MOLECULAR CHARACTERIZATION OF CASSAVA (*Manihot esculenta* Crantz.) PLANTS REGENERATED THROUGH ORGANOGENESIS AND SOMATIC EMBRYOGENESIS

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Thesis submitted in partial fulfillment of the requirement for the award of the degree of Masters of Science (Genetics) in the School of Biological Sciences of the University of Nairobi

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DECLARATION

This thesis is my original work and has not been submitted to any University for the award of any degree.

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DEDICATION

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LIST OF ABBREVIATIONS AND ACRONYMS

AB : Axillary bud

BAP : 6-benzylamino purine

CAM : Axillary bud enlargement medium

CBB : Cassava bacterial blight

CBM : Cassava basal medium

CBSD : Cassava brown streak disease

CEM : Shoot elongation medium

CIM : Somatic embryo induction medium

CMD : Cassava mosaic disease

CTAB : Cetyl trimethyl ammonium bromide

$CuSO_4$: Copper II sulphate

DNA : Deoxyribonucleic acid

dNTPs : Deoxyribonucleoside triphosphates

FAO : Food and Agriculture organization

FEC : Friable embryogenic calli

GD : Gresshoff and Doy medium

KALRO: Kenya Agricultural and Livestock Research Organization

KEPHIS: Kenya Plant Health Inspectorate Service

MS : Murashige and Skoog medium

MSN : Somatic embryo emergence medium

NAA : Naphthaleneacetic acid

NEPAD: New Partnerships for African Development

OES : Organized embryogenic structures

PCR : Polymerase chain reaction

RAPD : Random Amplified Polymorphic DNA

RFLP : Restriction Fragment Length Polymorphism

SE : Somatic Embryos

SSR : Simple sequence repeats

ABSTRACT

Cassava (*Manihot esculenta* Crantz.) is an important food crop in sub-Saharan Africa due to its high carbohydrate content. Occurrence of somaclonal variation in tissue culture derived cassava plants makes it necessary to assess the genetic stability of *in vitro* regenerated plants. The objective of this study was to evaluate the fidelity of cassava plants regenerated through direct organogenesis and somatic embryogenesis. Three cassava cultivars (TME14, TMS60444 and Kibandameno) were evaluated *in vitro*. Axillary buds (AB) from nodal cuttings were induced on Murashige and Skoog (MS) supplemented with 10 mg/L benzyl amino purine (BAP) and the shoots obtained rooted on basal MS. Somatic embryos (SE) from axillary buds were induced on MS with 12 mg/L picloram while friable embryogenic calli from SE were induced on Greshoff and Doy (GD) medium with 12 mg/L picloram. The obtained embryos and calli were matured on MS with 1 mg/L naphthaleneacetic acid (NAA) and resulting shoots elongated on MS with 0.4 mg/L BAP before rooting on basal MS. Average induction frequencies of axillary buds, somatic embryos and friable embryogenic calli were 74%, 96% and 22% respectively. Plant regeneration frequencies varied from 67% for somatic embryos to 100% for axillary bud derived plants. The effect of subculture period on genetic stability of axillary bud-derived regenerants and micropropagated plants was also assessed using 10 simple sequence repeat (SSR) markers. The nodal cuttings were subcultured onto fresh MS after every five weeks for a total period of thirty weeks. To validate the genetic homogeneity, five randomly selected plants obtained from the different *in vitro* regeneration processes and stages along with cassava donor mother plants were used. DNA was extracted from the young leaf tissues using a modified cetyl trimethylammonium bromide (CTAB) method. All the SSR profiles of DNA from micropropagated and axillary bud regenerants were monomorphic and comparable to the respective donor mother plants from 1st to

5th subculture. At the 6th subculture, similarity indices between the clones and the mother plants ranged from 0.95 to 1.0. Only three out of forty-five plants evaluated from somatic embryogenesis were true-to-type. The similarity indices ranged from 0.955 to 0.96, 0.905 to 0.96, and 0.81 to 0.96, for plants regenerated from primary SE, secondary SE and FEC, respectively. Secondary SE had the least genetic variation among the three stages of somatic embryogenesis. The somatic embryogenesis protocol used in this study may not be favorable in mass propagation due to undesirable genetic variation. However, the study showed that direct organogenesis from the axillary buds is a more reliable method for regeneration of true-to-type plants and can be exploited in clonal mass propagation, germplasm conservation and for improvement of cassava through genetic engineering. Further work should involve efforts to minimize somaclonal variation generated through somatic embryogenesis.

Key words: Cassava, axillary buds, somatic embryogenesis, somaclonal variation, SSR markers, genetic variability

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background information

Cassava (*Manihot esculenta* Crantz), 2n = 36, is a perennial woody shrub mainly cultivated in the tropical and subtropical countries primarily for its edible, large swollen secondary roots that contain a high carbohydrate content of up to 85% (Taylor *et al.*, 2004). It is a staple food for over 800 million people globally providing a cheaper source of carbohydrates (Burns *et al.*, 2010). Its ease of cultivation and ability to tolerate unfavorable environmental conditions (such as drought and poor soil fertility) makes cassava an important food security crop in sub-Saharan Africa. New Partnerships for African Development (NEPAD) identified the crop as a potential 'poverty fighter' in Africa (Ogero *et al.*, 2012). Cassava's potential of hunger alleviation also lies in its flexibility of harvest time and ability to be transformed into various forms and stored for a long time before use (Saelim *et al.*, 2006; Moyib *et al.*, 2007). Besides its starchy roots, consumption of cassava leaves and tender shoots for vitamins A and B, iron, calcium and proteins have also been reported in many parts of Africa (Nweke, 2005; Mapayi *et al.*, 2013).

According to FAOSTAT (2013), world cassava harvest production stands at 277 million metric tonnes from 20.3 million hectares; of which Latin America, Asia and Africa account for 10%, 32% and 56%, respectively. However, due to various abiotic and biotic constraints affecting cassava production, world average yields of only 12.8 tonnes per hectare have been reported against the expected optimum yields of 80 tonnes per hectare (FAOSTAT, 2013; Howeler *et al.*, 2013). The abiotic factors affecting cassava production include unfavorable climatic conditions, nutrient poor soils, early water stress, planting of unimproved low-yielding cultivars and root

deteriorations due to poor post-harvest handling practices (Fermont *et al.*, 2009; Bull *et al.*, 2011). Biotic factors which include: cassava mosaic disease (CMD), cassava brown streak disease (CBSD), cassava bacterial blight (CBB) and insect pests like whitefly (*Bemisia tabaci*) and green mites (*Mononychellus tanojoa*), are responsible for heavy yield losses in cassava (Hillocks and Jennings, 2003; Bull *et al.*, 2011; Howeler *et al.*, 2013).

The major problem with most vegetatively propagated plants like cassava is the accumulation of pests and diseases in the planting materials over a period of time (Mussio *et al.*, 1998). Availability of clean and healthy planting materials is a critical necessity in enhancing cassava production and tissue culture has proven to be the best source of obtaining clean planting materials. This is because the technology has the potential of producing thousands of high quality and disease-free cassava propagules unlike the conventional method of using stem cuttings (Ogero *et al.*, 2012). This technology can achieve production of clean planting materials even from infected mother plants through culture of young meristems frequently devoid of systemic pathogens due to undifferentiated vascular system (Cruz-Cruz *et al.*, 2013). This circumvents the accumulation and transmission of pests and diseases from one season to the other (Acedo, 2006; Wasswa *et al.*, 2010). In addition, tissue culture provides year-round availability of planting materials hence bypassing the ‘season-time’ limitation. It also provides an avenue for multiplication, transformation and conservation of cassava elite varieties that can be used in plant breeding.

Clonal fidelity is however an important prerequisite during micropropagation of any crop species (Jin *et al.*, 2008; Ribeiro, 2012). A major limitation often encountered with tissue culture is the

presence of somaclonal variation among clones of a donor mother plant; which arises due to various factors such as exposure to plant growth regulators (Venkatachalam *et al.*, 2007). Other factors responsible for the occurrence of somaclonal variations include the type of regeneration system used (Skirvin *et al.*, 1994), source of explant (Ahloowalia and Sherington, 1985), type of media components and culture duration (Cassells and Curry, 2001). During *in vitro* regeneration of some cassava genotypes, loss of beneficial traits has also been reported, mainly through somatic embryogenesis system (Beyene *et al.*, 2016). According to Alves *et al.* (2004), understanding the mechanisms leading to somaclonal variation, the appropriate procedures for preventing its occurrence and the development of early detection methods are important factors for ensuring uniformity in the production of micropropagated plantlets.

One strategy for detecting somaclonal variants is the use of molecular markers; which are part of the genome, thus excludes both environmental effects and misidentifications (Borba *et al.*, 2005). Simple sequence repeat (SSR) markers have successfully been used in detection of genetic differences or similarities in several micropropagated plants, including bananas (Martin *et al.*, 2006) and medicinal plants such as *Spilanthes calva* DC (Razaq *et al.*, 2013). They have also been used to detect the genetic uniformity among somatic embryogenesis derived plantlets (Sharma *et al.*, 2007; Orbović *et al.*, 2008; Zhang *et al.*, 2010). The high reproducibility, simplicity, and low cost of the experimental procedures of SSR compared to other molecular markers such as restriction fragment length polymorphism (RFLP) and random amplified polymorphic DNA (RAPD) makes it more appropriate for such studies. SSR markers have also been found to be very appropriate for characterizing genetic diversity of cassava genotypes due to their high abundance and uniform dispersion across the genome (Moyib *et al.*, 2007). This

study therefore used SSR markers to determine the genetic stability of cassava plants regenerated through nodal organogenesis and somatic embryogenesis.

1.2 Problem statement and justification

Improvement of cassava varieties through introduction of beneficial traits e.g. disease resistance from elite cassava varieties or introgression of favorable genes from other wild relatives is mainly done through *in vitro* transgenic technologies and not classical breeding. This is due to the unsynchronized flowering patterns, high levels of heterozygosity and low natural fertility exhibited in cassava growth. Hence tissue culture is pivotal in both the improvement and mass micropropagation of the staple crop. Conventionally, propagation of cassava is performed vegetatively using stem cuttings and unavailability of enough planting materials is caused by low multiplication rate in addition to infestation by diseases transmitted through successive generations. Therefore, the use of alternative techniques that allow for rapid multiplication and provision of clean planting materials to farmers is very important. Among these alternatives, micropropagation allows for the clonal production of high-quality plants in a short period of time and in reduced physical space as well as ensuring phytosanitary quality.

However, somaclonal variation often encountered among clonally propagated plants *in vitro* is a major limitation. Somaclonal variation from *in vitro* regenerated plants presents a serious challenge to conservation of the genetic fidelity of germplasm. This is due to the random and unpredictable nature of the mutations that alter various alleles during the *in vitro* propagation that might lead to activation of lethal genes or deactivation of integral genes. For example, Beyene *et al.* (2016) reported loss of natural resistance to cassava mosaic disease (CMD) in somatic embryogenesis-derived plants. They reported successful transfer of genes responsible for

producing siRNAs against cassava brown streak disease (CBSD) into TME 204 (a cultivar with natural resistance to cassava mosaic disease). However, the resultant somatic embryogenic regenerated TME 204 plants lost their natural resistance to CMD and the loss of resistance was as a result of somatic embryogenesis process.

Therefore checking of the genetic fidelity in tissue culture derived cassava plantlets before introduction into the environment could help conserve the genetic makeups of the different cassava varieties. One strategy for detecting somaclonal variants is the use of molecular markers; these markers are part of the genome, and thus can be used to avoid environmental effects and, consequently, misidentifications.

1.3 Objectives

1.3.1 Overall objective

To determine the tissue culture regeneration system of cassava with the least genetic variation between the regenerants and donor mother plants.

1.3.2 Specific objectives

(i) To determine the genetic stability of axillary bud-derived cassava plants using SSR markers

(ii) To determine the effect of subcultures on genetic stability of *in vitro* micropropagated plants and axillary bud-derived regenerants using SSR markers

(iii) To determine the genetic variability of cassava plants regenerated from somatic embryos and friable embryogenic calli using SSR markers

1.4 Null hypotheses

i. There is no genetic variability in axillary bud-derived cassava plants

ii. Repeated subculturing of micropropagated and axillary bud-derived plants does not cause genetic variability in the respective plants

iii. There is genetic stability among cassava plants regenerated from somatic embryos and friable embryogenic calli

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Origin and distribution of cassava

Cassava (*Manihot esculenta* Crantz) is a perennial root crop which grows as a shrub and belongs to the family *Euphorbiaceae* (Zhang and Gruissem, 2004). It is a food security crop majorly grown in sub-Saharan Africa, Asia and Latin America (El-Sharkawy, 1993). Cassava originated from Brazil and Paraguay, although the first evidence of its cultivation was found in El Salvador around 6000 B.C (O'Hair *et al.*, 1998; Agro, 2012). Due to its ability to grow in adverse climatic conditions and nutrient-poor soils, cassava quickly became a staple food for the South American and Caribbean natives. The crop was first introduced in Africa by the Portuguese traders from Brazil in the 16th Century (Nweke, 2005; Kosh-Komba *et al.*, 2015). Today, cassava is distributed throughout tropical and sub-tropical countries.

According to FAOSTAT (2013), cassava is cultivated worldwide on an area of about 20.4 million hectares out of which Africa, Asia and Latin America contribute 66%, 22% and 12%, respectively. Furthermore, out of the 277 million metric tonnes of fresh cassava harvested in 2013; Africa, Asia and Latin America contributed 56%, 32% and 10%, respectively (FAOSTAT, 2013). In Latin America, Brazil has the largest cassava cultivated area of 1.5 million hectares followed distantly by Colombia at 0.2 million. Of the 17 Asian countries where cassava is grown, Thailand and Indonesia have the largest cultivated areas of 1.4 and 1.1 million hectares, respectively (FAOSTAT, 2013). In Africa, cassava is grown in 40 countries with West Africa leading in its production followed closely by central Africa. Nigeria leads in cultivation of cassava both in Africa and globally with a total cultivated area estimated at 3.8 million hectares (FAOSTAT, 2013). Other major producers in Africa include Ghana, Angola and Democratic

Republic of Congo with 0.8, 1.2 and 2.1 million hectares, respectively, under cultivation. In eastern Africa, Tanzania leads with an area of 0.8 million hectares followed closely by Mozambique at 0.7 million. Kenya is ranked 9th in eastern Africa, 21st in Africa and 35th globally with an estimated cultivated area of 0.07 million hectares (FAOSTAT, 2013). In Kenya, it is predominantly grown in Coastal, Eastern and Western regions.

2.2 Genetics and wild relatives of cassava

Flow cytometry has been used to reveal that cassava is a diploid plant with a total of 36 chromosomes (2n = 36) (Awoleye *et al.*, 1994). Its haploid genome is 772 mega base pairs long. Cassava, alongside 90 other plant species make up the genus *Manihot* with *Manihot esculenta* Crantz being the most cultivated for its starchy roots (Fregene *et al.*, 1997). The progenitors of cassava have been reported by Carvalho and Schaal (2001) to be *M. esculenta ssp. Peruviana* and *Manihot esculenta ssp. flabellifolia.* Other wild relatives of the crop include; *M. triphylla, M. pilosa, M. glaziovii* (Carvalho and Schaal, 2001; Lokko *et al.*, 2006). *Manihot glaziovii* grows natively in Brazil and Columbia but has an exotic range in African countries including; Kenya, Nigeria, Sierra Leone, Tanzania, Singapore, Senegal, Uganda and Gambia (Orwa *et al.*, 2012).

2.3 Economic importance of cassava

Cassava is a staple food for an estimated 800 million people worldwide (Howeler *et al.*, 2013). In Africa it ranks second after maize as a major staple food. Its starchy roots (85% carbohydrates), ranking fourth after maize, sugarcane and rice, act as valuable sources of calories especially in less developed countries experiencing the challenge of malnutrition (Mapayi *et al.*, 2013). Due to its ability to produce considerable yields under erratic rainfall conditions and

nutrient poor soils, cassava is a major food security crop in sub-Saharan Africa (Ogero *et al.*, 2012). Since the roots can stay underground for two to three years before harvesting, cassava has been critical during civil unrest when displaced persons return to their farms (Bokanga, 2000). This has been reported in times of civil unrest in countries like Uganda, Angola, Rwanda, Burundi, Liberia and Mozambique (Bokanga, 2000). However, in other African countries such as Malawi and Tanzania, cassava is the main source of food during severe drought.

Only 30% of cassava roots is consumed after peeling, washing and boiling in Africa while the remaining percentage is processed into a variety of commercial products including dry chips and flour, cooked pastes, roasted or steamed granules and beverages (Bokanga, 2000). Most of these products are consumed locally within the country of production and the remaining portion of dried cassava chips and other industrial products are exported for foreign exchange (Bokanga, 2000). Apart from the roots, cassava leaves are also consumed as a vegetable in some African countries to compensate for the lack of adequate proteins and vitamins in the roots (Mapayi *et al.*, 2013). This is due to the abundance of vitamins (A, B and C), iron, calcium and proteins in cassava leaves. Apart from its use as food, cassava is also used as livestock feed and for biofuel production mainly in the Asian and Latin American countries (Wangsomnuk *et al.*, 2013). Its use in the pharmaceutical industries has also been reported by Acedo (2006).

