OPTIMIZATION OF REGENERATION AND *AGROBACTERIUM*-MEDIATED TRANSFORMATION PROTOCOLS FOR SELECTED KENYAN CASSAVA (*Manihot esculenta* Crantz) GENOTYPES

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other University or for any other award.

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To my mother, Susan Katumbi. “All that I am, and all that I shall be, I owe it to my prayerful mother”.

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ABBREVIATIONS AND ACRONYMS

2,4-D	2,4-dichlorophenoxyacetic acid
ACMV	African cassava mosaic virus
ANOVA	Analysis of variance
BAP	6-Benzylaminopurine
Bar	Bialaphos
bp	Base pairs
CaMV	Cauliflower mosaic virus
CMD	Cassava mosaic disease
CTAB	Cetyl trimethyl ammonium bromide
DNA	Deoxy-ribonucleic acid
DMSO	Dimethyl sulfoxide
dNTPs	Dinucleotide triphosphate
FAO	Food and Agriculture Organization
FEC	Friable embryogenic callus
GA_3	Gibberellic acid
GFP	Green Fluorescent Protein
GUS	β-glucuronidase
ILL	Immature leaf lobes
KALRO	Kenya Agricultural and Livestock Research Organization
LB	Lysogeny broth
MANOVA	Multivariate analysis of variance
MS	Murashige and Skoog medium
NAA	α Naphthaleneacetic acid
OD	Optical density
PCR	Polymerase Chain Reaction
Picloram	3,5,6- trichloro-4-aminopicolinicacid
PP2A	Protein Phosphatase 2A
Sdw	Sterile distilled water
PPD	Post-harvest physiological deterioration
SSA	Sub-Saharan Africa
T-DNA	Transfer DNA
UV	Ultraviolet
X-Gluc	5-bromo-4-chloro-3-indoyl glucuronic acid
YEP	Yeast Extract Peptone

ABSTRACT

Cassava (*Manihot esculenta* Crantz) is a tropical root crop that serves as a staple food and a vital source of income to small holder farmers in the tropics. Despite its contribution to food security, cassava production and utilization is faced by several challenges that include post-harvest physiological deterioration, insect and disease susceptibilities and accumulation of cyanogenic glycosides. Cassava crop improvement by conventional breeding has failed to address these constraints because of unsynchronized flowering, lack of resistance genes, high heterozygosity, allopolyploidy and poor seed set. Genetic transformation which begins with the establishment of embryogenic callus cultures can be used as one of the ways to complement these challenges faced by cassava breeders. This study investigated the effects of explant source (immature leaf lobes and meristematic stem segments), auxins (2, 4-dichlorophenoxyacetic acid (2, 4-D) and picloram), and photoperiod (0/24 and 16/8 light/ dark) on callogenesis and embryogenesis in five African cassava genotypes (KME 1, 08/080, 08/354, 08/274 and *TMS 60444*). Callus formation and embryogenesis were successfully achieved in both explant sources. The leaf explants recorded significantly higher frequencies ($p < 0.05$) of somatic embryogenesis compared to the stem explants in all the five genotypes. This study showed that the 0/24 light/ dark photoperiod was superior to the 16/8 light/ dark cycle for both callogenesis and embryogenesis. Although statistically insignificant, 8 mg/l 2, 4-D was the best concentration for the induction of embryogenesis in 08/354, TMS 60444, 08/274 and 08/080 while 10 mg/l gave the best results for genotype KME 1. For picloram, 10 mg/l showed the best results for embryogenesis across all genotypes. This study also determined the effects of varying formulations of BAP (6-Benzylaminopurine), NAA (α-Naphthalene acetic acid) and GA_3 (Gibberellic acid) on somatic embryo maturation and plant recovery of the selected cassava genotypes Embryos in the cotyledonary stage were incubated in maturation medium supplemented with five different combinations of plant growth regulators: BAP, NAA, and GA_3. Significant differences ($p < 0.05$) were recorded in shoot formation frequencies with combination 2 mg/l BAP, 0.01 mg/l NAA, 1.5 mg/l GA_3 and combination 1 mg/l BAP, 0.02 mg/l NAA, 1.5 mg/l GA_3 giving the highest rates. Transformability was determined by carrying out a histological *GUS* (β-glucuronidase) assay on callus transformed using *Agrobacterium tumefaciens* strain EHA 101 harbouring plasmid pTF 102 with a *GUS* visual marker gene and a bialaphos selectable marker gene. All the genotypes were found amenable to *Agrobacterium* mediated transformation with TMS 60444 and 08/274 recording the highest transformabilities of 73.33 % and 68.33 % respectively. A positive polymerase chain reaction (PCR) amplification targeting the *GUS* gene confirmed the transfer of the transgenes into cassava cells. The validated regeneration and transformation protocols reported here can be used for the integration of desired traits in African cassava genotypes.

CHAPTER ONE
INTRODUCTION

1.1 Background information

Cassava (*Manihot esculenta* Crantz) is a tropical root crop that serves as a source of carbohydrates for more than 800 million people worldwide and its cultivation is estimated to cover more than 18.9 million hectares worldwide (FAO, 2011, Mtunguja *et al.,* 2016). In developing world, cassava is one of the four most important food crops along with rice, sugarcane and maize. It is a vital source of food and income to resource poor farmers in the tropics and a staple food crop for nearly 200 million people in sub-Saharan Africa (Nyaboga *et al.,* 2013; Piero *et al.,* 2015a). In addition to its use as food, cassava is used in the manufacture of industrial products such as adhesives for laundry purposes, papers, alcohols, dextrin, textile sizing and wood glue (Okezie and Kosikowski, 1982; Anyanwu *et al.*, 2015).

Cassava is native to the Southern border of the Amazon basin (Allem, 1994). It was introduced to Africa in the mid-16th century by Portuguese traders (Olsen and Schaal, 1999; Ngugi, 2013). In Kenya, cassava is most widely grown in Nyanza, Western and Coastal regions by on a subsistence basis (Were *et al.,* 2004).

The global production of cassava is about 256 million tones, of which Africa accounts for 146 million tones (FAOSTAT, 2012; Okoya *et al.,* 2016). Due to its increasing use by the starch industry and its potential use for biofuel production, the global demand for cassava is rapidly growing (Jansson *et al.,* 2009; Larsson *et al.,* 2015). The main value of this tuber crop resides in its efficient carbohydrate production, ability to persist in the soil for long without decaying and its ability to grow in marginal conditions (Alves, 2002). Although greatest yields are obtained on loamy sandy soil under humid tropical conditions, cassava can tolerate marginal eroded soils and it adapts easily to acidic soils that are prevalent in the tropics (Nweke *et al.,* 2002).

