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Efficient conditions for in vitro establishment and regeneration of disease-free Ugandan farmer-preferred cassava genotypes

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Cassava (*Manihot esculenta* Crantz) is majorly devastated by two viral diseases, cassava brown streak disease (CBSD) and cassava mosaic disease (CMD), resulting in 100% yield loss. Being a clonal plant, nodal cuttings (NC) and shoot apical meristems (SAMs) are the best explants for production of disease free planting materials. In this study, NCs and SAMs were used to determine reliable indicators for successful *in vitro* establishment of cassava. Eight cassava genotypes were used for the study. Leaf samples were collected from 30 stakes of each of the eight genotypes planted in the screen house. The leaf samples were pooled and screened for presence and/or absence of CBSD and CMD by PCR using virus specific primers. Nodal cuttings were excised from screen house grown plants, surface sterilized to rid-off contaminants and established on Murashige and Skoog (MS) Medium. Using the sprouted stakes, 5-mm sized SAMs were excised, surface sterilized and reduced to 0.5-1 and 2-3 mm sizes. The SAMs were established on MS medium with varying concentrations of plant growth regulators (0.5, 1, 2) ml/L Benzylaminopurine (BAP) and (2, 4) ml/L Naphthalene acetic acid (NAA), Kinetin (K) and BAP respectively. PCR results revealed the pooled leaf samples were free of both CBSD and CMD for all genotypes. Establishment and regeneration of NCs was possible with MS medium for all genotypes. For the SAMs, the concentrations of (2, 4) ml/LBAP followed by 2 ml/LNAA facilitated their establishment and regeneration in comparison to KIN.SAMs of 2-3 mm sizes regenerated better than 0.5 - 1 mm size. Both NCs and SAMs of the different genotypes produced leaves, nodes, roots and there was an increase in plant length. These parameters are critical indicators for *in vitro* establishment and regeneration of cassava.

Key words: Cassava genotypes, cassava diseases, shoot apical meristems, nodal cuttings, growth regulators.

INTRODUCTION

Cassava (*Manihot esculenta* Crantz) of the family Euphorbiaceae, is the most important staple food crop grown by low-income African farmers. It is a vegetatively propagated crop and is recycled over seasons. The use

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of planting material from previous generation to establish the next generation provides an easy way for disease-causing pathogens, particularly viruses to pass from one generation to the next resulting in yield reduction, poor seed quality, and lack of available disease-free planting materials (Legg et al., 2011). There has been a decline in production of cassava from 5.5 million metric tonnes (2005) to 2.5 million metric tonnes (FAOSTAT, 2018), as a reult of cassava brown streak disease (CBSD) and cassava mosaic disease (CMD) epidemics (Alicai et al., 2007; Beyene et al., 2016).

The devastating CBSD is caused by at least two viral species: Cassava brown streak virus (CBSV) and Ugandan cassava brown streak virus (UCBSV), both belonging to genus Potyviridae. The CBSD infected tuberous roots are necrotic, rendering the tuber unfit for human consumption (Mbanzibwa et al., 2009). The CMD is caused by cassava mosaic geminiviruses (family Geminiviridae, genus Begomovirus) (Legg and Thresh, 2000). In Uganda, the dominant strains are; African cassava mosaic virus (ACMV) and the East African cassava mosaic virus Uganda variant (EACMV-UG2) (Harrison et al., 1997). Both diseases are transmitted by the whitefly vector (*Bemisia tabaci*) (Maruthi et al., 2005) and through the use of infected cuttings (Thresh et al., 1994) during vegetative propagation. A number of local and improved cultivars are grown by farmers, for their inherent preferred traits including high yield, taste (bitter/sweet), early maturity or long storage in soil, softness, high dry matter content, good root tuber qualities (for cooking and flour), and ready market for fresh roots (Otim-Nape et al., 2001). Unfortunately, many of the landraces were lost to CMD, and improved cultivars bred for resistance to CMD are all susceptible to CBSD (Abaca et al., 2012).

In Uganda, CBSD continues to spread and devastate cassava production and productivity (Alicai et al., 2019). With the help of the eye, foliar and stem symptoms are used by trained field assistants to determine the presence or absence of CBSD disease, of the plants using a known scale of 1-5 (Alicai et al., 2007, 2019). However, research has shown that some genotypes show severe foliar symptoms and others show mild symptoms, even though the plant is carrying the disease (Maruthi et al., 2005; Maruthi et al., 2019). The differences in the foliar symptoms is attributed to factors such as; variety, age of the plant, the viral species present and the environment in which the crop is growing (Maruthi et al., 2019). The levels of expression of the disease in the leaves vary from genotype to genotype. This necessitates validation of the health status of the plant material picked, from different agroecological zones, making it possible to establish virus free materials in tissue culture (Maruthi et al., 2019),

Plant tissue culture (PTC) methods have played an integral part of plant science research because it enables detailed study of plant development and facilitates bulking up of the plant materials (Thorpe, 2007). PTC relies on a phenomenon known as totipotency, which allows a single plant cell to grow, divide and differentiate into a complete new plant. PTC involves the cultivation of cells, tissues, organs in an appropriate nutrient medium, in a sterile environment to facilitate growth and maintain it, in vitro (Thorpe, 2007; George et al., 2008; Lone et al., 2020). PTC techniques have made it possible to eliminate plant pathogens like bacteria and fungus. However, it has not been the same for virus infections in plants (Baker and Jeyaprakash, 2015).

According to Nakabonge et al. (2020), the use of meristem tip culture in combination with hot water treated removed the cassava mosaic viruses (CMV) and reduced the UCBSV in the system of the plant. To further certify the absence of viruses in the explants to be used, molecular biology techniques like the polymerase chain reaction (PCR) are being used. PCR is a simple technique used to amplify and detect DNA and RNA sequences. It allows for enzymatic replication of DNA, amplification of specific regions of a DNA strand, making it possible to detect the viral sequences that are associated with a specific disease (Mullis and Faloona, 1987; Sambrook and Russell, 2001). The demand by farmers to access disease-free planting materials is on the increase, as there is need to certify these materials and accelerate their production in large quantities (Maruthi et al., 2019). In order to understand the response of each genotype and what can be used to measure their ability, with regard to bulking up of materials, nodal cuttings (NC) and the shoot apical meristems (SAMs) were used as explants and morphological features like roots, leaves and so on were documented.

Shoot apical meristems (SAMs) are a population of small, isodiametric cells with embryogenic characteristics found at the extreme end of the shoot axis. SAMs are considered a virus free region, given that most plant viruses do not infect it (Mochizuki and Ohki, 2015). In sweet potatoes (Wondimu et al., 2012) and sugar cane (Cha-um et al., 2006), sizes ranging from (0.5 - 0.7) mm and (0.5 - 1.5) mm respectively have been used for producing disease-free planting materials. Establishment and maintenance of SAMs is facilitated by plant growth regulators (PGR) like auxins and cytokinins that regulate plant growth and development (Donald, 1994; Ha et al., 2010; Murray et al., 2012; Fouracre and Poethig, 2019). Cells arising from the peripheral zones of the apical meristem contribute to formation of organs. The SAMs continuously regenerate itself and also produces leaves, lateral buds as well as stem tissues (Donald, 1994; Ha et al., 2010; Murray et al., 2012; Fouracre and Poethig, 2019), which further facilitates micropropagation or clonal propagation of plants. Thus, plants can be propagated either through the sexual or asexual developmental life cycle.