2.4 Constraints facing cassava production

Despite its ability to produce reasonable yields in nutrient poor soils, cassava faces various challenges during its growth period that impact negatively on both the quality and quantity of yields (Zhang and Gruissem, 2004). These challenges are of both biotic and abiotic origins.

Cassava is attacked by several arthropod pests including cassava mealybugs (*Phenacoccus manihoti*), whiteflies (*Bemisia tabaci*), green spider mites (*Mononychellus tanajoa*), cassava stem borer (*Chilomina clarkei*) and cassava scales (*Aonidomytilus albus*), which cause severe yield losses to the farmers (Hillocks and Jennings, 2003; Taylor *et al.*, 2004; Bull *et al.*, 2011). Apart from damaging the plants, these pests act as vectors of major diseases of cassava such as cassava mosaic disease (CMD), cassava bacterial blight (CBB), anthracnose, cassava root rot and cassava brown streak disease (CBSD) (Poubom *et al.*, 2005; Bellotti, 2008; Night *et al.*, 2011). These diseases are responsible for huge losses in cassava production due to their ease of transmission through planting materials from one season to another (Howeler *et al.*, 2013). Poor soil fertility, unfavourable climatic conditions and poor post-harvest handling techniques have also been reported by Fermont *et al.* (2009) as major constraints to cassava production.

2.5 Varieties of cassava in Africa

There are different cassava varieties grown in Africa with each country having its preferred set(s). Table 1 shows cassava varieties cultivated in Africa (Info-net, 2012; KEPHIS, 2015).

Table 1. Cassava varieties grown in Kenya and some other parts of Africa (Info-net, 2012)

Country	Varieties	Attributes
Kenya	"Guso"	Resistant to (ACMD); Sweet
	"Mucericeri"	Very sweet
	"Kaleso" ("46106/27") , "Karembo" ("KME-08-05") , "Karibuni" ("KME-08-01"), "Shibe" ("KME-08-04"), "Siri", "Tajirika" ("KME-08-02")	Tolerant to ACMD and cassava brown streak disease (CBSD); sweet
	“Kibandameno”*	Very susceptible to ACMD; very sweet; low in cyanide
	"2200", "Tereka", "Serere", "Adhiambo lera", "CKI", "TMS 60142", "BAO", "Migyeera", "SS 4", "MH 95/0183", "MM 96/2480", "MM 96/4884", "MM 96/5280", "MM 96/5588", "MM 97/2270"	Farmer preferred in the Western region; Have high pest and diseases resistance
Nigeria	"TMS 90257", "TMS 84537", "TMS 82/00058", "TMS 82/00661", "TMS 30001", "TMS 8 1/00110", "TMS 4(2)1425"	Have high pest and diseases resistance; and low cyanogenic glucosides content
	"NR8212", "NR 8082", "NR 8083", "NR 83107"	Have high pest and diseases resistance; and high cyanogenic glucosides content
	"NR 8208","TMS 91934"," TMS 30555", "NR 41044","TMS 50395", "TMS 30001", "TMS 30572"	Have moderate resistance to pests and diseases
	"TMS 60444"*	Very susceptible to pests and diseases. Model cultivar for research on cassava
Uganda	"Migyeera", "NASE 1 to 12", "SS 4", "TMS 4(2)1425", "TMS 192/0067", "TMS 50395", "Uganda MH 97/2961","TME 14"*	Tolerant to African Cassava mosaic Disease (ACMD)
Tanzania	"Kachaga", "191/0057", "191/0063", "191/0067", "MM 96/0876", "MM 96/3075B", "MM 96/4619", "MM 96/4684", "MM 96/5725", "MM 96/8233", "MM 96/8450", "SS 4","TMS 4(2)1425", " TME 14"*	Tolerant to African Cassava mosaic Disease (ACMD)

ACMD: African Cassava Mosaic Disease, **CBSD**: Cassava Brown Streak Disease
(*) represent cultivars used in the study

2.6 *In vitro* propagation of cassava

To ensure production of healthy planting materials void of accumulated pests and diseases, *in vitro* propagation of cassava plays a pivotal role. The two major methods of *in vitro* cassava regeneration are organogenesis and somatic embryogenesis (Taylor *et al.*, 2004; Zhang and Gruissem, 2004; Ogero *et al.*, 2012; Nyaboga *et al.*, 2015).

2.6.1 Organogenesis

Shoot organogenesis in cassava has been reported from apical meristems cultured on MS supplemented with both auxins and cytokinins at varying concentrations. Acedo (2006) successfully regenerated shoots from apical meristems of Philippine cassava cultivars cultured for six weeks on liquid or solid MS medium supplemented with 0.25 mg/L gibberellic acid (GA3), 0.1 mg/L benzylaminopurine (BAP) and 0.2 mg/L naphthalene acetic acid (NAA). Regeneration of cassava by culturing inter-nodal cuttings (2-3 cm) on MS medium supplemented with sucrose and gelling agent has been reported by Zhang and Gruissem (2004). In their report, the nodal regeneration medium (CBM) was composed of MS salts with vitamins, 2 μM $CuSO_4$, 2% sucrose and 0.3% gelrite. This medium has been widely used in *in vitro* regeneration of cassava plants from field cuttings in various laboratories (Saelim *et al.*, 2006; Bull *et al.*, 2009; Nyaboga *et al.*, 2013). However, no study has been carried out to test the fidelity of plants regenerated *in vitro* by organogenesis.

2.6.2 Somatic embryogenesis

For *de novo* cassava regeneration, somatic embryogenesis is the most routinely used method. Reports of successful generation of primary somatic embryos from immature leaves, shoot apical or axillary meristems, and floral tissue cultured on picloram supplemented MS medium

have been reported (Zhang and Gruissem, 2004). Saelim *et al.* (2006) induced somatic embryogenesis in explants (young leaf lobes, apical buds and lateral buds) of two Asian cassava cultivars by culturing in induction medium consisting of MS, 2% sucrose, 2 μM $CuSO_4$, 0.6% bactoagar and 12 mg/L picloram. Nyaboga *et al.* (2013) while working with eight African cultivars reported successful induction of somatic embryos in a medium consisting of MS, 2% sucrose, 2 μM $CuSO_4$, 0.8% Noble agar and 12 mg/L picloram.

Due to the multiplicity of origin encountered in generation of both primary and secondary somatic embryos, a transgenic cassava regeneration protocol through friable embryogenic callus (FEC) was first described by Taylor *et al.* (1996). They reported successful FEC generation by culturing primary somatic embryos of cultivar TMS60444 on a medium supplemented with Gresshoff and Doy (1972) (GD) salts and vitamins and picloram. Later, Taylor *et al.* (1997) demonstrated that by inclusion of both NAA and picloram, FEC could be induced from other cassava cultivars. FEC has since been adapted for cassava transformation by various researchers due to the uniformity of its cells (Zhang and Gruissem, 2004; Bull *et al.*, 2009; Zainuddin *et al.*, 2012; Nyaboga *et al.*, 2013; Apio *et al.*, 2015).

2.7 Somaclonal variation

The first occurrence of somaclonal variation was reported by Braun (1959) in crown gall tumors of tobacco. Extensive studies have since been done to determine the factors leading to somaclonal variation. Type of *in vitro* regeneration system, culture duration, types of phytohormones used in the regeneration media, sources of explants and tissue types are some factors reported to contribute to this phenomenon (Larkin and Scowcroft, 1981; Karp, 1994;

Rani and Raina, 2000). The variations may be genetically stable or epigenetic and as much as the mechanisms leading to somaclonal variations have not been fully understood, they are believed to be as a result of variations in chromosomal and DNA sequences, DNA methylations and transposon activations (Neelakandan and Wang, 2012; Krishna *et al.*, 2016). Somaclonal variation has since been reported in many studies that sought to produce true-to-type regenerants through both micropropagation and somatic embryogenesis processes of plant tissue culture. Some of the plants include; Banana (Ray *et al.*, 2006), Potato (Sharma *et al.*, 2007), Papaya (Kaity *et al.*, 2009), Sweet cherry (Piagnani and Chiozzotto, 2010), Arabidopsis (Jiang *et al.*, 2011), Oil palm (Rival *et al.*, 2013) and African violet (Matsuda *et al.*, 2014). There has not been any report however on the occurrence of this phenomenon in cassava plants regenerated *in vitro*.

2.7.1 Advantages

Genetically variable plants produced as a result of somaclonal variation aid in widening the gene pool for that particular species. Hence providing a quicker platform for selection of beneficial traits for crop improvement such as disease resistance, drought tolerance and salinity tolerance by plant breeders (Leva *et al.*, 2012). While working with salt-tolerant *Brassica juncea L,* Jain *et al.* (1991) reported overproduction in proline by a somaclone enabling it to adapt better than the parent to a saline environment. Barden *et al.* (1986) also reported successful generation of tomato (*Lycopersicon esculentum*, Mill.) somaclones that were resistant to tobacco mosaic virus. In addition, occurrence of chimerism in such plants is highly unlikely since they are raised through tissue culture of cells and the changes occur at the molecular level (Evans, 1989). In ornamental industry the variations have exploited in breeding for more appealing products for commercial purposes (Krishna *et al.*, 2016).

2.7.2 Disadvantages

In propagation systems such as germplasm conservation, where clonal uniformity is paramount, genetic variability caused during the *in vitro* cultures present a serious challenge (Jin *et al.*, 2008). Such genetic changes could result in depression of yields, reduced vigour and loss of resistance to pests and diseases (Rani and Raina, 2000). Even in cases where the mutants display acquisition of beneficial traits, there exist a very low probability that the genetic changes are stable and could be passed to the successive generations (Karp, 1994). While evaluating the stability of potato (*Solanum tuberosum* L.) somaclones' resistance to late blight, Cassells *et al.* (1991) reported breakdown in resistance in successive generations. Due to their unpredictable occurrences, the frequencies of obtaining somaclonal variants from various plant regeneration systems are quite low to be useful.

2.8 Molecular markers used for characterization of cassava

Various molecular markers have been used in studying the genetic diversity of cassava and proximity of its genome to that of the wild relatives such as *Manihot glaziovii* (Olsen and Schaal, 2001). These markers include Restriction Fragment Length Polymorphsms (RFLPs) (Fregene *et al.*, 1997); Random Amplified Polymorphic DNA (RAPD) (Carvalho and Schaal, 2001), and Simple Sequence Repeats (SSRs) (Chavarriaga-Aguirre *et al.*, 1998; Moyib *et al.*, 2007).

The first RFLP analysis in cassava was reported by Fregene *et al.* (1997) while working with the F1 generation from a cross between TMS 30572 and CM 2177-2 elite cassava cultivars from Nigeria and Colombia, respectively. This analysis revealed 132 RFLP markers that contributed to the construction of cassava's genetic linkage map alongside 30 RAPD and 3 SSR markers.

Due to their inexpensiveness and ability to provide unlimited number of polymorphism needed for classification, PCR-based techniques [RAPD, SSR, AFLP and ISSR (inter simple sequence repeats)] are widely preferred to RFLP (Mba *et al.*, 2001; Lokko *et al.*, 2006). It is the use of expensive enzymes and radioactive labeling dyes that makes RFLP less preferred in genetic studies involving a lot of samples (Kumar *et al.*, 2010). PCR-based techniques are however simple, fast, cost-effective and require minute amounts of DNA samples (Asante and Offei, 2003). In addition, the use of PCR- based markers for detection of somaclonal variation in micro-propagated plants have been reported in various plants (Ray *et al.*, 2006; Joshi and Dhawan, 2007; Sheidai *et al.*, 2008; Kumar *et al.*, 2010; Wangsomnuk *et al.*, 2013).

Carvalho and Schaal (2001) used RAPD markers to characterize Brazilian cassava collection alongside SSR markers. Asante and Offei (2003) used four RAPD primers and generated 41 distinct bands from 50 cassava genotypes and used the information to group the plants. Zacarias *et al.* (2004) characterized 35 cassava cultivars in Mozambique using 311 amplified fragments from twenty RAPD primers and clustered the cultivars in different distinct groups. Of the PCR based molecular techniques, Rahman and Rajora (2001) reported that RAPD and AFLPs are not suitable in the detection of somaclonal variation. This is because they are dominant markers making them less informative in evaluation of diploid organisms like cassava.

The onset of utilization of SSR markers in cassava was after Chavarriaga-Aguirre *et al.* (1998) managed to isolate and characterize 14 simple sequence repeats in cassava. Later, Mba *et al.* (2001) used a subset of the 14 SSRs and an additional 172 SSR markers to evaluate the genetic diversity of about 600 accessions of cassava world germplasm genebank at the International

Center for Tropical Agriculture, Colombia. Due to their simplicity and co-dominance, SSRs have been confirmed to be the most preferred molecular marker in cassava characterization (Chavarriaga-Aguirre *et al.*, 1998; Mba *et al.*, 2001).

Despite the wide use of SSR markers in the characterization of various cassava cultivars, reports of their use in detection of somaclonal variation in the crop do not exist. However, a number of studies on detection of somaclonal variation in several plants species including *Spilanthes calva* (Razaq *et al.*, 2013), *Solanum tuberosum* (Zarghami *et al.*, 2008), Kiwi fruit (Palombi and Damiano, 2002), date palm (Kumar *et al.*, 2010), poplar tree (Rahman and Rajora, 2001) and cotton (Jin *et al.*, 2008) using SSR markers have been reported. Therefore, this study used SSR markers to assess the genetic fidelity of cassava plants regenerated *in vitro* through micropropagation and somatic embryogenesis.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Plant materials and initiation of nodal cuttings into tissue culture

Three cassava cultivars namely Kibandameno, TMS60444 and TME14 were used in this study. The stem cuttings of each cultivar were obtained from Kenya Agricultural and Livestock Research Organization (KALRO) and established in pots containing sterile soil in a glass house at the School of Biological Sciences, University of Nairobi. Four weeks later, nodal segments of healthy plants were collected and used as explants to establish *in vitro* plants of the three cultivars. The explants were washed under running tap water for 10 minutes. Twenty node cuttings from young stems of each of the cultivar were singly sterilized, first with 70% ethanol for 5 minutes followed with 5% sodium hypochlorite for 15 minutes. Nodes were finally rinsed five times with sterile double-distilled water. The nodes were dried for 5 minutes by placing them on sterile paper towels in a laminar flow hood and the necrotic tissues removed under aseptic conditions using sterile scalpel. Three nodal cuttings were planted in baby jars containing cassava basal medium (CBM; Murashige and Skoog salts with vitamins, 2 µM CuSO4, 2% sucrose, 0.3% Gelrite, pH 5.8) for initiation. The baby jars were incubated at 26 – 28 °C under 16/8 hours photoperiod in the tissue culture growth chamber at the School of Biological Sciences, University of Nairobi.

3.2 Culture media preparation

Murashige and Skoog (1962) and Gresshoff and Doy (1974) commonly known as MS and GD, respectively were used in this study. The MS and GD media, both with vitamin supplements,

were sourced from Duchefa Biochemie, Netherlands. For media solidification, Gelrite™ (Duchefa Biochemie, Netherlands) and Noble agar™ (Sigma-Aldrich, co., Spain) were used. A total of six different types of media compositions were used, each for the different stages of cassava tissue culture (Table 2). The pH of each medium was measured using Orion 3 Star® bench pH meter and adjusted to 5.8 using either 1M NaOH or 1M HCl. All the media were sterilized by autoclaving in glass jars for fifteen minutes at a temperature of 121 °C and pressure of 15psi.

Table 2. Composition of media used in cassava tissue culture

Name of medium	Composition
Cassava basal medium (CBM)	1 × MS[a] salts with vitamins, 2 µM $CuSO_4$, 2% sucrose, 0.3% Gelrite, pH 5.8
Axillary bud enlargement medium (CAM)	1 × MS salts with vitamins, 2 µM $CuSO_4$, 50 µM 6-benzylamino purine (BAP), 2% sucrose, 0.8% Noble agar, pH 5.8
Somatic embryo induction medium (CIM)	1 × MS salts with vitamins, 2 µM $CuSO_4$, 50 µM picloram, 2% sucrose, 0.8% Noble agar, pH 5.8
Friable embryogenic calli medium (GD)	1 × GD[b] salts with vitamins, 50 µM picloram, 2% sucrose, 0.8% Noble agar, pH 5.8
Somatic embryo emerging medium (MSN)	1 × MS salts with vitamins, 5 µM Naphthaleneacetic acid (NAA), 2% sucrose, 0.8% Noble agar, pH 5.8
Shoot elongation medium (CEM)	1 × MS salts with vitamins, 2 µM $CuSO_4$, 2 µM BAP, 2% sucrose, 0.8% Noble agar, pH 5.8

[a]MS, Murashige and Skoog (1962); [b]GD, Gresshoff and Doy (1974)

3.3 Experimental design

In axillary bud induction, embryo induction and regeneration experiments, a treatment comprised of three replicates each containing 10 explants. This set was then repeated three times and culture vessels (Petri plates or culture bottles) arranged in culture shelves following a complete random design (CRD).

3.4 Induction of axillary bud from nodal explants

Using a sterilized scalpel, 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 explants were cultured for 4 - 10 days at 28 ± 2°C in the dark (Bull *et al.*, 2009). The enlarged axillary buds were removed from the nodal explants with sterile syringe needles under a binocular microscope and transferred to fresh CAM at a density of 10 buds per Petri plate. The axillary buds were used in the subsequent steps for shoot induction and generation of somatic embryos.