Cassava is clonally propagated using stem cuttings. After planting, new roots are produced and axillary buds sprout to form the shoot system. Macro-propagation is faced by challenges like low multiplication rates and accumulation of viral and bacterial diseases which reduce the productivity of the crop (Rani and Raina, 1998). Cultivation of cassava is beset by many problems such as pest and disease susceptibilities, accumulation of cyanogens (Piero *et al.,* 2015a), post-harvest physiological deterioration, and a low nutritional quality and commercial value of the starch (Ceballos *et al.,* 2004; Sayre *et al.,* 2011; Nyaboga *et al.,* 2013). Improvement of the cassava germplasm through traditional breeding methods has been hampered by the non-availability of necessary genes in the germplasm, high heterozygosity, allopolyploidy, low fertility as well as unsynchronized flowering making it difficult and time-consuming (Thro *et al.,*1996; Ceballos *et al.*, 2012).

Genetic transformation techniques that can complement for the limitations of conventional breeding (Taylor *et al.,* 2004) are hindered by the requirement of a reproducible transformation and regeneration system of cassava (Raemarkers *et al.,*1998). This is because the ability to regenerate *in vitro* is often limited to certain tissues and developmental stages. Therefore, a method for efficient transfer and stable integration of the transgenes into cassava genomic DNA as well as a method for identifying and selecting transformed cells are essential for cassava transformation (Fregene and Puounti-Kaerlas, 2002).

This study optimized a regeneration protocol for five selected Kenyan cassava genotypes and assessed their *agrobacterium* mediated transformability. Callus induction was carried out by culturing explants in callus induction media supplemented with different concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D) and picloram. The appropriate media composition for embryo maturation was determined by subculturing callus in Murashige and Skoog (MS) medium supplemented with varying concentrations of plant growth regulators NAA (α - Naphthaleneacetic acid), BAP (6-Benzylaminopurine) and GA_3 (Gibberellic acid). The plantlets were successfully hardened and transferred to the soil. *Agrobacterium* infectivity was determined through a histochemical transient *GUS* (β-glucuronidase) assay.

1.2 Problem statement

The global demand for cassava is rapidly growing due to its increasing use by the starch industry and its good potential for biofuel production. Because of its high-yield per unit of land, low input and maintenance requirements and its adaptability to marginal soils, cassava is an ideal food security crop for smallholder farmers. Despite its contribution to the world economy, cassava production and utilization is faced by several challenges that include post-harvest physiological deterioration, insect and disease susceptibilities and accumulation of cyanogenic glycosides. Considerable efforts have been deployed towards mitigating these challenges by conventional breeding. This strategy has however failed to address some of the constraints facing the production and utilization of cassava such as low protein content of cassava products, the presence of cyanogenic compounds in the tubers and post-harvest physiological deterioration (Cellebos *et al.,* 2012; Nyaboga *et al.*, 2015). Genetic engineering which can be used as a complementary approach however needs an optimized regeneration and transformation protocol.

1.3 Justification and significance of the study

To alleviate the shortcomings associated with conventional breeding, genetic engineering can be exploited not only as an alternative approach, but also to complement the efforts in traditional breeding towards the introduction of agronomically useful traits into the various cassava genotypes. Genetic transformation holds potential to reducing these problems by allowing the transfer of genes from various sources for generating transgenic plants possessing the desired traits. Additionally, genetic transformation is advantageous because of its capacity to precisely transfer single or even quantitative traits without the problems of genetic linkage encountered in conventional breeding.

Despite the success achieved in genetic modification of few cassava genotypes, especially the model cultivar TMS 60444, transgenic cassava production is still difficult for farmer preferred genotypes. The development of efficient regeneration systems compatible with transformation methods is a pre-requisite for successful application of genetic engineering. Additionally, different plant genotypes and different explants respond differently to *in vitro* culture treatments. Immature leaf lobes are the most commonly used explant type for cassava regeneration and transformation. Their occurrence and availability per plant is however limited necessitating the exploration of other explant types such as stem explants for improved regenerability and transformability. This will allow introgression of desired traits into the cassava germplasm with a higher efficiency. This study assessed the regenerability and transformability of selected Kenyan cassava genotypes.

1.4 Research questions

1. What is the influence of explant type, auxins and photoperiod on the frequencies of callogenesis and somatic embryogenesis in the selected cassava genotypes?
2. What is the influence of varying combinations of phyto-hormones on somatic embryo maturation and plant recovery in the selected cassava genotypes?
3. Are the selected Kenyan cassava genotypes amenable to *Agrobacterium tumefaciens* mediated transformation?

1.5 Objectives

1.5.1 General objective

To determine the amenability of selected Kenyan cassava genotypes to *in-vitro* regeneration and *Agrobacterium* mediated transformation.

1.5.2 Specific objectives

1. To determine the effect of explants, auxins, and photoperiod on callogenesis and somatic embryogenesis of the selected Kenyan cassava genotypes.
2. To determine the effects of varying formulations of NAA, BAP and GA_3 on somatic embryo maturation and plant recovery of the selected cassava genotypes.
3. To determine *Agrobacterium* mediated transformability of the selected Kenyan cassava genotypes.

CHAPTER TWO
LITERATURE REVIEW

2.1 Biology of cassava

Cassava (*Manihot esculenta* Crantz), is a dicotyledonous, perennial woody shrub of the family Euphorbiacae and Subfamily Crotonoideae. The Euphorbiacae family include agriculturally and economically important crops such as castor bean, jatropha (*Jatropha curcas*) and rubber (*Hevea braziliensis*) (Abdulla *et al.,* 2011).

The genus Manihot has about 98 known species that range from subshrubs (group Stipularis) to shrubs (Tripartitae and Graciles) and trees (group Glaziovìannae; Rogers and Appan, 1973; Nassar, 2005). The domesticated crop (*M. esculenta* Crantz) is a shrub that grows to 1-4 m in height. The stem branches in a dichotomous or trichotomous manner and the branching point exhibits a terminal inflorescence (Alves, 2002).

Cassava, also known as tapioca, yucca, manioc or mandioca is an outcrossing plant and is considered allopolyploid (2n=36). Cassava is vegetatively propagated by stem cuttings and the main carbohydrate storage organ in cassava is the root (Nassar, 1978). The plant lacks the typical dicot taproot system but instead adventitious roots arise from the base of the cut surface. Some of the adventitious roots remain fibrous to supply water and nutrients to the plant while others transform to storage roots (Wheatley and Chuzel, 1985; Morris and Taylor, 2010).

The mature cassava storage root (Figure 1.1) comprises three sections: the bark/periderm, the peel/cortex and the Parenchyma (Hunt *et al.*, 1977; Cabral *et al.*, 2000). The parenchyma is the starchy edible part of the root that constitutes about 85 % of the total root mass. The periderm is a thin layer that makes up about 3 % of the total root mass. The peel (cortex) is removed prior to processing and accounts for approximately 11-20 % of the root weight (Montagnac *et al.,* 2009a).

