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Genetic Stability of Cassava Plants Regenerated Through Organogenesis Using Microsatellite Markers

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Abstract: Tissue culture technology of cassava (*Manihot esculenta* Crantz) is a viable alternative to currently adopted techniques for mass propagation, germplasm conservation and genetic improvement. However, somaclonal variation is a common phenomenon in tissue culture which makes it mandatory to monitor the genetic stability of plants. Therefore, the objective of this study was to evaluate the genetic stability of cassava plants regenerated from axillary bud explants through direct organogenesis using simple sequence repeat (SSR) markers. High shoot regeneration (81.2 – 90.0%) occurred in MS medium supplemented with 10 mg/L 6-bnzylaminopurine (BAP) and multiple shoots (2 – 4 shoots per from axillary bud explant) were formed for all the three cultivars (TME14, TMS60444 and Kibandameno) tested. High frequency of rooting (100%) was obtained after transferring the plantlets to cassava basic medium (CBM) and the rooted plants were successfully hardened and acclimatized in the glasshouse with 100% survival rate. Three-month old plants exhibited normal morphological characters comparing with the mother plant. A total of 10 SSR markers were used to validate the genetic homogeneity amongst five randomly selected plants along with the donor mother plants. DNA fingerprints of axillary bud regenerated plants displayed monomorphic bands similar to mother plant, indicating homogeneity among the regenerated plants with donor mother plant. The effect of subculture frequency on genetic stability of axillary bud-derived regenerants and micropropagated plants was also assessed using SSR markers. All the SSR profiles from axillary bud regenerants and micropropagated plants were monomorphic and comparable to mother plants from 1st to 5th subculture, confirming the genetic stability among clones and mother plants. At the 6th subculture, similarity indicators between the progenies and the mother plants ranged from 0.95 to 1.0 and such a similarity indicated a very low polymorphism. The dendrograms generated through Unweighted Pair Group Method with arithmetic mean (UPGMA) analysis of the 6th subculture revealed 96% similarity amongst axillary bud regenerants and micropropagated plants with donor mother plant. This low polymorphism ratio between mother plants, axillary bud regenerants and micro-propagated plants indicates the little effect of somaclonal variations, the high genetic similarity between mother plants and progenies and demonstrates the reliability of this propagation system for cassava. These results suggest that direct organogenesis from the axillary buds is the safest method for regeneration of true-to-type plants and this system can be used for clonal mass propagation, germplasm conservation and genetic transformation of cassava.

Keywords: Cassava, Axillary Buds, Genetic Stability, Simple Sequence Repeats, Micropropagation

1. Introduction

Cassava (*Manihot esculenta* Crantz) is a staple food crop for more than a billion people in 105 sub-tropical and tropical countries [1]. The tuberous storage roots are rich in carbohydrates and can be cooked or processed for human consumption. Several agronomic traits make cassava suitable as a subsistence crop for rural farmers. It is drought-tolerant, grows well on low-nutrient soils and requires minimal resource input to cultivate [2, 3]. In addition, the tubers can be left in the soil for up to 3 years after maturation without decay and harvested when needed [4], making it very useful food security crop. With the ongoing climate change and

looming global energy crisis, cassava is also an ideal crop for bioenergy generation, biomaterial production and animal feed due to its high biomass productivity and high starch quantity and quality [5]. The global cassava harvest yield is about 277 million metric tonnes, of which Latin America, Asia and Africa account for 10%, 32% and 56%, respectively [6]. This production is far much below the yield potential of cassava under near-optimum climatic conditions. Cassava is vegetatively propagated using field stem cuttings and low production is caused by unavailability or limited access to planting materials and infection by diseases transmitted through successive generations [7].

To increase cassava production, biotechnology interventions are required to supply quality clean planting materials, development of high yielding and disease resistant varieties using both conventional breeding and transgenic technologies [8-10]. Plant tissue culture is recognized as one of the key areas of biotechnology because of its potential use in mass propagation hence season-independent production of planting materials [11], *in vitro* conservation and as a tool for genetic improvement. For clonal propagation of elite materials, *in vitro* conservation and *Agrobacterium*-mediated genetic transformation, a reliable and efficient *in vitro* system to regenerate phenotypically and genotypically identical plants is a pre-requisite. However, tissue culture environment and influence of culture conditions like plant growth regulators, culture media, type of explant, temperature, pH etc. induces somaclonal variation during the culture process ultimately leading to genomic changes in regenerated plants [12]. Even at optimal levels, frequent transfers of cultures during micropropagation can result in genetic variation, thus questioning the clonal fidelity of regenerated and micropropagated plants. The occurrence of somaclonal variation is a potential drawback when the propagation of an elite variety is intended, where clonal fidelity is required to maintain the advantages of desired elite genotypes (e.g., superior growth, starch properties, disease resistance, and other quality traits). In order to make this technology commercially viable, it is important to verify that plants obtained by micropropagation are true-to-type to the parent plant from which they were derived.

In comparison to various morphological, cytological and protein markers used in the detection of variation in tissue culture raised plants, molecular markers are more stable, heritable and highly reproducible. Several molecular markers such as RAPD, ISSR, SSR, RFLP-ISSR have been used to detect genetic uniformity and identify any potential somaclonal variations in plants produced through micropropagation [13 - 15]. Of these, simple sequence repeat (SSR) markers have advantages of high genomic abundance throughout the genome, co-dominant, locus-specific, greater reproducibility, high level of polymorphism, informative and strong discriminatory power [16, 17]. Many reports have documented the assessment of genetic fidelity of micro-propagated plants using microsatellite markers [17 - 19]. However, there is no report on assessment of genetic integrity of *in vitro* regenerated cassava plants by SSR markers.