3.5 Induction of shoots and regeneration of plantlets from axillary buds

To induce shooting from the axillary buds, plates containing the axillary buds were incubated under 16/8 h photoperiod at 28 ± 2 °C and buds 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 after four weeks. The developed shoots were transferred to CBM medium for rooting and subsequent growth. After 5 weeks in CBM, some well rooted plantlets were randomly selected and acclimatized in the glasshouse. Leaf samples of acclimatized axillary-bud regenerants as well as mother plants were collected for DNA extraction and SSR analysis. The remaining axillary-bud regenerants were used to

determine the effect of subculture frequency on genetic variation of plants regenerated through organogenesis.

3.6 Effect of subculture frequency on genetic variation of axillary-bud regenerants and *in vitro* micropropagated plantlets

To initiate *in vitro* micropropagated plants, nodal segments of three to five-week old plants planted in pots containing sterile soil 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.

Axillary-bud regenerants and *in vitro* micropropagated plants were subcultured by aseptically cutting the stems (at least two nodes per cutting) using a scalpel. The stem cuttings were planted on CBM by submerging the lower nodes into the media. After every five weeks thereafter the cultures were transferred to a fresh CBM medium. This was done until the 6th subculture cycle to determine the effect of subculture frequency on the induction of somaclonal variation. Leaf samples from each of the subculture cycle for both axillary-bud regenerants and micropropagated plants were collected for DNA extraction and SSR analysis. Leaf samples were collected from five different randomly selected plants per cassava cultivar (Kibandameno, TME14 and TMS60444), at each of the six subcultures. Samples from mother plants of all the three cultivars were used as controls.

3.7 Somatic embryogenesis

3.7.1 Generation of primary somatic embryos

To generate primary somatic embryos, enlarged axillary buds were aseptically excised from the explants with sterile syringe needles under a binocular microscope and transferred to CIM media (MS salts with vitamins, 2 μM CuSO4, 12 mg/L picloram, 2% sucrose and 0.8% Noble agar at pH 5.8) at a density of 10 per Petri plate. The plates with axillary buds were incubated at 28 ± 2 °C in the dark for 18 - 28 days. Each plate was examined regularly under a binocular microscope after two weeks of culture for formation of organized embryogenic structures (OES) and this was considered as primary embryos.

3.7.2 Generation of secondary somatic embryos

To generate secondary somatic embryos, the OES were first separated into clusters of 8 - 12 embryos under a binocular microscope by use of a sterile scalpel and a pair of forceps. The embryo clusters were sub-cultured onto fresh CIM medium (CIM2) at a density of 10 per Petri dish plate, for 2 - 4 weeks at 28 ± 2 °C in the dark. Each plate was examined regularly under a binocular microscope for embryo proliferation.

3.7.3 Generation of friable embryogenic calli

To generate friable embryogenic calli (FEC), secondary somatic embryos from CIM2 were divided into 8 – 12 small clusters under a binocular microscope using a sterile scalpel and a pair of forceps. The 8 – 12 clusters were transferred aseptically and placed onto Gresshoff and Doy (GD) medium (GD salts with vitamins, 12 mg/L picloram, 2% sucrose and 0.8% Noble agar at

pH 5.8) and cultured for three weeks at 28 °C under a 16/8 hour photoperiod. The number of explants forming FEC were recorded and sub-culturing of FEC onto fresh GD media was done after every three weeks (Nyaboga *et al.*, 2015).

3.8 Embryo maturation and regeneration of cotyledon stage embryos into plantlets

Clusters of primary and secondary somatic embryos and friable embryogenic calli were separately transferred onto embryo emergence medium (MSN) consisting of MS salts with vitamins, 1 mg/L NAA (Naphthaleneacetic acid), 2% sucrose and 0.8% Noble agar at pH 5.8. The clusters were cultured for two weeks at 28 °C under 16/8 hour photoperiod. They were routinely subcultured onto fresh MSN media after every two weeks until green cotyledons were formed.

Matured green cotyledons on MSN medium were transferred onto the shoot elongation medium (CEM; MS salts with vitamins, 2 µM CuSO4, 0.4 mg/L BAP, 2% sucrose and 0.8% Noble agar at pH 5.8). The cotyledons were cultured for two weeks at 28 °C under 16/8 hour photoperiod. They were routinely subcultured onto same fresh CEM media after every two weeks until shoots were formed. The developed shoots were transferred to a hormone-free CBM for rooting and subsequent growth of plantlets at 28 °C under 16/8 hour photoperiod.

3.9 Acclimatization of plants in the glasshouse

Plantlets were gently removed from the culture bottles after five weeks in CBM (MS salts with vitamins, 2 µM CuSO4, 2% sucrose and 0.3% gelrite at pH 5.8) and washed in lukewarm water to remove all the media attached to the roots. The plantlets were transplanted into plastic pots containing sterilized forest soil. The plantlets were transferred to a glasshouse for

acclimatization. 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 microenvironment 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 and assessed using morphological characteristics compared with the mother plants.

3.10 Imaging

Images of nodal explants, axillary buds, somatic embryos, friable embryogenic calli and regenerated plants were taken using a Canon HD camera (IXY 90F), Japan.

3.11 Statistical data analysis

The percentage data for axillary bud induction, embryo induction, embryo emergence, shoot elongation and regeneration of plants were subjected to one way analysis of variance (ANOVA) and means separated by Tukey's HSD test at $p \leq 0.05$. These analyses were computed in GenStat® computer software 15th edition. Prior to ANOVA, the percentages data were arcsine transformed based on the relation $Y = \text{arcsine} \sqrt{p}$, where p = the proportion obtained by dividing the respective percentage value by 100 as described by Rangaswami (2007).

3.12 Molecular characterization of regenerants and micropropagated plants

Plants from each of the three cultivars (Kibandameno, TME14 and TMS60444) were randomly selected for determining the occurrence of genetic variation among axillary bud-derived regenerants, and micropropagated plants from different cycles of subculture. Genetic variation was also determined in plants regenerated from different stages of embryogenic tissues namely

primary somatic embryos, secondary somatic embryos and FEC were used. Genetic variation between mother plants (a field-grown plant used as an explant source for culture initiation) and five randomly selected *in vitro* regenerants (about 3 months old established in soil under glasshouse conditions) were assessed by PCR-based SSR markers.

3.12.1 DNA extraction

Extraction of genomic DNA from cassava leaves of mother plants and selected regenerants was done using Cetyl trimethylammonium bromide (CTAB) protocol (Sharma *et al.*, 2008) with modifications. Leaf samples were weighed (200 mg) and crushed to form a homogenous paste in 700 µl CTAB buffer (2% CTAB, 1.4 M sodium chloride, 0.2 M EDTA, 1 M Tris-HCl and 4% Polyvinyl pyrrolidone, with a final pH of 7.5) and 150 µl of 20% sodium dodecyl sulphate (SDS). The homogenate was transferred into 1.5 ml eppendorf tube and incubated at 55 °C water-bath for 20 minutes. The tube was inverted 4 – 5 times after every five minutes during the incubation period to ensure uniform distribution of the crushed leaf tissues in the buffer. The sample was then spun in a Heal-Force® micro-centrifuge for 15 minutes at 12,000g. The supernatant was transferred to a new eppendorf tube preceding addition of equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) and gently mixed by inversion. The mixture was centrifuged at 10,000g for 7 minutes in the same micro-centrifuge. The top aqueous layer was transferred to a new eppendorf tube followed by addition of 50 µl of 7.4 M ammonium acetate and 2 volumes of ice cold absolute ethanol. The sample was incubated at -20 °C for 20 minutes to precipitate nucleic acids from the solution. The mixture was centrifuged at 10,000g for 10 minutes to pellet the precipitated nucleic acids. The supernatant was discarded and 500 µl of a wash solution (75% (v/v) ethanol and 15 mM ammonium acetate) was added to wash the pellet.

The washing step was repeated twice. After every wash, the mixture in eppendorf tube was centrifuged for 5 minutes at 10,000 g and the supernatant discarded. The pellet was then air dried for 10 minutes and dissolved in 70 µl TE (Tris-EDTA) buffer (10mM Tris-HCL and 1mM EDTA). Approximately 3 µL of 10 mg/ml ribonucleaseA was added to the dissolved nucleic acids and incubated for 30 minutes at 37 °C in a water bath. The DNA was stored at -20 °C until use.

3.12.2 DNA quantification and purity

The concentration and purity (A_{260}/A_{280} ratios) of the extracted genomic DNA was determined by a spectrophotometer (UV–Visible Elico spectrophotometer, India). DNA concentration and purity were estimated by electrophoresis on a 0.8 % agarose gel. The isolated DNA samples were diluted in sterile double-distilled water to produce working concentrations of 10 ng/µL. DNA was obtained from five different plants of each mother cultivar per experiment.

3.12.3 Simple sequence repeat (SSR) analysis

To determine the genetic fidelity of regenerated plants, 11 SSR primers (Table 3) designed by Mba *et al.* (2001) were used. The primers were synthesized by Inqaba Biotec (South Africa) and reconstituted according to the manufacturer's instructions in sterile nuclease free water.

Table 3. Eleven (11) SSR primer codes and sequences used for the study

Primer code	Forward primer sequence	Reverse primer sequence	Repeat motiff	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	$(CT)_{11}CG(CT)_{11}(CA)_{18}$	298
SRY 100	ATCCTTGCCTGACATTTTGC	TTCGCAGAGTCCAATTGTTG	$(CT)_{17}TT(CT)_{7}$	210
SRY 103	TGAGAAGGAAACTGCTTGCAC	CAGCAAGACCATCACCAGTTT	$(GA)_{22}$	272
SRY 35	GCAGTAAACCATTCCTCCAA	CTGATCAGCAGGATGCATGT	$(GT)_{3}GC(GT)_{11}(GA)_{19}$	282
SRY 45	TGAAACTGTTTGCAAATTACGA	TCCAGTTCACATGTAGTTGGCT	$(CT)_{27}$	228
SRY 78	TGCACACGTTCTGTTTCCAT	ATGCCTCCACGTCCAGATAC	$(CT)_{22}$	248
SRY 50	CCGCTTAACTCCTTGCTGTC	CAAGTGGATGAGCTACGCAA	$(CA)_{6}(N)_{6}(GA)_{31}$	271
SRY 175	TGACTAGCAGACACCGGTTTA	GCTTAACAGTCCAATAACGATAAG	$(GA)_{38}$	136

3.12.3.1 PCR amplifications and visualization of amplified fragments

Amplifications were carried out in MJ Mini™ personal Thermal Cycler (Bio-Rad, Singapore) in a total volume of 20 µl reaction mixture containing 20 ng of genomic DNA, 4 µl of 5X PCR buffer, 15 mM $MgCl_2$, 0.2 mM dNTPs, 1 unit Taq polymerase (Bioline, USA) and 0.1 µM of forward and reverse SSR primers. The PCR cycling consisted of an initial denaturation at 95 °C for 5 minute, 30 cycles of denaturation at 94 °C for 30 seconds, annealing at 54 °C for 30 seconds and extension at 72 °C for 1 minute and a final extension at 72 °C for 7 minutes.

The amplified PCR products were analyzed on a 2 % agarose gel (prepared by heat-dissolving 1g Agarose powder (Sigma Aldrich, USA) in 49ml of 1X Tris-Acetate EDTA buffer. pH 8.0) for 65 minutes at 60 V using 1X Tris-Acetate EDTA buffer. The gel was stained with ethidium bromide (0.5 µg/mL). The sizes of the amplicons were estimated by comparison with 100 bp molecular weight marker (Bioneer, Inc.). Gels were visualized under UV transilluminator and photographed by Easy Doc Plus gel documentation system (England).

3.12.3.2 Scoring and analysis of bands

The PCR reaction for each SSR primer was performed at least twice, and only clear and reproducible bands were used in data analysis. The bands were scored as presence (1) and absence (0) for each regenerant and mother plant and were transformed into a binary matrix. From binary data, DendroUPGMA server (Garcia-Vallvé *et al.*, 1999) was used in calculating matrix distances between the regenerants and their respective mother plants of the three cultivars. The distance matrices were generated based on Jaccard's similarity coefficient (Jaccard, 1908).

Similarity index between two samples i and j was obtained by the Jaccard formula;

$S_{ij} = a / (a + b + c)$

$D_{ij} = 1 - S_{ij}$

Where a is the number of DNA band(s) present in both plants i and j, b is the number of DNA band(s) present in i and not in j and c is the number of DNA band(s) present in j and not in i and D is the distant coefficient. Similarity matrices were subjected to cluster analysis of unweighted pair group method with arithmetic mean (UPGMA) and dendrograms constructed using FigTree software (Version 1.4.2).

CHAPTER FOUR

4.0 RESULTS

4.1 Induction of axillary buds and regeneration of plants

Nodal explants obtained from micropropagated plantlets (Figure 1A) formed axillary buds (Figure 1B) when incubated on MS supplemented with 10 mg/L BAP after 4 – 10 days of culture. 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 4). There was no significant difference in shoot induction frequencies among the three cultivars. Multiple shoots were formed from the axillary buds after four weeks of culture in axillary bud induction medium (CAM; MS salts with vitamins, 10mg/l BAP, 2 µM CuSO4, 2% sucrose, 0.3% Gelrite, pH 5.8) (Figure 2A). Cultivar TME14 produced the highest number of shoots (3.85) per axillary bud explant compared to Kibandameno and TMS60444 (Table 4). High frequency of rooting (100%) was obtained after transferring the plantlets to cassava basal medium (CBM; MS salts with vitamins, 2 µM CuSO4, 2% sucrose and 0.3% gelrite at pH 5.8).

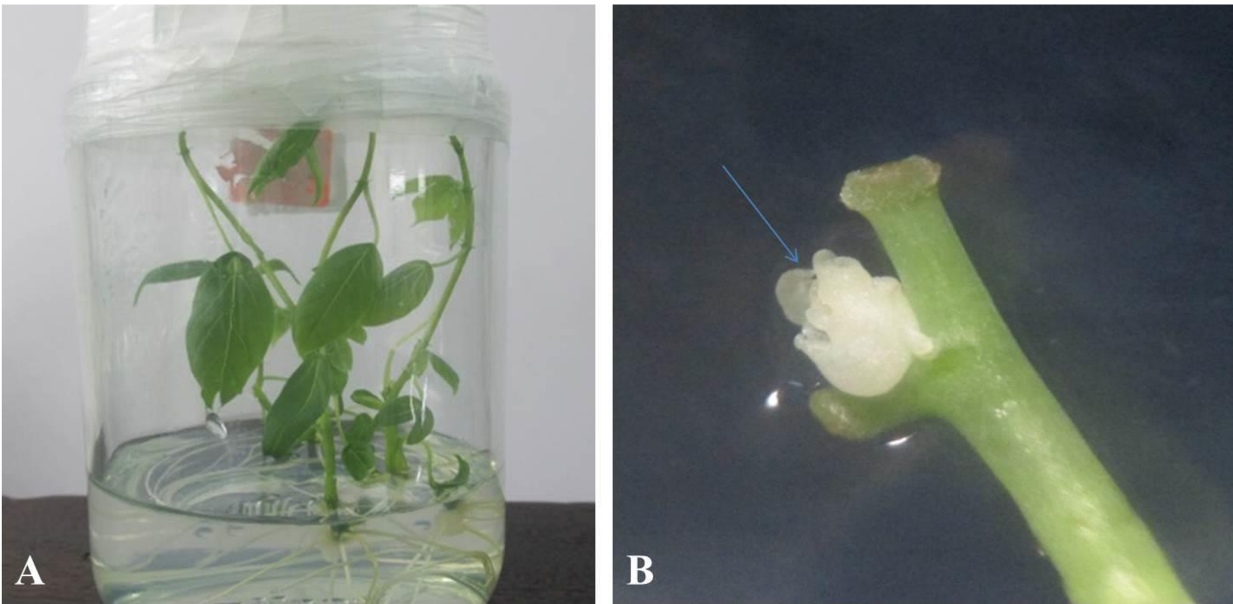

Figure 1. Plantlet regeneration and induction of axillary buds in cassava. **A**. Regenerated plantlets of cassava (cv. TME14) after four weeks on cassava basal medium (CBM) without growth regulators ; **B**. Axillary bud (arrow) forming from a cassava (cv. TMS60444) nodal cutting after seven days of culture on axillary bud induction medium (CAM) supplemented with 10mg/l BAP.

Table 4. Average frequencies of axillary bud and shoot induction and number of shoots produced per axillary bud explants from 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 ± 20.88	2.09 ± 0.35a
TME14	78.41 ± 24.6b	90.00 ± 11.55	3.85 ± 0.75b
TMS60444	91.06 ± 17.86b	83.85 ± 23.14	2.55 ± 0.44a
F	*75.29*	*1.24*	*5.71*
d.f	*2,42*	*2,32*	*2,32*
P	*<0.001*	*0.957*	*0.022*

Data on axillary bud and shoot induction frequencies were *arcsine transformed* before ANOVA. Means followed by the same letters in the same column are not significantly different at $P<0.05$ according to *Tukey's HSD* test. (±) represents standard deviation of the mean.

