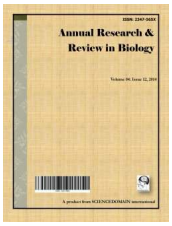

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Potential of Somatic Embryogenesis in Elimination of East Africa Cassava Mosaic Virus from Infected Cassava Cultivars in Kenya

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Authors' contributions

This work was carried out in collaboration between all authors. Authors JMW and ENN designed the study. Author GKM carried out the laboratory experiments, performed statistical analysis and wrote the first draft of the manuscript. Authors JMW and ENN supervised the laboratory experiments and offered technical advice. All authors read, reviewed and approved the final manuscript.

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ABSTRACT

Cassava mosaic disease (CMD) is an economically important disease limiting production of cassava (*Manihot esculenta* Crantz) in sub-Saharan Africa. Use of virus-free planting material is among the strategies for management of CMD. However, obtaining clean planting material for farmer-preferred varieties is often difficult. This study evaluated the efficacy of somatic embryogenesis to produce disease-free cassava planting materials from CMD-infected cultivars TME 14, Ex-Mariakani, Sagalato, Kibandameno and TMS 60444. Axillary buds of East Africa cassava mosaic virus (EACMV)-infected cassava nodal cuttings were cultured on MS salts with vitamins supplemented with 12 mg/l picloram for generation of primary somatic embryos (SE) which were subcultured onto the same fresh medium for generation of secondary SE. Primary and secondary SE were cultured separately onto MS supplemented with 1 mg/l naphthaleneacetic acid (NAA) for induction of cotyledons and subsequent regeneration of plants on MS supplemented with 0.4 mg/l 6-benzylaminopurine (BAP). Polymerase chain reaction (PCR) was used to discern the presence of EACMV in regenerated plants. Plants regenerated from primary and secondary

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somatic embryos were 87.6% and 93.5% virus free, respectively, with the PCR technique of viral particle detection. The virus-free plants acclimatized in the glasshouse showed absence of viral symptoms morphologically. These findings demonstrated the effectiveness of somatic embryogenesis in elimination of EACMV from infected cassava plants to produce clean planting materials.

Keywords: Manihot esculenta Crantz; EACMV; somatic embryogenesis; virus elimination; polymerase chain reaction.

1. INTRODUCTION

Cassava (*Manihot esculenta* Crantz) is a species native to tropical America and was brought to Africa and Asia by Portuguese people in the 16th century. The crop has been widely adopted in the tropics and mid-altitude areas because of its ability to tolerate drought, grow in nutrient poor soils and requirement of minimal management practices [1]. About 80% of cassava dry weight is starch, primarily the roots [2,3], and provides staple food to more than 800 million people worldwide [4]. Cassava leaves which are used as a vegetable are a good source of protein and vitamins [5], while roots are rich in carbohydrates [6]. In Coast, Nyanza and Western regions of Kenya, the root cover is peeled, cut into small pieces, sun-dried and mixed with sorghum or maize and then milled to make polenta-like dish or porridge [7].

In spite of cassava being among the top preferred crops worldwide, its production is constrained by many biotic and abiotic elements. Pests such as whiteflies, cassava green mites, cassava mealybug, the variegated grasshopper and cassava viruses are the major constraints in cassava production [8]. According to Ntawuruhunga et al. [9], pests and diseases account for 49.6% loss in cassava production with drought and weeds contributing 22.9% and 14.7% losses, respectively. Of all cassava production constraints, cassava mosaic disease (CMD) caused by cassava mosaic geminiviruses (CMGs) is the most destructive in sub-Saharan Africa [10].

Cassava mosaic geminiviruses (CMGs) are spread by the whitefly vector *Bemisia tabaci* and transmitted by infected cassava cuttings through vegetative propagation [11]. During the late 1920s and early 1930s, CMD had spread virtually to all cassava-growing regions of the Africa mainland and its islands, including areas from southern Kenya, through coastal Tanzania to river Zambezi in Mozambique and Malawi [12]. Symptoms of CMD occur in the form of blotchy yellow vein chlorosis, regular or irregular mosaic, mottling, misshapen and twisted small sized leaflets, and overall dwarfism of a plant [13,1] but are mild where the cassava plant is tolerant, or the viral strain is less virulent [13]. However, there is a little spread of the disease to resistant varieties and susceptibility of plants decreases with age [13]. The significance of the disease manifests in several morphological and cytological alterations [14] that result from dieback and rot of the cassava tuber [12]. Cassava mosaic disease causes production losses worth more than US$1 billion every year and thus becomes a threat to food as well as income security for over 30 million farmers growing the crop in East and Central Africa [15]. Approximately 82% yield losses occur annually as a result of CMD in pandemic-affected areas [16]. In Kenya, the disease has reduced the projected cassava production potential yield of 90 tons/ha to current 11 tons/ ha which is a serious concern [17]. Regions where severity of CMD is dreadful have experienced nearly total crop failure, prompting farmers to stop cassava farming, especially of highly susceptible cultivars [16].