Among the farmer-preferred cassava cultivars in East Africa, TME14 and Kibandameno are landraces ranked highest by farmers. They are being scored excellent for plant establishment, early maturation, high yield, mealiness, flavor, cooking qualities and market value [20, 21]. Additionally, TME14 is resistant to cassava mosaic disease (CMD) and Kibandameno has a sweet pleasant flavor, thus making them ideal selection for growers. So far, *in vitro* regeneration of plants using axillary buds as explants and clonal fidelity of micropropagated plants of cultivars TME14 and Kibandameno has not been done. The aim of this study therefore, was to assess the genetic fidelity of cassava plants regenerated from axillary buds using SSR markers. In addition, this study evaluated the effect of subculture frequency on genetic fidelity of axillary derived and micropropagated cassava plants to clarify the variation pattern and frequency of somatic variation in the course of the subculture.

2. Material and Methods

2.1. Plant Materials

Three cassava cultivars namely TMS60444, TME14 and Kibandameno were used in the present study. The stem cuttings of each cultivar were obtained from Kenya Agricultural and Livestock Research Organization (KALRO) and established in pots in the glasshouse at the School of Biological Sciences, University of Nairobi. After 4 weeks, nodal segments of the healthy plants were collected, sterilized and used for establishment of *in vitro* plantlets on cassava basic medium (CBM; MS [Murashige and Skoog] salts with vitamins, 2 μM CuSO4, 2% sucrose, 0.3% Gelrite, pH 5.8) for subsequent experiments in tissue culture laboratory at the School of Biological Sciences, University of Nairobi.

2.2. Induction of Axillary Bud from Nodal Explants

Using a sterilized scalpel, 30 nodal explants of l0 mm in length from each of the three cultivars were cut from 2 - 3 week old *in vitro* plantlets, and placed horizontally on the 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. The explants were cultured for 4 - 10 days at 28°C in the dark. The enlarged axillary bud were removed from the nodal explants with sterile syringe needles under a binocular microscope as described by Zainuddin *et al*. [22] and Nyaboga *et al*. [23] and transferred to fresh CAM at a density of 5 per Petri dish plate.

2.3. Induction of Shoots and Regeneration of Plants

To induce shooting from the axillary buds, plates containing the axillary buds were cultured on CAM media at 28°C under 16/8 h photoperiod and transferred onto fresh CAM media every 14 days. The percentage of axillary buds

regenerating to plants was recorded. The number of shoots formed per axillary bud was also recorded for each cultivar. The developed shoots were transferred to a cassava basic culture medium (CBM) for rooting and subsequent growth. After 5 weeks in CBM, well rooted plantlets were acclimatized in the glasshouse. The remaining plants were subcultured by aseptically cutting the stems (at least two nodes per cutting) using a scalpel and planting on CBM medium by submerging the lower node into the medium. After every five weeks thereafter the cultures were transferred to a fresh CBM medium. The cultures were maintained until the 6th subculture to determine the effect of age of culture in the induction of somaclonal variation *in vitro*. Leaf samples from each of the subculture cycle were collected for DNA extraction and SSR analysis.

2.4. Effect of Subculture Frequency of in Vitro Micropropagated Plants

Nodal segments of three to five-week old plants planted in pots in glasshouse were used for establishment of *in vitro* plantlets on CBM. Shoots were developed after 5 weeks of culture at 28°C under 16/8 hour photoperiod. Subculturing was done by aseptically cutting the stems (at least two nodes per cutting) using a scalpel and planting on CBM medium by submerging the lower node into the medium. After every five weeks thereafter the cultures were transferred to a fresh CBM medium. The cultures were maintained until the 6th subculture to determine the effect of age of culture in the induction of somaclonal variation *in vitro*. Leaf samples from each of the subculture cycle were collected for DNA extraction and SSR analysis.

2.5. Acclimatization of the in Vitro Regenerated Plants to the Glasshouse

A total of 12 well rooted plants per cultivar were carefully removed from the culture bottles and washed in excess water to remove agar attached to the roots. The cleaned plants were transferred to plastic pots containing sterilized forest soil. The plants were watered with tap water and transparent polythene bags were used to cover the plants in the pots. The bags were held at the base of each pot by a rubber band to create an air tight micro environment for the plantlets. One edge of the polythene bags was cut open using a pair of scissors after 10 days, the second edge after 20 days and the entire polythene bag was removed after 30 days. The number of surviving plants was recorded. The acclimated plants of all the three cultivars were grown in the glasshouse for three months, where they were assessed at the morphological level for the occurrence of off-types.

2.6. Genetic Stability of Axillary Bud-Derived and Micropropagated Plants

Five plants from each of the three cultivars were randomly selected for determining the occurrence of microsatellite DNA somaclonal variation among the axillary bud-derived regenerants, different cycles of subculture of axillary bud-derived and micropropagated plants.

2.6.1. DNA Extraction

Genomic DNA was extracted from mother plants, axillary bud-regenerated plants, different subculture cycles (starting from subcultures 1 until subculture 6) of axillary bud-regenerated and micropropagated plants (Figure 1) using the modified cetyltrimethylammonium bromide (CTAB) extraction protocol as described by Sharma *et al*. [24]. Aliquots of each sample were quantified on 0.8% agarose gel along with a standard concentration of DNA, constructed from phage lambda DNA, to verify the quantity and quality of the extracted DNA. The concentration of each sample was estimated by visual comparison of the intensity of the two bands (DNA samples and standards). The DNA samples were then diluted to a working concentration of 10 ng/µL.