Cassava leaves are alternate and palmately lobed with the number of leaf lobes ranging from three to nine. During flowering, cassava produces leaves that are reduced in size and lobe number. The leaves closest to the inflorescence are mostly simple and have no lobes. The adaxial surface of cassava leaves has a waxy epidermis that gives the leaves a shiny appearance (Cerqueira, 1989).

Cassava inflorescences are terminal and monoeceous. Female flowers open 1-2 weeks before the male flowers and are normally cross-pollinated by insects making cassava a highly heterozygous crop. Cassava fruits are capsular with three locules, while the seeds have a caruncle which varies in size (Nassar, 2007).

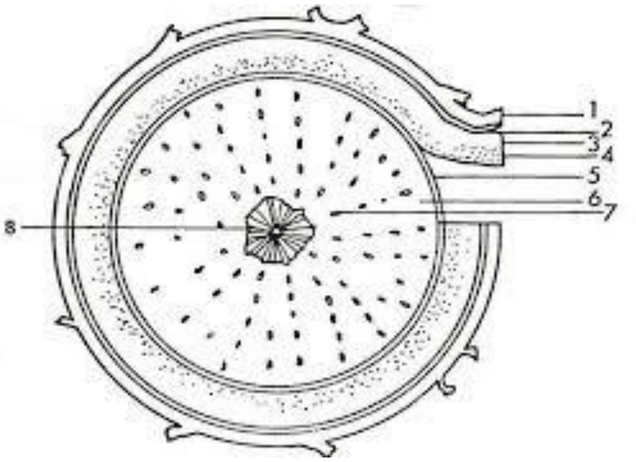

Figure 1.1: Transverse section of a cassava storage root; 1: bark/periderm, 2: sclerenchyma, 3: cortical parenchyma, 4: phloem, 2-4: peel, 5: cambium, 6: storage parenchyma, 7: xylem vessel, 8: xylem vessels and fibres, 5-8: edible parenchyma. Source: Hunt *et al.,* (1977); Cabral *et al.,* (2000).

2.2 Botanical and geographical origin of cassava

According to Allem (1994), wild populations of *Manihot flabellifolia* are the progenitors of the current commercial cultivars and landraces of cassava. More recently, studies using Single nucleotide polymorphism (SNPs) and simple sequence repeat (SSR) markers have indicated that cassava was likely domesticated from the wild Manihot species, *Manihot esculenta* ssp. Flabellifolia (Olsen and Schaal, 1999; Olsen, 2004).

To establish the phylogeographical origin of cassava, Olsen and Schaal (1999) studied the locus of the *glyceraldehyde-3-phosphate dehydrogenase* (G3pdh) gene from several progenitor species. The findings of the study provided a clear insight that cassava was domesticated from wild *M. esculenta* populations along the southern border of the Amazon basin.

According to archeological evidence, cassava domestication dates back to 5000-7000 years BC (Lathrap, 1970). During the 16th Century Portuguese traders introduced cassava into West Africa from Brazil (Jones, 1959). An increase in trade led to cultivation of the crop in central regions of Africa and by the 18th Century, it was farmed in the provinces of East Africa where plants were introduced from Madagascar via Indian Ocean trade routes. Cultivation expanded rapidly and cassava is currently cultivated in approximately 40 African countries (Jennings, 1976; Hahn *et al.*, 1979; Hillocks, 2002).

2.3 Economic importance of cassava

2.3.1 Cassava as food

Starch accounts for approximately 80 % of the root dry weight of cassava (Montagnac *et al.*, 2009a). In sub-Saharan Africa, cassava tubers account for almost two-thirds of the direct human caloric intake, whereas rice, wheat, and maize account for only one-third of total calories consumed (FAO, 2011). It is a food staple for approximately 500 million people in about 105 countries (FAO, 2008) and amongst the top four most important crops (with rice, sugarcane and maize) in the developing world (FAOSTAT, 2009).

Cassava is processed by removing the skin after which it can be eaten raw or further processed to make other food products. However, only the cassava cultivars with low cyanogenic glycoside content are eaten raw. The cultivars having high cyanogenic glycoside content are processed and cooked before consumption. The preparation methods differ between continents and countries. Boiling, mashing, frying and drying are widely used to produce granules, flour and chips (Balagopalan, 2002).

In some areas where cassava is grown, the leaves are also consumed, usually as vegetable or as a sauce accompaniment to the main staple which may, or may not be cassava. Unlike the roots that are essentially carbohydrate, cassava leaves are good sources of minerals, are rich in ascorbic acid, vitamin A, methionine, lysine and contains significant amounts of riboflavin. The leaves can therefore provide a supplement to the starchy diets prevalent among the economically disadvantaged in the tropical world (Balagopalan, 2002).

2.3.2 Cassava as animal feed

Cassava is undoubtedly the only alternative that can replace a considerable portion of maize in the livestock feed industry. It therefore has a prime of place in tropical agriculture. Various parts of the cassava plant including the roots, leaves and stem of cassava are used to feed animals (Mathur *et al.*, 1969). The importance of cassava in animal nutrition arises because of the deficiency of dietary energy in the form of soluble carbohydrates. The deficiency is more acute in the tropics where forage crops are more fibrous, coarse, bulky and less palatable than in the temperate zones (Balagopalan, 2002). It is the high amylase content of cassava that makes it a good source of carbohydrate for animal diets. The animals can be fed with fresh roots but the roots are sometimes boiled prior to feeding animals to reduce chances of cyanide toxicity (Omole, 1977).

According to Mathur *et al.* (1969), replacement of cereals with cassava up to 50 – 100 % does not affect the milk quantity and quality of dairy animals. The roots, however, have low protein content (0.7-1.3 % fresh weight), but this short-coming is overcome by supplementing them with protein additives such as soya or microbial techniques (Omole, 1977). In many developing countries, cassava leaves are used as forage especially during the dry season when other feeds are scarce (Balagopalan, 2002).

2.3.3 Industrial use of cassava

In Asia and South East Asia, cassava is grown mainly for the manufacture of industrial products such as sweeteners, acids, alcohols and biodegradable plastics. Cassava starch has the potential to offer strong competition to the industrial use of maize, wheat, rice and potato. This is because cassava starch has unique properties which make it ideal for many applications in the food, textile and paper industries where flour and starch from the other crops have had great monopoly (Ren, 1996). Due to its high amylopectin content, cassava starch forms clear, non-gelling pastes with little retro-gradation tendency. Compared to maize, wheat and potato starch, cassava starch has the lowest gelatinization temperature and hence consumes less energy during processing (Ziska *et al.,* 2009).