Unlike bacterial and fungal diseases, viral diseases have no effective chemical control on infected plants [18]. The supply of virus-free planting materials is therefore important for sustainable crop production and is a prerequisite for the international exchange of germplasm to avoid risks of introducing diseases to uninfected areas [18]. Various methods are available for the elimination of viruses from plants, including chemotherapy, electrotherapy, thermotherapy and meristem culture, which are reported to have recorded partial successes in controlling viral diseases in plants [19,20]. Currently, 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 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. Tissue culture techniques such as somatic embryogenesis have

been applied to a number of crops to eliminate viruses' efficiently. However, different studies reported varied efficiencies of somatic embryogenesis in elimination of virus from several crops. According to Damba et al. [1] disease free planting materials were generated from African cassava mosaic virus (ACMV) infected plants through somatic embryogenesis. Similarly, Gribaudo et al. [21] 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. However, somatic embryogenesis method has not been used to eliminate viruses from Kenyan cassava cultivars. Therefore, the aim of this study was to determine the efficacy of somatic embryogenesis in elimination of EACMV from infected cassava cultivars in Kenya. This will enable CMD management through dissemination of virus-free farmer-preferred but CMD susceptible cultivars.

2. MATERIALS AND METHODS

2.1 Collection of Cassava Cultivars Infected with East Africa Cassava Mosaic Virus (EACMV)

Stems of popularly grown cassava cultivars (TME14, Ex-Mariakani, Sagalato, Kibandameno) in Kenya and the model cultivar TMS60444 exhibiting EACMV symptoms were collected from the Kenya Agricultural and Livestock Research Organization (KALRO), Biotechnology Centre, Nairobi. The information on popularity of the cultivars was also obtained during cultivar collection. Three stem cuttings were collected per cultivar and established in pots in a glasshouse at the School of Biological Sciences, University of Nairobi.

2.2 Detection of East Africa Cassava Mosaic Virus (EACMV)

Sprouted cassava cuttings were diagnosed to confirm EACMV infection using PCR with virus specific primers as described by Fondong et al. [22].

2.2.1 DNA extraction

Extraction of DNA from leaves of cassava cultivars infected with East Africa cassava mosaic virus was conducted using a modified Sodium dodecyl sulfate (SDS) based extraction protocol of Dellaporta et al. [23]. In the modified protocol, liquid nitrogen was excluded in the DNA extraction process; 200 mg of leaf sample was directly ground in 700 µl of extraction buffer containing 700 mM NaCl and 20 mM of βeta-mercaptoethanol and 150 µl SDS. The ground samples were incubated at 55℃ for 15 minutes then centrifuged at 1200 rpm for 5 minutes. Supernatants were transferred into a new sterile Eppendorf tube and 250 µl of chloroform: isoamylalcohol (24:1) was added and mixed well. The mixture was spun at 13800 rpm and upper aqueous layer was transferred into a new sterile Eppendorf tube into which 50 µl of ammonium acetate and 500 µl of absolute ethanol were added. The tubes were inverted slowly and incubated at -20℃ for 45 minutes before centrifuging at 13800 rpm for 5 minutes. The precipitated DNA pellets were washed with 700 µl of wash buffer (90% ethanol). The DNA pellets were aseptically dried for 10 minutes at room temperature and dissolved in 60 µl sterile double-distilled water. The quality of genomic DNA was checked on 0.8% agarose gel and the quantity estimated relative to known concentrations of lambda DNA (NEB N3011S, New England Biolabs, Ipswich, MA).

2.2.2 PCR analysis for specific detection of EACMV

The extracted DNA was subjected to PCR using primer pair EAB555F (5'-TACATCGGCCTTTGAGTCGCATGG-3') and EAB555R (5'-CTTATTAACGCCTATATAAACACC-3'), amplifying a 550 bp product. The reactions were performed in a total volume of 12.5 µl consisting of 2.5 µl 10 X Taq buffer, 0.25 µl of 10 mM dNTPs, 0.75 µl of 25 mM $MgCl_2$, 0.5 µl (25 pmol) each of forward and reverse primers, 1 µl (50 ng/µl) DNA template and 7 µl sterile distilled water. Amplifications were performed in a MJ Mini^{TM} personal Thermal Cycler using the following thermocycling conditions: Initial denaturation of 94℃ for 3 minutes followed by 30 cycles of denaturation at 94℃ for 30 seconds, annealing at 58℃ for 30 seconds, extension at 72℃ for 45 seconds and final extension of 72℃ for 5 minutes.