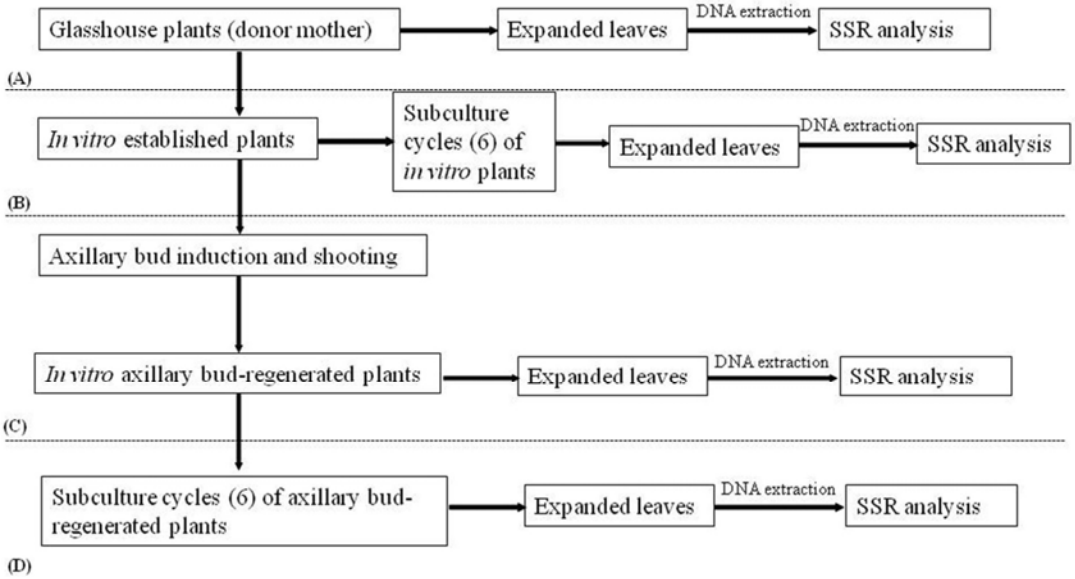

Figure 1. *Processes used to obtain the leaf samples used in SSR analysis. (A) Mother plants, (B) subculture cycles of micropropagated plants, (C) plant regeneration from axillary bud via direct organogenesis, and (D) subculture cycles of axillary bud regenerated plants.*

2.6.2. Simple Sequence Repeat (SSR) Markers Analysis

A set of 11 simple sequence repeat primers (Table 1) were screened for their ability to amplify DNA from axillary bud regenerants, different subculture cycles of regenerants and micropropagated and donor mother plants to differentiate somaclones. Amplifications were carried out in a total volume of 20 µl a mixture containing 20 ng of genomic DNA, 4 µl of 5X PCR buffer containing 15 mM $MgCl_2$ and 0.2 mM dNTPs, 1 unit Taq polymerase (Bioline, USA) and 0.1 µM of forward and reverse SSR primers from Inqaba Biotec (South Africa). The PCR consisted of an initial DNA denaturation at 95°C for 5 minute, 30 cycles comprising denaturation at 94°C for 30 seconds, annealing at 54°C for 30 seconds, extension at 72°C for a minute and a final extension at 72°C for 7 minutes. The amplified DNA fragments were separated on a 2.5% agarose (Sigma Aldrich, USA) gel using 1X Tris-Acetate EDTA buffer and stained with ethidium bromide (0.5 µg/mL). The sizes of the amplicons were estimated by comparison with the standard molecular DNA using 100 bp ladder (Bioneer, Inc.). Gels were visualized under UV and photographed by a Gel Documentation System. To identify the somaclonal variants, only the clear polymorphic fragments from the DNA amplification were considered. The bands were counted based on the presence or absence in the gels. Variations in the electrophoretic profile occurring within regenerants and mother plants of the same cultivar were considered as possible somaclonal variations. From the generated binary data, DendroUPGMA server [25] was used in calculating matrix distances between the regenerants and their respective donor mother plants. The distance matrices were generated based on Jaccard's similarity coefficient [26]. Similarity matrices were then subjected to cluster analysis of unweighted pair group method with arithmetic mean (UPGMA) and dendrograms constructed using FigTree software (Version 1.4.2).

Table 1. *List of SSR primers used for assessment of genetic stability in axillary bud-derived and micropropagated cassava plants.*

Primer code	Forward primer sequence	Reverse primer sequence	Repeat motif	Product size (bp)
SRY 106	GGAAACTGCTTGCACAAAGA	CAGCAAGACCATCACCAGTTT	(CA)24	270
SRY 3	TTAGCCAGGCCACTGTTCTT	CCAAGAGATTGCACTAGCGA	(CA)17	247
SRY 9	ACAATTCATCATGAGTCATCAAC	CCGTTATTGTTCCTGGTCCT	(GT)15	278
SRY 51	AGGTTGGATGCTTGAAGGAA	CGATGCAGGAGTGCTCAACT	(CT)11CG(CT)11(CA)18	298
SRY 100	ATCCTTGCCTGACATTTTGC	TTCGCAGAGTCCAATTGTTG	(CT)17TT(CT)7	210
SRY 103	TGAGAAGGAAACTGCTTGCAC	CAGCAAGACCATCACCAGTTT	(GA)22	272
SRY 35	GCAGTAAACCATTCCTCCAA	CTGATCAGCAGGATGCATGT	(GT)3GC(GT)11(GA)19	282
SRY 45	TGAAACTGTTTGCAAATTACGA	TCCAGTTCACATGTAGTTGGCT	(CT)27	228
SRY 78	TGCACACGTTCTGTTTCCAT	ATGCCTCCACGTCCAGATAC	(CT)22	248
SRY 175	TGACTAGCAGACACCGGTTTA	GCTTAACAGTCCAATAACGATAAG	(GA)38	136