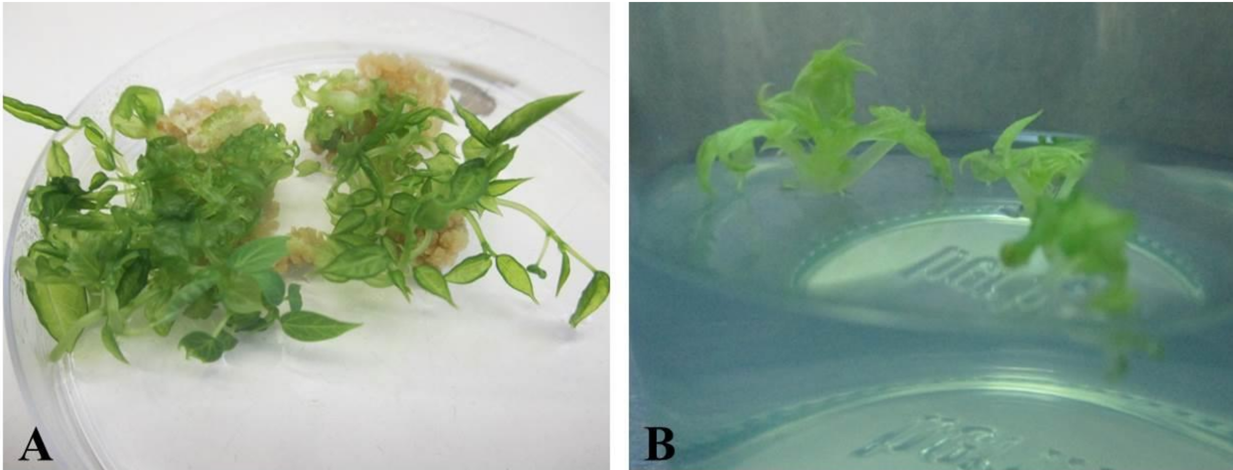

Figure 2. Induction of shoots from axillary buds of cassava **A.** Multiple shoots forming from axillary buds (cv. TME14) after three weeks of culture on CAM medium with 10mg/l BAP, in a 16 hour photoperiod, **B**: Freshly cultured cassava shoots (cv. TMS60444) in CBM without growth regulators

4.2 Regeneration of plants from nodal cuttings in successive subcultures

The nodal cuttings cultured on cassava basal medium (CBM; MS salts with vitamins, 2 µM CuSO4, 2% sucrose, 0.3% Gelrite, pH 5.8) formed roots after seven days followed by formation of new leaves from the tenth day. Successive subcultures did not affect the frequency of regeneration for both axillary bud-derived and micropropagated cassava plantlets in the six subcultures for cultivars Kibandameno, TME14 and TMS60444 as shown in Table 5 and Table 6. Over 90% of the initiated cuttings in each subculture successfully regenerated into cassava plantlets.

Table 5. Average regeneration frequencies of micro-propagated plantlets in three cassava cultivars from six successive subcultures

Cultivar	**Frequency of regeneration (%)**					
	Subculture 1	Subculture 2	Subculture 3	Subculture 4	Subculture 5	Subculture 6
Kibandameno	91.50 ±12.02	90.00±14.11	91.32± 3.44	97.91± 2.95	93.77± 2.92	91.12± 2.44
TME14	92.67± 7.02	98.00± 2.83	88.25± 9.89	97.62± 3.37	93.08± 2.00	89.23± 6.89
TMS60444	90.00±14.14	94.44± 7.86	98.50± 2.12	93.55± 2.05	90.50± 6.36	93.50± 2.11
F	*0.54*	*0.84*	*1.71*	*1.13*	*0.45*	*0.55*
d.f	*2,42*	*2,42*	*2,42*	*2,42*	*2,42*	*2,41*
P	*0.47*	*0.27*	*0.19*	*0.22*	*0.36*	*0.41*

Data was *arcsine t*ransformed before ANOVA. (±) represents standard deviation of the mean.

Table 6. Average regeneration frequencies of axillary bud-derived plantlets in three cassava cultivars from six successive subcultures

Cultivar	**Frequency of regeneration (%)**					
	Subculture 1	Subculture 2	Subculture 3	Subculture 4	Subculture 5	Subculture 6
Kibandameno	90.69 ±11.11	92.45±8.75	89.15± 7.70	93.43± 3.66	88.75± 7.32	89.52± 7.09
TME14	93.64± 5.23	97.00± 5.63	92.59± 8.99	92.65± 8.93	92.81± 4.32	89.73± 9.77
TMS60444	91.32±10.16	90.39± 8.48	96.55± 10.40	94.56± 7.89	93.56± 4.25	91.22± 5.40
F	*1.23*	*1.33*	*0.13*	*0.679*	*1.40*	*1.38*
d.f	*2,42*	*2,40*	*2,41*	*2,41*	*2,42*	*2,42*
P	*0.33*	*0.21*	*0.34*	*0.74*	*0.86*	*0.91*

Data was *arcsine t*ransformed before ANOVA. (±) represents standard deviation of the mean

4.3 Generation of primary somatic embryos

Cultivars Kibandameno, TME14 and TMS60444 did not record any significant difference in the frequency of organized embryogenic structures (OES) formation after 14 days of culture on embryo induction medium (CIM; MS salts with vitamins, 12mg/l picloram, 2 μM $CuSO_4$, 2% sucrose, 0.3% Gelrite, pH 5.8) (Table 7). The OES started to form on the cultured axillary buds from the seventh day. In all the three cultivars, observed OES were at different embryo developmental stages even from the same explant. Majority were at the late globular stage while some were at the early globular stage (Figure 3).

Table 7. Average frequencies of organized embryogenic structures (OES), secondary somatic embryos and friable embryogenic calli formed in the three cassava cultivars

Cultivar	Mean frequency of OES formation at 2 weeks (%)	Frequency of secondary somatic embryo formation (%)	Frequency of FEC production (%)
Kibandameno	94.44±5.41	94.44±5.41	23.43±3.14b
TME14	100±0	100±0	11.41±2.05a
TMS60444	96.22±2.87	96.22±2.87	33.51±3.32c
F	*0.62*	*0.83*	*266.85*
d.f	*2,27*	*2,32*	*2,27*
P	*0.55*	*0.42*	*<0.001*

Data was *arcsine* transformed before ANOVA. (±) represents standard deviation of the mean.

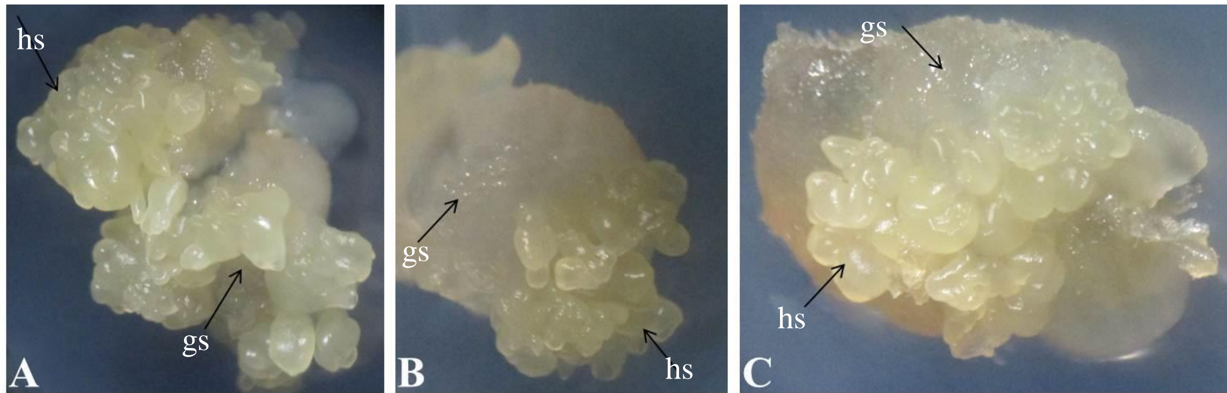

Figure 3. Primary somatic embryos from axillary buds of cassava after two weeks of culture on CIM medium with 12mg/l picloram. **A**: Kibandameno, **B**: TME14 and **C**: TMS60444. Arrows show organized embryogenic structures at different developmental stages [Globular stage (gs) and heart stage (hs)] within the same explants.

4.4 Generation of secondary somatic embryos

After two weeks of culture of organized embryogenic structures (OES) in fresh embryo induction medium (CIM2) which had the same composition as CIM, frequency of proliferation of secondary embryos was similar for cultivars TME14, TMS60444 and Kibandameno (Table 7). Secondary embryos were observed as finger-like projections in all directions (Figure 4). In some explants of cv. TMS60444 and TME14, the embryos differentiated into glassy cotyledons by the fourteenth day since transfer onto the CIM2 medium (Figure 4).

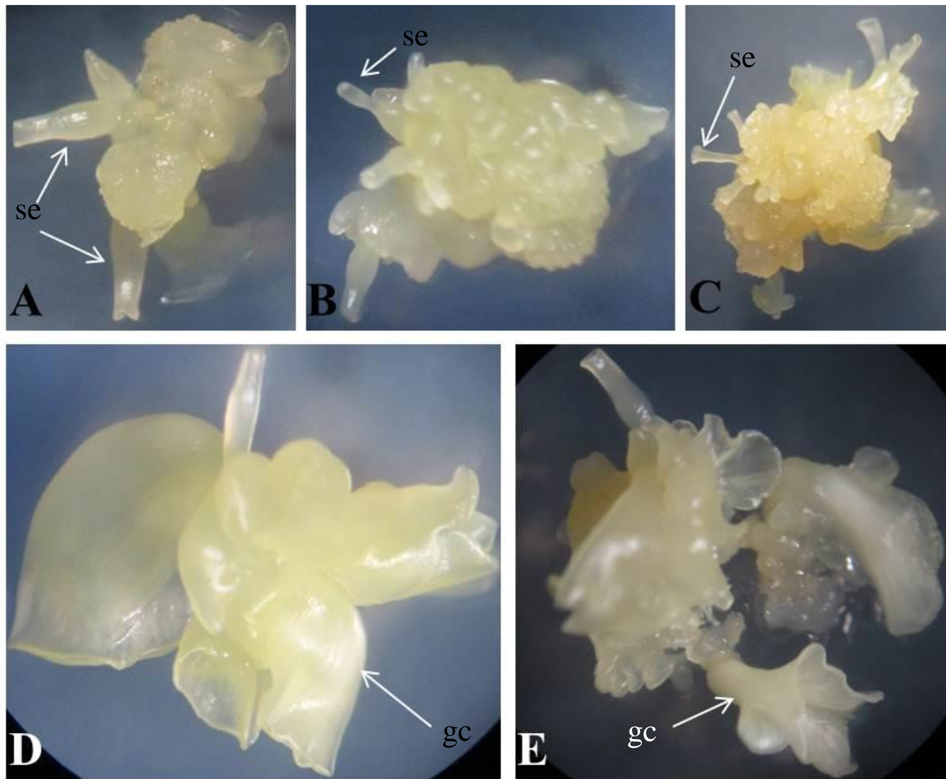

Figure 4. Secondary somatic embryos on embryo induction medium (CIM2) **A-C**: Somatic embryos (se) proliferating after 2 weeks of culture (cv. Kibandameno, TME14, and TMS60444 respectively). **D-E**: Glassy cotyledons (gc) forming from secondary somatic embryos of cultivars TME14 and Kibandameno respectively

4.5 Generation of friable embryogenic callus (FEC)

Four weeks after culturing secondary somatic embryos on GD medium (GD salts with vitamins, 12 mg/L picloram, 2% sucrose and 0.8% Noble agar at pH 5.8), the cultivars recorded significant difference ($P \leq 0.05$) in the frequency of friable embryogenic calli production (Table 7) with TMS60444 having the highest. However, the amount of friable embryogenic callus (FEC) produced by the fourth week was little in all the cultivars hence they were cultured for two more weeks on GD medium (Figure 5). Onset of FEC formation was first observed in cultivar TMS60444 after three weeks of culture on GD medium. In TME14 however, initiation of FEC delayed until the fourth week on GD medium. Generally frequency of FEC production in all the

three cultivars was low with cultivar TMS60444 recording the highest frequency of 33.51% (Table 7).

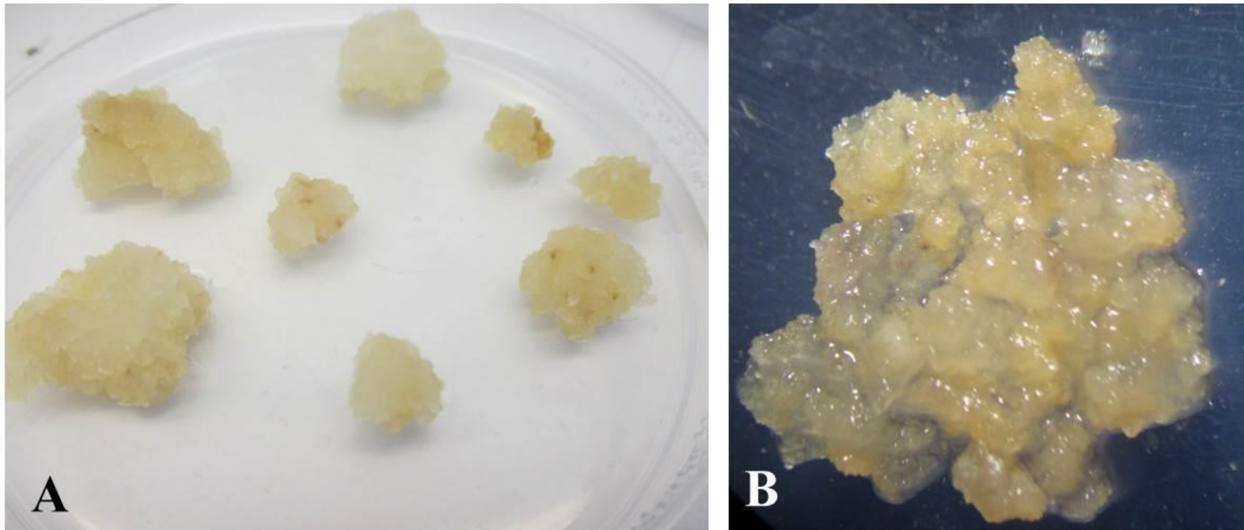

Figure 5. Friable embryogenic calli (FEC) after six weeks of culture of somatic embryos on GD medium with 12 mg/L picloram. **A**: FEC developing from embryo clusters (cv. TME14) **B**: Crushed FEC clusters (cv. Kibandameno)

4.6 Embryo maturation and regeneration of cotyledon stage embryos into plantlets

The frequencies of embryo emergence from primary embryos, secondary embryos and friable embryogenic calli (FEC), after two weeks of culture on embryo emergence medium (MSN; MS salts with vitamins, 1mg/L NAA, 2% sucrose and 0.8% Noble agar at pH 5.8) are shown in Table 8.

Primary and secondary embryos recorded high frequencies (88.23%-100%) of maturation on the emergence medium irrespective of the cassava cultivar. FEC however recorded a significantly lower ($P \leq 0.05$) emergence frequency for all the cultivars. Figure 6 shows green cotyledons emerging from friable embryogenic calli cultured on MSN.

Table 8. Cotyledonary-stage embryo emergence and plant regeneration frequencies of three cassava cultivars

Embryogenic stage	Cultivar	Frequency of cotyledonary stage embryos emergence	Frequency of plant regeneration
Primary SE	Kibandameno	100±0a	67.46±3.64
	TME14	100±0a	78.89±7.70
	TMS60444	100±0a	84.44±16.78
Secondary SE	Kibandameno	92.21±7.23a	89.68±9.01
	TME14	93.64±5.53a	86.03±14.3
	TMS60444	88.23±3.56a	88.57±10.3
FEC	Kibandameno	61.57±2.72b	72.22±4.81
	TME14	62.99±6.66b	75.48±4.31
	TMS60444	65.06±7.06b	79.37±18.03
F		*440.34*	*2.67*
d.f		*2,87*	*2,86*
P		*<0.001*	*0.296*

Data was *arcsine* transformed before ANOVA. Means followed by the same letters in the same column are not significantly different at *P<0.05* according to *Tukey's HSD* test. (±) represents standard deviation of the mean.

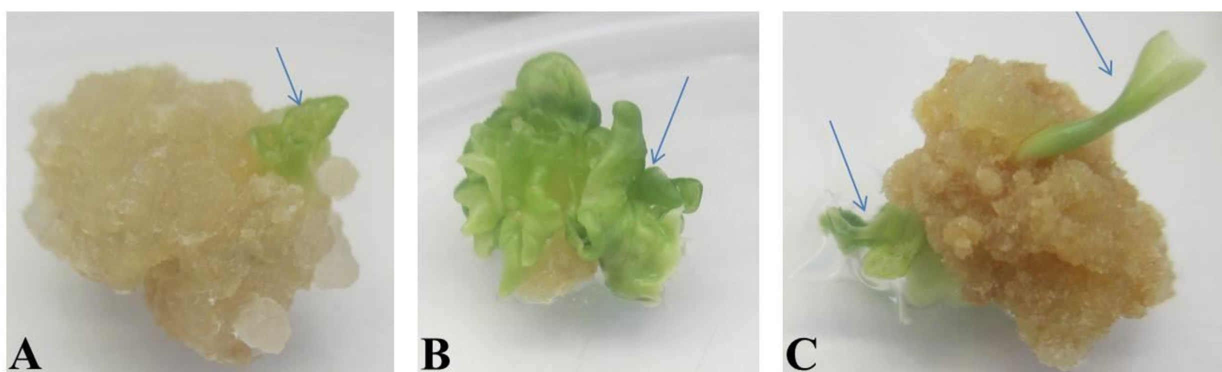

Figure 6. Green cotyledons (arrows) emerging from friable embryogenic callus (FEC) of three cassava cultivars after two weeks of culture on embryo emergence medium with 1mg/L NAA. **A** (cv.Kibandameno), **B** (cv.TMS60444) and **C** (cv. TME14)

Regeneration frequencies of elongated shoots derived from friable embryogenic calli (FEC), primary and secondary somatic embryos cultured on CBM;(MS salts with vitamins, 2 μM CuSO4, 2% sucrose, 0.3% Gelrite, pH 5.8) are shown in Table 8. There was no significant difference in regeneration frequencies produced among the three cultivars (Kibandameno, TME14 and TMS60444).

