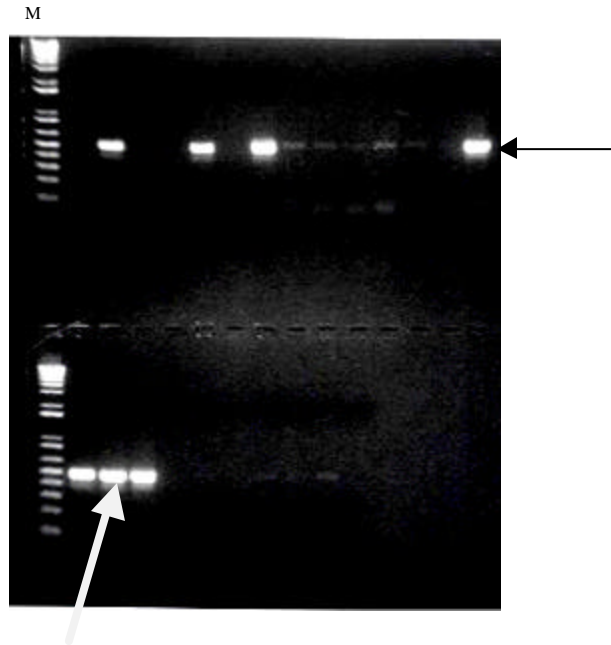


Fig. 1: Amplified cDNA bands of CMV isolates (arrowed) of approximately 500 base pairs, M = DNA marker

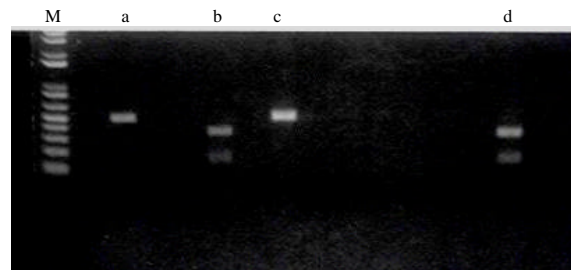


Fig. 2: Restriction enzyme digests of CMV cDNA with *Msp1* showed typical CMV 1 pattern of 2 bands of approximately 350 and 150 bp each (lanes b and d) and two isolates (lanes a and c) remained undigested, M= DNA marker

DISCUSSION

The results of this study provide evidence that CMV occurred in *Musa* spp. and other crops in mixed cropping. The CMV isolates were recovered from leaf samples from crops over a wide geographical area thus suggesting possible spread by human activities through exchange and sale of planting materials over long distances. The CMV isolates from *Musa*, *Dioscorea*, *Capsicum* and *Occimum* species all belonged to CMV I. Subgroup I isolates have been previously reported to occur predominantly in the tropics and sub-tropics causing severe symptoms and epidemics. Subgroup II typified by isolate ToRS is prevalent in the temperate regions (Haase *et al.*, 1989).

Losses due to CMV can be high where there is super-infection by other viruses or where there is poor management. *Pepper veinal mottle virus* is endemic in pepper in the areas surveyed. Severe or heart-rot isolates of CMV which are far more damaging than the common isolates of the virus, do not occur in all banana-producing areas. It is therefore important to avoid the introduction of severe CMV isolates into new areas where they can cause significant damage (Bouhida and Lockhart, 1990).

Some CMV isolates could not be transmitted to test plants from *Musa* leaf tissues. Transportation of leaf samples over long distances and storage conditions while in transit may have led to the poor state of those leaf tissues. Monoclonal antibodies alone could not be used in serological tests to differentiate CMV isolates into serogroups. Many of the isolates reacted positively with polyclonal antibodies in DAS-ELISA but escaped detection with the monoclonal antibodies in TAS-ELISA. Also, isolates that reacted with Mab 490 with affinity for CMV II showed CMV I restriction pattern after enzyme digest. Similarly Bashir *et al.* (2006) could not use CMV II MAB to differentiate 13 CMV isolates infecting cucurbits in Northwest region of Iran. Monoclonal antibodies have a narrow specificity but could be highly sensitive. IC-RT-PCR technique was used successfully to amplify CMV cDNA. The *Msp* I restriction pattern of 11 CMV isolates produced 2 DNA fragment sizes of approximately 350 and 150 bp. This is typical of CMV subgroup I isolates as earlier reported (Rizos *et al.*, 1992; Singh *et al.*, 1995). Two of the CMV isolates were distinct and were not digested by *Msp*1 and *Eco*R1 restriction enzymes. The symptoms induced on test plants by the virus isolates were similar to those reported elsewhere for CMV (Varveri and Boutsika, 1999). Chatzivassiliou *et al.* (2004) had reported that the elimination of weeds that consists the main alternative virus and vectors' reservoirs, is generally suggested as an effective procedure for managing virus epidemics. Even though difficult to apply due to the numerous host species involved, the control measure had been successful in controlling CMV in lettuce and celery crops. Our diagnostic survey on home gardens showed evidence of CMV infections. It is important to raise awareness of farmers to disease management strategies. The occurrence of CMV in *Musa* spp. and the intercropped vegetables may pose a potential risk of CMV epidemic, although the epidemiological impact of CMV in Nigeria has not been fully assessed or is poorly understood the virus has all the time to evolve and spread within the species. The mixed cropping system practiced in subsistent farming systems as in the case in Southern Nigeria enhances virus spread and in the absence of control measures the results are severe virus infections and crop losses. Selective intercropping and improved cultural practices is important to reduce the risk of potential CMV epidemics. Alternatively breeding for resistance still remains the most effective way of combating viral diseases and should be further exploited.

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