3. Results

3.1. Induction of Axillary Buds and Regeneration of Plants

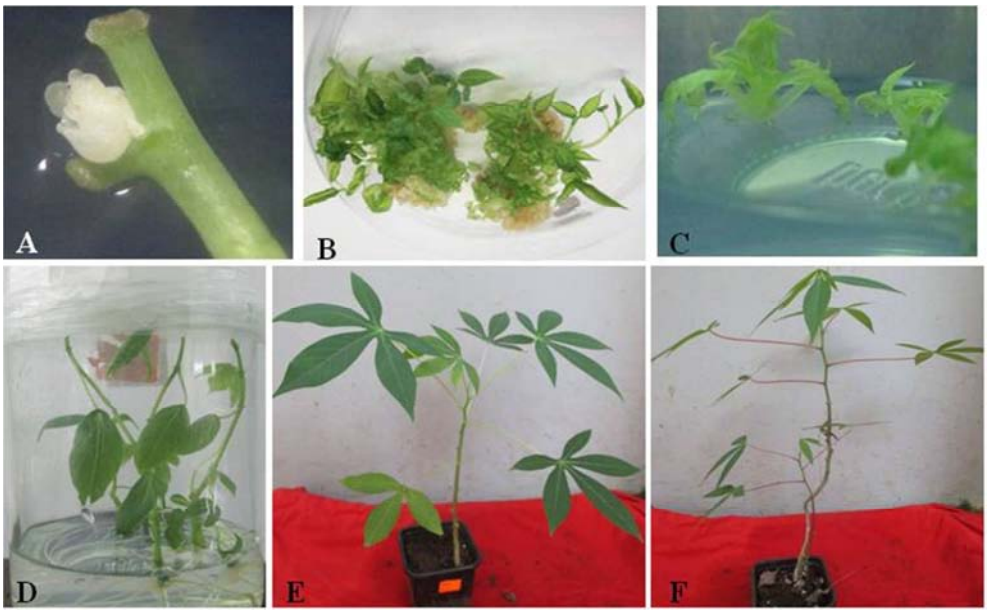

Figure 2. *In vitro regeneration of cassava using axillary bud explants. (A) Induced axillary bud (cultivar TMS60444) from nodal explant after 10 days of culture on CAM in darkness, (B) Multiple shoots forming from axillary buds after three weeks of culture on CAM, (C) shoots in CBM medium, (D) rooted plantlets in CBM medium after three weeks of culture, (E) acclimatized axillary bud regenerated plant of cultivar TMS60444 in a glasshouse, and (F) acclimatized axillary bud regenerated plant of cultivar Kibandameno in the glasshouse.*

In this study, nodal explants incubated on MS supplemented with 1 mg/L BAP induced axillary buds (Figure 2A) in all the three cultivars after 4 – 10 days. Axillary bud induction frequencies in cultivars TMS60444 and TME14 were significantly higher ($p \leq 0.05$) compared to Kibandameno, with TMS60444 recording the highest induction frequency (Table 2). There was no significant difference among the three cultivars with regards to shoot induction frequencies. Multiple shoots were formed from the axillary buds after four weeks of culture in medium containing 1 mg/L BAP (Figure 2B). Cultivar TME14 produced the highest number of shoots (3.85) per axillary bud explant compared to Kibandameno and TMS60444. High frequency of rooting (100%) was obtained after transferring the plantlets CBM media (Figure 1C and D).

Table 2. *Average frequencies of axillary bud and shoot induction and number of shoots produced per axillary bud explants the three cassava cultivars.*

Cultivar	Axillary bud induction frequency in (%)	Shoot induction frequency (%)	Average no. of shoots produced per axillary bud explant
Kibandameno	54.84 ± 22.74a	82.19 ± 20.88a	2.09 ± 0.35a
TME14	78.41 ± 24.6b	90.00 ± 11.55a	3.85 ± 0.75b
TMS60444	91.06 ± 17.86b	83.85 ± 23.14a	2.55 ± 0.44a

Data was arcsine transformed before ANOVA. Means followed by the same letters are not significantly different at $p \leq 0.05$ according to Tukey's HSD test. (±) represents standard deviation of the mean.

3.2. Acclimatization and Phenotypic Characterization of Plants in the Glasshouse

All plants produced new leaves after two weeks of transfer to sterile soil mixed with manure and the plants were successfully acclimatized in the glasshouse (Figure 1E and F) with 100% survival rate was obtained. Visual assessments of 3-month-old axillary bud-derived plants of the three cultivars growing in the glasshouse showed that the plants were phenotypically normal and identical with their donor mother plants.