Following extensive research by IITA and CIAT during the 1980s and 1990s, cassava flour can be used as a partial substitute for wheat in bread (up to 10-15 %), biscuits and snacks (40-100 %). Fructose syrup and fructose crystals made from cassava starch can be used as food sweeteners (Goossens, 2004). Cassava flour is also used in canned and powdered soups, instant desserts, sausages and processed meat, sauces, as a thickener, filler, binder, stabilizer and texture improver. The low protein and lipid content of cassava flour are also a value contributing to its neutral flavor and white colour (Awoyale *et al.,* 2016).

Cassava starch has been widely used in the manufacture of paper mainly on account of its low price. Cassava has been found to provide a high colour (whiteness), low dirt and fibre content, and uniformity of lots. In the textile industry, cassava starch is used for sizing, finishing and printing. Approximately 80 % of the starch used in textiles is used in sizing where individual fibres of yarn are shaped into a warp (Bokanga and Djoussou, 1998).

Cassava starch is saccharified to simple sugars used in the production of sugar alcohols such as sorbitol, mannitol and maltol that have a wide range of applications in industry and in medicine (Ren, 1996). The starch is also used in the production of dextrins used as envelope gums, bottle-labeling adhesives, postage stamps adhesives, in making cardboard boxes and photographic mounting materials (Cock, 1985).

In the agricultural sector, cassava starch has been found to have the potential for use as a gelling agent for *in vitro* culture medium. A study by Maliro and Lameck (2004), reported shoot proliferation of stem nodal sections of *Uapaca kirkiana* and *Faidherbia albida* using tissue culture medium gelled with cassava flour. Cassava tubers are peeled, washed and grated. Using a blender, the grated cassava is made into a paste. The paste is then strained into a clean plastic bucket using a cheese cloth and the solution obtained topped up with distilled water. The starch solution is left to stand for 24 h and the supernatant poured off to obtain a clean starch paste. The starch is then dried at room temperature, crushed and used as a gelling agent (Maliro and Lameck, 2004).

Due to the increasing gap between the energy requirement of the industrialized world and inability to meet such needs from the limited sources of energy like fossil fuels, there is a growing interest in using crops such as cassava as a source of biofuel (Balat and Balat, 2009; Jansson *et al.*, 2009). Production of bio-ethanol is sustainable, eco-friendly, economically viable and feasible with available technologies (Chandel *et al.*, 2007; Tillman *et al.*, 2009; Londo *et al.*, 2010). This realization led to 8 % increase in bio-fuel production from 2005 to 33 thousand million liters in 2009. The ability of cassava to grow on marginal soils (Dixon *et al.*, 2002), high productivity and yield (Ziska *et al.*, 2009), low management cost and requirement of minimum labor

(Chiwona-Karltun *et al.*, 1998) have placed it among the candidates for bio-fuel production (Balat and Balat, 2009; Jansson *et al.*, 2009).

2.4 Global cassava production

The global production of cassava has almost doubled over the past three decades to about 230 million metric tons in 2010. Over half of this is grown in Africa, a third in Asia and 14 % in Latin America. Nigeria is the largest producer, growing 38 million metric tons in 2010 (FAO, 2010). Other major producers are Brazil, Indonesia, Thailand, and the Democratic Republic of Congo. Today, thousands of cassava cultivars are spread throughout the tropics and subtropics of Africa, Asia, and South America and grown in over 105 countries (FAO, 2011). Africa, where cassava is grown primarily for food, is the largest producer with yields estimated to exceed 118 million tonnes per year (Figure 2.1; FAOSTAT, 2009).

Cassava is mainly produced by small scale farmers on marginalized lands within the tropics. Such small scale agricultural systems coupled with other constraints of cassava production and utilization create challenges such as uneven quality of goods, unreliability of supply and a costly marketing structure. Additionally, labor costs for harvest are high for the highly perishable storage roots which have to be processed into a storable form shortly after harvest. A combination of these factors including pest and disease infestation has impeded maximal increase in the global production of cassava (FAO, 2011).

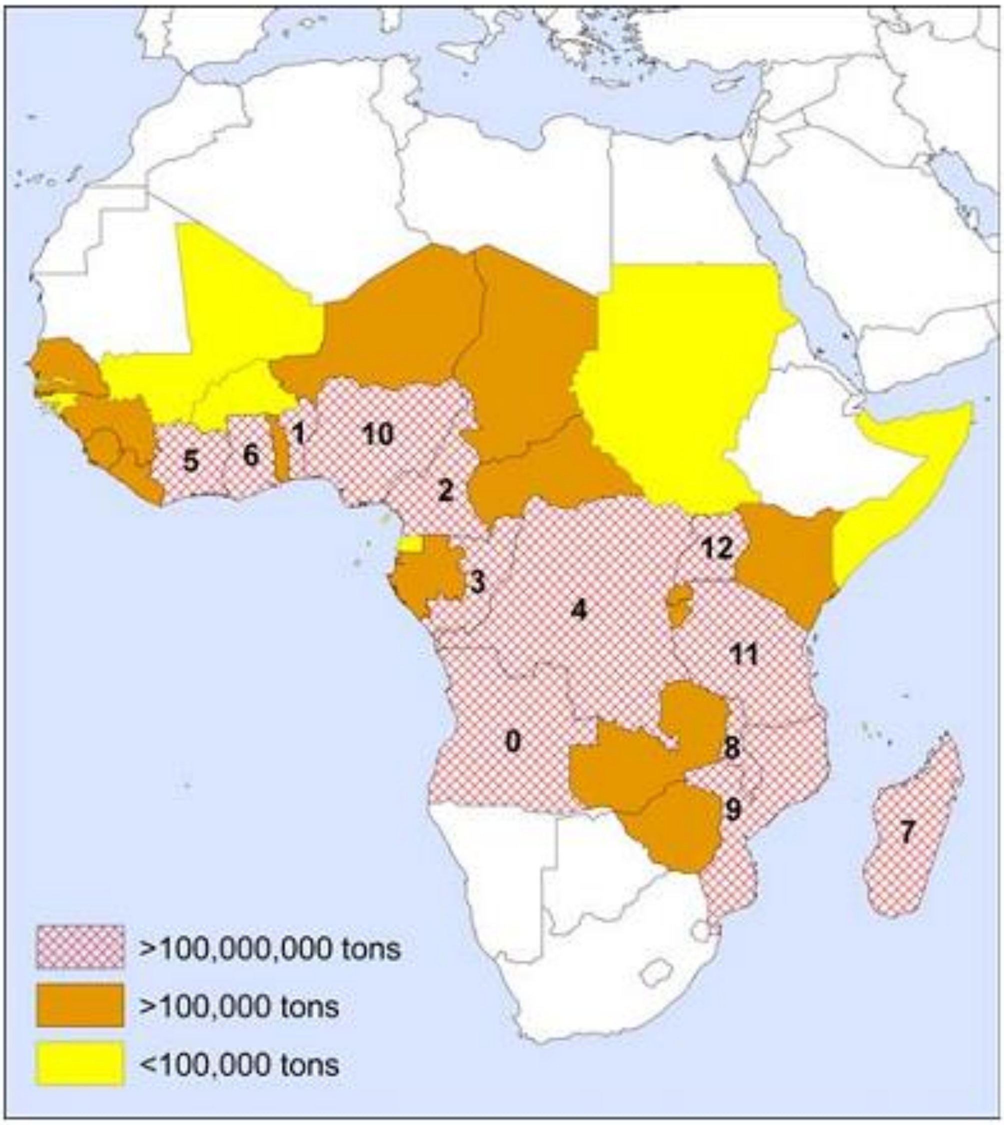

Figure 2.1: Cassava production in Africa. Country labels: Angola (0), Benin (1), Cameroon (2), Congo (3), Democratic Republic of Congo (4), Côte d'Ivoire (5), Ghana (6), Madagascar (7), Malawi (8), Mozambique (9), Nigeria (10), Tanzania (11) and Uganda (12). Source: FAOSTAT (2009); Bennett (2015).