The sexual cycle of new plants arise from the fusion of

the parental gametes forming zygotic embryos within seeds, each embryo having a new combination of genes due to meiosis, resulting into a new individual plant (George et al., 2008). In the asexual or vegetative cycle, selection of the mother plant or stock plant is crucial. The genes of the mother plant are copied exactly at mitosis, resulting into a new plant that is considered a clone of the mother plant. The asexual approach is considered a more reliable method for true-to-type *in vitro* propagation (George et al., 2008). Micro propagation is a method used for rapid proliferation of tiny stem nodal cuttings, facilitating conservation of germplasm and massive production of disease-free planting materials (Thorpe, 2007; George et al., 2008).

The response of the explant used *in vitro* is dependent on its physiological state, the genotype, the culture media and the health status of the explant (Isah, 2019). The medium is critical because it provides the appropriate nutrients that facilitate growth of the plant. Plant growth involves cell volume increase, cell division, and developmental programmes that specify tissue and organ identity (Lastdrager et al., 2014). Growth of the explants can be measured by using parameters such as number of leaves and roots because they act as reliable indicators for photosynthetic activity and the support system of the plant respectively. The number of nodes can be used to ascertain the number of plants that can be obtained, therefore facilitating the multiplication of plants all year round (Isah, 2019).

Earlier reports on cassava sprouting and regeneration were focused on the use of the stem cuttings (EL-Sharkawy, 2004). Majority of the reports to date have been demonstrated through somatic embryogenesis in the presence of plant growth regulators for Kenyan cultivars (Nyaboga et al., 2013), Ugandan cassava genotypes (Apio et al., 2015), Ghanaian cultivars (Elegba et al., 2021) and so on, in an effort to develop genetic transformation systems for the crop. However, there is no clear documented information on the multiplication rate and regenerative ability of plants generated from in vitro nodal cuttings (NC) and shoot apical meristems (SAMs), as an incentive for farmers. This study was conducted to identify indicators suitable for growth and multiplication of disease-free Ugandan cassava genotypes using nodal cuttings (NC) and shoot apical meristems (SAMs).

MATERIALS AND METHODS

Cassava genotypes

The experiments were conducted at the screen house setup at the National Crops Resources Research Institute (NaCRRI). A total of eight (8) cassava genotypes, seven (7) improved varieties: NASE 3, NAROCASS 1, NAROCASS 2, NASE 13, TME 204, NASE 19, NASE 12 and one (1) land race: Alado, were collected from different agroecological zones of the country. The Northern (Nase 13, Nase 12, Alado, TME 204), Western (NAROCASS 1, NAROCASS 2), Eastern (Nase 3) and Central (Nase 19). These genotypes were selected for their resistance to cassava mosaic disease (CMD) (Nase 3, Nase 13, Nase 12 and TME 204), tolerance to cassava brown streak disease (CBSD) (Nase 19, NAROCASS 1 and NAROCASS 2) and susceptibility to both diseases (Alado) and to CBSD (TME 204). A total of 10 L of the insecticide Cypermethrin at 2 ml/L concentration was prepared. With the help of a 16-ml capacity knapsack sprayer, the prepared cypermethrin was sprayed in and out of the screen house to get rid of whiteflies, mites and other insects a day earlier. Using black forest soil, a total of thirty-one (31) four-nodes stakes were planted for each genotype. The total number of stakes planted was two hundred forty-eight (248). Each pot contained a stake that was watered every morning and evening. Their ability to sprout fully was monitored. The experiment was laid out in completely randomized design (CRD) and was repeated twice. Data on the number of stakes that fully sprouted was taken. Data was analysed using ANOVA at P≤0.05 with Genstat 12th Edition. These stakes were established as a source of explant material for the in vitro experiments in tissue culture and the disease diagnostics in the molecular laboratory.

Leaf samples collection

Leaf samples were collected from a total of 30 sprouted stakes for each of the eight (8) genotypes growing in the screen house. Leaf samples were picked from the top three leaves and placed in 1.5 ml of screw capped vials, which were placed in a styrofoam box containing ice packs and carried to the molecular laboratory for extraction. Leaf samples from 10 plants for each genotype were pooled to achieve one sample. Each genotype therefore had a total of three pooled samples for CMD and CBSD detection. Samples 1-3 represented NASE 19, 4-6 (NAROCASS 2), 7-9 (Alado), 10-12 (TME 204), 13-15 (NASE 12), 16-18 (NAROCASS 1), 19-21 (NASE 3) and 22-24 (NASE 13). Samples for CMD and CBSD detection were handled separately. The total number of samples used was 24 for CMD and 26 for CBSD. For CBSD two samples, one from tissue-cultured and field grown TME 204 plant was added as internal and external checks.

Extraction of total nucleic acids for CMD and CBSD detection

Total nucleic acids were extracted from leaf samples following the cetyl trimethyl ammonium bromide (CTAB) method as described by Lodhi et al. (1994) with some modifications. Firstly, One hundred (100) ml of the CTAB extraction buffer (100 mM TrisHCl, 20 mM EDTA, 1.4 mM NaCl, 5%β-Mercaptoethanol) was prepared. To the CTAB extraction buffer, 0.1 mg/ml of proteinase K enzyme was added before adding to the samples. Secondly, 0.2 g of asymptomatic mature cassava leaves were collected and placed in 1.5 ml screw capped vials, 700 µl of CTAB extraction buffer were added to each sample and homogenized using a Tissue lyser (Model Percellys 24, bertin technologies, France, 2013). The samples were incubated at 65°C for 30 min, and mixed by inversion every 10 min. The samples were kept for 10 min at room temperature, then 700 µl of 24:1 mix of chloroform: Isoamyl alcohol was added to each sample, mixed by inversion for 10 min and centrifuged at 12000 rpm for 10 min. The 550 µl of the upper aqueous phase was transferred to a clean 1.5 ml Eppendorf tube. This step was repeated and 0.7 volume of the upper aqueous phase was added to ice cold (-20ºC) isopropanol, mixed gently then incubated at -20ºC for 1 h, spun for 10 min at 13000 rpm, after which the isopropanol was decanted. To purify the pellet, 500 µl of 70% ethanol was added to the samples, washed by tapping, then centrifuged at 13000 rpm for 10 min. The ethanol was decanted and the samples left to air dry for 40 min. The same approach was used for CBSD samples. The air-dried pellets were resuspended in

300 µl of nuclease free water and stored at -20ºC for CMD analysis and in 50 µl of nuclease free water for CBSD analysis. The purity and quality of each nucleic acid sample was assessed using a NanoDrop spectrophotometer (Model 2000C, Thermo Scientific, Waltham, MA).

