# Elimination of African Cassava Mosaic Virus (ACMV) and East African Cassava Mosaic Virus (EACMV) from cassava (*Manihot esculenta* Crantz) cv. 'Nwugo' via somatic embryogenesis.

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## ABSTRACT

The presence of African cassava mosaic virus (ACMV) and other cassava mosaic disease (CMD) viruses in cassava (*Manihot esculenta* Crantz) can cause severe damage leading to huge economic loss to infected cassava plants; therefore, the use of virus-free cuttings for multiplication cum cultivation is highly desirable. In this study, the use of somatic embryogenesis for the cleansing of 'Nwugo' cultivar with mixed infections of African cassava mosaic virus and East African cassava mosaic virus – Cameroon strain (EACMV-CM) was investigated. Polymerase chain reaction (PCR) was employed to confirm the presence of these viruses using specific primers as described in the materials and methods. Regenerated plantlets via somatic embryos showed absence of these viruses demonstrating that somatic embryogenesis is a promising technique to adopt for elimination of viruses from infected plants especially those propagated vegetatively.

Key words: ACMV, EACMV, Manihot esculenta, somatic embryogenesis, Nwugo, PCR.

### INTRODUCTION

Cassava (Manihot esculenta Crantz) is a shrub 1-5 m high which is cultivated for its starch-containing tuberous roots (Cock, 1985). The crop is widely propagated in the tropics and the starchy roots provide food for over 500 million people in developing countries (Roca et al., 1992). One of the greatest problems confronting this all important crop in Africa is Cassava Mosaic Disease (CMD). In Africa, this disease is caused by the viruses - African Cassava Mosaic Virus (ACMV) and East African Cassava Mosaic Virus (EACMV). They are transmitted by the whiteflies (Bemisia tabaci), and sometimes through contaminated instruments and infected planting material. Presence of these viruses can cause losses of up to 40% to 50% of total yields in cassava throughout the continent (Thresh et al., 1994; Otim-Nape et al., 1996).

The most widely used method for virus elimination is meristem tip culture. This technique takes advantage of the fact that many viruses fail to invade the meristematic region. The use of this method is not 100% efficient in that its efficiency depends on the size of the meristem tip as well as the ability of the operator to excise the dome shaped meristem tip unwounded.

There are reports on successful elimination of virus from infected plants through the use of somatic embryogenesis. Parmessur et al. (2002) reported successful elimination of virus and phytoplasma from sugarcanes (Saccharum officinalis L.) infected with sugarcane yellow leaf virus and sugarcane yellow phytoplasma using the technique of somatic embryogenesis. D' Onghia et al. (2001) also reported elimination of citrus psorosis virus from citrus by somatic embryogenesis using infected stigma and style as explants. Similarly, Gribaudo et al. (2006) in their study on the use of different techniques to eliminate infected Grapevine (Vitis vinifera L.) from Grapevines rupestris stem pitting-associated virus discovered that somatic embryogenesis produced almost 100% virus -free plants over other methods

including meristem tip culture. Another advantage somatic embryogenesis has over meristem tip culture is its ability to generate multiple plantlets per explant unlike meristem-tip culture that produces only one plantlet per explant.

At present, there is little or no literature on virus elimination in any of the cassava genotypes via somatic embryogenesis. Hence, this paper examines the possibilities of obtaining virus-free plants from infected Nigerian local line 'Nwugo' cultivar through the use of somatic embryogenesis.

### MATERIALS AND METHODS

Plant material / Source of explant: The experiment was conducted at the International Laboratory for Tropical Agricultural Biotechnology (ILTAB), Donald Danforth Plant Science Center, St. Louis, Missouri, USA. The in vitro cultures of the 'Nwugo' cultivar used for this study was raised at the tissue culture laboratory of National Root Crops Research Institute (NRCRI), Umudike, Abia State, Nigeria before being exported to the danforth center laboratory in USA as mentioned above. This 'Nwugo' cultivar is one of the farmer's preferred cassava genotypes often cultivated by local farmers in Abia State. Clean 'Nwuqo' cultivar cuttings when cultivated in uninfected field produces good harvest. One of the greatest challenges confronting rural farmers with this genotype is its susceptibility to CMDs especially that caused by African cassava mosaic virus. The cultivar was chosen based on its responses to tissue culture in terms of production of friable embryogenic callus (FEC) - an indication that the genotype may be a good candidate for cassava genetic transformation experiments.