2.5 Constraints to cassava production and consumption

2.5.1 Biotic stress

According to Herrera-Campo *et al.* (2011), the production and utilization of cassava is curtailed by a number of biotic and abiotic stresses, which can cause yield losses of up to 90% or total crop failure. Some of the biotic factors include; cassava mosaic virus disease, cassava brown streak virus disease (Vanderschuren *et al.,* 2009; Yadav *et al.,* 2011), green mite (Yaninek, 1994), cassava mealy bug and cassava bacterial blight (Banito *et al.,* 2010).

Cassava Mosaic Disease (CMD) is probably the most significant biotic constraint to cassava production in Africa. The true incidence and severity of CMD is however difficult to quantify (Sseruwagi *et al.,* 2004). Cassava Mosaic Disease is caused by whitefly-transmitted begomoviruses (family Geminiviridae) for which several species have been identified throughout cassava growing regions of Africa (Stanley and Gay, 1983; Hong *et al.,*1993; Berrie *et al.,* 2001; Bull *et al.,* 2003; Bull *et al.,* 2006). Cassava Mosaic Disease is characterized by a yellow-green mosaic of the leaves, leaf distortion, stunted growth and decrease in the size of root. Cassava brown streak virus (CBSV) which is also transmitted by whiteflies (Maruthi *et al.,*2005; Alicai *et al.,* 2007; Legg *et al.,* 2011) is a devastating disease that causes damaging losses to root production and quality (Pennisi, 2010; Appel, 2011).

Cassava bacterial blight (CBB), caused by *Xanothomonas axonopodis* pv. Manihotis (Xam) is present in all areas where cassava is cultivated. Cassava Bacterial Blight has been estimated to cause yield losses of over 50 to 75 % depending on the environmental conditions (Wydra and Verdier, 2002).

2.5.2 Abiotic stress

Cassava is a long season crop capable of growing in diverse agro-ecological zones. This hardiness contributes largely to its exposure to abiotic stresses and partially explains its limited average yield (Wydra and Verdier, 2002). Most frequently, the crop is grown in drought-prone regions, low fertility soils, in alkaline or poor acidic soils. Despite the fact that cassava is a long season crop able to grow in diverse agro-ecological zones, this is a serious constraint to cassava production (Howeler, 2002). In many regions, drought and salinity are widespread, and are expected to cause serious salinization of more than 50% of all arable lands by the year 2050 (Ashraff, 1994). In particular, it is predicted that water deficit will continue to be a major abiotic factor affecting global crop yields. Approximately 20% of the worlds cultivated land is affected by salinity, which results in loss of 50% agricultural yield (Bartels and Sunkar, 2005). The use of inferior planting material, for example, diseased cuttings or unimproved varieties compounded by inefficient planting densities, poor weed, pest and disease management is also a challenge to cassava production (Hillocks, 2002).

According to Sanchez *et al*. (2006), the most critical constraint to cassava production, utilization and commercialization is the short shelf-life of its storage roots due to rapid post-harvest physiological deterioration (PPD). This occurs 24 to 48 h after harvest and is characterized by an initial blue/ black discoloration of the vascular parenchyma due to the oxidation of phenolic compounds by reactive oxygen species (Sanchez *et al.,* 2006). This is then followed by a general discoloration of the storage parenchyma rendering the roots unmarketable and unpalatable (Morante *et al.,* 2010). Postharvest Physiological Deterioration (PPD) significantly affects crop losses, root quality, economic costs, marketability, consumer availability and commercial processes (Page and Beeching, 2011).

2.5.3 Poor nutritional content of cassava

Cassava roots are excellent sources of carbohydrates. A diet consisting principally of cassava is, however, deficient in protein, vitamins and micronutrients (Table 1.1). Cassava has the lowest amount of protein content among major crops at 0.9 grams per 100 grams of edible portion and several essential amino acids, including sulfur-containing ones, are in very low abundance (Montagnac *et al.*, 2009b). Additionally, these sulfur-containing amino acids may further be depleted as they are required for cyanide detoxification in the body by rhodanese. Additional food sources are therefore required to ensure a diet balanced with proteins, (Cock, 1985).

Table 1.1: Approximate quantity of selected nutrients, vitamins and minerals per 100 g tissue of cassava (Montagnac *et al.*, 2009b).

Nutrient	Roots	Leaves
Vitamin A (µg)	5-35	8300-11800
Vitamun C (mg)	14.9-50	60-370
Protein (g)	0.3-3.5	1-10
Carbohydrate (g)	25.3-35.7	7-18.3
Zinc (ppm)	14	71

2.5.4 Cyanogenic glycosides

All organs of the cassava plant apart from the seeds contain cyanogenic glycosides linamarin and lotaustaralin. The cassava plant also contains hydrolytic enzymes capable of breaking the cyanogenic glycosides to release potentially toxic amounts of free cyanide in the form of cyanohydrin and hydrogen cyanide (HCN) upon hydrolysis by the endogenous enzyme linamarase (White *et al.,* 1998). Under normal circumstances however, the cyanogenic glycosides are separated from the hydrolytic enzymes. Free cyanide is only produced when cell disruption processes that bring the enzyme in contact with the substrate occur (Piero *et al.,* 2015a; Piero *et al.,* 2015b). While most plants produce small amounts of cyanide associated with ethylene production, cassava produces cyanogenic compounds in high quantities that they may function as translocatable forms of reduced nitrogen or as chemical defense molecules against insects and herbivores (Poulton, 1990).