3.3. Genetic Stability of Axillary Bud-Derived Plants

Of the 11 SSR primers used for checking the fidelity of *in vitro* generated plants, 10 SSR primers generated clear, reproducible and distinguishable patterns of bands in the size range 130 – 850 bp for the mother plants and their clonal derivatives. The number of bands for each primer varied from 2 to 4 with an average of 2.7 bands per primer (Table 3). All the bands were monomorphic for all the axillary bud-regenerants and mother plants (Figure 3A).

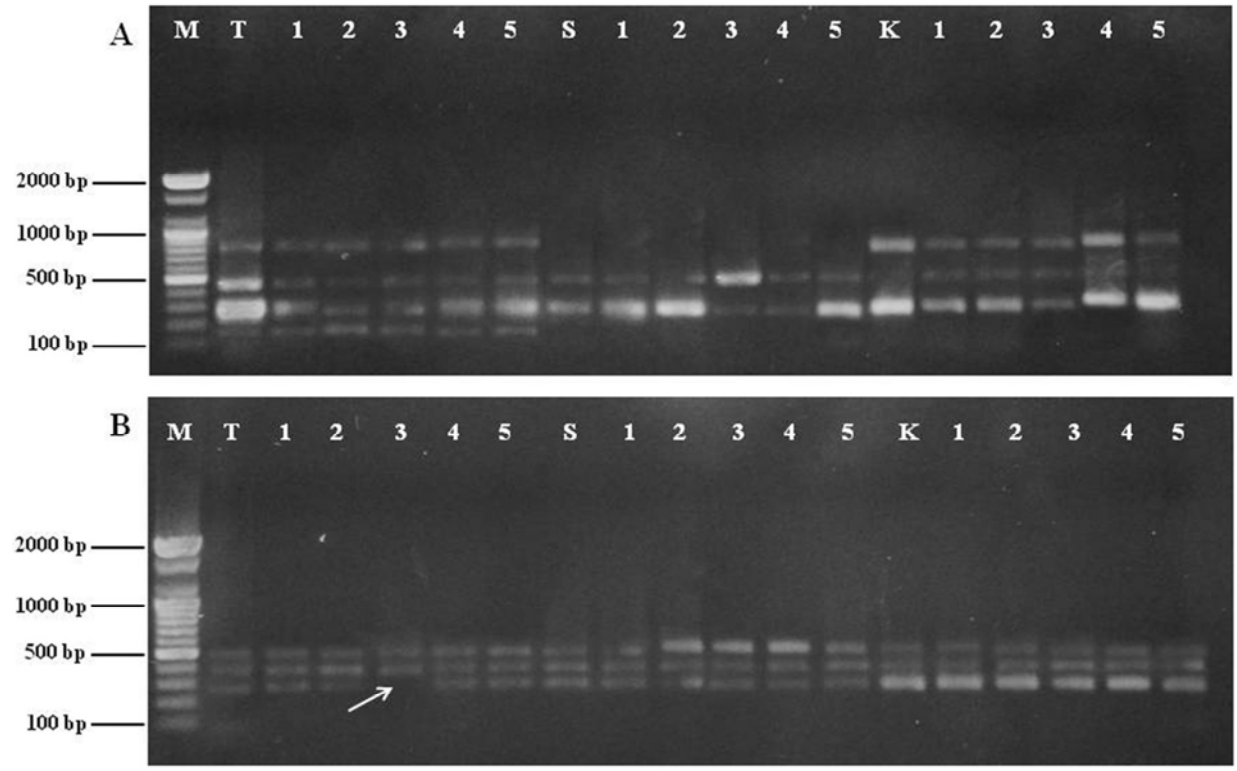

Figure 3. *Assessment of genetic fidelity of axillary bud regenerated and after 6th subculture. (A) SSR profiles of axillary bud regenerated plants alongside their mother plants after amplification using primer SRY51, and (B) SSR profiles of 6th subculture of micropropagated plants alongside their mother plants after amplification using primer SRY35. Lanes: M- 100 bp DNA marker; T, S, and K- Donor mother plants of cultivars TME14, TMS60444 and Kibandameno, respectively; 1 to 5 - regenerants from each cultivar. Arrow indicates the somaclonal variant from regenerants of cultivar TME14.*

Table 3. *Number of monomorphic and polymorphic bands, and the size range of PCR products amplified from axillary bud-derived regenerants and donor mother plants.*

Primer code	Total number of amplified bands	No. of monomorphic bands	No. of polymorphic bands	Percentage monomorphism	Range of amplicon sizes (bp)
SRY 106	2	2	0	100	200 - 270
SRY 3	2	2	0	100	170 - 200
SRY 9	2	2	0	100	180 - 290
SRY 51	4	4	0	100	190 - 500
SRY 100	3	3	0	100	200 - 450
SRY 103	3	3	0	100	290 - 500
SRY35	3	3	0	100	290 - 480
SRY 45	4	4	0	100	130 - 450
SRY 78	2	2	0	100	280 - 400
SRY 50	2	2	0	100	190 - 280
Total	27	27	0		

A similarity matrix based on Jaccard's coefficient revealed that the pair-wise value between the mother plants and the axillary bud-derived regenerants was 1 (Table 4), indicating 100% similarity. Dendrogram analysis based on the Jaccard's similarity coefficient revealed 100% genetic similarity among the mother plants and its derivatives (axillary bud-derived plants) (Figure 4). In the dendrogram, the axillary bud-derived plants were grouped into three clusters based on the mother plant cultivar (Figure 4). SSR analysis revealed true-to-type plants within each cluster viz., cluster I: TME14 mother plant to regenerants 1 - 5; cluster II: TMS60444 mother plant to regenerants 1 - 5; and cluster III: Kibandameno mother plant to regenerants 1 – 5.