Media Composition / Preparation: Culture Murashige and Skoog (MS) (Murashige and Skoog, 1962) and Gresshoff and Doy (GD) (Gresshoff and Doy 1972) basal media are prepared from constituent chemicals (Sigma Chemical Co., St. Louis) as detailed at http://www.phytotechlab.com/. Macro- and micronutrients, vitamins and Fe-EDTA stock solutions are prepared at 25x, 1000x, 1000x, and 200x concentrations respectively and stored at 4°C until required. Stocks are mixed, sucrose added at 20 g/l, pH adjusted to 6.1 with 1 N NaOH and Difco Noble Agar added at 8 g/l before autoclaving at 121°C for 20 min. Napthalene acetic acid (NAA) and Benzylaminopurine (BAP) are added prior to autoclaving but picloram and antibiotics when needed are added from filter sterilized stock solutions after media is autoclaved and cooled to 42°C, as determined by the use of a FLUKE 62 Mini IR Thermometer (Fluke Biomedical, Everett, WA). All GD and MS based media containing picloram are dispensed into 10 cm x 15 mm plates at 25 ml per dish while MS based regeneration and whole plant media are poured into 10 cm x 25 mm Petri dish at 40 ml per dish. Cultures were incubated at  $28^{\circ}$ C under a 16/8 hour photoperiod at 75 µMs/ms<sup>2</sup>.

All tissue manipulations were performed under a stereomicroscope (Olympus SZ61).

The *in vitro* cultures were multiplied first by subculturing them on to Murashige and Skoog basal medium containing 20 g/l sucrose (MS2 Agar) according to Murashige and Skoog (1962). These plantlets were later transferred to the green house at the Donald Danforth Plant Science Center after undergoing post flask management for phenotypic expression as shown in Figs. 1(a-d) which served as source of mother plants for further *in vitro* studies.

From the phenotype of this 'Nwugo' cultivar growing in the green house, it became obvious that the cultivar has virus infection. The plants were showing severe symptoms like twisting of the leaves with pale green or yellow areas which are commonly small and distorted. Generally, the disease leads to overall reduction in yield and stem size. The type of virus infection was confirmed when PCR analysis was conducted using DNA samples extracted from the young leaves and specific primers for ACMVCM-AC1-F with the sequence

5'- CGG ATG GCT CGC TTC TTG AAT TGT C -3'

ACMVCM-AC-R 5'- TCT GTA GGG AGC TGC ATC AGA ATG G-3' and

EACMV-CM-AC1 F2 with the sequence 5'- TGT GAG GGA GAT GAA GAG CGC ATT -3'

EACMV-CM-AC1 R2 5' - AGG GCC AGC ATT TAG CTC AGG TAT -3'. These primers were purchased from Integrated DNA technologies (IDT). For detailed information see

http://www.idtdna.com/Catalog/Usage/Page1.aspx.

**Micropropagation:** Nodal cuttings were collected from the infected plants growing at the green house and initiated on Murashige and Skoog (MS) basal medium (1962), supplemented with 20 g/l sucrose and 8 g/l Difco noble agar following the protocol described by Taylor *et al.* (2012). This medium was used for mass production of *in vitro* plantlets that supplied the immature leaf lobes (explants) initiated for production of somatic embryos.

Induction and maturation of somatic embryos: Immature leaf lobes excised from six weeks old in vitro derived plantlets with the aid of a surgical needle were used for callus induction. Studies has shown that immature leaf lobes with unexpanded leaves have greater capabilities for somatic embryos induction (Reamakers et al., 1993). These explants were cultured on 5.22 g/l DKW medium (somatic embryo induction medium supplied by Phyto-Technology Laboratories Shawnee Mission, KS) supplemented with 20 g/l sucrose, 8 g/l noble agar and 50 µM picloram. The pH was adjusted to 5.7 ± 0.1 before autoclaving at 121°C for 15 minutes. The media were poured into 9 cm diameter Petri dishes under a sterile laminar flow hood; 25 ml of the medium were poured per Petri dish and allowed to cool and solidify.