The most prevalent cyanogenic glycoside in cassava is linamarin (95 %) with lesser amounts of lotaustralin. The total amount of cyanogenic glycoside is dependent on cultivar, cultural practice, environmental conditions and plant age (White *et al.,* 1995). Cultivars with <100 mg HCN equivalents kg^{-1} fresh weight in their roots are called ‘sweet’ while cultivars with >450 mg HCN equivalents kg^{-1} FW are classified as ‘bitter’ cassava. At relatively high concentrations, ingested or inhaled cyanide is an extremely potent and rapidly acting metabolic poison (Wheatley and Schwabe, 1985). Most processing procedures (sun drying, heap fermentation, production of farinha in Brazil and gari in West Africa), and cooking efficiently reduces released HCN to safe levels. Nonetheless, people, especially children, may, particularly during periods of social disruption caused by conflict or famine, consume inadequately processed cassava resulting in acute intoxication. The symptoms manifest as vomiting, dizziness, tropical ataxic neuropathy, stunting in children, and 21 konzo, an irreversible paralysis of the legs particularly in nutritionally compromised individuals (Andersen *et al.,* 2000).

2.6 *In vitro* plant regeneration of cassava

The first breakthrough in cassava regeneration was achieved by Kartha *et al*. (1974) who developed shoots from apical meristems of five cassava genotype through meristem culture. Cassava regeneration techniques include somatic embryogenesis, organogesis, meristem culture, protoplast culture (Kartha *et al.,* 1974; Hankoua *et al.,* 2005; Nyaboga *et al.,* 2014; Ngugi *et al.,* 2015).

2.6.1 Somatic embryogenesis

Somatic embryogenesis is the most commonly used regeneration method in cassava, where embryogenesis is restricted to meristematic and embryonic tissues. Somatic embryogenesis is the production of embryolike structures from somatic cells. The somatic embryo is an independent bipolar structure which is not vascularly connected to the tissue of origin. The structure of somatic embryos resembles that of zygotic embryos (Raemakers *et al.,* 1993). Explants such as meristems and shoot tips (Puonti-Kaerlas, 1998), immature leaf lobes (Li *et al.,*1996; Taylor *et al.,* 1996a) and cotyledons of zygotic embryos (Stamp and Henshaw, 1982) are used to induce the formation of somatic embryos. A cyclic embryogenesis system can be established by constant sub culturing of somatic embryos, where the embryos rarely pass the torpedo stage, until transferred to germination medium. Anthers and immature inflorescences can also be used for the induction of somatic embryos (Puonti-Kaerlas, 1998).

2.6.2 Friable embryogenic callus

Friable embryogenic callus (FEC) is a less organized embryogenic tissue produced from organized mature somatic embryos. Friable embryogenic callus are the most recent and efficient regeneration system of cassava (Taylor *et al.*, 1996b). To produce

FEC’s, somatic embryogenic tissues are cultured on Gresshoff and Doy (1972) basal medium instead of the conventional Murashige and Skoog basal medium (1962). To enhance the callus proliferation rate, the FEC’s are cultured in liquid Schenk and Hildebrandt (1972) medium (Taylor *et al.,* 1996a).

2.6.3 Organogenesis

Organogenesis involves induction and development of shoot and root primordia directly from the explant without an intervening callus stage by culturing explants on medium supplemented with cytokinins (Li *et al.,* 1996; Mussio *et al.,* 1998). This gives rise to multiple unipolar shoots that are excised and transferred to a different medium to induce root formation and development. Direct organogenesis provides a fast multiplication and has hence become a method of choice for *in-vitro* propagation. Additionally, this method is fast and not prone to somaclonal variation associated with long callus culture (Das and Pal, 2005). Organogenesis has been used as the regeneration system for transformation of sugarcane (Enríquez-Obregón *et al.,* 1998), sunflower (Schrammeijer *et al.,* 1990) and yams (Nyaboga *et al.,* 2014).

2.6.4 Meristem culture

Meristem culture and regeneration of cassava are not only a source of explants for cassava transformation but also serve as a means for viral decontamination as well as in multiplication of cassava. Most frequently, plant cells divide faster than viruses and therefore meristems are usually devoid of viruses (Schopke *et al.,* 1993). Meristems are the tissue of choice of all the different explants used for regeneration because they represent ‘growth centres’ of plants (Fregene and Pounti Kaerlas, 2002). Meristem culture is easy, fast and relatively genotype-independent. It has been largely used for germplasm preservation, micropropagation, transformation and eliminating viruses and other pathogens from plant materials (Kartha *et al.,* 1974; Bajaj, 1983; Konan *et al.,* 1994).

2.6.5 Protoplast culture

Theoretically, protoplasts can be isolated from any part of the plant. The possibility of isolating protoplasts capable of sustained division and plant regeneration is, however, still restricted to a limited number of plant species and explant sources (Blackhall *et al.,* 1995). The leaf mesophyll is frequently employed as source material for protoplast isolation. Other explants also used for the same are hypocotyl, stem, petiole, cotyledon, florets, callus suspensions and somatic embryos (Blackhall *et al.,* 1995). There have been several reports of successful isolation and culture of cassava protoplasts (Mabanza and Jonard, 1983; Mabanza, 1984; Szabados *et al.,* 1987; Nzoghe, 1989). The regeneration competence of protoplasts is governed by the plant genotype, the ontogenetic state of the explant source and the cultural environment (Blackhall *et al.,* 1995). One of the disadvantages of this system is the long span of time from explant inoculation to suspension culture then to regenerated plantlets (20 weeks), which may result in somaclonal variation and loss of regeneration capacity (Makwarela and Rey, 2006).

2.7 Conventional breeding of cassava

Cassava production and consumption is constrained by a diverse set of biotic and abiotic factors that need to be ameliorated for adequate commercial exploitation. This realization led to the establishment of the International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria in 1972 and the Centro Internacional de

Agricultura Tropica (CIAT) in Colombia, under the guidance of the Consultative Group on International Agricultural Research (CGIAR) to gather and improve the cassava germplasm (Hillocks, 2002; Ceballos *et al.*, 2004).

Cassava breeders have employed selective breeding strategies to re-engineer plants to produce traits/ qualities considered desirable to farmers and/ or consumers. The deployment of novel statistical models, the increasingly abundant genetic information and bioinformatics tools has enabled the introgression of desirable traits into various cassava genotypes (Ceballos *et al.,* 2012). Conventional breeding programs have resulted in the introgression of important traits into the cassava germplasm with improvements recorded for virus resistance (Hahn *et al.,*1980; Okogbenin *et al.,* 2007), PPD (Morante *et al.,* 2010), bacterial blight resistance (Vanderschuren *et al.,* 2009) and starch quality (Ceballos *et al.*, 2012).

Several biological attributes of the crop impede the elucidation of breeding goals of yield increase, root quality improvement, and disease resistance in cassava. Consequently, conventional breeding has not been able to resolve the problems of low protein content of cassava products and the presence of cyanogenic compounds in the tubers (Puonti-Kaerlas, 1998). Cassava is characterized by high levels of heterozygosity within populations that makes the recovery of improved lines through backcrossing virtually impossible. Additionally, cassava is monoecious and challenges in synchronization of flowering between parental lines coupled with limited flowering make conventional breeding programs laborious, difficult and time consuming (Jennings and Iglesias, 2002; Ceballos *et al.*, 2004).