Polymerase chain reaction (PCR) for detection of CMGs

Total nucleic acids was used as a template for the standard PCR reaction to detect cassava mosaic Geminiviruses (CMGs) using ACMV and EACMV-UG2 specific primers (ACMV-AL1/F and ACMV-ARO/R) and (ACMV-CP/R3 and uv-AL1/F1) respectively. Fragments produced for ACMV and EACMV-UG2 are about 1000 and 1500 bp, respectively (Harrison et al., 1997). The PCR reaction was performed in a volume of 20 µL containing 12.4 µl of nuclease free water, 2.5 µl of 5X PCR reaction buffer, 1.5 µl of 25 mM $MgCl_2$, 1.0 µl of 10 mM dNTPs (2.5 mM), 10 pmoles of forward primer, 10 pmoles of reverse primer, 0.2 µl of 5U/µl of Taq polymerase and 2 µl of DNA template was added. Two master mixes were prepared for ACMV and EACMV with two different primer pairs for 26 samples each respectively. The PCR conditions were as follows: a one cycle of the denaturation step at 94°C for 2 min, 55°C for 1 min, 72°C for 2 min followed by the annealing step with 30-35 cycles at 94°C for 1 min, 55°C for 1.30 min, 72°C for 2 min and lastly one extension cycle 94°C for 1 min, 55°C for 1 min, 72°C for 10 min and held at 4°C for ACMV and EACMV. The reaction was run for 3 h. The PCR products were electrophoresed in a 1.2% agarose gels stained with ethidium bromide and run at 80 V for 1 h in X1 Tris-Acetate-EDTA (TAE) buffer of pH 8. The gels were visualized under UV light and photographed using a GelDoc system (Model: uGenius3, SYNGENE, UK). The absence of the expected band sizes, indicated absence of the Gemini viruses in the sample.

RT-PCR for CBSD detection of CBSVs

To prepare the cDNA that was used in the PCR reaction, 50 µl of total nucleic acid for each sample plus 7.5 µl of the DNase mix (DNase 1 and DNase 1 buffer) was added to each sample and mixed well. The twenty six (26) samples together with the controls were left to incubate at 37ºC for 30 min. The samples were left at room temperature for 2 min. 1 µl of 0.5M EDTA was added and heated at a temperature of 75ºC for 10 min. The RNA was quantified using a NanoDrop spectrophotometer (Model 2000C, Thermo Scientific, Waltham, MA). To a sterile RNase-free microfuge tube, 1 µg of the RNA sample, 2 µl of the primer d(T)23 VN and a calculated amount of nuclease-free water was added to achieve 8 µl and were mixed together. The sample RNA/d(T)23VN was denatured for 5 min at 65ºC, spun and placed on ice, to generate the first strand cDNA, 10 µl of the ProtoScript II Reaction Mix (2X) and 2 µl of the ProtoScript II Enzyme Mix (10X) were added to achieve a volume of 20 µl. The 20 µl cDNA synthesis reaction was incubated at 42ºC for 1 h and the enzyme deactivated at 80ºC for 5 min. This was done for 28 samples. The reverse transcription reaction product was used for PCR reaction to detect cassava brown streak viruses (CBSVs) using the CBSVDF2 and CBSVDR primers, the fragment sizes obtained for UCBSV and CBSV are 437 and 343 b p respectively (Mbanzibwa et al., 2010). The PCR reaction was performed in a volume of 20 µL containing 6 µl of nuclease free water, 10 µl of One Taq Quick-Load 2X Master Mix with Standard buffer, 1 µl of 10 µM forward primer, 1 µl of 10 µM Reverse primer and 2 µl of the template. The samples were gently mixed. The PCR conditions used were as follows: 94°C denaturation step of 30 s followed by 30 cycles of 30 s at 94°C, 30 s at 51ºC and 30 s at 72ºC; then the final elongation step of 10 min at 72°C and held at 4ºC. The reaction ran for 1 h 46 min. The PCR products were electrophoresed in a 1.2% agarose gels stained with ethidium bromide and run at 80 V for 1 h in X1 Tris-Acetate-EDTA (TAE) buffer of pH 8. The gels were visualized under UV light and photographed using a GelDoc system (Model: uGenius3, SYNGENE, UK). The absence of the expected band sizes, indicated absence of the viruses in the sample.

Establishment of nodal cuttings (NC) in vitro

Nodal cuttings were excised from the fully grown tested disease free cassava plants in the screen house. NC was surface sterilized using 2% of 3.85 m/v Sodium hypochlorite (NaOCl), 2-3 drops of Tween-20, and swirled for 10 min to ensure microbes are removed. The NC was rinsed using tap water and transferred to sterile conditions under the laminar flow hood for further sterilization. To the 500 ml of 2% NaOCl, 4-5 drops of tween-20 was added, stirred and dispensed into a jar carrying NC of each genotype. These were further swirled for 10 min and rinsed five times with sterile distilled water under a laminar flow hood. The edges of the nodal cuttings which were affected by bleach were cut off. The remaining explants was placed on Murashige and Skoog (MS) basal medium with vitamins (Duchefa Biochemie), with 2% of sucrose (Merck) and 3 g of gelrite (Duchefa Biochemie) and monitored for their response *in vitro*. This was done for all eight genotypes. A total of 31 NC were used for each genotype. The cultures were placed in a growth room at temperature of 28 ± 2°C and photoperiod of 16 h of light and 8 h of darkness. Completely randomized design was used. The experiment was repeated twice. Data on sprouting, number of leaves, nodes, length of plants were documented. Data was analysed using ANOVA at P≤0.05 with Genstat 12th Edition.

Establishment of shoot apical meristems (SAMs) *in vitro*

The shoot apical meristems (SAMs), 5 mm in size, were excised from the 2 months old mother plant confirmed to be disease-free. The SAMs were surface sterilized using 1% of 3.85 m/v Sodium hypochorite (NaOCl), 2-3 drops of Tween-20, and swirled for 5 min to remove microbes. The SAMs were rinsed using tap water and transferred to sterile conditions under the laminar flow hood for further sterilization. To the 200 ml of 1% NaOCl, 2-3 drops of tween-20 were added, stirred and dispensed into the jar carrying the SAMs of each genotype. These were further swirled for 5 min and rinsed three times with sterile distilled water under the laminar flow hood. The protective layers of the SAMs were excised under a dissecting microcospoe using a hypodermal needle to achieve, 2-3 mm size explants and placed on MS basal medium supplemented with Benzylaminopurine (BAP) at varying levels (0.5, 1 and 2 ml/L). This was done for eight genotypes. Using 90 x 15 cm sized petri dishes, 5 explants of 2-3 mm sized SAMs were plated. Four plates were setup for each genotype. A total of 20 SAMs 2-3 mm were established for each genotype. The subsequent experiments were conducted using 0.5 – 1 mm size SAMs, which were placed on MS basal medium with vitamins (Duchefa Biochemie) containing three plant growth regulators: Benzylaminopurine (BAP), Naphthalene acetic acid (NAA) and Kinetin (KIN) at two concentration levels (2 and 4 ml/L), 2% sucrose (Merck) and 3 g of gelrite (Duchefa Biochemie) and monitored for their response in vitro. This was done for all the six genotypes. Each petri dish 90 x 15 cm size contained 4 explants. Three petri dishes were set up for each genotype. A total of 12 SAMs of 0.5-1 mm were established for each genotype. The cultures were placed in a growth room at temperature of 28 ± 2°C and photoperiod of 16 h of light and 8 h of darkness. Completely randomized design was used. Each experiment was repeated twice. Data on sprouting, number of leaves, nodes, roots and plant length were taken. Data was analysed using ANOVA at P≤0.05 with Genstat 12th Edition.

Figure 1. Establishment of cassava stakes in the screen house. a) Uniform size stakes for planting, b) Stakes of cassava planted in soil, c) Sprouted stakes of Nase 12, with the yellow arrow indicating a plant that dried out after sprouting.

RESULTS

Establishment of nodal cuttings (NC) in the screen house

The cassava stem cuttings (stakes) were established insterilized soil and watered morning and evening to ensure their growth. Stakes from all the genotypes sprouted (Figure 1). Significant differences were observed among the genotypes (P = 0.017) for sprouting at 5% CI. According to the results obtained, Alado and TME 204 had the highest number of stakes that sprouted while Nase 13 had the least (Table 1).