The immature leaf lobes were extracted with the help of a dissecting microscope, using a sterile forceps, syringe and needle. The leaf lobes were exposed using the forceps, excised with the needle and placed on media with the abaxial side and midrib of the explants placed in contact with the medium. Ten Petri dishes were raised with each having 12 pieces of the leaf lobe plated on it. The cultured Petri dishes were incubated under dim light at  $28 \pm 2^{\circ}$ C for 21 to 30 days before transferring them to another induction medium (Gresshof and Doy (GD) medium) for production of friable embryogenic callus.

Production of friable embryogenic callus (FEC): Friable embryogenic callus was initiated from high quality three week old organized embryogenic structures (OES) formed from cassava induction medium (DKW) following the method described by Taylor et al. (1996). These embryogenic tissues were cultured on Gresshoff and Doy medium modified by addition of 20 g/l sucrose, solidified with 8 g/l difco noble agar and 50 µM picloram (GD2 50P). The picloram (synthetic growth regulator) was added after allowing the medium to cool to a temperature of 45°C under laminar flow hood. Clusters of OES were placed on GD2 50P medium with each Petri dish receiving a total of five (5) clusters. The cultured plates were incubated in the dark or under dim light at a temperature of 28 ± 2°C. Formation of FEC was achieved through sub- culturing of the cultured somatic embryos every 2 - 3 weeks with minimal production of non embryogenic callus (NEC) (Bull et al., 2009; Taylor et al., 2001; 2012).

**Maturation and germination of somatic embryos:** The two kinds of somatic embryos – organized embryogenic structures and friable embryogenic callus were transferred separately to stage 1 regeneration medium without antibiotics for maturation of the embryos. Stage 1 regeneration medium (MS2 5NAA) consists of Murashige and Skoog salts and vitamins supplemented with 20 g/l sucrose, 5  $\mu$ M  $\alpha$ -naphthalene acetic acid (NAA) and solidified by 8 g/l noble agar. This is followed by subsequent transfer to stage 2 regeneration medium (MS2 0.5NAA) in which the concentration of the NAA is reduced to 0.5 µM after three weeks. The plates containing the tissues were taken to the growth room and incubated at 28°C with 16 h light and 8 h darkness for maturation into cotyledons under a dim fluorescent light with light level of 10  $\mu$ Ms/m<sup>2</sup>.

After three weeks the cotyledons that developed from the stage 1 or stage 2 were moved to germination medium (MS2 2BAP). This medium consists of MS based salts and vitamins modified by 2  $\mu$ M 6benzylaminopurine (BAP) in place of NAA. Somatic embryos that have developed to possess two distinct, green cotyledons, a swollen hypocotyls and meristem region were transferred to the germination medium making sure that any adhering callus were removed and the embryos pressed into the medium such that the underside of the cotyledons were in contact with the medium without being submerged.

**Rooting of plantlets and multiplication:** After three to four weeks germinated cotyledons that have developed plantlet with expanded leaves were transferred to Murashige and Skoog basal medium (MS2 agar) for rooting and further shoot development. From these regenerated plantlets, leaf samples were collected for PCR analysis for confirmation of presence or absence of the viruses using specific primers as described above.

PCR analysis for virus detection: Leaf samples were collected from the infected plants growing in the green house and from in vitro plantlets for DNA extraction. Similarly, samples were collected from OES, FEC, cotyledons and leaves from regenerated plantlets via somatic embryogenesis for DNA extraction and PCR analysis. Leaf tissues were pulverized using ceramic bead and the MP Fastprep machine for 40 sec at 4.0 m/s, and the extraction of the DNA done with the aid of a Qiagen DNAeasy Plant Mini kit following the manufacturer's instructions. The primers used were ACMV and EACMV-CM primers. The concentration and quality of the extracted DNA were determined using Nanodrop equipment and running of 1 µL of the samples on gel electrophoresis respectively.

