Effectiveness Of Somatic Embryogenesis In Eliminating The Cassava Mosaic Virus From Infected Cassava (Manihot Esculenta Crantz) Plant Materials

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Abstract: Cassava (*Manihot esculenta* Crantz) is a staple food for many people in the tropical regions. However, yield of cassava has reduced of late due to high incidence of cassava mosaic disease (CMD) which is caused by the cassava mosaic virus (CMV). This necessitated the study on the production of disease free cassava materials from CMD cassava plants through somatic embryogenesis. CMV infected cassava leaves were cultured for callus tissue induction and somatic embryos (SE) generation on modified MS media supplemented with 2, 4–D. The SE maturation was carried out on modified MS media supplemented with Benzyl Amino Purine (BAP). Callus tissue initiation and induction started ten (10) days after plating (DAP), SE were generated 35 DAP and survival rate of explants was 90.2 %. Maturation of SE occurred 60 DAP and the number of somatic embryos per explant ranged from 5 – 14. Polymerase Chain Reaction (PCR) and Enzyme Linked Immunosorbent Assay (ELISA) were used to detect the presence of CMV on leaves, callus tissues and SE. East African Cassava Mosaic Virus (EACMV) and African Cassava Mosaic Virus (ACMV) were two different strains of CMV detected in the leaf, callus tissue and SE from CMD cassava explants. The SE that was generated from CMV infected leaves of cassava showed 87.5% virus free with the PCR technique of viral particle detection. The outcome of the study demonstrated the effectiveness of somatic embryogenesis in eliminating the ACMV from infected materials and EACMV from infected cassava plants to produce viral free planting materials.

Index Terms: African cassava mosaic virus, cassava mosaic disease, enzyme linked immunosorbent assay, east african cassava mosaic virus, polymerase chain reaction and somatic embryogenesis.

1. INTRODUCTION

Cassava (*Manihot esculenta* Crantz) known as a food security crop in Africa originated from the South and East of Amazon basin. It is cultivated as cash crop in Nigeria and Ghana (SESRTCIC, 2007) with 60% of cassava cultivated in Ghana sold as a cash crop (Nweke *et al.,* 2002). Cassava mosaic disease (CMD) is restricted to cassava growing areas in the tropics. About 15 % - 20 % yield losses occurs in Africa with up to 82 % yield losses in CMD pandemic-affected areas (Legg *et al*., 2006). CMV is a begomoviruses which is transmitted by the whitefly of the family *Aleyrodidae* and Order *Hamoptera* (Brown *et al.,* 1995). The CMD was first reported in 1894 in Tanzania and has since been recorded in all the cassava growing areas of Africa (Calvert and Thresh, 2002). In Ghana, incidence of CMD is 71 % and an estimated loss caused by the disease is 27 - 30 % (Cudjoe *et al.,* 2003).

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According to Thresh (2001), the single most important disease that is responsible for significant reduction in the yield of cassava is the CMD, caused by the CMV. Several attempts have been made in the past to control CMV with partial success. For complete elimination of virus in plants, pathologist suggested methods such as chemotherapy, electrotherapy, thermotherapy and meristem culture, which are reported to have recorded partial successes in controlling viral diseases in plants (Thresh, 2003; Fajinmi *et al.,* 2011). However, tissue culture techniques such as somatic embryogenesis have been applied to a number of crops to eliminate viruses. Somatic embryogenesis from stigma and style cultures of citrus was used to eliminate citrus psorosis virus from three citrus species (D'Onghia *et al.,* 2001). According to Quainoo *et al.,* (2008) disease free cocoa planting materials were generated from cocoa swollen shoot virus infected plants through somatic embryogenesis. Fan leaf viruses and leaf roll-associated viruses from grapevines were eliminated through somatic embryogenesis (Goussard *et al.,* 1991; Goussard and Wiid, 1992). The aim of this research was to produce virus-free planting material from CMV-infected cassava plants through somatic embryogenesis.