2.8 Genetic engineering of cassava

Genetic engineering offers several advantages over conventional breeding. For instance, it is possible to precisely transfer single or even quantitative traits without the problems of linkage encountered in traditional breeding. With genetic engineering, it is possible to extend the genetic pool of useful gene sources beyond the species level (Fregene and Puonti-Kaerlas, 2002). Some of the impediments of cassava conventional breeding such as the crops inherent heterozygous nature, inbreeding depression and the polygenic and recessive nature of many desirable traits are therefore overcome by genetic engineering (Ceballos *et al.*, 2004; Ceballos *et al.*, 2010). Consequently, genetic engineering offers an attractive alternative for introgression of desired traits into lines of agronomic importance (Bull *et al.*, 2011).

The first published attempt to transform cassava was that of Calderon, (1988). He inoculated leaf pieces, stem pieces and embryogenic cells with Agrobacterium containing plasmids with the coding sequences for neomycin phosphotransferase II (nptII), phosphorinotricin acetyltransferase (*bar*) or β-glucoronidase (*gus*). He isolated callus lines expressing the phenotype expected from the transformation tissue. Subsequent reports of transient gene expression then followed (Arias-Garzon and Sayre, 1993; Luong *et al.*, 1995).

The first report of stable cassava transformation was by Li *et al.* (1996) using *Agrobacterium*-mediated transformation of somatic cotyledons. At the same time, Taylor *et al.* (1996a) reported the regeneration of transgenic plantlets from friable embryogenic callus (FEC) transformed by micro-particle bombardment.

Arias-Garzon (1997) and White *et al.* (1998) were the first to transform cassava with a gene of agronomic importance after successfully introgressing into the cassava genome the gene encoding hydroxynitrile lyase (HNL). Siritunga *et al.* (2004) also transformed cassava with the gene encoding HNL driven by the 35S promoter. The rate of acetone cyanohydrin breakdown in the transformed plants was 41-75 % faster than wild-type cassava (Siritunga *et al.,* 2004). Additional reports of the genetic transformation of cassava have followed (Sarria *et al.,* 2000; Zhang and Puonti-Kaerlas, 2000).

The BioCassava Plus project team established through the Melinda and gates foundation Grand Challenges in Global Health projects has developed a number of transgenic cassava lines with value-added traits, such as virus resistance and increased vitamin A, iron, and zinc contents. In this project, the The Donald Danforth Plant Science Centre partnered with the Kenya Agricultural and Livestock Research Organization (KALRO) and some of these lines are being investigated in pilot field trials (Sayre *et al.,* 2011). Ogwok *et al.* (2012) reported transgenically imparted resistance to CBSD through RNA interference (RNAi) technology in cassava under field conditions.

Like all other crops, the improvement of cassava by genetic engineering requires the development of an efficient regeneration protocol hence efficient transfer and stable integration of the transgenes into plant genomic DNA is essential, as well as a means for identifying and selecting transformed cells (Fregene and Pounti-Kaerlas, 2002).

2.9 Role of reporter genes in plant transformation

Reporter genes are used for the identification of transgenic plants by visual detection of transformed tissues. The products of the reporter genes can either be detected directly or they catalyze specific reactions whose products are detectable. Examples of such visually identifiable reporter genes include *Escherichia coli* β -glucuronidase gene (*GUS*), green fluorescent protein (GFP), and luciferase (Ow *et al.*, 1986; Cubbit *et al.*, 1996; Ruijter *et al.*, 2003).

2.9.1 β -Glucuronidase

β -glucuronidase is a hydrolase that cleaves a variety of glucuronides. The enzyme, which was initially isolated from E-coli K12, is encoded for by the *gus*A or *uid*A gene. Higher plants have little or no detectable GUS activity at the assay conditions. Gus substrates are available for histochemical, spectrometric and fluorometric assays (Ruijter *et al.*, 2003). The spectrometric assay uses p-nitrophenyl- β-D-glucuronide or 4-methyl umbeliferyl glucuronide as a substrate. The substrate is cleaved by GUS to yield 4-methylumbeliferron which is a blue fluorescent compound. Histochemical analysis is used for the localization of gene activity in intact cells and tissues. For the histochemical assay, β -Glucuronidase uses an external substrate, 5-bromo-4-chloro-3-indolyl glucuronide (X-gluc) for histochemical localization resulting in a blue precipitate at the site of enzyme activity (Jefferson, 1987; Schmidt *et al.*, 1992).

The *gus* gene is commonly used in plants because it is stable, its assay is simple and it tolerates many commonly used chemicals and assay conditions like temperature and pH (Kavanagh *et al.*, 1988). Plants expressing *gus* are normal, healthy, and fertile and the tissue extracts continue to show high levels of *gus* activity after prolonged storage.

The β -Glucuronidase enzyme can be fused with selectable marker genes such as *npt II* to allow visualization and the selection of transformed plant cells. Additionally, *gus* does not interfere with the physiology and metabolism of the plant. It is, therefore, safe for the environment and the consumer (Ruijter *et al.,* 2003).

2.9.2 Green Fluorescent Protein (GFP)

The green fluorescent protein (GFP) is a naturally accuring protein found in jelly fish, *Acquoerea Victoria*. The bioluminesce has been used as a universal reporter in a broad range of heterologous living cells and organisms (Prasher *et al.,* 1992). GFP can be continually monitored over time and unlike GUS does not require the use of a substrate as the protein product is itself fluorescent. Wild type GFP fluoresces green at 507nm upon excitement by ultraviolet light at 395 nm or at 475 nm under blue light (Ahlandsberg *et al.,* 1999). The GFP reporter system allows monitoring of plant cells expressing it directly within growing plant tissue. The GFP reporter system is advantageous in that it is non-destructive can be detected 1.5 hours following gene integration and can be tracked over extended time period via digital imaging. Although successful in some plant transient expression assays based on strong promoters or high copy number viral vectors, GFP will require further improvement of expression efficiency and fluorescence to be useful as a marker in intact plants (Sheen *et al.,* 1995; Rizzuto *et al.,* 1995; Stewart, 2001).

2.9.3 Luciferase

The luciferase reporter gene system uses bioluminescence to detect gene expression. Both animal and plant luciferases have activities detectable in plant cellular extracts and can therefore be used. Although the assay can be performed easily, relatively expensive luminometry equipment is needed (Thomson *et al.,* 1991). The bacterial luciferase which has its origin from *Vibrio harveyi* uses a flavin and an aldehyde of at least eight carbon chain length as substrates. Luciferase activity leads to light emission at 490 nm wavelength (Herrera-Estrella *et al.,* 1994; Kost et al., 1995).