The reaction was performed in a total volume of 25  $\mu$ L consisting of 22  $\mu$ L of master mix (Invitrogen Master Mix Kit purchased from Invitrogen Corporation Carlsbad, CA, USA), 1  $\mu$ L each of the forward and reverse primers and 1  $\mu$ L of the DNA template. The PCR profile (conditions) were as follows: one cycle at 95°C for 4 min, 34 cycles at 95°C for 35 minutes, 30 sec at 58°C for the general amplification, 72°C for 1min and final extension step at 72°C for 10 min. The PCR products were detected by electrophoresis in 1.8% agarose gel, stained with 5  $\mu$ L ethidium bromide, visualized and photographed on an UV transilluminator Alpha Imager (Alpha Innotech Corporation, San Leandro, CA, USA).

### RESULTS

The phenotypic expression of the infected 'Nwugo' cassava genotype from which explants used for the study were collected is shown in Figure 1(a-d). This was clear indication that the cultivar was severely infected as confirmed by PCR analysis. Figure 2 (a-f) shows the immature cassava leaf lobes that served as explants for the production of the somatic embryos. It also reveals the two types of somatic embryos - organized embryogenic structure and friable embryogenic callus produced through the

induction role of synthetic auxin (picloram) on immature leaf lobes (explants) derived from in vitro raised infected 'Nwugu' cultivar plated on DKW and GD2 50 P media respectively. Over 50% of the immature leaf lobes produced OES on DKW induction medium from which FEC were derived after transferring them to GD2 50P medium. The developments of the somatic embrvos into cotyledons, their maturation and subsequent germination of the cotyledons / regeneration into plantlets are presented in Figure 3 (a-f). The weaning of the regenerated 'Nwugo' plantlets in the humidity chamber and their further growth and development in the Green house at Danforth center are displayed in Figure 4. Figure 5 shows the result of PCR analysis conducted for confirmation of presence or absence of African cassava mosaic virus using specific primer for the virus. Similarly, the PCR analysis result conducted with the same samples as shown in Figure 5 but with a different primer specific for EACMV-CM strain in place of ACMV is presented in Figure 6. All the twenty regenerated plantlets showed absence of the viruses (Fig.4) and this was confirmed by the PCR analysis conducted on ten plants among the twenty that regenerated via somatic embryogenesis (lanes 5-14) in Figures 5 and 6.



Fig. 1: Infected 'Nwugo' cultivar that supplied the explants - (a) Infected Nwugo cultivar *in vitro ; (b-d) Different stages of infected Nwugo cultivar growing in the Green house.* 

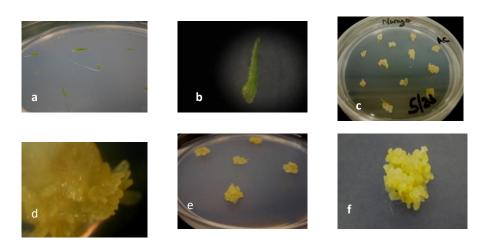


Fig. 2: Induction of somatic embryos - (a & b) immature leaf lobes on DKW induction medium; (c) callus formation; (d) Somatic embryo (OES) on DKW; (e & f) Somatic embryo (FEC) on GD2 50P medium.

#### DISCUSSION

Although virus eradication is often performed for cassava clonal selection through traditional sanitation techniques (meristem tip culture and thermotherapy), this technique takes advantage of the fact that some viruses are unable to replicate in this region (Parmessur et al., 2002). The success of meristem tip culture resides in the ability to dissect the meristematic dome with one or two leaf primordial from the mother plant and its successful regeneration. But results from such techniques can be quite variable depending on the number, type of viruses, starting material, skill of personnel and other factors. Therefore, not all meristem tips established are guaranteed to be virus-free. Beside the use of meristem tip culture as means of eliminating viruses from infected plants, there are reports now on the use of somatic embryogenesis for virus eradication. Studies on attempts to cleanse Grapevine rupestris stem pitting-associated virus and other viruses using different virus elimination techniques showed that somatic embryogenesis gave the best results (Gribaudo *et al.*, 2006). There are several reports on the use of somatic embryogenesis for successful eradication of viruses from infected plants (D' Onghia *et al.*, 2001; Parmessur *et al.*, 2002; Gribaudo *et al.*, 2006) but none has been reported on cassava at present.