Table 4. *Similarity matrices of mother plants of cultivars TME14, TMS60444 and Kibandameno and their respective axillary bud-derived regenerants based on Jaccard's similarity coefficient of SSR data.*

	ME	E1	E2	E3	E4	E5	MS	S1	S2	S3	S4	S5	MK	K1	K2	K3	K4	K5
ME	1	1	1	1	1	1	0.769	0.769	0.769	0.769	0.769	0.769	0.741	0.741	0.741	0.741	0.741	0.741
E1		1	1	1	1	1	0.769	0.769	0.769	0.769	0.769	0.769	0.741	0.741	0.741	0.741	0.741	0.741
E2			1	1	1	1	0.769	0.769	0.769	0.769	0.769	0.769	0.741	0.741	0.741	0.741	0.741	0.741
E3				1	1	1	0.769	0.769	0.769	0.769	0.769	0.769	0.741	0.741	0.741	0.741	0.741	0.741
E4					1	1	0.769	0.769	0.769	0.769	0.769	0.769	0.741	0.741	0.741	0.741	0.741	0.741
E5						1	0.769	0.769	0.769	0.769	0.769	0.769	0.741	0.741	0.741	0.741	0.741	0.741
MS							1	1	1	1	1	1	0.792	0.792	0.792	0.792	0.792	0.792
S1								1	1	1	1	1	0.792	0.792	0.792	0.792	0.792	0.792
S2									1	1	1	1	0.792	0.792	0.792	0.792	0.792	0.792
S3										1	1	1	0.792	0.792	0.792	0.792	0.792	0.792
S4											1	1	0.792	0.792	0.792	0.792	0.792	0.792
S5												1	0.792	0.792	0.792	0.792	0.792	0.792
MK													1	1	1	1	1	1
K1														1	1	1	1	1
K2															1	1	1	1
K3																1	1	1
K4																	1	1
K5																		1

Lanes ME, MS and MK represent mother plants of cassava cultivars TME14, TMS60444 and Kibandameno respectively. E1-E5, S1-S5 and K1-K5 represent axillary bud-derived regenerants of cultivars TME14, TMS60444 and Kibandameno, respectively.

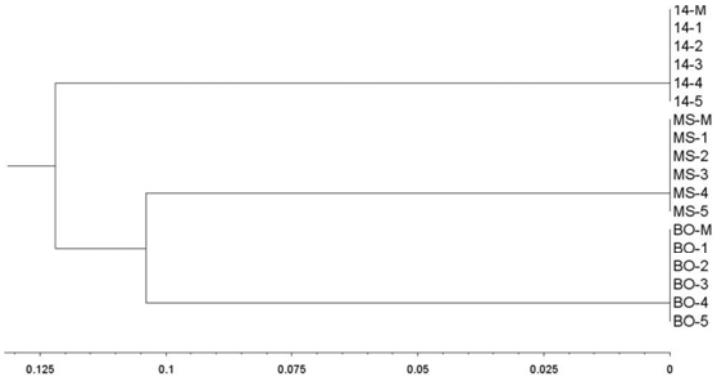

Figure 4. *Dendrogram showing genetic relationships of axillary bud regenerated plants (1 - 5) and the mother plants (14-M, MS-M and BO-M) of three cassava cultivars by UPGMA cluster analysis from SSR data. 14-M, MS-M and BO-M represents cultivars TME14, TMS60444 and Kibandameno, respectively. 14-1 to14-5, MS-1 to MS-5 and BO-1to BO-5 represent regenerants of cultivars TME14, TMS60444 and Kibandameno, respectively.*

3.4. Effect of Subculture Frequency on Genetic Stability of Axillary Bud-Derived Regenerants and Micropropagated Plants

The effect of subculture frequency on genetic variations of axillary bud-regenerated plants was analyzed using 10 SSR markers. The number of bands varied from 2 to 4, with an average of 3 bands per SSR primer. These 10 SSR primers generated a total of 162 amplicons from all the six subcultures of axillary bud-derived plants, and the band sizes ranged from 130 - 850 bp. The banding pattern of PCR amplified products from plants of 1st – 5th subculture was monomorphic. The genetic similarities of the mother plant and subcultured plants based on SSR markers varied from 0.955 (mother plant and subcultured plants of 6th generation) to 1 (subcultured plants of 1st – 5th generations) with an average value of 0.9775. Dendrogram analysis based on the Jaccard's similarity coefficient revealed 100% genetic similarity among the mother plants and its derivatives from 1st to 5th subcultures. At the 6th subculture, the mother plants and axillary bud regenerants were highly similar (similarity coefficient level was 1) for cultivars TME14 and Kibandameno. Only one variant was observed at the 6th subculture for cultivar TMS60444 (Figure 5) at genetic similarity of 0.955 and the polymorphism level was 3.70%. This polymorphism was observed in one of the regenerants of cultivar TMS60444 using primer SRY78.