2.2.3 Agarose gel electrophoresis of amplified PCR products

The amplified PCR products were analyzed using agarose gel electrophoresis. Exactly 6 µl of the product was mixed with 6X gel loading buffer (2 µl) and loaded onto the wells on 1% agarose (w/v) gel in 1X TAE (Tris-Acetate-EDTA) buffer.

The loaded samples were electrophoresed at 60 V for 60 minutes. The amplified products separated by agarose gel were stained with ethidium bromide solution (2 µl EtBr/100 ml 1X TAE buffer) for 40 minutes and visualized using the DNR-Imaging System with UV-transilluminator.

Following PCR amplification, EACMV-positive plants of all the cultivars were used in initiation of *in vitro* plantlets for somatic embryogenesis.

2.3 EACMV Elimination through Somatic Embryogenesis

2.3.1 Preparation of culture medium

The culture medium used for initiation of EACMV-infected cassava plants was cassava basic medium (CBM; Murashige and Skoog [MS] salts with vitamins supplemented with 2 µM $CuSO_4$, 2% sucrose, 0.3% Gelrite, pH 5.8), prepared following the protocol described by Nyaboga et al. [24].

2.3.2 Sterilization and initiation of nodal cuttings of EACMV-infected plants into tissue culture

Two nodes were cut from stems of EACMV-infected cassava plants growing in the glasshouse using sterile scalpel blades. The nodal cuttings were washed three times using tap water containing two drops of Tween 20 to remove debris and sequentially rinsed three times with sterile double-distilled water. The nodal cuttings were soaked in 5% sodium hypochlorite solution for 10 minutes and then rinsed thrice with sterile double-distilled water and aseptically dried. The edges of the scorched ends of the nodes were carefully cut under sterile conditions, and each node was individually initiated on CBM medium and incubated in a growth chamber at 28℃, 16/8 photoperiod. *In vitro* EACMV-infected cassava plantlets were subcultured after every 5 weeks for generation of enough plants for induction of axillary buds and subsequent production of somatic embryos.

2.3.3 Induction of axillary buds

Using a sterilized scalpel, nodal explants of l0 mm in length were cut from 4 weeks old *in vitro* EACMV-infected plantlets and placed horizontally on cassava axillary bud induction medium (CAM). The CAM was made up of MS salts with vitamins, 2 µM $CuSO_4$, 10 mg/L 6-benzylaminopurine (BAP), 2% sucrose and 0.8% noble agar at pH 5.8. Petri plates with nodal explants were wrapped with aluminium foil (for darkness) and cultured for 6 - 10 days at 28℃ in the growth chamber.

2.3.4 Induction and maturation of somatic embryos

Enlarged axillary buds were cultured on MS medium supplemented with 50 µM picloram, 20 g/l sucrose and 8 g/l noble agar and the pH was adjusted to 5.7 before autoclaving at 121℃ for about 20 minutes at 15 psi. About 25 ml of the medium was poured into 9 cm diameter Petri plates under a sterile laminar flow hood and allowed to cool. About 5 to 6 axillary buds were cultured in each Petri plates and incubated at 28℃ for 25 days. The primary somatic embryos formed were divided into two batches. The first batch was transferred onto same fresh medium for another 25 days for development of secondary somatic embryos. The second batch was transferred onto cotyledon emergence and regeneration media to form cotyledons and plantlets, respectively.

2.3.5 Germination of somatic embryos, rooting and multiplication of plantlets

Primary and secondary somatic embryos were transferred separately to stage 1 regeneration medium (MS salts and vitamins supplemented with 5 µM α-naphthalene acetic acid (NAA), 20 g/l sucrose and solidified by 8 g/l noble agar) for maturation of the embryos. The matured embryos were transferred to stage 2 regeneration medium (MS salts and vitamins supplemented with 0.5 µM α-naphthalene acetic acid (NAA), 20 g/l of sucrose and solidified by 8 g/l noble agar). Incubation was carried out in the growth room at 28℃ under 16/8 hours photoperiod to form cotyledons. After three weeks, the developed cotyledons were transferred to germination medium (MS salts and vitamins supplemented with, 2 µM 6-benzylaminopurine (BAP), 20 g/l sucrose and solidified by 8 g/l noble agar) to form shoots. After four weeks, the formed shoots with expanded leaves were transferred to CBM medium for rooting and further shoot development. Further sub-culturing was conducted after every 5 weeks.

2.4 Polymerase Chain Reaction Analysis for Virus Detection in Regenerated Plantlets

Leaf samples were collected from plantlets regenerated from primary and secondary somatic

embryos for PCR analysis to confirm the presence or absence of EACMV using specific primer pair EAB555-F/EAB555-R. The extraction of DNA and PCR analysis for specific virus detection was performed following the protocol described in sections 3.2.