2.0 MATERIALS AND METHODS

2.1 Experimental site and source of explant

The study was conducted in the plant house and Spanish laboratory at the University for Development Studies, Ghana and the Molecular Biology and Virology Laboratories at the Crop Research Institute, Ghana. Four local cassava cultivars infected with cassava mosaic virus were used to induce somatic embryos. These cultivars are Ankrah (AN), Biabasse (BB), Nagbagu Sule (NS) and Buyadoo (BD). Freshly harvested stem cuttings of these cultivars were obtained from Savannah Agriculture Research Institute (SARI) and Waribogu in the Tolon District of Northern Region and Gomoa Ojobi in

the Gomoa East District of Central Region of Ghana. Cutting from these materials were planted in 20 litre buckets containing loamy soil and compost (decomposed goat droppings and grasses) in the ratio of 2:1 respectively and allowed to sprout in a plant house at an average temperature of 28.3 ºC.

2.2 Experimental Design

A single factor experiment laid out in a completely randomized design was used. The factor was cassava cultivar with four levels as AN, BB, NS and BD which formed the treatments. There were five (5) replications, each consisting of 20 Petri dishes of 90 mm in diameter with five (5) leaf cuttings per Petri dish.

2.3 Stock Solutions and Media Preparation

The Murashige and Skoog (MS) media supplemented with 8 mg 2, 4–D. was used. MS conversion and maturation media was prepared by adding the desired amount of MS stock solutions and supplemented with 0.5 mg BAP. The pH of the culture media was adjusted to 5.8 after 8 g of agar was added and the medium was sterilized by autoclaving at a temperature of 121 ^{0}C with a pressure of 0.15 Kpa (1 bar) for 15 minutes. The developing somatic embryo cultures were kept in a growth room under 16 hour light and 8 hour dark cycle at 25 ^{0}C. Sub culturing was carried out at every 2 weeks.

2.4 Detection of cassava mosaic virus

2.4.1 Sample collection

Leaves from cassava plants infected with CMV were harvested from the field and plant house. Two and a half grams (2.5 grams) each of cassava leaf and callus samples for PCR viral indexing and 1 gram of somatic embryos were placed in sterile eppendorf tubes and stored on ice before being transported to Crop Research Institute, Kumasi- Ghana.

2.4.2 Enzyme Linked Immunosorbent Assay (ELISA) and Polymerase Chain Reaction (PCR)

The presences of virus in the sampled materials were detected by ELISA standards using a modified procedure as described by Clark and Adams (1977). Total **g**enomic cassava DNA was extracted from cassava leaf, callus and somatic embryo samples using a modified Egnin *et al.* (1998) protocol. DNA quality from the samples used was checked on an ethidium bromide stained agarose gel. Primers for CMV detection manufactured by Sigma, UK are presented in table 1. The product is a 360 base pair and runs from position of 350bp to 800bp. A master mix containing Taqpoltmerase and dNTPs was supplied by Qiagen (Multiplex PCR kits), UK. The PCR programme was 94 ºC for 4 minutes (denaturation), 35 cycles of 94 ºC for 30 seconds, 50 ºC for 30 seconds and 72 ºC for 1 minute (annealing), followed by 72 ºC for 7 minutes (extension).

Table 1: Primers used to detect viral DNA fragments in cassava samples

Primer	Sequence → (5´ 3´)	Strand	Target Genome	Amplicon Size
ACMV F_1	ATGTCGAAGCGAC CAGGAGAT	Virion-Sense	Cp	368bp
ACMV R_1	TGTTTATTAATTGC CAATACT	Complementary	Cp	368bp
ACMV AL1/F	GCGGAATCCCTAA CATTATC	Virion-Sense	ALI	520bp
ACMV ARO/R	GCTCGTATGTATC	Complementary	AR2	520bp
UV AL1 / F1	CTCTAAGGCCTG TGTCTTCTGGGAC TTGTGTG	Virion-Sense	ALI	650bp
UV CP/ R_3	GTTACGGAGCAAC ATGCAAT	Complementary	CP	650bp

Primer Sources: Zhou *et al*. (1997) and Fondong *et al*., (2000)

2.5 Data collection and analysis

Explants survival, mass of callus tissues, days to somatic embryos formation, number of embryogenic cells, days to somatic embryo formation and days to somatic embryos maturation were recorded. Plant source for explants were carefully examined for viral signs and symptoms which were selected and leaf sample taken for confirmation of the presence of CMV by ELISA and PCR procedures.. Plant leaves from plant house and tissue culture materials (callus tissues and somatic embryos) were taken through ELISA and PCR for the viral indexing. Quantitative data were subjected to analysis of variance (ANOVA) with Genstat Discovery (Edition 3). Probability level of $P \leq 0.05$ was considered significant for all analyses and Standard Error of Differences (SED) was used to separate means.