4.7 Acclimatization of plants

Plants transferred to the glass house after five weeks of culture on cassava basal medium (CBM) performed better during the acclimatization process. The plants from this category recorded high rates (85%) of survival as shown in Table 9. Plantlets cultured on CBM for only three weeks before transfer to the glass house however recorded low survival rates (21%) for all the cultivars. Figure 7 shows healthy regenerated cassava plants at the 20th and 30th days in the glass house. Phenotypically, all the tissue culture derived plants were similar to the mother plants regardless of the sources of explants i.e. primary embryos, secondary embryos, FEC, nodal cuttings (Figure 8).

Table 9. Response of cassava tissue culture generated plants to the acclimatization process after 30 days.

Cultivar	Duration of plantlets *in vitro* (weeks)	Survival rate after 30 days *ex vitro* (%)
TME 14	3	24.75
	5	87.5
TMS60444	3	18
	5	83.2
Kibandameno	3	24.75
	5	83.5

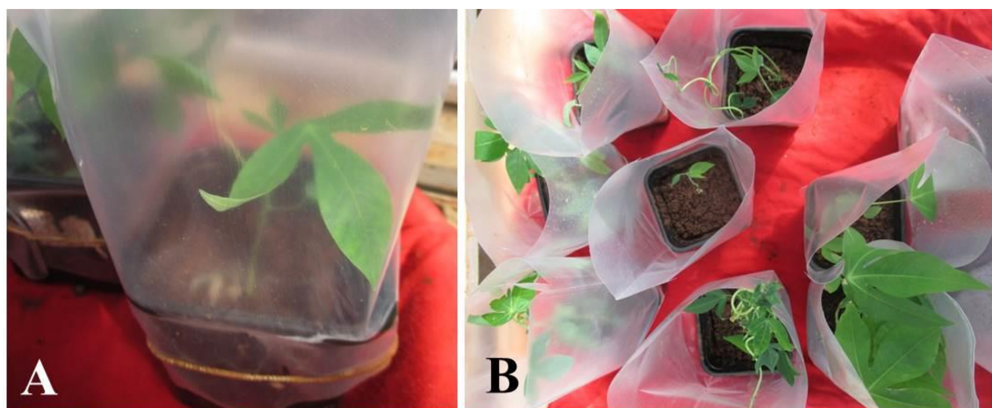

Figure 7. Acclimatization of cassava plants. **A**: Plantlet in a pot covered with a polythene bag 20 days post transfer, **B**: Fully opened polythene bags covering plantlets 30 days post transfer

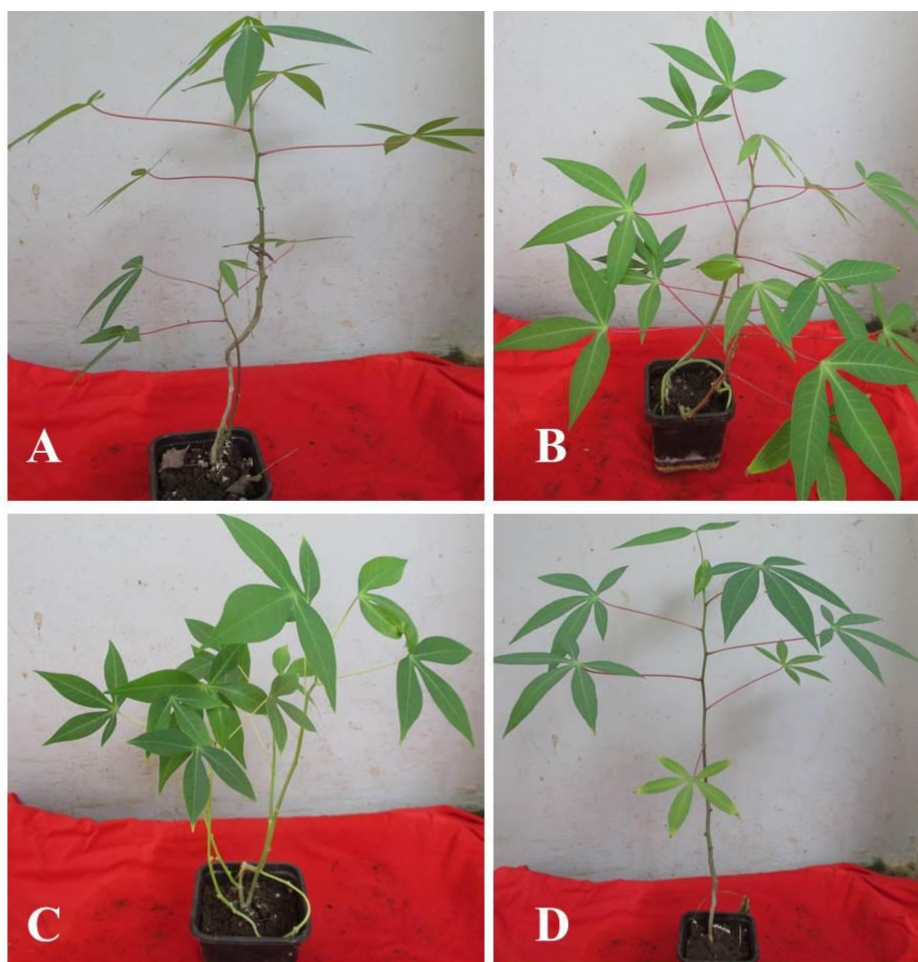

Figure 8. Acclimatized 12 weeks old cassava plants in the glass house. The plants were obtained from (**A**) First nodal subculture (cv. TME14), (**B**) friable embryogenic callus (cv. Kibandameno), (**C**) Primary somatic embryos (cv. TME14) and (**D**) Secondary somatic embryos (cv. TMS60444). .

4.8 Molecular analysis

4.8.1 DNA quantification

Each sample yielded an average DNA concentration of 2676.13ng/µl and an A_{280}/A_{260} ratio of 1.84. The concentration and quality was not dependent on the cassava cultivar.

Table 10. Average spectrophotometer readings obtained from three cultivars of cassava

Cultivar	Nucleic acids Conc.(ng/ul)	A260/A280
Kibandameno	2509.7 ± 21.78a	1.85±0.01a
TME14	2817.3 ± 11.36a	1.82±0.02a
TMS60444	2701.4 ± 8.36a	1.85±0.01a
F	*1.61*	*0.62*
d.f	*2,27*	*2,27*
P	*0.22*	*0.44*

Means followed by the same letters in the same column are not significantly different at $P<0.05$ according to *Tukey's HSD* test. (±) represents standard deviation of the mean.

4.8.2 Simple sequence repeats (SSRs) screening

Out of the 11 primers (Table 3) screened in the preliminary study using DNA of mother plants of the three cultivars, SRY175 did not produce any bands when its PCR products were electrophoresed hence was not used in the analysis. The ten primers that produced distinct and scorable bands were hence used for the molecular analysis of the regenerated plantlets.

4.8.2.1 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 (Table 3). The number of bands amplified 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 (Table 11).

Table 11. Number of monomorphic bands, polymorphic bands and the size range of PCR products amplified from axillary bud-derived regenerants (sixth subculture) 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 - 850
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	1	1	50	280 - 400
SRY 50	2	2	0	100	190 - 280
Total	**27**	**26**	**1**		

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 (Tables 12 and 13).

Table 12. Similarity matrices of mother plants of cultivars TME14, TMS60444 and Kibandameno and their respective axillary bud-derived regenerants (fifth subculture) 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.

Table 13. Similarity matrices of mother plants of cultivars TME14, TMS60444 and Kibandameno and their respective axillary bud-derived regenerants (sixth subculture) 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	0.808	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	0.808	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	0.808	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	0.808	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	0.808	0.741	0.741	0.741	0.741	0.741	0.741
E5						1	0.769	0.769	0.769	0.769	0.769	0.808	0.741	0.741	0.741	0.741	0.741	0.741
MS							1	1	1	1	1	0.955	0.792	0.792	0.792	0.792	0.792	0.792
S1								1	1	1	1	0.955	0.792	0.792	0.792	0.792	0.792	0.792
S2									1	1	1	0.955	0.792	0.792	0.792	0.792	0.792	0.792
S3										1	1	0.955	0.792	0.792	0.792	0.792	0.792	0.792
S4											1	0.955	0.792	0.792	0.792	0.792	0.792	0.792
S5												1	0.76	0.76	0.76	0.76	0.76	0.76
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 corresponding mother plants of cassava cultivars TME14, TMS60444 and Kibandameno respectively. **E1-E5**, **S1-S5** and **K1-K5** represent regenerants of cultivars TME14, TMS60444 and Kibandameno respectively.

Similarity calculated using Jaccard's similarity coefficient revealed 100 % genetic similarity among the mother plants and its derivatives from 1st to 5th subcultures are as shown in Table 12. At the 6th subculture, the mother plants and axillary bud regenerants were highly similar (similarity coefficient level was 1) for cultivars TME14 and Kibandameno.

One variant observed at the 6th subculture for cultivar TMS60444 from the banding profile of SRY78 primer is shown in Figure 9. As a result of the polymorphism, the variant was clustered separately from the mother plant and regenerants of the same cultivar (TMS60444) as shown by the dendrogram in Figure 10.

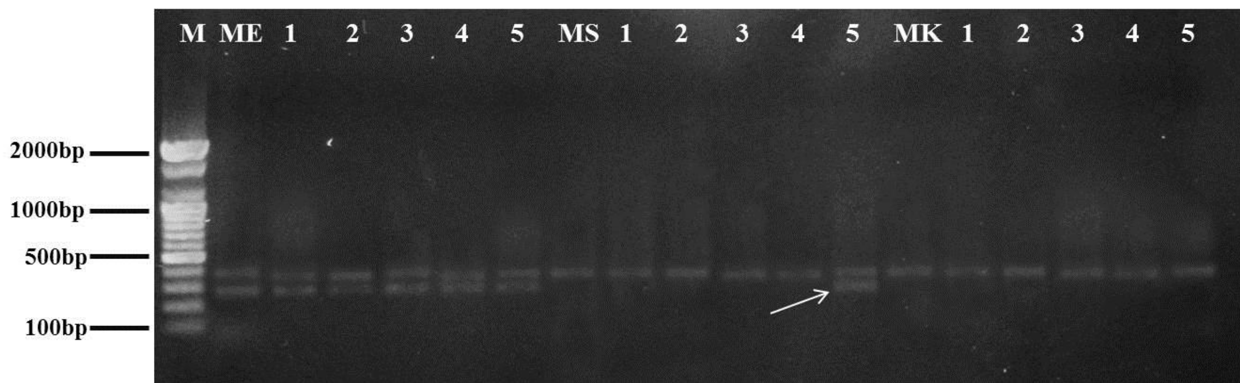

Figure 9. SSR profiles of 15 cassava regenerants from the sixth subculture (axillary bud –derived plantlets) alongside their mother plants after amplification using SRY78 primer. Lanes: **M**- 100bp DNA marker; **ME**, **MS**, **MK**- Donor mother plants of cultivars TME14, TMS60444 and Kibandameno respectively; 1 to 5- regenerants from each cultivar. Arrow indicates the somaclonal variant from regenerants of cultivar TMS60444

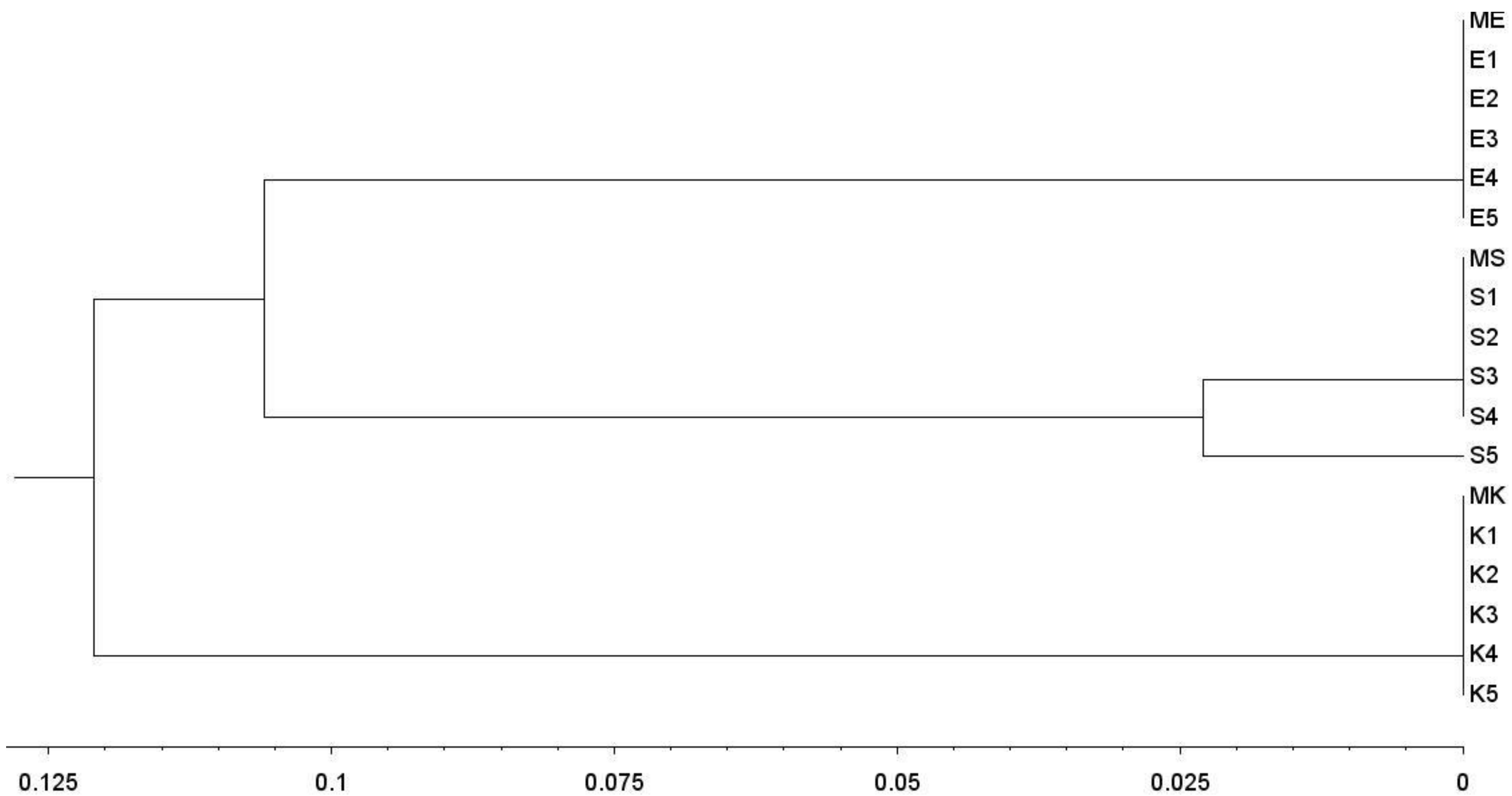

Figure 10. Dendrogram showing genetic relationships of axillary bud regenerated plants (1 - 5) from the sixth subculture and the mother plants (ME, MS and MK) of three cassava cultivars by UPGMA cluster analysis from SSR data. ME, MS and MK represents cultivars TME14, TMS60444 and Kibandameno, respectively.

For the micropropagated plants, the banding profiles of PCR amplified products from plants derived from 1st – 5th subculture were monomorphic and similar to the mother plants. 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 unlike in the sixth subculture where similarity ranged from 95% to 100% (Table 14).

Table 14. Similarity matrices of mother plants of cultivars TME14, TMS60444 and Kibandameno and their respective micropropagated regenerants (sixth subculture) 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	0.96	1	1	0.769	0.769	0.731	0.769	0.769	0.769	0.741	0.741	0.741	0.741	0.741	0.741
E1		1	1	0.96	1	1	0.769	0.769	0.731	0.769	0.769	0.769	0.741	0.741	0.741	0.741	0.741	0.741
E2			1	0.96	1	1	0.769	0.769	0.731	0.769	0.769	0.769	0.741	0.741	0.741	0.741	0.741	0.741
E3				1	0.96	0.96	0.731	0.731	0.692	0.731	0.731	0.731	0.704	0.704	0.704	0.704	0.704	0.704
E4					1	1	0.769	0.769	0.731	0.769	0.769	0.769	0.741	0.741	0.741	0.741	0.741	0.741
E5						1	0.769	0.769	0.731	0.769	0.769	0.769	0.741	0.741	0.741	0.741	0.741	0.741
MS							1	1	0.952	1	1	1	0.792	0.792	0.792	0.792	0.792	0.792
S1								1	0.952	1	1	1	0.792	0.792	0.792	0.792	0.792	0.792
S2									1	0.952	0.952	0.952	0.75	0.75	0.75	0.75	0.75	0.75
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 corresponding mother plants of cassava cultivars TME14, TMS60444 and Kibandameno respectively. **E1-E5**, **S1-S5** and **K1-K5** represent regenerants of cultivars TME14, TMS60444 and Kibandameno respectively obtained from the sixth nodal subculture.