In our study, somatic embryogenesis produced virusfree plantlets from 'Nwugo' cultivar infected with mixed infections of African cassava mosaic virus and East African cassava mosaic virus Cameroon strain. Somatic embryogenesis in cassava produces two kinds of somatic embryos – organized embryogenic structures and friable embryogenic callus as reported by Raemakers *et al.* (1996). Both somatic embryos have the capacity to regenerate into cassava plantlets when transferred into appropriate media. In the present study, we obtained regenerated plantlets from both OES and FEC that showed absence of the viruses as confirmed by the PCR results carried out (Figs. 5 and 6)

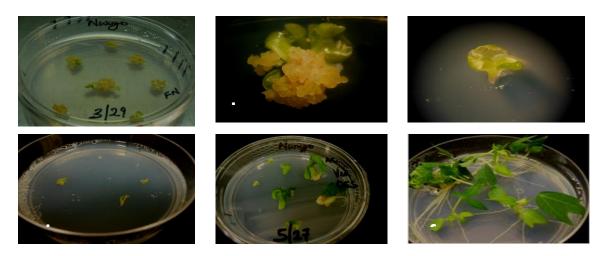


Fig.3: Procedure for regenerating plantlets via somatic embryogenesis – (a & b) cotyledons developing from FEC on MS2 0.5 NAA (stage 2 medium); (c & d) expanded developed Cotyledons on MS2 2BAP (germination medium); (e) germinating cotyledons on MS2 2 BAP;







Fig.4: Regenerated Nwugo plantlets showing absence of the viruses at different growth stages in the Green house at Danforth Plant Science Center St. Louis, USA.

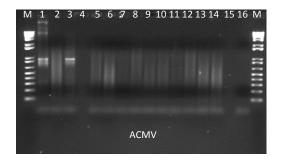


Fig. 5: PCR result shows – lane M (1kb+ ladder), lane 1 (positive control – 900 base pairs), Lane 3 (infected Nwugo sample) and lanes 5 to 14 (samples from regenerated Nwugo via somatic embryogenesis indicating absence of the virus).

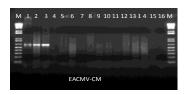


Fig. 6: PCR result shows – lane M (1kb+ ladder), lane 1 (positive control – 600 base pairs), Lanes 2 & 3 (infected Nwugo sample) and lanes 5 to 14 (samples from regenerated Nwugo via somatic embryogenesis indicating absence of the virus).

The successful elimination of both viruses by somatic embryogenesis in this study also confirms the efficiency of this technique in eradicating viruses in single and mixed infections. This is in conformity with the work of Parmessur et al. (2002) who reported successful elimination of both sugarcane yellow leaf virus and sugarcane vellows phytoplasma from infected plants by tissue culture of callus derived from leaf rolls. These findings open new opportunities to overcome the old strategy of 'detect-and-destroy' so far adopted by plant breeders. With this technique, plant material infected with virus and other pathogens could be cultured in vitro for elimination of the pathogens, and released for evaluation by plant breeders. Somatic embryogenesis technique requires little skill, and plantlets free from pathogens can be generated within six months. However, it is important that the tissue culture technique is backed with sensitive molecular diagnostic tools to ascertain absence of pathogens. The possibility of somaclonal variation should also be taken into account through subjecting the regenerated plantlets to further screening by plant breeders. This study has shown that somatic embryogenesis is a promising technique for virus elimination. It has been applied by some laboratories towards virus eradication of infected crops like sugarcanes, citrus and grapevines and as such can be employed for efficient virus elimination in

cassava varieties infected with viruses. We believe that this should be a welcome development by existing cassava improvement programmes as well as a panacea to ongoing efforts toward virus elimination in cassava a vegetatively propagated crop.

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