3.0 RESULTS

3.1 Survival of explants on MS induction medium

Significant differences were observed in survival rate of the explants among the cultivars. The survival rate of Buyadoo was significantly higher ($P < 0.001$) than Ankrah but NagbanguSule and Biabasse were similar (Figure 1)

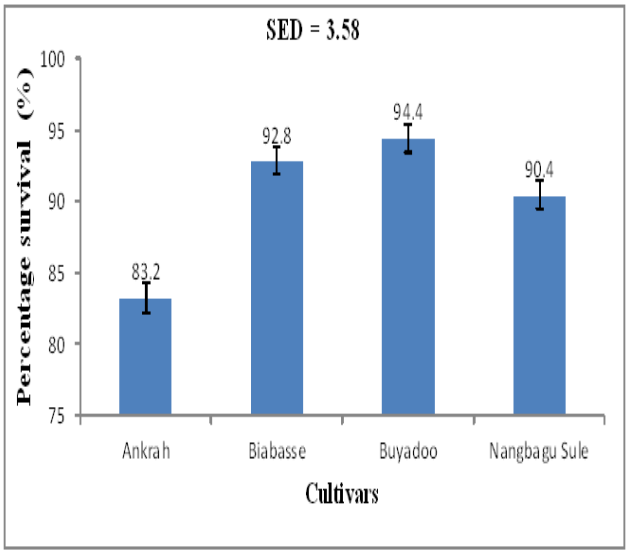


Figure 1: Number of survived explants of four cassava cultivars 14 days after plating on MS induction medium. Bars represent S.E.D

3.2 Days to somatic embryo formation

Significant differences ($P < 0.001$) were observed in days to somatic embryo formation of explants among the cultivars. The days to somatic embryo formation of Ankrah was significantly faster than Biabasse (Figure 2).

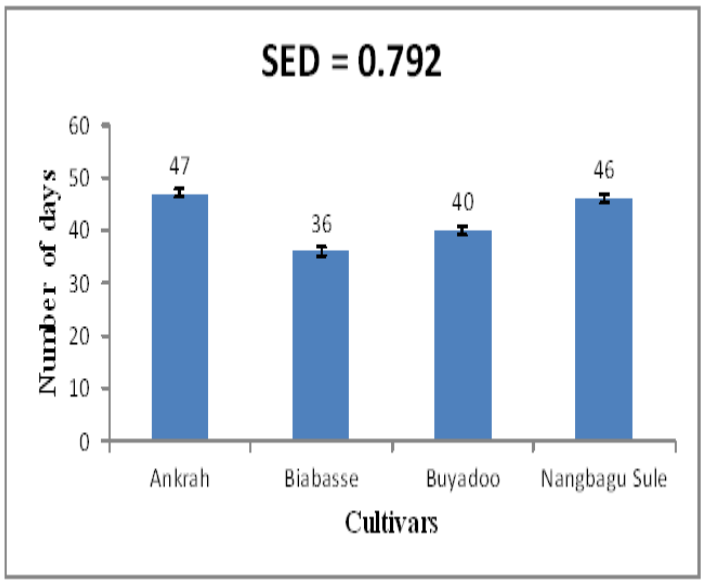


Figure 2: Days to somatic embryo formation of the four cultivars. Bars represent S.E.D

3.3 Number of somatic embryos per explant

Significant differences were observed in the number of somatic embryos formed per explant among the cultivars. The number of somatic embryos per explant of Biabasse was significantly higher ($P < 0.001$) than NangbaguSule, Buyadoo and Ankrah (Figure 3).