Polymorphism was observed between nodal micropropagated and mother plants of cultivar TME14 and TMS60444 (Figure 11). 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 12).

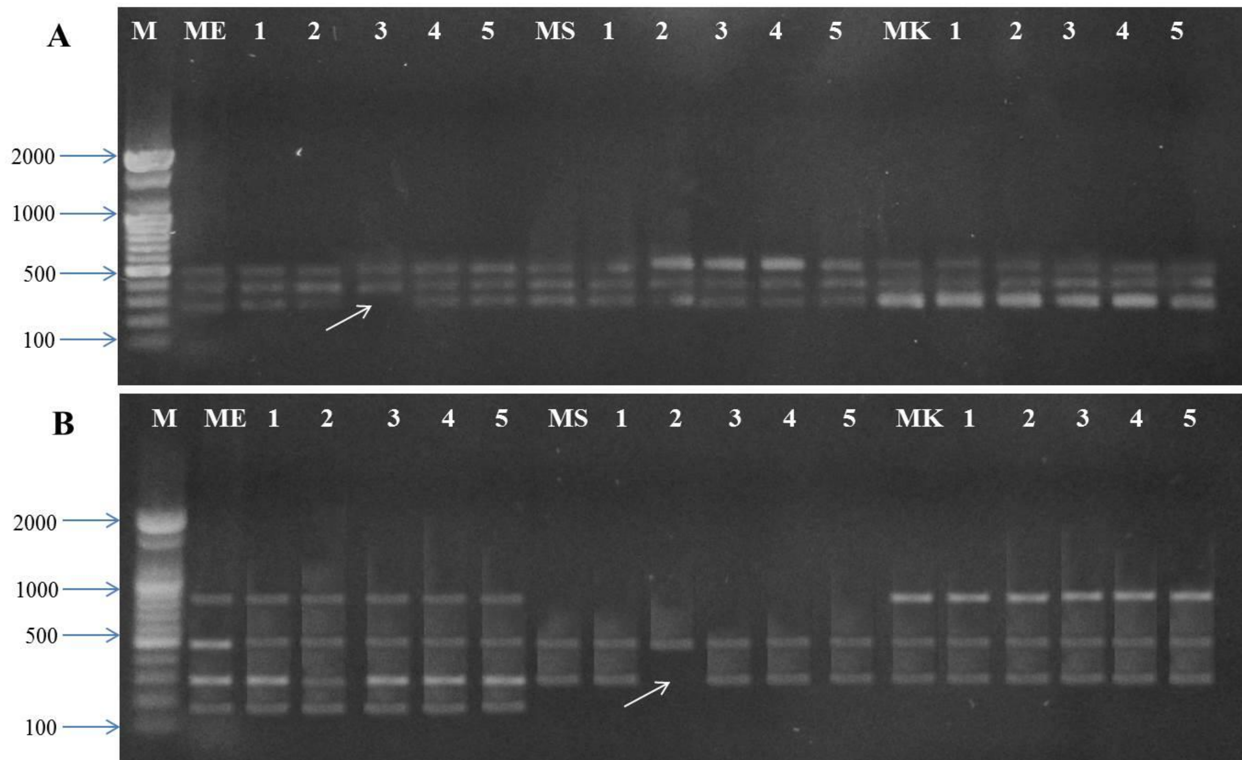

Figure 11. SSR profiles of 15 cassava regenerants (node micropropagated) in the sixth subculture alongside their mother plants after amplification using primer **A**) SRY35 and **B**) SRY51. Lanes: **M**- 100bp DNA marker; **ME**, **MS**, **MK**- Donor mother plants of cultivars TME14, TMS60444 and Kibandameno respectively; **1 to 5**- regenerants from each cultivar. Arrows indicate the two somaclonal variants from cultivars TME14 and TMS60444 respectively.

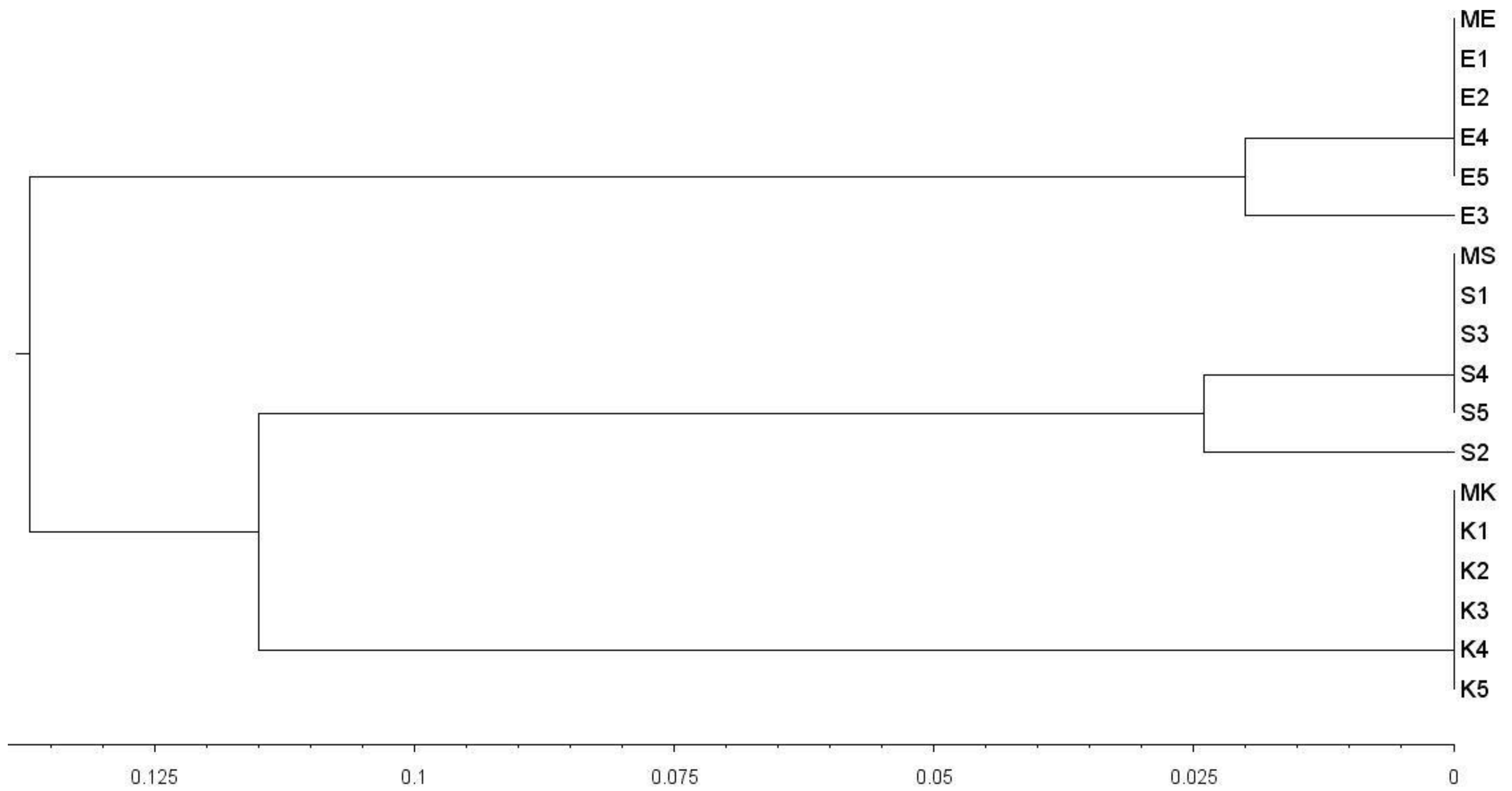

Figure 12. Dendrogram showing genetic relationships of cassava micropropagated plants (1 - 5) from the sixth subculture and the mother plants (**ME**, **MS** and **MK**) of three cassava cultivars by UPGMA cluster analysis from SSR data. **ME**, **MS** and **MK** represents cultivars TME14, TMS60444 and Kibandameno, respectively.

4.8.2.2 Genetic variability of primary and secondary somatic embryo-derived plants

The 10 SSR primers used to test possible microsatellite DNA instability in plants regenerated from primary somatic embryos generated a total of 401 distinct and clear amplification products (bands) ranging from 130 to 850 bp in size (Table 15). The number of bands for each primer ranged from 2 – 4, with an average of 3 bands per SSR primer. Eight out of 27 bands (alleles) were polymorphic with primer SRY45 having the highest number of polymorphic bands (Table 15).

Table 15. Total number of amplified products, number of polymorphic bands and percentage polymorphism in cassava donor mother plants and somatic embryo and FEC-derived regenerants using 10 SSR primers

Primer code	**Total amplified bands**	**No. of polymorphic bands (% polymorphism)**			**Size range (bp)**
		Primary embryos	Secondary embryos	FEC	
SRY 106	2	0 (0)	0 (0)	0 (0)	200 - 270
SRY 3	2	1 (50)	1 (50)	0 (0)	190 - 200
SRY 9	2	1 (50)	0 (0)	0 (0)	180 - 290
SRY 51	4	1 (25)	1 (25)	1 (25)	190 - 850
SRY 100	3	1 (33.33)	1 (33.33)	1 (33.33)	200 - 450
SRY 103	3	1 (33.33)	1 (33.33)	2 (66.67)	290 - 500
SRY35	3	0 (0)	0 (0)	3 (100)	290 - 480
SRY 45	4	2 (50)	2 (50)	3 (75)	130 - 450
SRY 78	2	1 (50)	1 (50)	0 (0)	280 - 400
SRY 50	2	0 (0)	0 (0)	0 (0)	190 - 280
Total	27	8 (29.63)	7 (25.93)	10 (37.04)	

The values in () represent percentage polymorphism
FEC: Friable embryogenic calli

The genetic similarities of the donor mother plants and primary somatic embryo-derived plants based on 10 SSR markers varied from 0.905 (mother plant and TMS60444 regenerate S3) to 1 (mother plant and TMS60444 regenerate S1) with an average value of 0.953 (Table 16). Both the highest and lowest similarity of the regenerated plants to the donor mother plants was observed in cultivar TMS60444. Only one primary somatic embryo-derived regenerants (S1) of cultivar TMS60444 showed similar SSR banding profiles with the mother plant hence recorded a similarity coefficient of 1 (Table 16).

Table 16. Genetic similarity matrices of donor mother plants and primary somatic embryo-derived plants of cultivars TME14, TMS60444 and Kibandameno based on Jaccard's similarity coefficient from SSR markers

	ME	**E1**	**E2**	**E3**	**E4**	**E5**	**MS**	**S1**	**S2**	**S3**	**S4**	**S5**	**MK**	**K1**	**K2**	**K3**	**K4**	**K5**
ME	1.000	0.960	0.960	0.960	0.960	0.960	0.769	0.769	0.808	0.692	0.731	0.808	0.741	0.769	0.704	0.778	0.704	0.778
E1		1.000	0.920	0.920	0.920	0.920	0.800	0.800	0.840	0.720	0.760	0.840	0.769	0.800	0.731	0.741	0.731	0.808
E2			1.000	0.920	1.000	0.920	0.731	0.731	0.769	0.654	0.692	0.769	0.704	0.731	0.667	0.741	0.667	0.741
E3				1.000	0.920	0.920	0.800	0.800	0.769	0.720	0.760	0.769	0.769	0.800	0.731	0.808	0.731	0.741
E4					1.000	0.920	0.731	0.731	0.769	0.654	0.692	0.769	0.704	0.731	0.667	0.741	0.667	0.741
E5						1.000	0.800	0.800	0.840	0.720	0.760	0.840	0.769	0.800	0.731	0.808	0.731	0.808
MS							1.000	1.000	0.955	0.905	0.952	0.955	0.792	0.750	0.750	0.760	0.826	0.760
S1								1.000	0.955	0.905	0.952	0.955	0.792	0.750	0.750	0.760	0.826	0.760
S2									1.000	0.864	0.909	1.000	0.760	0.720	0.720	0.731	0.792	0.800
S3										1.000	0.857	0.864	0.708	0.667	0.667	0.680	0.739	0.680
S4											1.000	0.909	0.750	0.708	0.783	0.720	0.783	0.720
S5												1.000	0.760	0.720	0.720	0.731	0.792	0.800
MK													1.000	0.955	0.955	0.957	0.955	0.957
K1														1.000	0.909	0.913	0.909	0.913
K2															1.000	0.913	0.909	0.913
K3																1.000	0.913	0.917
K4																	1.000	0.913
K5																		1.000

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

The dissimilarities between donor mother plants and the primary embryo-derived plants were observed as loss of bands/alleles. For instance using primer SRY45, a 450 bp band was absent in regenerated plantlet K1 of cultivar Kibandameno but was present in the mother plant MK (Figure 13). The dendrogram generated using UPGMA analysis based on Jaccard's genetic similarities of the SSR markers clustered each cultivar as an entity at a similarity coefficient of 0.86 (Table 16; Figure 14). For cultivar TME14 and Kibandameno, all the primary somatic embryo-derived regenerants evaluated were genetically different from the mother plants (Figure 14).

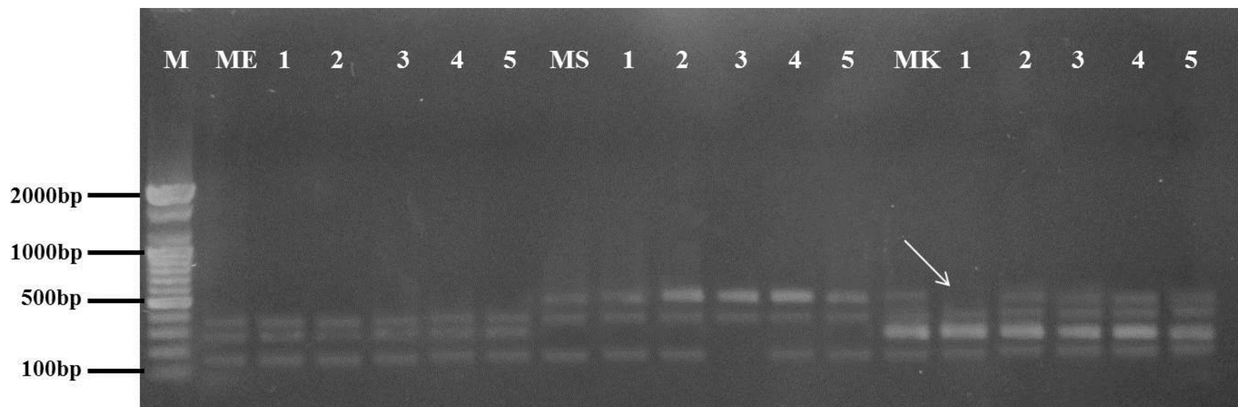

Figure 13. SSR profiles of 15 cassava regenerants obtained from primary embryos alongside their mother plants after amplification using primer SRY45. Lanes: **M**- 100bp DNA marker; **ME**, **MS**, **MK**- Donor mother plants of cultivars TME14, TMS60444 and Kibandameno respectively; **1 to 5**- regenerants from each cultivar. Arrow shows the somaclinal variant of cv. Kibandameno.

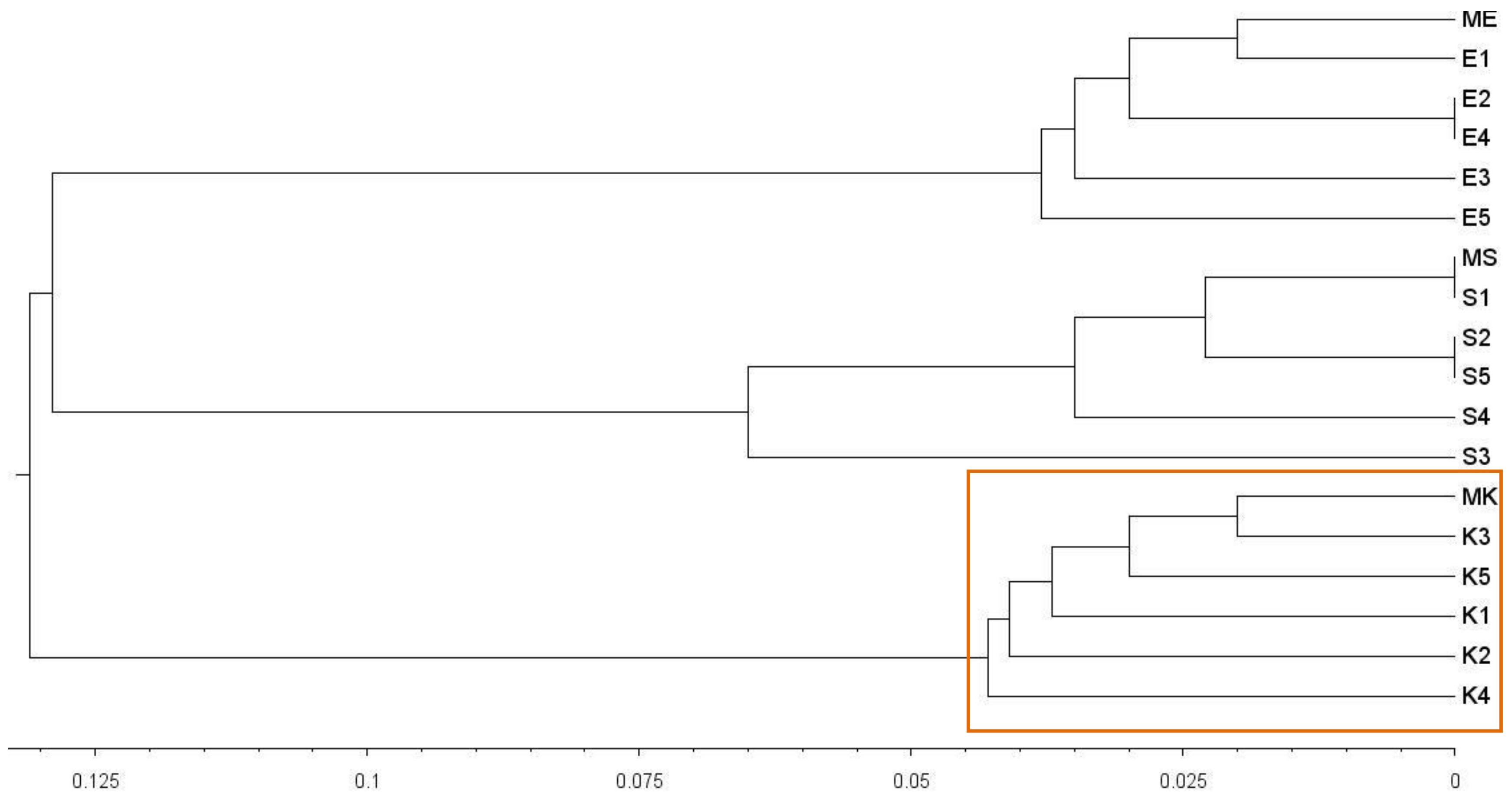

Figure 14. UPGMA dendrogram based on Jaccard's disimilarity indices from SSR data set showing genetic relationship among cassava regenerants (**E1-E5, S1-S5, K1-K5**) of cv. TME14, TMS60444 and Kibandameno respectively that were regenerated from primary somatic embryos. **ME**, **MS**, **MK** represents the respective donor mother plants.