The EACMV elimination efficiency was determined by PCR in plantlets regenerated from primary and secondary somatic embryos. The efficiencies for primary and secondary embryos were calculated separately as the percentage of plantlets that tested negative (virus free-plantlets) for EACMV against the total number of plantlets tested (virus-free and virus-positive plantlets) after PCR analysis of extracted DNA.

2.5 Hardening and Acclimatization of Regenerated Plants in the Glasshouse

After five weeks of establishment in CBM medium, a total of 60 virus-free plantlets (30 plantlets from each stage of regeneration and 6 plantlets per cultivar) in regenerants from primary somatic embryos and secondary somatic embryos were transferred to a glasshouse at the University of Nairobi. Plantlets with expanded leaves, shoots and well developed roots were removed from the glass jars and rinsed with warm water (double distilled at 10℃) to clean the agar media from the roots. Each plantlet was established in a 2 L plastic pot in which a sterile potting mix (forest soil, red sand and completely decomposed husk from coffee) had been added and covered with polythene to increase the humidity. The plants were kept in a 70% shaded glasshouse for 3 weeks before the polythene was removed. The hardened plants were regularly observed for CMD symptoms for 3 months in the glasshouse.

2.6 Data Analysis

Data on survival rates among the cultivars, duration of somatic embryos formation, induction frequencies of OES, percentage average germination of cotyledonary-stage embryos, number of established plants per 6 OES clusters, number of plants regenerated that tested positive and/ or negative for EACMV and virus elimination efficiency were subjected to analysis of variance (ANOVA) using GenStat 10th Edition, and a probability level of $p \leq 0.05$ was considered while computing the standard error of means (SEM). The means were separated by Tukey's LSD test ($p \leq 0.05$), where the means which were insignificantly different ($p \geq 0.05$) were assigned the same letter.

3. RESULTS AND DISCUSSION

3.1 Detection of EACMV in Plants of Selected Cassava Cultivars

The viral status of mother plants of selected cassava cultivars was confirmed by symptom expression in the glasshouse and PCR analysis. The established cassava cultivars used as source explants for somatic embryogenesis developed symptoms such as yellow to green chlorotic mosaic on the leaves, mottling then leaf curling and distortion (Fig. 1). The symptoms observed for CMD are similar to those reported by Were et al. [25]. Leaf chlorosis (Fig. 1A), leaf curling (Fig. 1B) and stunting (Fig. 1C) were also observed. Thresh and Cooter [10] also observed chlorotic lesions, leaf curling, stunting and drying up of CMD-infected cassava plants. Similar observations were made by Were et al. [25] on popularly grown cassava cultivars in Kenya which were CMD-infected.

All the sprouted plants in the glasshouse tested positive for the presence of EACMV by PCR amplification of a 550 bp fragment specific to EACMV (Fig. 2). This confirmed the findings of Sing'ombe et al. [26] that EACMV is present and severe in local genotypes in Kenya. Therefore, the CDM-infected cassava cultivars were considered to be suitable source material for testing the efficiency of somatic embryogenesis to eliminate viruses from infected cassava.

3.2 Survival Rates of EACMV-Infected Nodal Explants in Culture Medium

There were significant differences ($p \leq 0.05$) in the survival rates of explants among all the cultivars tested ranging from 66.7 to 93.3% (Fig. 3A). Cultivars Sagalato and TMS 60444 recorded the highest survival rate while Kibandameno had the least. These variations in survival rates could be attributed to the cultivar differences and variations in cultivar response to *in vitro* culture conditions. These findings concur with the assertion of Sidorov [27] that survival rate of culture explants may vary due to cultivar differences and specific nutritional requirement for optimal growth.

Fig. 1. Symptoms of cassava mosaic disease on an infected cassava cultivar that supplied the explants for somatic embryogenesis. (A) Infected *in vitro* plantlets; (B) and (C) Different stages of infected plants growing in the glasshouse

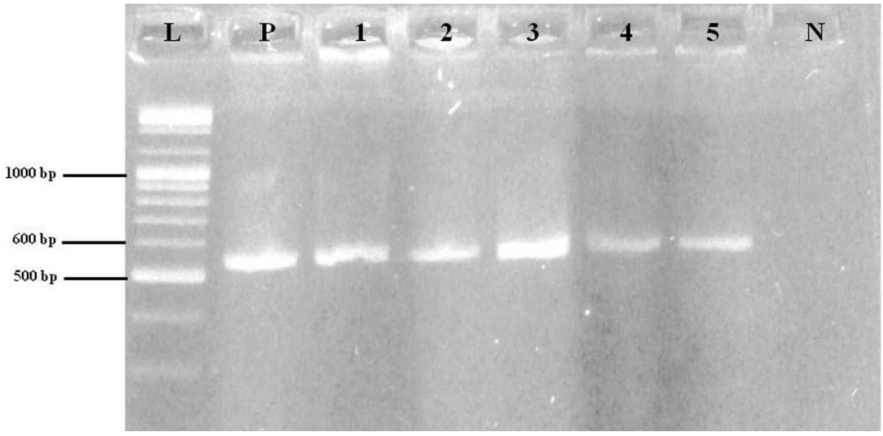

Fig. 2. Agarose gel electrophoresis to detect EACMV in leaves of CMD-infected cassava cultivars. Primer EAB555-F/EAB555-R was used to amplify 550 bp of EACMV replicase gene