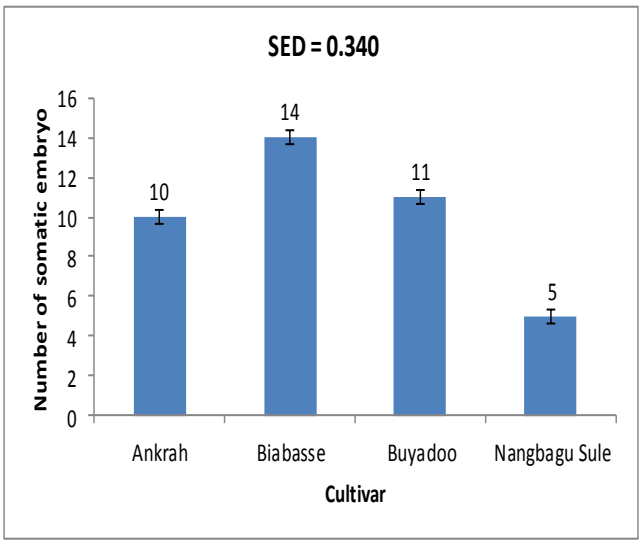
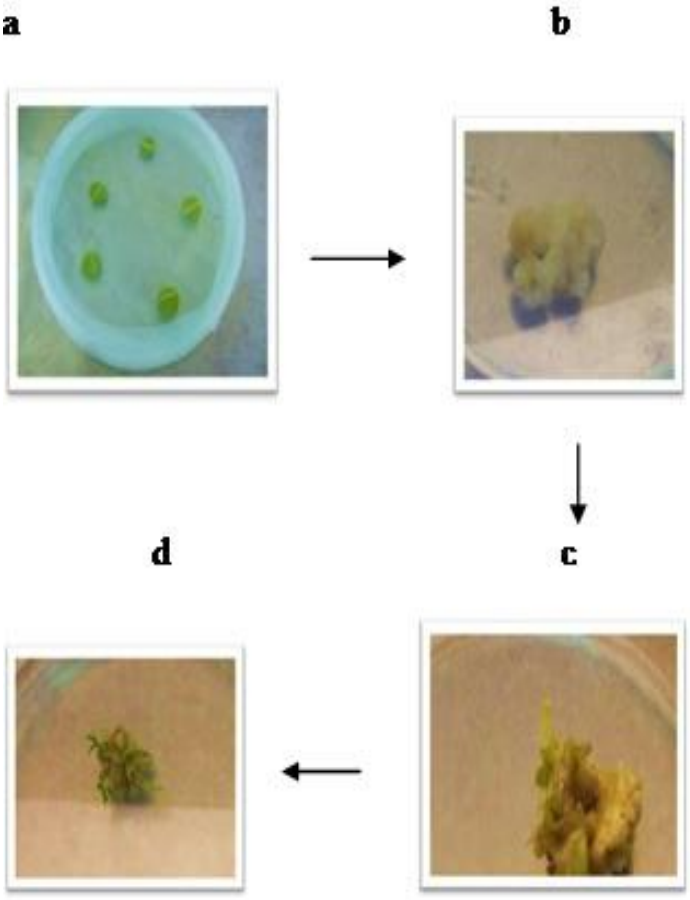


Figure 3: Number of somatic embryos per explant of four cassava cultivars. Bars represent S.E.D



a – Leaf cuttings
b – Callus tissues formed
c – Somatic embryos formed
d – Germinated somatic embryos

3.4 Detection of cassava mosaic virus

Two different strains of CMV namely EACMV and ACMV were detected in the samples tested.

Table 2: Detection of CMV strains by ELISA technique

Samples	Tested samples	ACMV	EACMV	ACMV and EACMV	Positive to CMV	Negative to CMV
Field	48 (100%)	8(16.67%)	3(6.25%)	31(64.58)	42(87.5)	6(12.5%)
Plant house	24(100%)	4(16.67%)	2(8.33%)	12(50%)	18(75%)	6(25%)
Callus	20(100%)	0(0%)	0(0%)	0(0%)	0(0%)	20(100%)
Somatic embryos	20(100%)	0(0%)	0(0%)	0(0%)	0(0%)	20(100%)

***Figures in the parenthesis is the corresponding percentage of frequency of CMV detected**

ACMV-F_1/ACMV-R_1 primer, ACMV was detected in eight (8) samples were positive for the presences of ACMV (Figure 4). The primer indicated 680 base pair fragments Lane M, 100 base pair DNA ladder (Axygen), lane N (Negative control), lanes 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23 and 24 were cassava samples from four cultivars.