CBSD and CMD detection

Leaf samples collected from all screen house-grown cassava genotypes tested negative for the presence of the African Cassava Mosaic Virus (ACMV) with a band size of 1000bp and the East African Cassava Mosaic Virus (EACMV) with a band size of 1500bp (Figure 2a). Sample 24 was lost during processing to obtain DNA (Figure 2a). Similarly, all leaf samples analyzed for CBSD were negative for the presence of the cassava brown streak viruses (CBSVs) (Figure 2b). A field Sample 26 from CBSD symptomatic plant used as a check, tested positive for CBSD (Figure 2b). A disease-free tissue culture lab Sample 25, was used as an internal check (Figure 2b). The band size of 437bp represented CBSVs, while the Uganda cassava brown streak virus (UCBSV) were represented by the band size of 343bp (Figure 2b).

Responses of nodal cuttings (NC) to *in vitro* conditions

All the eight genotypes were subjected to the steps as illustrated in Figure 3. Most of the nodal cuttings sprouted to produce leaves, nodes and roots (Figure 3g) and for others, bacterial contamination was observed (Figure 3e). Significant differences were observed among the genotypes for production of leaves (P = <.001). The genotype NASE 19 had the highest average number of leaves (3.9±0.35) while NAROCASS 1 had the least (2 ± 0.14) (Figure 4a). Significant differences were observed among the genotypes for the number of nodes observed (P =<.001). The highest average number of nodes was observed in NASE 19 (4.0 ± 0.44) and least in NAROCASS 1(1.4±0.13) (Figure 4b). Similarly, significant differences were observed among the genotypes for the ability to produce roots (P = <.001). NASE 12 had the highest average number of roots (7.2 ±0.56), while NASE 3 had the least (0.9 ±0.43) (Figure 4d). No roots were produced by NAROCASS 1 (Figure 3h; Figure 4d). Also, significant differences were observed among the genotypes for plant length (P = 0.005). NASE 13 had the highest average of plant length (2.24±0.25) and NAROCASS 1 had the least (0.55 ± 0.18) (Figure 4c).

Table 1. Percent sprout of the selected farmer-preferred cassava genotypes.

SN	Genotype	Sprouting (%)*
1.	Alado	100
2.	Nase 19	94
3.	Nase 12	97
4.	Nase 13	81
5.	Nase 3	97
6.	NAROCASS 1	94
7.	NAROCASS 2	97
8.	TME 204	100

*Mean values of sprouted stakes of the genotypes from two experiments.

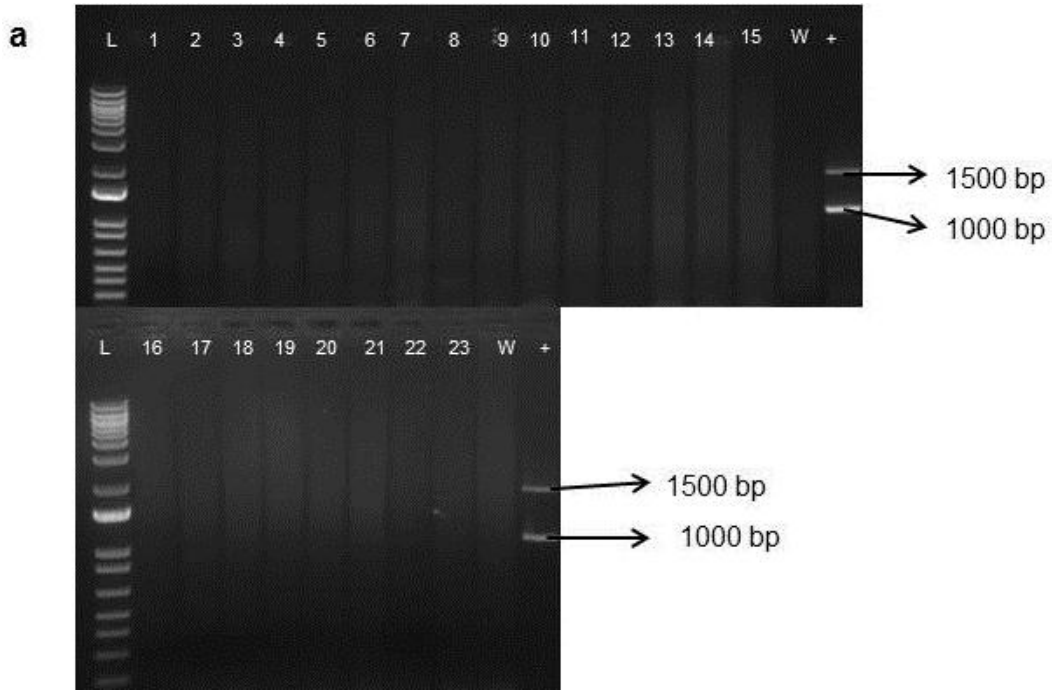

Figure 2a. PCR results for plants tested for the cassava mosaic disease (CMD). The plants were all negative for the disease. L – 1 Kb plus ladder, W – Negative check and + - Positive check, 1-23 leaf samples from test plants.

Responses of shoot apical meristems (SAMs) to *in vitro* conditions

In general, most SAMs obtained from all genotypes, sprouted and regenerated into plantlets (Figure 5) in the presence of plant growth regulators *in vitro* for 2-3 mm size (Figure 6a). In the case of 0.5 - 1 mm size, not all SAMs from genotypes regenerated (Table 2); this was dependent on the plant growth regulator (PGR) used, the concentration of the PGR and the genotype (Table 2). Preliminary experiments on SAMs conducted with 2-3 mm sized explant on varying concentrations of Benzylaminopurine indicated that the SAMs were able to regenerate (Figure 6a). Significant differences were observed in relation to effect of genotype ($P \leq 0.001$) while no significant differences were noted for the auxin levels ($P = 0.741$) and the interaction ($P = 0.610$) at 5% CI (Figure 6a). Highest regenerative ability was observed in TME 204, whereas NASE 12 had the least regenerative ability (Figure 6a). In the case of 0.5 – 1 mm sized SAMs, not all explants sprouted for the genotypes given the PGR concentrations used (Table 2). Overall, Nase 19 had plants sprout with all the PGR concentrations. The highest percentage of sprouting was in Nase 19 (100%)