Lanes are L: 100bp molecular marker, P: Positive control, N: Negative control, 1-5: Wells containing loaded amplified PCR products (1-TME14, 2- Ex-Mariakani, 3-Sagalato, 4-Kibandameno, and 5-TMS60444)

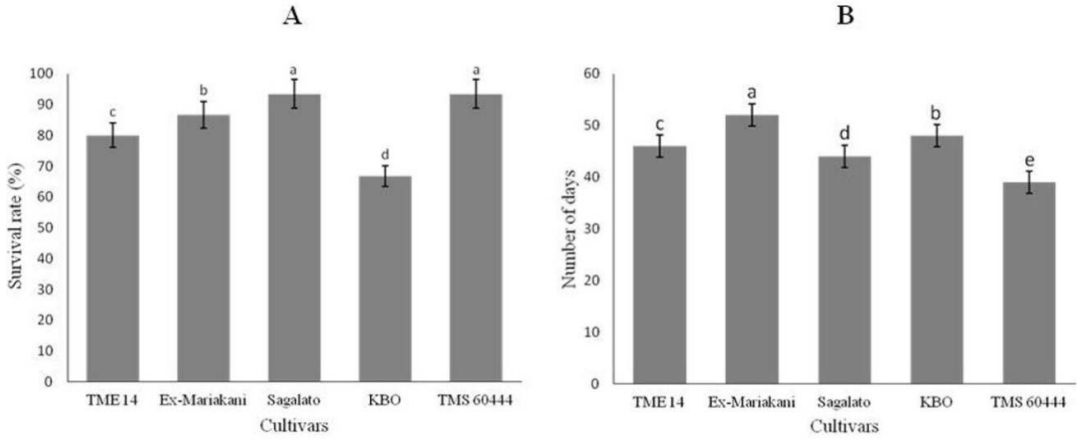

Fig. 3. (A) Survival rate of nodal explants of five cassava cultivars 14 days after planting on CBM medium, (B) Number of days to somatic embryo formation of axillary buds from five cassava cultivars. KBO represents cultivar Kibandameno

Survival rate was calculated as the percentage between the number of survived explants/cultured explants. Error bars represent standard error of means. Bars accompanied by the same letters are not significantly different by Tukey's LSD test ($p \leq 0.05$)

3.3 Induction of Somatic Embryos from Axillary Buds of Infected Nodal Explants

All cultivars investigated in the study produced OES containing somatic embryos. Organized embryogenic structures (OES) constituted a greater number of somatic embryos and less amount of surrounding non-embryogenic soft tissues. An increase in the numbers of OES resulted in an increase in the numbers of embryos obtained. Production of OES was significantly variable in some cultivars ($p \leq 0.05$) with production frequencies ranging from 66.7% to 89.5% (Table 1). The production of OES was highest in Sagalato (89.5%) followed by TME14 (85.7%), Kibandameno (77.8%), TMS60444 (77.7%) and was lowest in Ex-Mariakani (66.7%). However, previous reports on production of OES from TMS60444 and Kibandameno resulted in frequencies of more than 80% [24]. The variations in OES production frequencies observed in this study could be due the differential response of cultivars to *in vitro* culture conditions. This result suggests that genetic factors are important in the response of different cultivars to an *in vitro* culture. This is also in agreement with previous reports that the number of somatic embryos produced by different cassava cultivars is genotype-dependent [24]. However, lower production of OES reported in this study could be attributed to the fact that the mother (source) plants were EACMV-infected. Presence of virus in the mother plant may have limited the chances of axillary bud explants proliferation to OES due to limited number of cells which are viable but with EACMV.

Table 1. Induction frequencies of OES from different cassava cultivars infected with EACMV

Cultivar	No. of auxiliary buds cultured	OES production frequency (%)
TME14	21	85.71 ± 1.16
Ex-Mariakani	24	66.67 ± 0.33
Sagalato	19	89.47 ± 0.88
Kibandameno	18	77.78 ±1.20
TMS60444	22	77.73 ± 0.33

*Organized embryonic structure (OES) production frequencies were recorded by calculating the ratio of OES clusters/cultured axillary buds explants*100. Values are means of 3 independent experiments*

There were significant differences ($p \leq 0.05$) in the time required to induce somatic embryos among the five cultivars (Fig. 3B). The period ranged from a mean of 36 to 52 days. The number of days to somatic embryo formation of cultivar TMS60444 was significantly lower (36 days) than the other CMD-infected cultivars cultured. Ex-Mariakani had the least somatic embryo development rate requiring an average of 52 days. The time to somatic embryo formation observed in this study was longer (36 to 52 days) than that reported by Damba et al. [1] (36 to 46 days). The differences could be due to genotypic variations and severity of CMD infections in the tested cultivars.