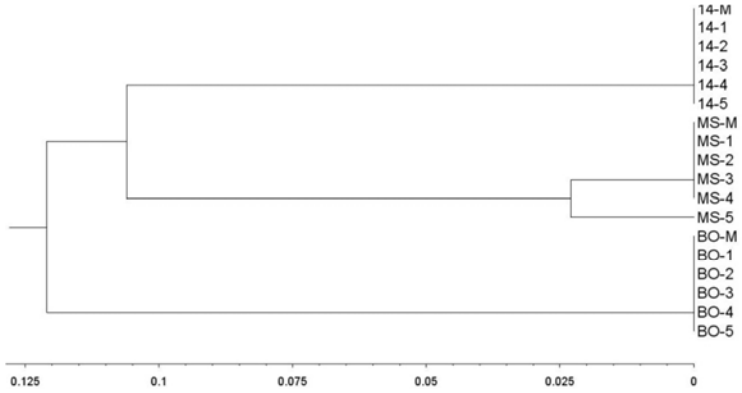

Figure 5. *Dendrogram showing genetic relationships of the 6th subculture of axillary bud regenerated plants (1 - 5) and the mother plants (14-M, MS-M and BO-M) of three cassava cultivars UPGMA cluster analysis from SSR data. 14-M, MS-M and BO-M represents cultivars TME14, TMS60444 and Kibandameno, respectively. 14-1 to14-5, MS-1 to MS-5 and BO-1to BO-5 represent 6th subculture of axillary bud regenerants of cultivars TME14, TMS60444 and Kibandameno, respectively.*

For the micropropagated plants, the banding profiles of PCR amplified products from plants of 1 – 5th subculture were monomorphic and similar to the mother plants. Considering the mother plants and their clones displayed monomorphic banding pattern, it can be suggested that *in vitro* regenerated clones from 1 – 5th subcultures maintained their genetic integrity. A similarity matrix based on Jaccard's coefficient revealed that the pair-wise value between the mother plants and micropropagated plants from first to fifth subcultures was 1, indicating 100% similarity. Polymorphism was observed in micropropagated and mother plants of cultivar TME14 and TMS60444 (Figure 3B). For the 6th subculture two progenies were grouped together with their respective cultivars TME14 and TMS60444 mother plants at a similarity level of 96% and 95%, respectively (Figure 6). In view of a 7-month period of *in vitro* propagation, such a similarity indicated a very low polymorphism.

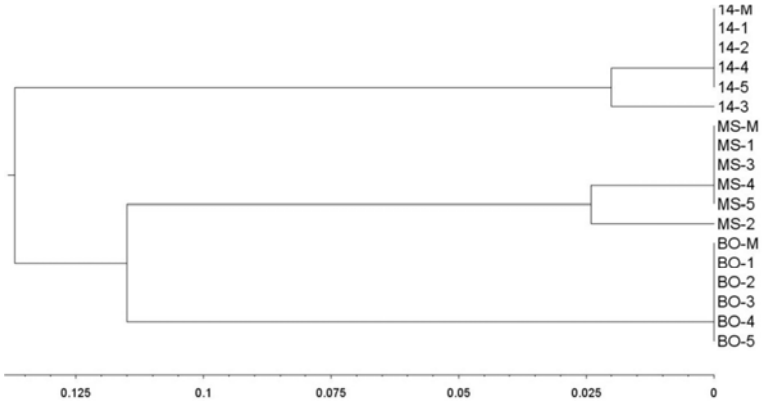

Figure 6. *Dendrogram showing genetic relationships of the 6th subculture of micropropagated plants (1 - 5) and the mother plants (14-M, MS-M and BO-M) of three cassava cultivars UPGMA cluster analysis from SSR data. 14-M, MS-M and BO-M represents cultivars TME14, TMS60444 and Kibandameno, respectively. 14-1 to14-5, MS-1 to MS-5 and BO-1 to BO-5 represent 6th subculture of micropropagated plants of cultivars TME14, TMS60444 and Kibandameno, respectively.*

4. Discussion

Cassava propagation using field stem cuttings as the classical method is not adequate for rapid and healthy multiplication. Cassava production is also faced with other challenges such as bacterial and viral diseases which lead to insufficiency of planting materials. The fact that farmers obtain cuttings from their previous crop or neighbours, suggests that without a deliberate program for restricting movement of infected material would perpetuate the spread of diseases [27]. An alternative method that proves to remediate the low coefficient of multiplication and infections of cassava is *in vitro* micropropagation. However, somaclonal variation in plants recovered from *in vitro* cultures has been reported in crop plants [28]. This may lead to phenotypic changes in the propagated clones and alternation in the agronomic performance [29]. Therefore, in the clonal regeneration, one of the most crucial concerns is to retain genetic stability of *in vitro* propagating material. Hence, a quality check up for true-to-type planting material at an early stage of development is considered to be very useful in plant tissue culture [30] and molecular markers serve as an important tool to check the genetic uniformity and true to type nature of micropropagated plants.

In this study, there were significant differences ($p \leq 0.05$) in the axillary bud induction frequencies among the three cultivars. This suggests the cassava cultivars exhibit a differential response in the ability to produce axillary buds from nodal explants, thus it is paramount to test individual cultivars for their axillary bud induction response. Shoots were successfully regenerated from axillary bud explants in all the three cultivars used in this study. Several studies have also reported *in vitro* nodal culture of cassava [31 - 33]. Among various methods of *in vitro* propagation, use of axillary buds is the most widely used system [32] owing to its simplicity and high multiplication rates. In addition, this system of *in vitro* propagation possesses low risk of genetic instability as reported in other crops due to existence of organized meristems [13]. Micropropagation of plants from the cultures of pre-formed structures such as axillary buds has been reported to maintain uniformity among regenerated plants [34].