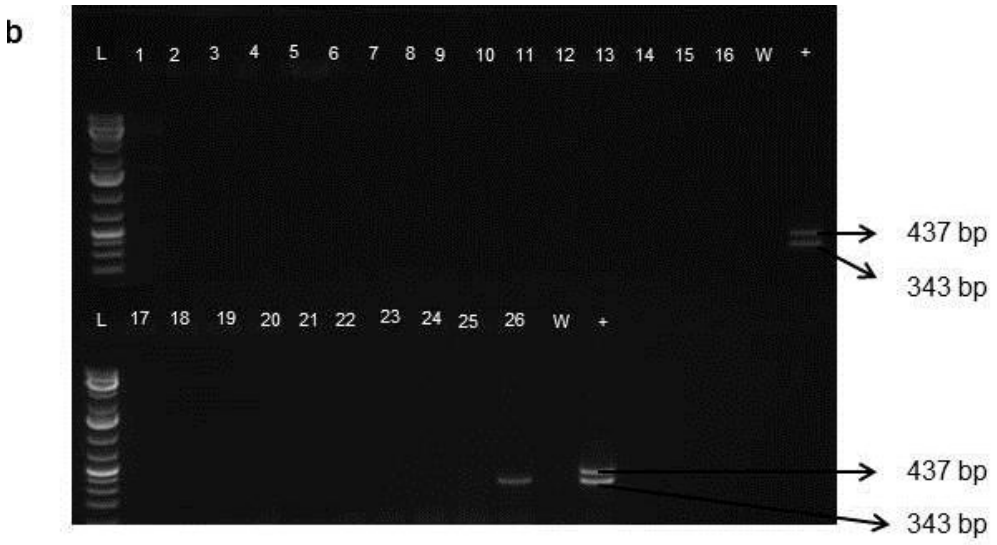

Figure 2b. PCR results for plants tested for cassava brown streak disease (CBSD). The plants were all negative for the disease, except for field sample 26 which showed symptoms of the disease. L- 1Kb plus ladder, W – Negative check and + - Positive check, 1 – 26, leaf samples from test plants.

in media with (2, 4) ml/L BAP (Table 2). Significant differences were observed due to the effect of the plant growth regulators ($P=1.19X10^{-20}$), genotypes ($P=2.98x10^{-43}$) and the interaction ($P=3.76x10^{-31}$) at 5% CI (Table 2).

Responses of SAMs (2-3) mm sized in vitro

Given the demand and response of these genotypes *in vitro*, five (5) genotypes out of the eight were selected as a sample representation, to ascertain the number of roots, nodes and leaves, as indicators for their establishment of 2-3 mm SAMs (Figure 6b, c, d). Explant sizes of 2-3 mm produced leaves with an average number of 4-6 leaves. Significant differences were observed in the number of leaves produced in presence of auxins (P≤0.001), genotype (P≤0.001) and the interaction between auxin and genotype (P≤0.001). The highest average number of leaves were produced by Alado (7.25±0.58) in media with 2 ml/L BAP, while the least average number leaves were produced by NASE 14 (4.29±0.45) at 1 ml/L BAP (Figure 5c and Figure 6b). Significant differences was also observed in the number of nodes produced in the genotypes (P≤0.001), and interaction of the auxin and the genotype (P=0.005). No significant differences were observed in number of nodes produced due to presence of the auxins (P = 0.435). The highest average number of nodes were observed in Alado (3.74±0.29) in media with 1 ml/L BAP and the least average number of nodes in NAROCASS 1 (0.33±0.16) at 1 ml/L BAP (Figure 5c and Figure 6c). Significant differences were observed in number of roots produced due to the auxins (P≤0.001) and the interaction between auxin and the genotype (P = 0.007). No significant differences were observed for the number of roots produced in relation to the genotype (P = 0.140). The highest average number of roots were observed in Alado (3.38±0.42) at 0.5 ml/L BAP, whereas NAROCASS 1 had the least average number of roots (0.33±0.22) at 2 ml/L BAP (Figure 5c and Figure 6d).

Responses of SAMs (0.5-1) mm sized in vitro

Given the responses of the 2-3 mm sized SAMs, subsequent experiments on SAMs were done on 0.5 -1 µM explant sizes for the six genotypes (Alado, NAROCASS 1, NAROCASS 2, TME 204, NASE 19 and NASE 12) on three different plant growth regulators (PGR) (Benzylamionpurine (BAP), Naphthaleneacetic acid (NAA) and Kinetin (KIN)). The responses of the genotypes varied with the PGRs used and their concentrations (Table 2). All genotypes produced leaves, nodes, roots and showed an increase in plant length (Figures 5d and 7). In some instances, depending on the plant growth regulator (PGR) used; no roots were produced by some genotypes (Figure 7). Overall, NASE 19 performed better than all other genotypes, while Benzylaminopurine (BAP) facilitated regeneration across all genotypes in comparison to the plant growth regulators (Figure 7). At 2 ml/L, BAP facilitated production of leaves generally in all the genotypes followed 4 ml/L BAP. Significant differences were noted in the ability of genotypes to produce leaves from the 0.5 -1 mm sized SAMs, in relation to the PGRs levels (P≤0.001), genotype (P≤0.001) and the interaction

Figure 3. Pictorial representation of collection of plant materials from screen house and the establishment of nodal cuttings in vitro. a) Collected plant material of Alado, b) Single plantlet of Alado, c) Nodal cuttings with no branches and leaves, d) Excising of the nodal cutting to achieve a single node e) Sprouted nodal cuttings of NAROCASS 1, seven days after in vitro establishment on MS medium, with the white arrow showing the bacterial contamination and f) Fully developed plants from nodal cuttings of NASE 13 after 28 days, g) A sample plant to show data collected on leaves, nodes, roots and plant length, h) A plant of NAROCASS 1 which developed roots, leaves, plant height and no roots.

(P≤0.001) (Figures 5d and 7a). Alado did not perform well compared to the rest of other genotypes. The interaction between the PGR levels and genotypes indicated that 2 ml/L BAP, 4 ml/L BAP and 2 ml/L NAA promoted the production of leaves across genotypes (Figures 5d and 7a).

Significant differences were noted in the ability of genotypes to produce number of nodes on the stem from the 0.5 -1 mm sized SAMs, in relation to the auxin levels (P≤.001), genotype (P≤0.001) and the interaction (P≤0.001) (Figure 7b). The auxin concentrations that facilitated the production of nodes were 2 ml/L NAA and the 2 ml/L BAP across the genotypes. At interaction of the auxin and the genotypes, 2 ml/L BAP, 4 ml/L BAP and 2 ml/L NAA facilitated production of nodes (Figure 7b). Significant differences were also noted in the ability of genotypes to produce roots from the 0.5 -1 mm sized SAMs, in relation to the auxin levels (P = 0.021) and the interaction (P≤0.001). No significant differences were noted in relation to the genotypes (P = 0.294) (Figure 7d). In most instances, production of roots was observed in most of the genotypes, in the presence of 2 ml/L BAP, 2 ml/LNAA and in some cases 2 ml/L KIN. The different levels PGRs impacted on the production of roots negatively in Alado compared to the rest of the genotypes (Figure 7d). The interaction of the auxin and genotypes indicated that 2 ml/L NAA, 2 ml/L BAP and 4 ml/L BAP facilitated growth of roots in the genotypes (Figure 7d). Significant differences were noted in the ability of genotypes to have their plant length increase from the 0.5 - 1 mm sized SAMs, in relation to the auxin levels (P = 0.009), genotypes (P≤0.001) and the interaction (P≤0.001) (Figure 7c). The PGRs at 2 ml/L NAA and 2 ml/L BAP promoted increase of the plant length. Alado had the highest plant length among the genotypes. The interaction of PGRs and genotypes facilitated increase in plant length among the genotypes at 2 ml/L BAP, 4 ml/L BAP and 2 ml/L NAA (Figure 5i; Figure 7c).