3.4 Regeneration of Cassava Plantlets from Primary and Secondary Somatic Embryos

All the cultivars tested were highly regenerative, producing an average of between 41 to 54 and 58 to 68 cotyledon-stage embryos (Fig. 4) per 6 clusters of OES for primary and secondary somatic embryos, respectively (Tables 2 and 3). Primary somatic embryos of TMS 60444 produced more cotyledon-stage embryos (Mean = 50) compared to other cultivars after 21days of culture on regeneration medium (Table 2). Secondary somatic embryos of TMS 60444 and Ex-Mariakani produced more cotyledon-stage embryos (68 each) compared to other cultivars after 21 days of culture on regeneration medium (Table 3). The percentage of green cotyledons forming shoots was cultivar dependent (Table 2 and 3). The average number of plants regenerated from 6 clusters of OES for primary embryos ranged from 5 to 9 (Table 2) while for secondary embryos, the range was between 7 and 12 plants (Table 3). TME 14 had significantly higher rate of germination ($p \leq 0.05$), producing 9 and 12 plants per 6 clusters of OES from primary and secondary somatic embryos, respectively. An average of 6 and 10 plantlets per cultivar were established from plants regenerated from primary and secondary somatic embryos, respectively. The data revealed that the number of established plants were dependent on the germination of cotyledonary stage embryos (Figs. 4B-E). Most of the secondary somatic embryos germinated into cotyledons that resulted in greater number of established plants. These observations concur with the findings of Damba et al. [1] who reported that secondary somatic embryos of cassava cultivars Ankrah, Biabasse, Nagbagu Sule, and Buyadoo had greater regeneration efficiency than primary somatic embryos. Also, Anuradha [28] asserted that the

significance of secondary somatic embryos depends on factors such as the maturation period as well as the number of germinated embryos.

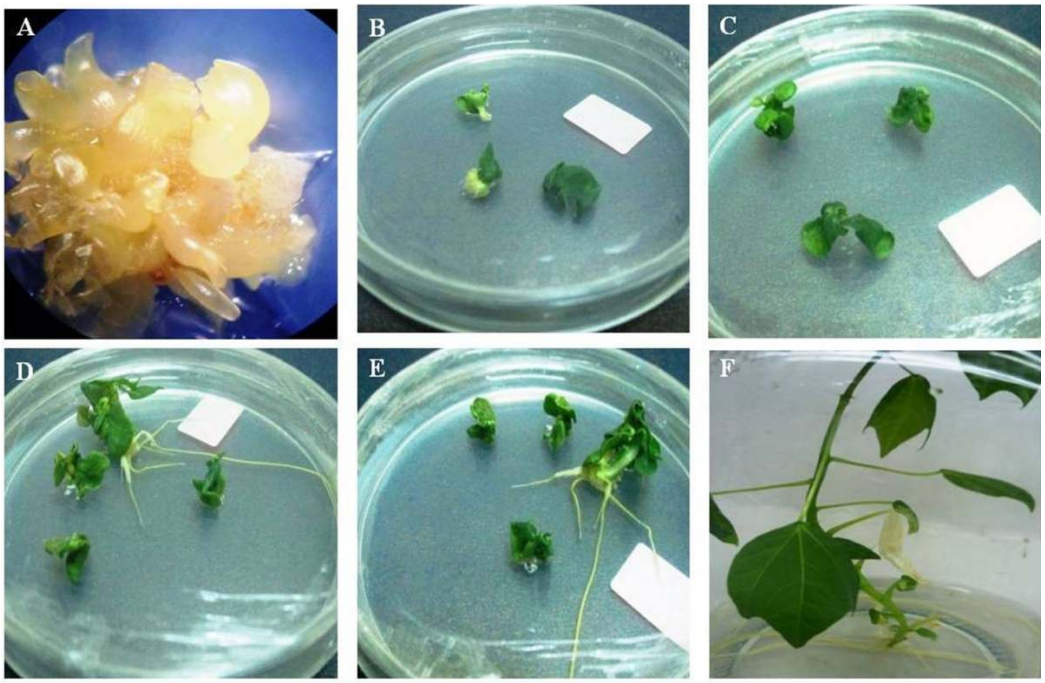

Fig. 4. Stages of cassava plantlets regeneration via somatic embryogenesis. (A) Cotyledons developing from somatic embryos on regeneration medium; (B) and (C) matured cotyledons on CEM medium; (D) and (E) germinating cotyledons on CEM medium; (F) developed plantlet on CBM medium