For analysis of secondary embryo-derived plants, all 10 tested SSR primers amplified clear and scorable bands with satisfactory intensity. A total of 399 distinct and clear bands were generated from the donor mother plants and secondary somatic embryo-derived regenerants. The size of the amplification fragments ranged from 130 to 850 bp. The number of bands for each primer ranged from 2 to 4, with an average of 3 bands per SSR primer (Table 15). Seven out of 27 bands were polymorphic with primer SRY45 producing the highest number of polymorphic bands (Table 15). The genetic similarities of the donor mother plants and somatic embryo-derived plants based on SSR markers varied from 0.92 to 1 with an average value of 0.96. The highest similarity of the regenerants to the donor mother plants of 1 was recorded by cultivars TMS60444 and Kibandameno (Table 17) while the lowest similarity of 0.92 was recorded by regenerants of cultivar TME14. Two secondary somatic embryo-derived regenerants, S2 and K4 of cultivars TMS60444 and Kibandameno, respectively, showed similar SSR banding profiles with the mother plant hence recorded a similarity coefficient of 1 (Table 17).

Table 17. Genetic similarity matrices of donor mother plants and secondary somatic embryo-derived regenerants of cultivars TME14, TMS60444 and Kibandameno based on Jaccard's similarity coefficient from SSR markers

	ME	**E1**	**E2**	**E3**	**E4**	**E5**	**MS**	**S1**	**S2**	**S3**	**S4**	**S5**	**MK**	**K1**	**K2**	**K3**	**K4**	**K5**
ME	1.000	0.960	0.960	0.920	0.920	0.960	0.769	0.731	0.769	0.731	0.731	0.731	0.741	0.778	0.704	0.778	0.741	0.778
E1		1.000	0.920	0.958	0.880	0.920	0.800	0.760	0.800	0.760	0.760	0.760	0.769	0.808	0.731	0.808	0.769	0.741
E2			1.000	0.880	0.958	0.920	0.731	0.692	0.731	0.692	0.692	0.692	0.704	0.741	0.667	0.741	0.704	0.741
E3				1.000	0.917	0.880	0.833	0.792	0.833	0.792	0.792	0.792	0.800	0.769	0.760	0.769	0.800	0.769
E4					1.000	0.880	0.760	0.720	0.760	0.720	0.720	0.720	0.731	0.704	0.692	0.704	0.731	0.769
E5						1.000	0.800	0.760	0.800	0.760	0.760	0.760	0.769	0.808	0.731	0.808	0.769	0.808
MS							1.000	0.952	1.000	0.952	0.952	0.952	0.792	0.760	0.826	0.760	0.792	0.760
S1								1.000	0.952	1.000	0.905	0.905	0.750	0.720	0.783	0.720	0.750	0.720
S2									1.000	0.952	0.952	0.952	0.792	0.760	0.826	0.760	0.792	0.760
S3										1.000	0.905	0.905	0.750	0.720	0.783	0.720	0.750	0.720
S4											1.000	1.000	0.750	0.720	0.783	0.720	0.750	0.720
S5												1.000	0.750	0.720	0.783	0.720	0.750	0.720
MK													1.000	0.957	0.955	0.957	1.000	0.957
K1														1.000	0.913	1.000	0.957	0.917
K2															1.000	0.913	0.955	0.913
K3																1.000	0.957	0.917
K4																	1.000	0.957
K5																		1.000

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

The dissimilarities between donor mother plants and the embryo-derived plantlets were observed as loss of alleles. Figure 15 shows the absence of a 250bp band in two regenerants of cv. TMS60444. The dendrogram generated using UPGMA based on Jaccard's genetic similarities of the SSR markers clustered each cultivar as an entity at a similarity coefficient of 0.87 (Figure 16).

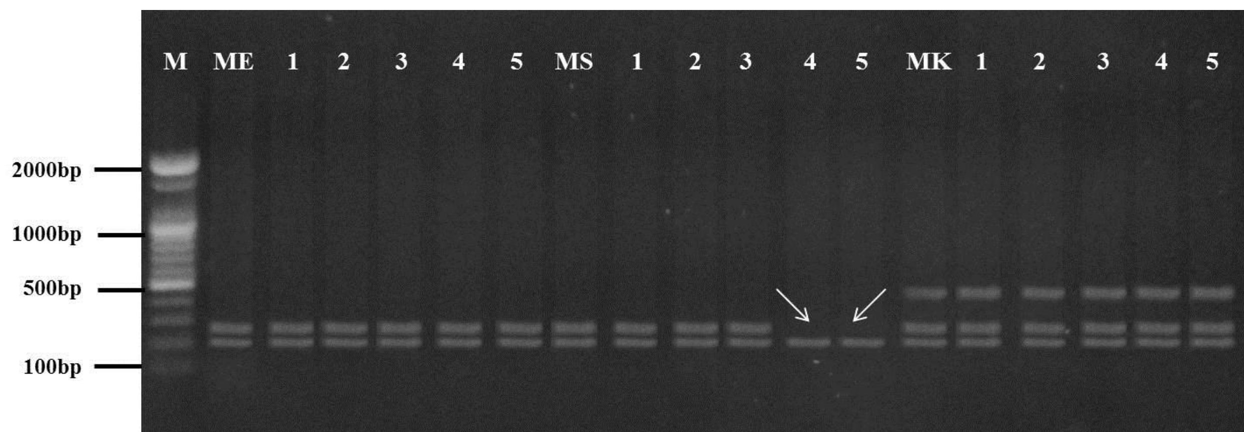

Figure 15. SSR profiles of 15 cassava regenerants obtained from secondary embryos alongside their mother plants after amplification using primer SRY100. Lanes: **M**- 100bp DNA marker; **ME**, **MS**, **MK**- Donor mother plants of cultivars TME14, TMS60444 and Kibandameno respectively; **1 to 5**- regenerants from each cultivar. Arrows show two somaclonal variants of cv. TMS60444

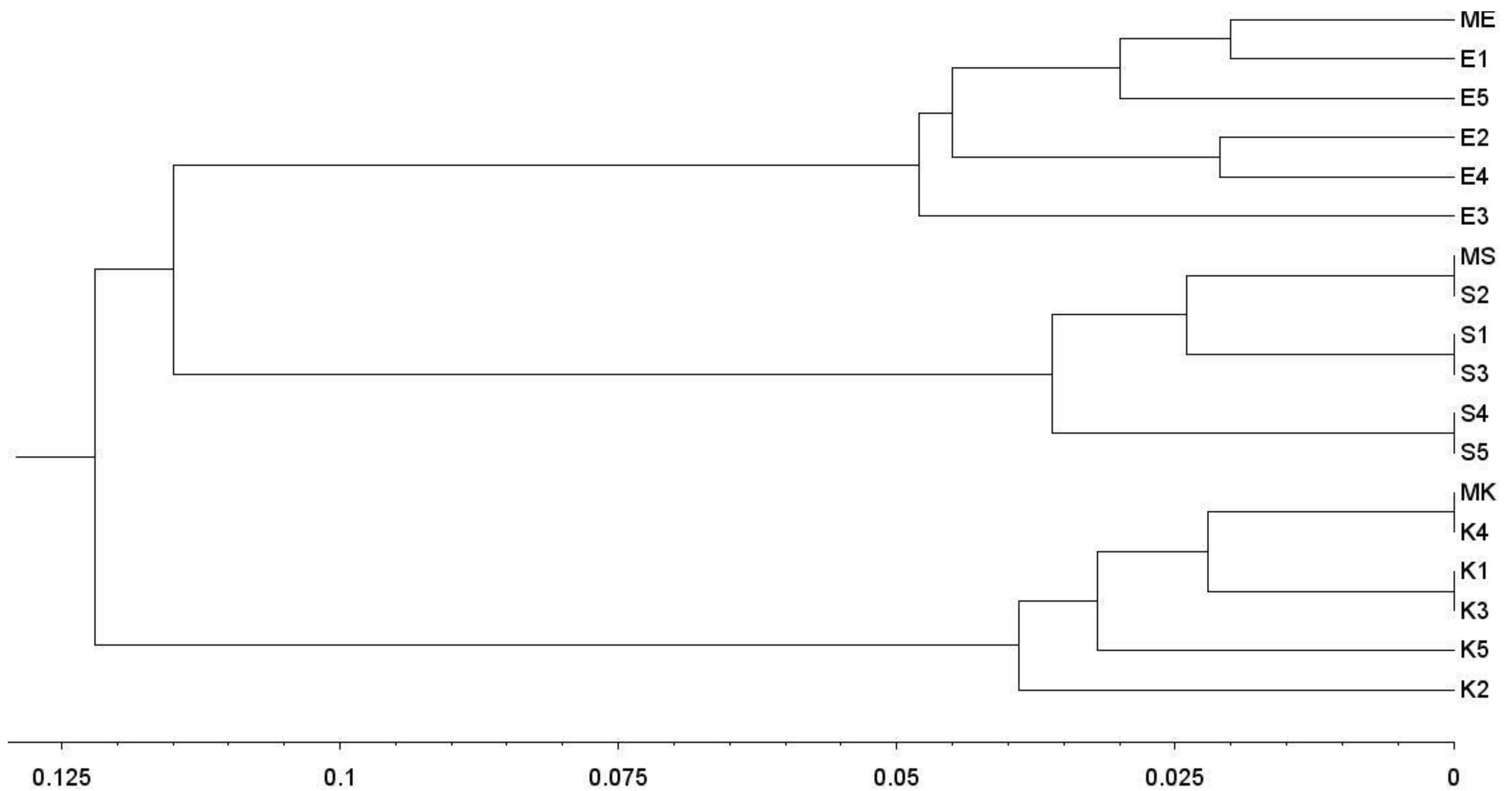

Figure 16. UPGMA dendrogram based on Jaccard's disimilarity indices from SSR data set showing genetic relationship among cassava regenerants (**E1-E5, S1-S5, K1-K5**) of cv. TME14, TMS60444 and Kibandameno respectively that were regenerated from secondary somatic embryos. **ME**, **MS**, **MK** represents the respective donor mother plants

4.8.2.3 Genetic variability of friable embryogenic calli-derived plants

All the 10 SSR primers produced a total of 384 bands ranging between 130 and 850 bp. The number of scorable bands, for each primer varied from 2 (SRY106) to 4 (SRY51 and SRY45) with an average of 3 bands per primer. Out of the 27 bands/alleles scored, ten were polymorphic with primers SRY35 and SRY45 recording the highest number of polymorphic bands (Table 15).

Jaccard's similarity indices between regenerated plants derived from all the three cultivars and their respective mother plants were less than 1 (Table 18). The highest similarity coefficient recorded between mother plants and regenerated plantlets was 0.96 for cultivar TME 14 while the lowest was 0.81 for cultivar TMS60444 (Table 18). Different degrees of genetic variations were detected between the mother plants and FEC-derived plants of different cassava cultivars. The dissimilarities between donor mother plants and the FEC-derived plants were observed as loss of bands in FEC-derived plants (Figure 17).

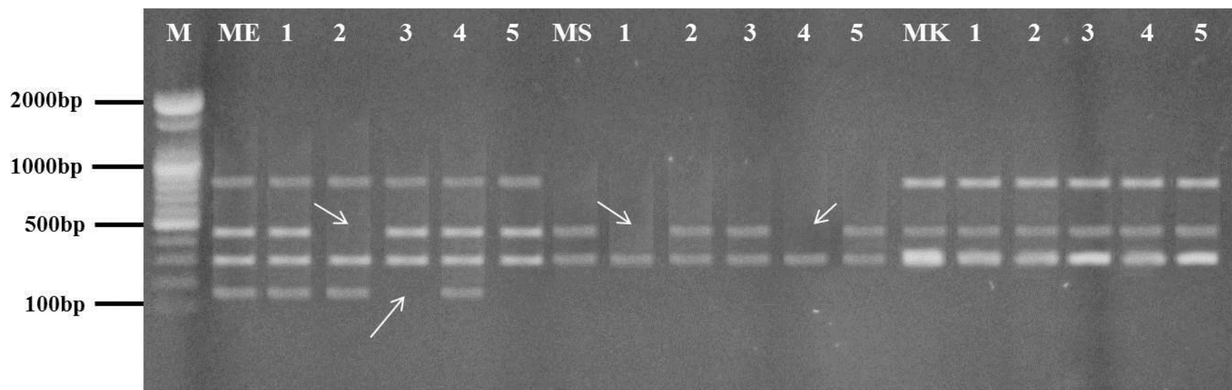

Figure 17. SSR profiles of 15 cassava regenerants obtained from friable embryogenic calli alongside their mother plants after amplification using primer SRY51. Lanes: **M**- 100bp DNA marker; **ME**, **MS**, **MK**- Donor mother plants of cultivars TME14, TMS60444 and Kibandameno respectively; **1 to 5**- regenerants from each cultivar. Arrows show somaclonal variants from cultivars TME14 and TMS60444.

Table 18. Genetic similarity matrices of donor mother plants and friable embryogenic callus-derived regenerants of cultivars TME14, TMS60444 and Kibandameno based on Jaccard's similarity coefficient from SSR markers

	ME	**E1**	**E2**	**E3**	**E4**	**E5**	**MS**	**S1**	**S2**	**S3**	**S4**	**S5**	**MK**	**K1**	**K2**	**K3**	**K4**	**K5**
ME	1.000	0.960	0.920	0.960	0.960	0.920	0.769	0.692	0.615	0.731	0.731	0.692	0.741	0.704	0.630	0.704	0.704	0.769
E1		1.000	0.958	0.920	0.920	0.880	0.731	0.654	0.640	0.692	0.692	0.654	0.704	0.667	0.654	0.667	0.667	0.731
E2			1.000	0.880	0.880	0.840	0.692	0.680	0.667	0.654	0.720	0.615	0.667	0.630	0.615	0.630	0.630	0.692
E3				1.000	0.920	0.958	0.800	0.720	0.640	0.760	0.760	0.720	0.769	0.731	0.654	0.731	0.731	0.800
E4					1.000	0.958	0.800	0.720	0.640	0.760	0.760	0.720	0.769	0.731	0.654	0.731	0.731	0.800
E5						1.000	0.833	0.750	0.667	0.792	0.792	0.750	0.800	0.760	0.680	0.760	0.760	0.833
MS							1.000	0.905	0.810	0.952	0.952	0.905	0.792	0.826	0.667	0.750	0.750	0.750
S1								1.000	0.895	0.950	0.950	0.810	0.708	0.739	0.583	0.667	0.739	0.667
S2									1.000	0.850	0.850	0.714	0.625	0.652	0.565	0.583	0.652	0.583
S3										1.000	0.905	0.857	0.750	0.783	0.625	0.708	0.783	0.708
S4											1.000	0.857	0.750	0.783	0.625	0.708	0.708	0.708
S5												1.000	0.708	0.739	0.652	0.739	0.667	0.667
MK													1.000	0.955	0.864	0.955	0.955	0.955
K1														1.000	0.818	0.909	0.909	0.909
K2															1.000	0.905	0.818	0.818
K3																1.000	0.909	0.909
K4																	1.000	0.909
K5																		1.000

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

Analysis of the coefficient of genetic similarity among the different plants indicated that all the regenerated plants had varied degree of genetic difference from the mother plant as well as among themselves (Figure 18).