DISCUSSION

The growth of plants *in vitro* is influenced by factors such as physiological state of the explant, the genotype, the health status of the plant and the culture media. The health status is a very crucial indicator as to how the plant responds *in vitro* (Isah, 2015, 2019). The results indicate that all the genotypes tested for the presence and absence of the cassava mosaic disease (CMD) and cassava brown streak disease (CBSD) were negative indicating that the pre-basic planting materials were free from viruses, therefore certified as healthy. However, it is

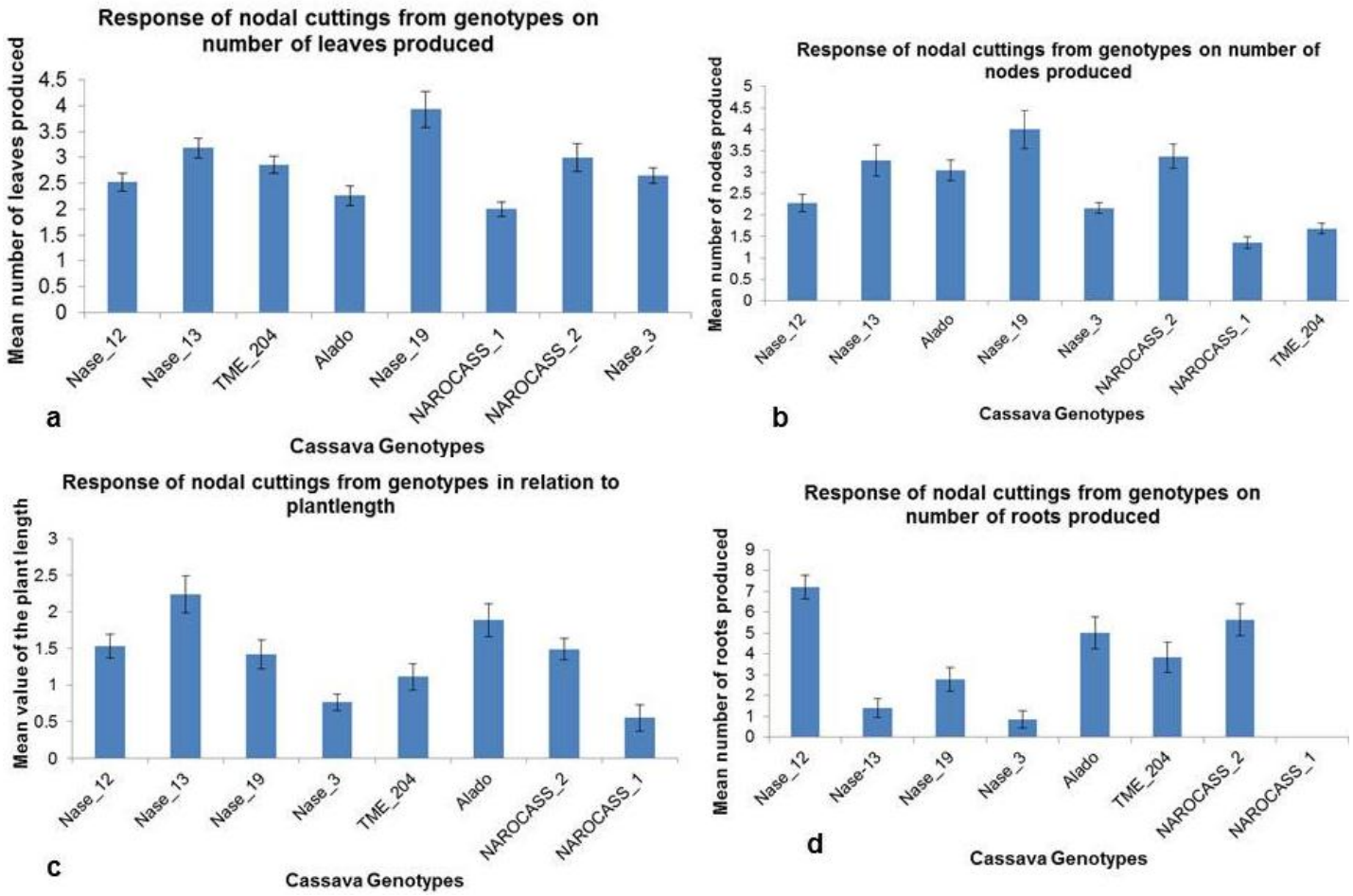

Figure 4. The average number of leaves, nodes, roots and plant height from selected cassava genotypes established in vitro using nodal cuttings. Values are mean ± SD of two independent experiments.

important to note that in both situations (nodal cuttings and shoot apical meristems), some explants were lost due to bacterial or fungal contamination as noted in Figure 3e for nodal cuttings and Table 2 for the 0.5-1 mm SAMs. The death of the explants indicated that the plant innate immune response was triggered on detection of microbe-associated molecular patterns (MAMPs) (He et al., 2007; Bolton, 2009), and the plants ability to recognize pathogen effectors through the resistance proteins was low (Bent and Mackey, 2007; Bolton, 2009); therefore, the available defense mechanisms were not as operative against the pathogens that attacked the explants (Katagiri, 2004; Bolton, 2009).

In some instances, it was noted that the potential of the explants to grow fully into complete plants, was curtailed or reduced, which was observed in plants with reduced plant height. Research in Arabidopsis has indicated that when allocation of resources towards defense responses associated with bacteria and fungus occur in plants *in vitro* (Heil et al., 2000), the plant is forced to adjust growth to reduced levels of carbon available (Bolton, 2009), resulting in poor growth and yields.

Growth in plants is linked to an increase in size associated with cell division and cell elongation (Lastdrager et al., 2014). Cell division and expansion is highly coordinated by a number of molecular networks, which are triggered depending on the environment of the plant (Gonzalez et al., 2012) and therefore facilitate developmental programs that specify tissue and organ identity (Lastdrager et al., 2014). There were differences observed in the responses of the explants to the *in vitro* growth culture conditions, which was evidenced in the parameters measured such as number of leaves, roots, nodes and plant length. The findings indicate that the growth pattern of the plants given the parameters used, was better in nodal cuttings (NC) than shoot apical meristems (SAMs). This could be attributed to the fact that architectural development of nodal cuttings is already set therefore the explant utilizes the available sucrose in the media for energy and biomass, allowing for production of new cells and transition from a vegetative to a generative phase (Rolland et al., 2006; Lastdrager et