Table 2. Regeneration of plantlets from primary somatic embryos

Cultivar	Av. no. of OES clusters	No. of cotyledonary-stage embryos	% average germination of cotyledonary-stage embryos	Aver. no. of regenerated plants/6 clusters of OES
TME 14	6	48 ± 1.13	65 ± 1.22	9 ± 1.15
Ex-Mariakani	6	45 ± 0.71	69 ± 1.81	7 ± 0.12
Sagalato	6	54 ± 0.22	59 ± 1.02	5 ± 0.91
Kibandameno	6	41 ± 1.64	57 ± 1.43	5 ± 1.37
TMS 60444	6	50 ± 1.42	64 ± 0.87	6 ± 0.09

Somatic embryos were cultured on regeneration medium and emerging green cotyledons were cultured on CEM medium. Values are means ± SD of three independent experiments

Table 3. Regeneration of plantlets from secondary somatic embryos

Cultivar	Av. no. of OES clusters	No. of cotyledonary-stage embryos	% average germination of cotyledonary-stage embryos	Aver. no. of regenerated plants/6 clusters of OES
TME 14	6	61 ± 1.18	75 ± 1.13	12 ± 1.16
Ex-Mariakani	6	68 ± 1.44	78 ± 1.46	12 ± 0.33
Sagalato	6	62 ± 0.12	69 ± 0.32	10 ± 0.88
Kibandameno	6	58 ± 1.43	59 ± 1.67	7 ± 1.20
TMS 60444	6	68 ± 0.75	75 ± 0.82	8 ± 0.33

Somatic embryos were cultured on regeneration medium and emerging green cotyledons were cultured on CEM medium. Values are means ± SD of three independent experiments

3.5 Effectiveness of Somatic Embryogenesis in Elimination of EACMV

Somatic embryogenesis offers a wider range of application such as multiplication of plants, regeneration of plantlets in biotechnological plant breeding programs as well as virus elimination [21,29]. Polymerase chain reaction analysis was used to detect EACMV in plants regenerated from primary and secondary somatic embryos. Molecular identification of viruses that infect plant material is currently achieved by amplification of partial or full genomic sequences by PCR. PCR is the more powerful technique due to its ability to recover viral sequences from very low viral titres and is now the preferred approach for virus detection. In the present study, EACMV-specific primers amplified a fragment of the expected size of 550-bp in virus-infected cassava regenerated plants, while no amplification was obtained with EACMV-free regenerated plants (Fig. 5). Results obtained from PCR analysis showed that 87.6% (Table 4) and 93.9% (Table 5) of plantlets regenerated from primary and secondary somatic embryos, respectively, tested negative for the presence of EACMV. Overall, PCR results revealed that EACMV can be eliminated through somatic embryogenesis because 90.8% of the regenerated plantlets tested virus-free from EACMV. In somatic embryogenesis, each cell is capable of regenerating into a new plant and there is separation between vascular system of the parent tissue and that of regenerated plantlets [30,31] and this result in the generation of disease free plants from infected materials.

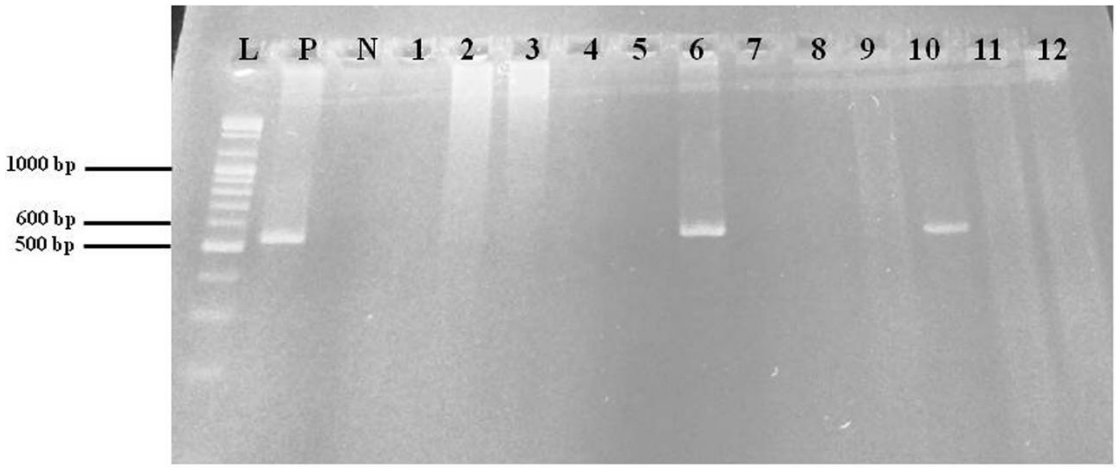

Fig. 5. Agarose gel electrophoresis of amplified PCR products to detect EACMV in DNA extracted from plants regenerated from primary somatic embryos. EAB555F/EAB555R primer pair specific to EACMV replicase gene was used to amplify a 550 bp fragment