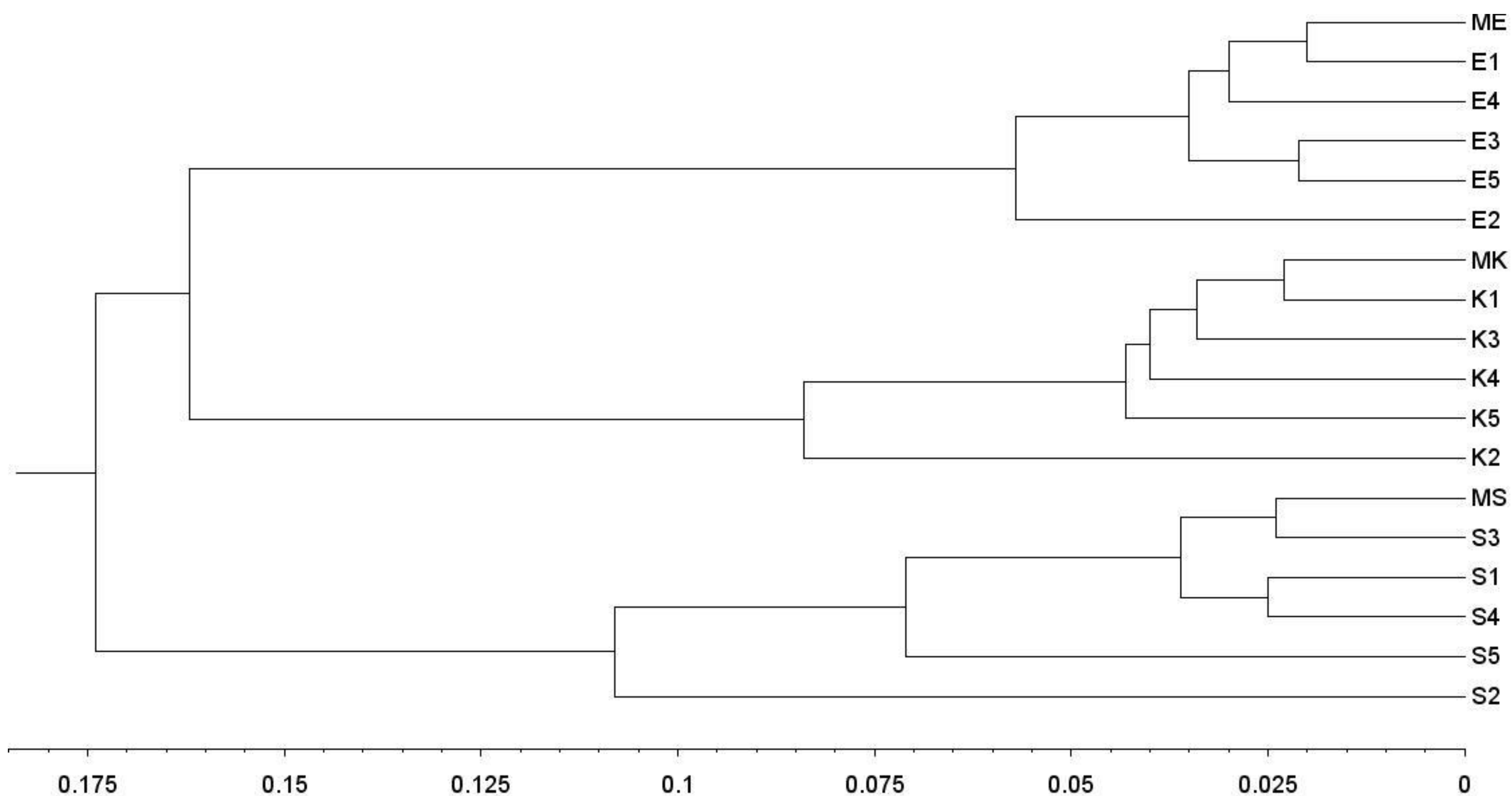

Figure 18. UPGMA dendrogram based on Jaccard's disimilarity indices from SSR data set showing genetic relationship among regenerants (**E1-E5, S1-S5, K1-K5**) of cv. TME14, TMS60444 and Kibandameno respectively obtained from friable embryogenic calli. **ME, MS, MK** represents the respective donor mother plants.

CHAPTER FIVE

5.0 DISCUSSION

The traditional use of field stem cuttings for cassava propagation poses a risk of spreading cassava diseases over a large geographical area within a short period of time. This is owed to the fact that farmers' sources of the cassava planting materials are neither restricted nor certified hence include use of cuttings from neighbours (Wasswa *et al.*, 2010). Such viral and bacterial diseases greatly reduce the yields obtained in cassava farming. *In vitro* micropropagation hence provides a suitable alternative to remediate the insufficiency of supply of disease-free cassava planting materials. Somaclonal variation has however been reported in crop plants recovered from *in vitro* cultures (Debnath, 2005) and the variations may affect the overall agronomic performance of the crop (Vázquez and Linacero, 2010). Retention of genetic stability among tissue culture regenerated plants is a crucial concern in clonal regeneration. Hence, assessment of the genetic stability of *in vitro* regenerants is highly recommended in plant tissue culture (Zilberman and Henikoff, 2007) and molecular markers have successfully been used to test the true-to-type nature of the regenerants.

Various explants of cassava plants can be used for micropropagation through organogenesis. In this study, axillary buds from nodal stem cuttings were used for production of plant material from elite mother plants of cassava cultivars Kibandameno, TME14 and TMS60444. Significant differences ($p \leq 0.05$) occurred in the axillary bud induction frequencies among the three cultivars. This suggests that different cassava cultivars respond differently to production of axillary buds from nodal explants, hence testing of individual cultivars for their axillary bud induction response is recommended before commencing the organogenesis process. Differential

response to similar tissue culture conditions by various types of explants due to differences in genotype have also been reported in other plants (Mehmood *et al*., 2013; Muktadir *et al*., 2016).

Shoots were successfully regenerated from axillary bud explants in all the three cultivars used in this study. Several studies have also reported successful nodal culture of cassava through tissue culture (Konan *et al.*, 2006; Medina *et al.*, 2006; Escobar *et al.*, 2009). Among various *in vitro* propagation methods, use of axillary buds is the most widely used system (Konan *et al.*, 2006) since it is simple and yields high rates of multiplication. In addition, incidences of genetic instability of plants regenerated through this system of *in vitro* propagation has been reported to be low in other crops due to existence of organized meristems (Martins *et al.*, 2004). Uniformity among plants regenerated from pre-formed structures such as axillary buds has also been reported (Ostry *et al.*, 1994).

The axillary bud-derived plants derived from 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 result is in agreement with the findings of Wang and Charles (1991) that suggested progenies from organized meristems illicit minimal or no variation with the donor mother plants since the cells do not go through callus stage. Basing clonal homogeneity of regenerated plants on morphological traits can be inaccurate; hence genetic fidelity assessment was done on the axillary bud-derived plants at molecular level using microsatellite markers. This marker system was selected due to its reliability, simplicity and efficiency in evaluating clonal uniformity in other crop plants (Rahman and Rajora, 2001; Marum *et al.*, 2009; Nookaraju and Agrawal, 2012) and in genetic diversity studies in cassava (Raji *et al.*, 2009; Mapayi *et al.*, 2013).

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 showed no polymorphism therefore indicating genetic uniformity of the regenerants and their respective donor mother plants. The monomorphic banding pattern in axillary bud-derived regenerants and mother plants suggested that all the tested plants were alike and similar to the donor mother plants genetically and no variations had 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 (Rahman and Rajora, 2001). Therefore, *in vitro* axillary bud-derived regenerants evaluated in this study were true-to-type and could be due to exclusion of intermittent callus stage. The results from this study support other findings (Kumar *et al.*, 2010; Bhatia *et al.*, 2011) on the use of axillary bud regeneration as one of the safest mode of micropropagation for preservation of genetic fidelity. 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 (Rani and Raina, 2000; Joshi and Dhawan, 2007; Kumar *et al.*, 2010; Bhatia *et al.*, 2011).

In vitro clonal propagation has been used in the production of seedlings that are identical to the mother plant after repeated sub-culturing (Ray *et al.*, 2006). Continuous maintenance of cultures may often result in chromosomal rearrangements and mutations (Cassells and Curry, 2001). As *in vitro* culture promotes genetic disturbances due to many factors, genetic stability confirmation is of immense importance in preservation of the desirable attributes of tissue culture regenerated

plants (Thiem *et al.*, 2013). Hence, it becomes vital to reveal the genetic steadiness of long term maintained cultures. Genetic stability of the micropropagated plants from different subculture cycles was also analyzed among 5 clones of each of the three cultivars alongside the respective 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 genetic stability 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 of the nodal micropropagated plants, 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. Such levels of similarities obtained after a period of seven months in *in vitro* culture indicated a very low polymorphism.

The longer the period of *in vitro* subculture, the higher the risk of genetic variation among micropropagated plants. Devi *et al.* (2015) reported an increase in genetic variations in between subcultures of *Nepenthes khasiana* from the first regeneration (5.65%) to the third regeneration (10.87%). 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 attributed to the stressful conditions such as high concentrations of sugars and phytohormones, low ventilation rate and low light availability, which the cultured plants are exposed to (Us-Camas *et al.*, 2014). Under such conditions, the cultured plant cells have to undergo dedifferentiation and continue dividing through a process that is error prone at the genetic level (Miguel and Marum, 2011). Therefore from the findings of this study, for

germplasm conservation, the *in vitro* propagated plants need to be transferred to the field after twenty five weeks of culture and fresh cultures initiated using new plants from the field.

In this study, different levels of genetic variation were detected between the donor plants and somatic (both primary and secondary) embryo-derived plants of different cultivars. Based on the polymorphisms observed, genetic variants detected amongst secondary embryo regenerants were fewer than primary embryo-derived plants. This contrasts with the reports of apparent positive correlation between genetic variations and number of multiplication cycles (Brar and Jain, 1998; Côte *et al.*, 2001), but supports the absence of mutations in somatic embryo-derived cocoa plants Fang *et al.* (2009). Cassava regenerated from somatic embryos (primary and secondary) did not involve the callus phase. The results contradict previous reports by other researchers that plants regenerated via direct somatic embryogenesis maintain genetic stability of the plantlets with least risk of genetic variation (Rani and Raina, 2000; Kumar *et al.*, 2010; Rai *et al.*, 2012). Variations observed in the banding profiles of SSR markers in the present study could be due to an array of causes at the molecular level such as DNA methylation, base deletions, additions or substitutions which may be attributed to tissue culture conditions. On the other hand, the role of epigenetic component of the genome in causing the variations cannot be disregarded. Sharma *et al.* (2007) also reported somaclonal variation among potato (*Solanum tuberosum*) regenerated through somatic embryogenesis.

Cassava has been reported to be recalcitrant to FEC induction (Liu *et al.*, 2011) and it has been reported that FEC induction in cassava is genotype-dependent, therefore, requiring optimization of protocols for efficient formation of FEC for each cultivar (Raemakers *et al.*, 2000; Chetty *et al.*, 2013; Nyaboga *et al.*, 2013). In the current study, this was clearly demonstrated by significant differences observed across the three cassava cultivars (TMS60444 - 33.51%,

Kibandameno - 23.43% and TME14 -11.41%). These observations demonstrate the possibility of presence of an underlying genetic control that influences the ability of a given genotype to induce FECs and hint at the presence of genes or alleles that repress formation of FECs.

In *Agrobacterium*-mediated transformation of cassava, utilization of FEC developed from somatic embryogenic structures as the target tissues has widely been adopted (Liu *et al.*, 2011; Taylor *et al.*, 2012). FEC is a preferred target tissue for genetic transformation since; the high population of totipotent cells located on the surfaces of preexisting FEC tissues are unicellular in origin thus reducing the likelihood of chimeras occurring and the large surface area they expose during transformation process owing to their small-sized cell clumps hence making access to target cells easier for the transforming agent and eventually, the selection of transformants. Further, FEC has the potential of producing large numbers of transgenic events (Bull *et al.*, 2009; Taylor *et al.*, 2012). Despite the wide adoption of FEC in genetic engineering of cassava (Liu *et al.*, 2011; Sayre *et al.*, 2011), no study has been done to assess the genetic stability of FEC-derived regenerants with reference to the donor mother plants.

In this study, all FEC-derived regenerants from the three cassava cultivars were genetically variable. The dendrogram, based on UPGMA method of cluster analysis demonstrated the genetic instability of FEC-derived regenerants from the mother plant. The genetic variability occurred due to loss of SSR alleles in all the three cultivars, which is a form of mutation mainly observed among eukaryotes (Chang *et al.*, 2002; Vigouroux *et al.*, 2002; Sibly *et al.*, 2003). Friable callus induction is a complex biological process that might evoke changes at the genetic sequence or gene expression levels (Ikeuchi *et al.*, 2013; Ma *et al.*, 2015) and this explains the variability observed in FEC-derived plants with respect to the mother plants. In addition, the transition of the somatic cells to embryonic cells, dedifferentiation, proliferation before

subsequent regeneration into plantlets involves a lot of genetic programming and reprogramming that could easily alter the genetic makeup of the cultured plant (Neelakandan and Wang, 2012). This genetic variability of FEC-derived regenerants with respect to mother plants presents a potential drawback to the use of FEC as the preferred target tissue in cassava genetic transformation. The study therefore provides valuable insights on the possible effect of somatic embryogenesis process on the genetic makeup of cassava.

Culturing of explants *in vitro* may induce the loss of cellular control, resulting in somaclonal variations (Pathak and Dhawan, 2012). The reasons accounting for somaclonal variations remain unclear, although factors such as culture medium composition (Bardini *et al.*, 2003), culture duration (Modgil *et al.*, 2005), phytoregulators (Biswas *et al.*, 2009), number of subcultures or transfers (Rodríguez López *et al.*, 2010), explant type, passage through the indirect callus phase (mass of undifferentiated cells with regeneration potential) (Miguel and Marum, 2011) are considered capable of inducing this variability *in vitro*. In this study, high genetic variation observed among FEC-derived plants versus somatic embryo-derived plants might have been as a result of longer total period of culture *in vitro*. High oxidative stress that plant tissues undergo during *in vitro* culture process as a consequence of the formation of reactive oxygen species has been reported to cause damage at the DNA level and may lead to instability of microsatellite (Jackson *et al.*, 1998). The detection of somaclonal variants in this study using SSR markers among morphologically indistinguishable plants underscores the need for genetic stability assessment of tissue culture-propagated plants.

Based on documented literature on *in vitro* propagation of cassava, no study exists on occurrence of somaclonal variation. The use of SSR markers in this study proved to be effective in revealing somaclonal variation in cassava regenerated through somatic embryogenesis.

CHAPTER SIX

6.0 CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

1. This study demonstrated that the use of 10 mg/L BAP is suitable for regeneration of plants from axillary buds of cassava and no genetic variation was induced. This indicates that direct organogenesis using axillary buds is a safe method for regeneration of true-to-type plants in cassava.
2. For supplying true-to-type seedlings and germplasm conservation, sub-culture frequency of both micropropagated and axillary bud-derived cassava should be limited to a maximum of five times, after five weeks intervals, to obtain clonally identical plants.

3a. Somaclonal variation occurred in plants regenerated from all stages of somatic embryogenesis i.e. primary somatic embryos on MS with 12mg/l picloram, secondary somatic embryos on MS with 12mg/l picloram and friable embryogenic calli (FEC) on GD with 12mg/l picloram.

3b. The use of SSR markers was effective in detection of somaclonal variants regenerants in cassava cultivars Kibandameno, TMS60444 and TME14 obtained through somatic embryogenesis and direct organogenesis.

6.2 Recommendations

1. Optimization of shoot induction from axillary buds needs to be done for different cultivars of cassava in order to increase the regeneration efficiency.
2. There is need to explore other hormones such as 2,4-dichlorophenoxyacetic acid (2,4-D), dicambia and Indole-3-acetic acid (IAA), for improvement of FEC production for each cassava cultivar.
3. SSR markers should be employed in early detection of somaclonal variation among cassava regenerants resulting from all the tissue culture processes of cassava to preserve their genetic fidelity with respect to the elite mother plants.
4. *In vitro* micropropagated and axillary-bud derived cassava plants should be transferred to the field after five successive sub-cultures of five weeks intervals or earlier and fresh cultures initiated from the field to limit the chances of genetic variation resulting from tissue culture.
5. Genetic engineering technologies aimed at improvement of cassava should target the use of induced axillary buds as explants for genetic transformation.
6. There is need to determine if the genetic variations induced in cassava tissue culture are heritable or epigenetic by growing the regenerated plants to maturity and evaluation of F1 plants.

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APPENDIX: Murashige and Skoog Medium (1962) composition

Nutrients	Concentrations (mg/l)
Macronutrients	
Ammonium Nitrate	1650
Potassium Nitrate	1900
Calcium Chloride, Anhydrous	332.2
Magnesium Sulphate	180.7
Potassium Phosphate Monobasic	170
Micronutrients	
Potassium Iodide	0.83
Boric Acid	6.2
Manganese Sulphate· H_2O	16.9
Zinc Sulphate· 7 H_2O	8.6
Molybdic Acid, Sodium Salt, 2 H_2O	0.25
Cupric Sulphate· 5 H_2O	0.025
Cobalt Chloride· 6 H_2O	0.025
Ferrous Sulphate· 7 H_2O	27.8
Na 2 -EDTA	37.26
Vitamins	
Nicotinic Acid	0.5
Pyridoxine ,HCl	0.5
Thiamine	0.1
Other components	
Myo-Inositol 100	100
Glycine	2
Sucrose	30000
Agar	8000