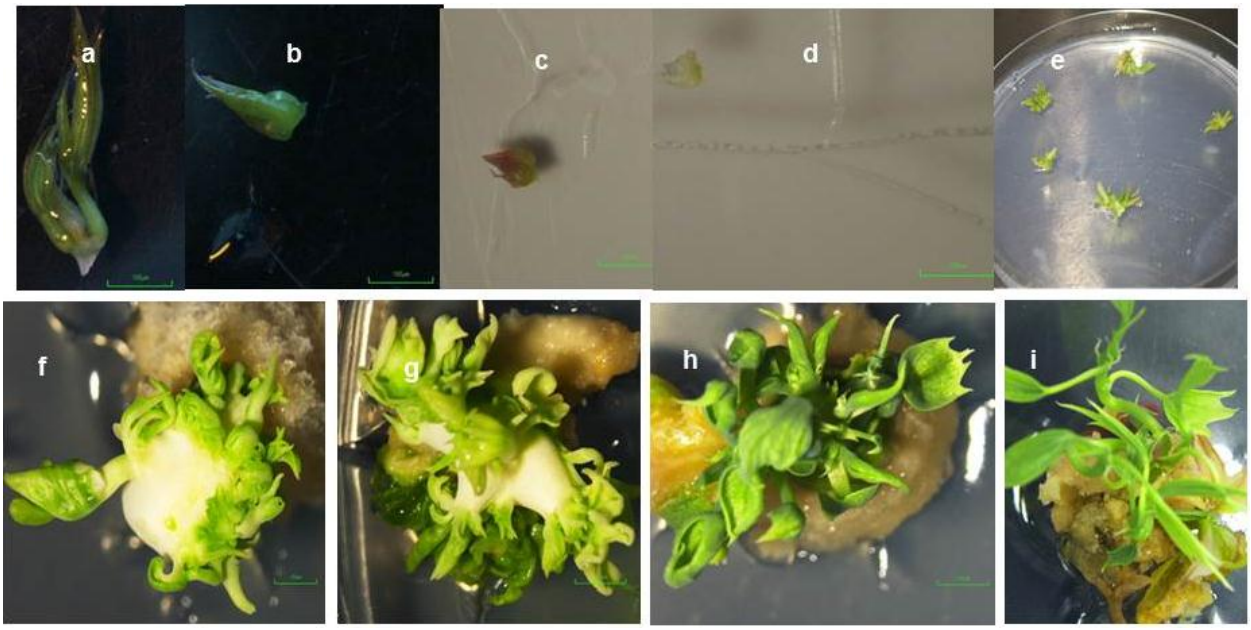

Figure 5. Different stages from collection of SAMs to regeneration into complete plants. a) The SAM explant excised from screen house-grown mother plant, b) SAMs to which leaf petioles have been removed c) 2-3 mm size of SAMs, d) 0.5-1 mm size SAMs, e) Sprouting SAMs, seven days after establishment, f & g) Progressive stages of germination and regeneration, g) A regenerated plant with defined leaves, h) A fully regenerated plant with well-defined stem and leaves.

al., 2014), while for the SAMs, cell division, cell differentiation and maturation into complete plants is possible in the presence of cytokinins and auxins (Murray et al., 2012).

Auxins are central to plant growth and development by moderating the activity of auxin response factors (ARFs), whose metabolism and transport are modulated by sugars, that induce phytochrome-interacting factors (PIFs) that promote growth (Leivar and Quail, 2011; Lastdrager et al., 2014). The study investigated the use of auxins like naphthalene acetic acid (NAA) and cytokinins like benzylaminopurine (BAP) and Kinetin (KIN) to promote growth and development of the SAMs despite the sizes used. The results show that for the different sizes of SAMs, the cytokinin BAP facilitated growth and development of the SAMs better than kinetin. A detailed study in *Arabidopsis thaliana*, indicated that the high cytokinin levels in SAMs, sustain the stem cell population (Shani et al., 2006; Murray et al., 2012), and can do so due to the SHOOT MERISTEMLESS (STM), homeodomain transcription factor which promotes cytokinin sysnthesis by increasing ISOPENTENYL TRANSFERASE (IPT) gene expression (Yanai et al., 2005; Murray et al., 2012). The larger sizes of SAMs (2-3 mm) established in BAP, responded better and faster with regard to growth than the smaller sizes (0.5-1 mm) grown in the same medium. This could be associated with the fact that when cytokinins are applied alone, they have the ability to increase the apical growth leading to initiation of primordia, indicating that cytokinins stimulate growth of SAMs (Yoshida et al., 2011; Murray et al., 2012). On the other hand, while there is a high level of auxins at the SAMs center, previous work suggests there is active repression of auxin responses alongside the high levels of cytokinin signaling (Bartrina et al., 2011). However, it is important to note that auxins play an important role in organ initiation and positioning at the SAM (Murray et al., 2012). This may explain the responses observed in the explants when the auxin NAA was used alone.

The auxins and cytokinins in these experiments were used singly; however, in each situation, depending on the concentration of the auxin and cytokinin, the SAMs were able to regenerate into complete plants by producing the leaves, roots and an increase in plant length was observed. The SAMs are indeterminate structures which continue to grow as long as the environmental conditions favour growth. They form phytomere which is a developmental unit consisting of many leaves, and a node to which the leaves are attached (Lazar, 2003). Similar observations were noted in the emergence of the SAMs despite the sizes used for the different genotypes. The development of leaves indicated the ability of the explants to photosynthesize; given that leaves are organs used by the plant to capture light energy, which further facilitates chemical reactions that sustain the life of the plant. For explants like nodal cuttings (NC), which are determinate, the emergence of leaves is genetically programmed (Lazar, 2003).

The findings indicate that the cytokinin BAP facilitated

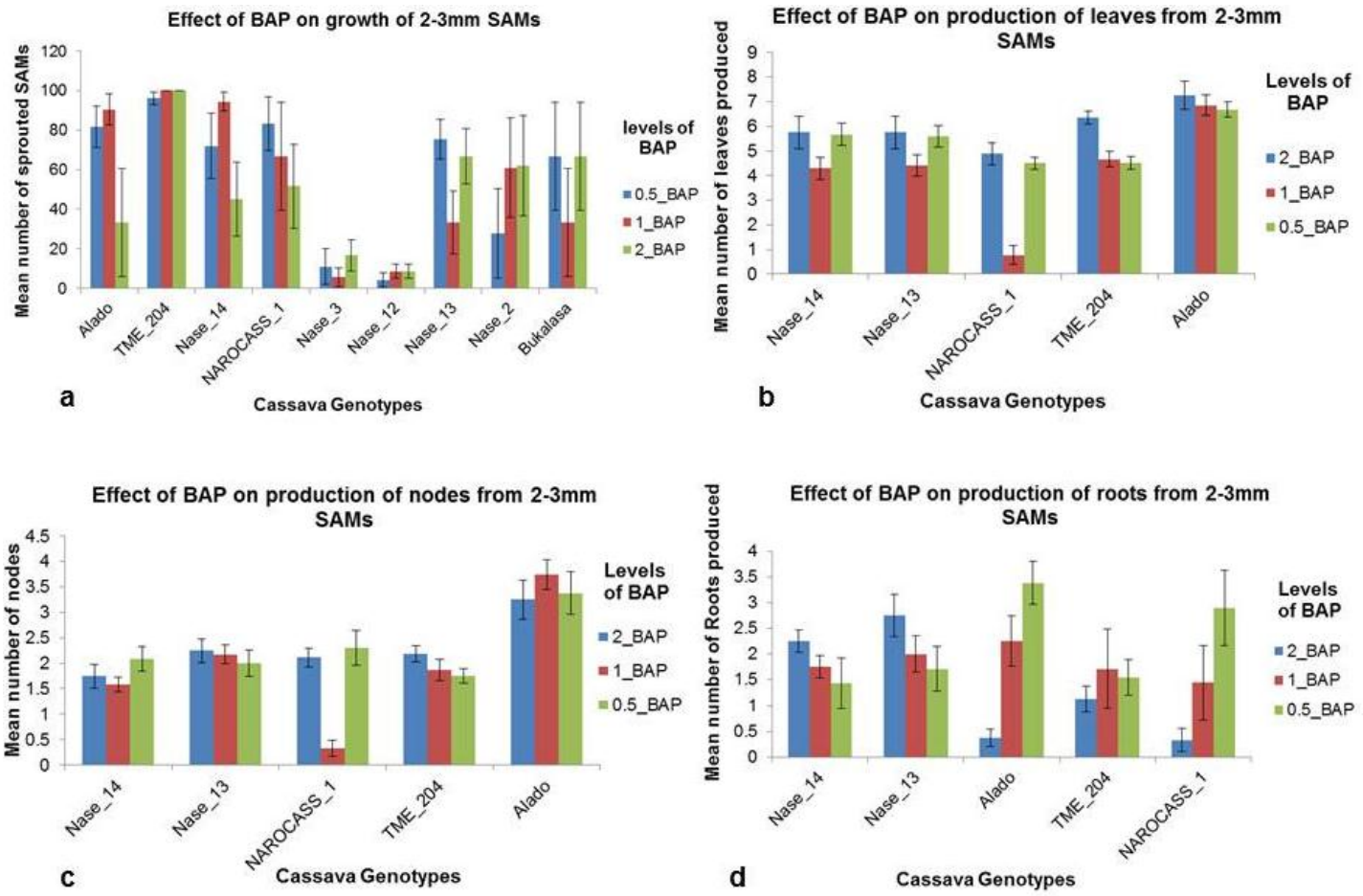

Figure 6. The regenerative ability of the Shoot Apical Meristems (SAMs) (2-3) mm sizes for eight cassava genotype in vitro. Values are means ± SD of two independent experiments.