Lanes are L: 100 bp molecular marker, P: infected positive control, 1 – 12: Wells containing loaded amplified PCR products (1, 2 and 3: TME 14, 4 and 5: Sagalato, 6 and 7: Ex-Mariakani, 8 and 9: Kibandameno, 10, 11 and 12: TMS 60444), N: Non-infected negative control. Lane 6 and 10 shows amplified 550 bp amplified PCR product specific to EACMV in plants of cultivars Ex-Mariakani and TMS 60444, respectively

Table 4. Efficiency of EACMV elimination in plants regenerated from primary somatic embryos

Cultivar	No. of plants regenerated	% tested positive for EACMV	% tested negative for EACMV	% virus elimination efficiency
TME 14	9	0.0	100	100
Ex-Mariakani	7	28.6	71.4	71.4
Sagalato	5	0.0	100	100
Kibandameno	5	0.0	100	100
TMS 60444	6	33.3	66.7	66.7
Average	6	12.4	87.6	87.6

Efficiencies were calculated as the percentage of the ratio of plantlets that tested negative for EACMV/total number of plantlets tested

Table 5. Efficiency of EACMV elimination in plants regenerated from secondary somatic embryos

Cultivar	No. of plants regenerated	% tested positive for EACMV	% tested negative for EACMV	% virus elimination efficiency
TME 14	12	0.0	100	100
Ex-Mariakani	12	8.3	91.7	91.7
Sagalato	10	100	100	100
Kibandameno	7	100	100	100
TMS 60444	8	25.0	75.0	75.0
Average	10	6.1	93.9	93.9

Efficiencies were calculated as the percentage of plantlets that tested negative for EACMV/ the total number of plantlets tested

Plants regenerated from primary somatic embryos had relatively lower average elimination efficiency (87.6%) compared to those regenerated from secondary somatic embryos (93.9%). From the axillary bud derived from EACMV-affected nodal explants, to the primary somatic embryos and later the secondary somatic embryos, the presence of EACMV was reduced, indicating that the progress of the virus was progressively impeded. This is in agreement with previous study by Quainoo et al. [32] who reported that somatic embryogenesis was capable of the progressive interruption of the movement of cocoa swollen shoot virus (CSSV) from primary to secondary somatic embryos.

In this study, all plants of cultivars TME14, Kibandameno and Sagalato regenerated from both primary and secondary somatic embryos were confirmed virus-free (100% elimination efficiency) by PCR analysis. The virus was detected in regenerated plants of cultivars Ex-Mariakani and TMS60444. Cultivar TMS60444 recorded the least EACMV elimination efficiency of 66.7% and 75% in plants regenerated from primary and secondary somatic embryos, respectively. These results indicate that elimination of viruses from infected cassava cultivars is genotype-dependent. The elimination efficiency in the different cultivars is not related to the differential response of genotypes to CMD infection. This is because both Kibandameno and TME14 recorded 100% elimination efficiency and it is clear that Kibandameno is highly susceptible to cassava mosaic viruses [26], while TME14 has 'R'(CMD-2) genes which makes it tolerant to cassava mosaic viruses [33,34]. Previous reports on somatic embryogenesis of four local cassava cultivars in Ghana resulted in virus elimination efficiencies of 80% [1]. Similarly, Nkaa et al. [35] was able to regenerate virus free "Nwugo" cultivar initially infected with African cassava mosaic virus (ACMV) via somatic embryogenesis. In the present study, EACMV PCR-negative regenerated plants were successfully weaned to the glasshouse (Fig. 6A and B). Visual observation of the plantlets in the glasshouse revealed the absence of symptoms of viral disease for up to 3 months (Fig. 6C).

Fig. 6. Glasshouse acclimatization of *in vitro* regenerated virus-free cassava plants of various cultivars. (A) Cassava plantlet during day 5 of acclimatization; (B) Cassava seedling after 21 days; (C) Survived cassava plants after 10 weeks

4. CONCLUSIONS

In conclusion, the results from this study indicate that production of somatic embryos from different cassava cultivars is genotype-dependent. This study has shown that somatic embryogenesis is a potentially promising technique for virus elimination in cassava and it has been demostrated to function for a range of cassava cultivars. This study is potentially useful with respect to future breeding work aimed at improving the crop in East Africa as a means to generate resistant cultivars to EACMV. It will also be of value for regenerating EACMV-free cultivars of cassava (TME 14, Ex-Mariakani, Sagalato, Kibandameno and TMS 60444) for distribution among farmers and global cultivar exchange programmes.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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