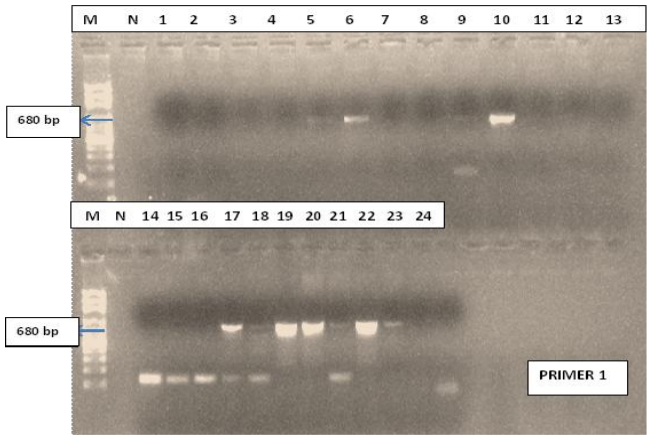


Figure 4: PCR analysis with ACMV-F1/ACMV-R1 primer.

ACMV was detected in six (6) samples with ACMVARO-R1/ACMV-ALI-F1 primer in samples tested (Figure 5). The primer indicated 800 base pairs fragments. Lane M was 100 base pair DNA ladder (Axygen), lane N (Negative control), lanes 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23 and 24 were cassava samples from four cultivars.

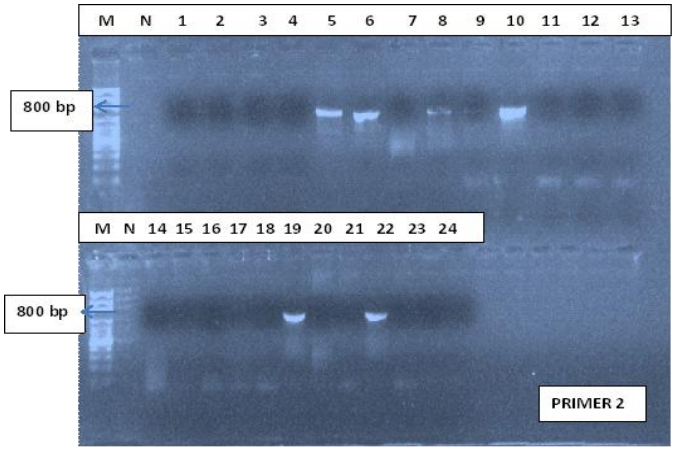


Figure 5: PCR analysis with ACMV-ARO-R/ACMV-ALI-F primer.

The UV-AL_1-F_1/UVCP-R_3 primer detected one sample positive for the presence of CMV (Figure 6). The primer indicated 780 base pairs fragments. Lane M, 100 base pair DNA ladder (Axygen), lane N (Negative control), lanes 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23 and 24 were cassava samples from four cultivars.

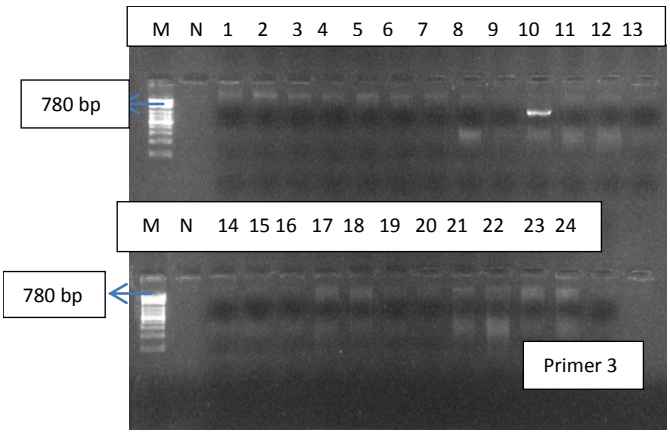


Figure 6: PCR analysis with UV-AL_1-F_1/UV CP-R_3primer.

4.0 DISCUSSION

4.1 Survival rate of explants

The survival rate of explants was 90.2 % and higher survival rate of cultures could be attributed to cultivar differences ($p < 0.001$). This finding supported the assertion of Dodds and Roberts (1995) that survival rate of cultured explants may vary due to cultivar difference and the basic nutritional requirement for optimum growth of tissues or cells of the species cultured.

4.2 Number of somatic embryos per explant

The number of somatic embryos per explant ranged from 5 to 14. The variation of number of somatic embryos per explant could be attributed to cultivar difference and the weight (size) of callus tissue. This was in agreement with the findings of Hankoua *et al.* (2005) who reported that genotypic differences contributed to the ability of immature leaf lobes and apical shoot meristems of cassava to form different number of primary somatic embryos per callus explant.