Table 2. Percentage sprout of 0.5-1mm sized SAMs from cassava genotypes.

	*Sprouting (%) of SAMs					
Plant growth regulator levels	**Nase_19**	**Nase_12**	**NAROCASS_2**	**NAROCASS_1**	**Alado**	**TME_204**
2_BAP	100	25	41.7	8.3	41.7	25
4_BAP	58.3	0	58.3	79.2	0	25
2_NAA	16.7	16.7	8.3	8.3	0	25
4_NAA	100	0	33.3	8.3	0	25
2_KIN	33.3	25	0	8.3	0	0
4_KIN	58.3	58.3	8.3	8.3	16.7	0

*Mean values of sprouted 0.5 -1 mm SAMs from two experiments Values are mean ± SD of two independent experiments. Data was analysed using ANOVA at P≤0.05 using Genstat 12th Edition.

the regeneration of the SAMs into complete plants better than kinetin, despite the fact that similar concentrations were used. In the case of BAP, both concentrations allowed for regeneration at different percentages while in the case of kinetin, 4 ml/L favoured regeneration. This could be associated with the fact that different concentration levels of each cytokinin, may result in expression of different transcription factors that promote different developmental pathways (Bhatia, 2015), as noted in both cytokinins. The responses observed among the genotypes with regard to the number of leaves, nodes, roots produced and plant length noted, which

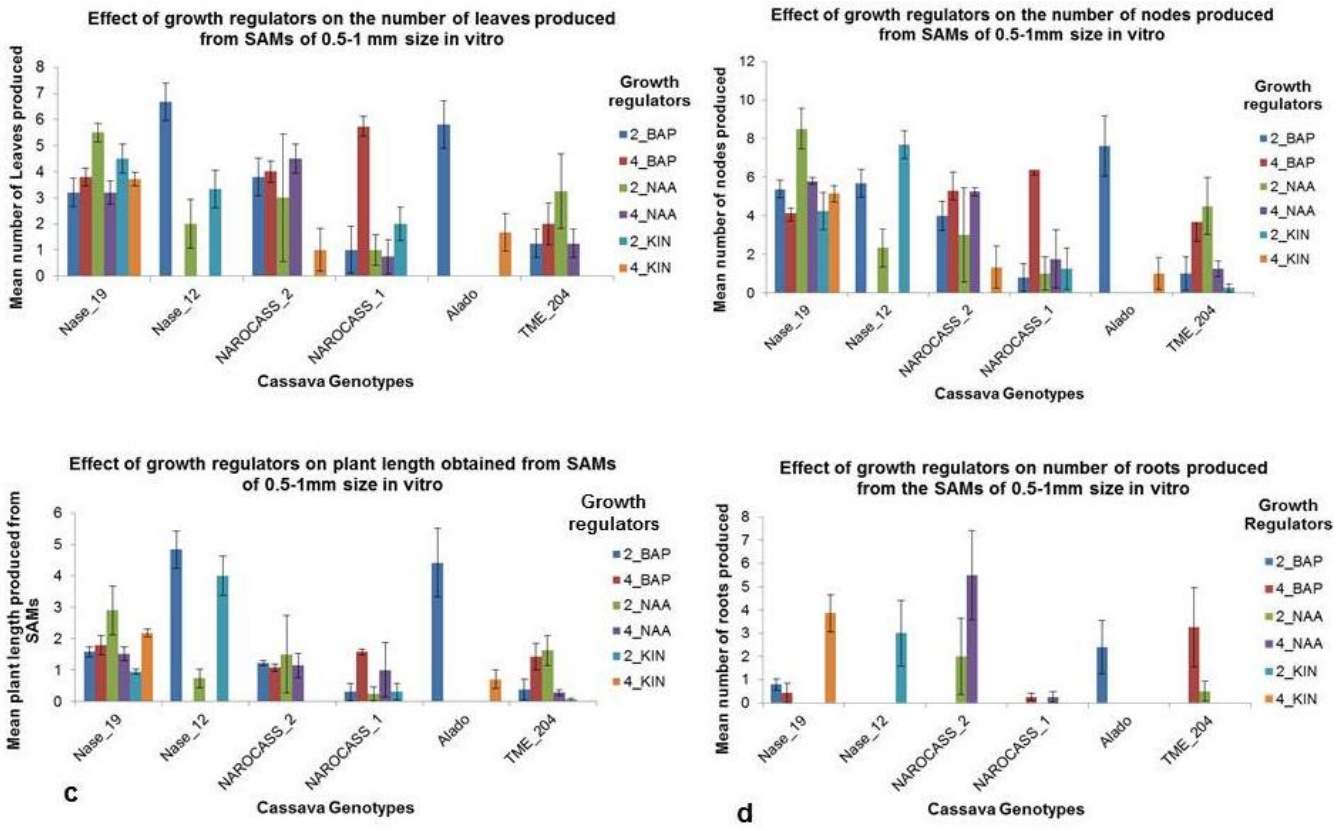

Figure 7. The average number of leaves, nodes, roots and plant length of six cassava genotypes established *in vitro* using SAMs (0.5-1 mm in size) on different concentrations of growth regulators. Values are means ± SD of two independent experiments.

were used to assess the growth and regenerative ability of the SAMs and NCs, suggest that the genetic differences for each cassava genotype, together with *in vitro* conditions like temperature and photoperiod (George et al., 2008) were crucial for regeneration.

The ability of the explants to produce roots plays an important role in absorption of water and minerals nutrients to facilitate plant growth and help the plant to be firmly anchored in the medium. In both explants used, it was noted that root emergence was observed 14 days after co-culture in the appropriate medium (Lazar, 2003). The findings obtained on the number of nodes and the plant height are important indicators as to how many explants can be generated, facilitating bulking up of plant materials all year round through micro propagation. SAMs can also be used for production of disease-free planting materials (George et al., 2008; Isah, 2015).

Conclusions

The number of leaves, roots, nodes and the plant length of cassava plants are reliable indicators of plant growth, development and establishment *in vitro*, for both the nodal cuttings (NC) and shoot apical meristems (SAMs). The auxin and cytokinin at the three concentrations, (2 and 4 ml/L BAP as well as 2 ml/L NAA) facilitated growth and development of the explants into complete plants singly. Plant growth regulators are essential for the development of SAMs, which is not necessary for the NCs. With the demand for certified materials on the rise, the information generated from this study, gives a clear picture of the vigor of different genotypes in vitro. These parameters can also be used to facilitate micro propagation and production of disease-free plants all year round.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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