The axillary bud-derived plants of the three cultivars were acclimatized and all the plants established in the glasshouse were phenotypically normal and identical with their donor mother plants indicating minimal or absence of somaclonal variations. This is not surprising given the fact that organized structures such as axillary meristems tend to produce micropropagated progeny with reduced or no variation among them due to their origin from pre-existing meristems and the absence of an intervening callus stage [35]. As clonal homogeneity based on morphological traits is not precise, we decided to investigate if there was any change in axillary bud-derived plants at molecular level using microsatellite markers. This marker system was selected as it is simple, more reliable and proved highly efficient in evaluating the clonal uniformity in other crop plants [17 - 19] and genetic diversity studies in cassava [11, 36].

Assessment of genetic stability in tissue-culture regenerated plants through molecular markers at early stage is desirable before their exploitation for routine propagation. In the present study, all SSR profiles of 15 axillary bud-regenerants and mother plants did not show any polymorphism indicating genetic uniformity of the plants. The monomorphic banding pattern in axillary bud-derived regenerants and mother plants suggested that all the plants were alike and similar to the mother plants genotypically and no variations occurred during tissue culture conditions. These monomorphic banding patterns between mother plants of the three cassava cultivars and their respective axillary bud-derived regenerants could be due to regeneration from organized tissues like axillary buds which have been reported to preserve the genetic fidelity of the regenerants in other crops [18]. Therefore, *in vitro* axillary bud-derived regenerants in the present study were true-to-type to the mother plants and could be due to regeneration of the plants from pre-existing meristem without any intermittent callus [37, 38]. The results from this study support the use of axillary bud regeneration as one of the safest mode of micropropagation for the production of true-to-type plants. There are also many reports in literature suggesting that plants regenerated through organized tissues like meristems maintain genetic integrity of the plantlets with a least risk of genetic variation [13, 37 - 40].

In vitro clonal propagation is used to produce seedlings identical to the mother plant after repeated sub-culturing. In general, clonal propagation through tissue culture should generate individuals identical to the mother plant from which they were sub-cultured. Continuous maintenance of cultures may often result in chromosomal rearrangements and mutations [41]. As *in vitro* culture promotes genetic disturbances due to many factors, confirmation of genetic stability is of particular importance to preserve the desirable attributes of *in vitro* regenerated plants and germplasm lines [42]. Hence, it becomes vital to reveal the genetic steadiness of long term maintained cultures. In this study, genetic stability of the micropropagated plants from different subculture cycles was analyzed among 5 clones of each of the three cultivars as well as the mother plants. The observation of monomorphic bands profiles among axillary bud-regenerants and mother plant of the same cultivar from 1st to 5th subculture reflects the maintenance of allele composition during successive cycles of *in vitro* propagation. For the axillary bud regenerants, only one variant was observed at the 6th subculture for cultivar TMS60444 at genetic similarity of 0.955 and the polymorphism level was 3.70%. Also at the 6th subculture, two progenies were grouped in the same cluster with their respective mother plants of cultivars TME14 and TMS60444 at a similarity level of 96% and 95%, respectively. In view of a 7-month period of *in vitro* propagation, such a similarity indicated a very low polymorphism.

Generally, the longer the subculture time is, the greater the risk of gene variations is. [43] Found genetic variation among the donor mother plant and the regenerated plants of *N. khasiana* from the first regeneration to the third regeneration was increased from 5.65% (first regeneration) to 10.87% (third regeneration). This may be due to an increase in duration of the regenerants under tissue culture conditions being exposed to various factors which induce somaclonal variations. In the present study, somaclonal variants detected in the 6th subculture showed that longer periods of maintaining plants *in vitro* could have an effect on their genetic stability. This might be explained by the stressful environment experienced by *in vitro*-raised plants such as high concentrations of sugars and plant growth regulators, low ventilation rate and low light availability [44]. Under these conditions, cultured plants cells are forced to change their molecular make ups in order to generate different cell types and cell division to generate tissues and organs require a precise coordination of genetic and epigenetic processes [45]. Therefore, for germplasm conservation, the *in vitro* propagated plants need to be transferred to the field after a given period of time and fresh cultures initiated using new plants from the field.

Extensive research has been carried out on the mechanism of somaclonal variation. The exact cause of somaclonal variation in *in vitro* cultures are still unknown, although it is believed that alterations in auxin-cytokinin concentrations and their ratio, duration of *in vitro* culture, *in vitro* stress due to unnatural conditions, altered diurnal rhythm and nutritional conditions [46] together or independently are responsible. Cultured plant tissues are also known to undergo high levels of oxidative stress due to reactive oxygen species formed within the cells and the latter is known to cause DNA damage, including that of microsatellite instability [47]. The detection of somaclonal variation using microsatellite DNA markers among morphologically indistinguishable micropropagated plants in this study underlines the need for testing tissue culture-propagated plants at the molecular level.

5. Conclusion

In the present study, no somaclonal variation was observed between axillary bud-derived and mother plants; indicating direct organogenesis from axillary buds is the safest method for the regeneration of true-to-type plants in cassava. As this protocol is genetically stable as validated by SSR markers and it keeps the integrity of the genotype under regeneration intact, axillary bud explants can be used for *Agrobacterium*-mediated genetic transformation of cassava in future for incorporation of various desirable traits like viral, fungal and bacterial disease resistance, prolonged shelf life, nutritional enhancement and reduction of cyanide level. From the results obtained in this study using SSR markers to evaluate the genetic homogeneity of cassava plantlets, we conclude that this micropropagation system for cassava can be carried out for a considerable length of time (for at least 7 months) without any risk of genetic instability.

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