4.3 Detection of cassava mosaic virus

Callus tissues and somatic embryos tested 100 % CMV viral free material with ELISA (Table 2). The results of the study with the ELISA technique indicated that 5 (6.94%) cassava samples tested revealed EACMV only, 12 (16.67%) of the 72 samples tested revealed ACMV and 43 (59.72) samples of the 72 samples tested revealed both EACMV and ACMV from plant house and field cassava plants. This confirmed the findings of Were *et al.* (2003) that ACMV and EACMV were found in West Africa. The 75 % samples from the plant house tested positive instead of 100% by ELISA technique on the field samples may probably be due to defense mechanism of the cassava plant to manage the CMV. This supported the findings of Fargette *et al*. (1988) and Rossel *et al*. (1988) who stated that some cassava varieties have the ability to localize the distribution of CMV so that whole branches or individual shoots may develop free of any detectable virus. This confirm to the finding of Were *et al. (*2003)that **t**wo cassava plants obtained from Kisangani in the Democratic Republic Congo infected by ACMV and UgV 'lost' ACMV when their virus status was checked at 6-month intervals for three years. Cassava samples with mosaic virus subjected to PCR revealed that samples that were free from CMV by ELISA serology showed the presence of the viral DNA in their callus and somatic embryos. This result showed that PCR technique was more sensitive than ELISA technique in detecting the viral particles at low titre levels. This confirmed the assertion of Henson and French (1993); Hadidi *et al*. (1995); Candresse *et al*. (1998) that PCR technique was proven to be 100-1000 fold more sensitive than serological methods like ELISA. The PCR is capable of distinguishing between plants with low viral titre and those that are totally viral free. The primers used in the detection of viral particles in the genomic DNA of diseased plant materials revealed that viral particles in the plant are not well distributed in the plant system. This confirmed the findings of Pacumbaba (1985), who reported that viral infection in cassava plant was incompletely systemic in nature. The PCR results revealed that CMV can be eliminated through somatic embryogenesis because of 87.5 % of somatic embryos tested virus free from CMV. This finding also supported the assertion of Chawla (2002) that somatic embryos generated have no vascular connection between the calluses and the mother plant hence the generation of disease free materials from infected materials. The results of the PCR revealed that Ankrah (sample ten) tested positive ACMV and EACMV. This could be attributed to the age of callus tissue on which the somatic embryo was induced. The outcome of this work confirmed the findings of Quainoo *et al.,* (2008) who reported that cocoa swollen shoot virus was transmitted to primary somatic embryos induced from the infected callus tissues. The results of the study also confirmed the finding of Quainoo (2011) who stated that the age of callus tissue and cotyledonary material affected the selection of CSSV-free cocoa somatic embryos. The transfers of virus particle from callus tissue to somatic embryos depended on time of somatic embryos formation on the callus tissues. The Ankrah sample that tested CMV positive by the PCR took a long time to form somatic embryos. The PCR technique with primer 3 detected viral DNA on Ankrah (sample ten) only and the remaining samples showed CMV free (Figure 8). The primer 3 was designed to detect the DNA of EACMV which targeted the CP and AL_1 of CMV viral DNA. The Ankrah sample (sample 10) probably contained the DNA of EACMV or UgV, UV-AL_1-F_1/UV CP-R_3primer which was designed to detect DNA of UgV. This results confirmed the works of Deng *et al*. (1997) and Zhou *et al*. (1997) who reported that a new virus variant has been detected and identified known as distinctive strain of EACMV (EACMV-UG) or Uganda variant cassava mosaic virus (UgV).

Conclusions

The study revealed that the number of embryogenic callus of explants that develop into somatic embryos was species or cultivar dependent. The number of somatic embryos formed per explant was determined to a large extent by the size of the callus tissue. PCR technique was more sensitive than ELISA technique in detecting viral contamination.

Recommendations

You are hanging yourself here be careful The study also recommended that viral indexing should be done in four (4) separate stages of cultures life, that is explant stage before plating, callus stage, somatic embryo stage and plantlet stage in future experiments. Universal primer should be used for the viral diagnostic test in future experiments.

Acknowledgments

Much thanks to International Institute of Tropical Agriculture (IITA) under the project of West African Agricultural productivity Programme (WAAPP) for their financial assistance.

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