The animal luciferase originates from the North American firefly *Photinus pyralis*. The luciferase is encoded for by the *luc* gene and uses luciferin and Adenine triphosphate (ATP) as substrates (Ow *et al.,* 1986). Although the assay is extremely sensitive, the enzyme is highly labile and difficult to assay accurately. Moreover, the reaction is complex and there is little potential for routine histochemical analysis (De Luca and McElroy, 1978).

CHAPTER THREE
MATERIALS AND METHODS

3.1 Collection and preparation of plant materials

Five selected cassava genotypes, namely KME 1, 08/354, 08/274 and 08/080 and TMS 60444 were obtained from the farms of KALRO- Mtwapa and KALRO - Embu. The genotype, TMS 60444 is a model genotype in cassava regeneration and transformation and was used as a control in the study (Ihemere, 2003). The other genotypes; KME 1, 08/354, 08/274 and 08/080 were selected on the basis of their susceptibility to African cassava mosaic virus and cassava brown steak virus. Fresh stem cuttings of 20 to 30 cm with 5 to 8 nodes were obtained from mature plants.

The cuttings were planted in pots to establish the mother stock at Kenyatta University Plant Transformation Laboratory (PTL) glasshouse (Figure 3.1A). Six-week old shoots were used to establish an *in-vitro* culture of the cassava cultivars. Cuttings (5 cm long) with a single node were surface-sterilized with 10 % household bleach containing 3.5 % sodium hypochlorite for 20 minutes with swirling then with 70 % ethanol for 5 minutes and rinsed three times with sterile distilled water containing 0.015 % Triton-X100.

3.2 *In vitro* micropropagation

After surface sterilization, the dead bleached ends of the nodal explants were cut off and the nodes were cultured on cassava micro-propagation media; MS basal salts with vitamins (Murashige and Skoog, 1962) supplemented with 3 % sucrose, 0.3 % Gelrite (pH 5.8). The cultures were incubated at 28 ºC under cool white fluorescent light 300 foot candle for 16 h /8 h light/dark cycle. The cultures were maintained on a two weekly sub-culture cycle as an *in vitro* source of ‘clean’ sterile plants (Figure 3.1B).

Figure 3.1: Bulking and propagation of cassava; A: Mother stock established at the Kenyatta University- PTL glasshouse in plastic pots; B: *In-vitro* cultures of cassava at the Kenyatta University- Plant Transformation Lab; Scale: 2.5 cm.

3.3 Regeneration of selected cassava genotypes

3.3.1 Callus induction and somatic embryogenesis

Dissected stem segments and immature leaf lobes measuring 5 to 10 mm long were placed in callus initiation media containing MS basal salts, Gamborg B5 vitamins (Gamborg *et al.*, 1968), 100 mg/l myo-inositol, 0.2 % sucrose, 0.5 mg/l $CuSO_4$, 50 mg/l casein hydrolysate (Schopke *et al.*, 1993) supplemented with 2, 4-dichlorophenoxyacetic acid (2,4 D concentrations 4, 6, 8, and 10 mg/l) and picloram (concentrations 4, 6, 8, and 10 mg/l) separately to determine the best concentration of each of the auxins for callus induction and somatic embryogenesis. In total, four different concentrations of 2,4 D and four different concentrations of picloram were evaluated. Prior to autoclaving, the pH of the media was adjusted to 5.7 and solidified with 0.8 % Difco-Bacto agar. Each treatment was replicated six times in Petri dishes (Sterilin™ Standard 90 mm diameter), containing 50 ml of induction medium and inoculated with 10 explants. The plates were then double-sealed with parafilm and separately incubated under two photoperiods (0 h/ 24 h light/dark and 16 h /8 h light/dark cycle) at a temperature of 28 °C to determine the best photoperiod for the induction of somatic embryogenesis. For each photoperiod, six plates were cultured for every auxin concentration and three replicates were done. The cultures were maintained in the callus induction media for 28- 42 days with a regular 2 weekly sub-culture.

3.3.2 Maturation of somatic embryos

Maturation refers to the development of the embryo at the globular stage into green cotyledonary embryos with defined shoot and root apices. Four week-old embryogenic calli were sub-cultured onto petri dishes (Sterilin™ Standard 90 mm diameter) containing 25 ml of various formulations of NAA (α-Naphthaleneacetic acid), BAP (6-Benzylaminopurine) and GA_3 (Gibberellic acid) to compare their effectiveness on maturation of somatic embryos. The following five combinations of maturation media were investigated; **M**: 3 mg/l BAP, 0.02 mg/l NAA 0.5 mg/l GA_3; **M1**: 3 mg/l BAP, 0.1 mM NAA 1 mg/l GA_3; **M2**: 2 mg/l BAP, 0.3 mM NAA 0.5 mg/l GA_3; **M3**: 2 mg/l BAP, 0.1 mM NAA 1.5 mg/l GA_3; **M4**: 1 mg/l BAP, 0.2 mM NAA 1.5 mg/l GA_3.

3.3.3 Desiccation and germination of somatic embryos

For the adsorption of phenolic compounds, the green cotyledonary embryos with defined shoot and root apices were transferred onto glass bottles containing 50 ml of hormone free desiccation medium. This medium comprised 0.8 % activated charcoal, 2% sucrose, MS salts and B5 vitamins solidified with 0.8 % (w/v) Difco-Bacto agar. After 7-14 days, the number of shooted embryos was recorded and transferred onto cassava micro propagation media which contained MS salts with vitamins (Murashige and Skoog, 1962) supplemented with 3 % sucrose, solidified with 0.3 % gelrite (pH 5.8) and maintained at 28ºC under cool white fluorescent light 300 foot candle for 16 h /8 h light/dark cycle.

3.3.4 Hardening of *in-vitro* regenerated plants

Plantlets with defined roots and shoots were transferred onto small pots filled with peat moss and covered with a plastic bag to regulate the humidity and temperature for two weeks at room temperature in the glasshouse. Water was added using a hand sprayer in mist form. After two weeks, plastic bags were gradually removed for the plantlets to get acclimatized to the glasshouse environment after which the surviving plants were transplanted to larger pots filled with a mixture of peat moss and soil (50/50 % v/v). These were later transplanted onto potted soil and the watering regime was reduced to once a week.

3.4 Transformation of regenerable cassava genotypes

3.4.1 Plasmid constructs and bacterial strain

Agrobacterium tumefaciens strains EHA 101 (Hood *et al.*, 1986) containing a standard binary vector pTF102 (11622 bp) maintained at the Kenyatta University Plant Transformation Laboratory was used in this study. The constitutive cauliflower mosaic virus promoter (35S CaMV) was used to drive both the *gus* reporter gene and the *bar* gene.

During *Agrobacterium tumefaciens* cultivation, the spectinomycin resistance gene was used for selection of bacterial plasmid pTF102 while the *bar* gene which encodes phosphoinothricin acetyltransferase was used as a selectable marker gene for selection of transgenic plants (Figure 3.2).

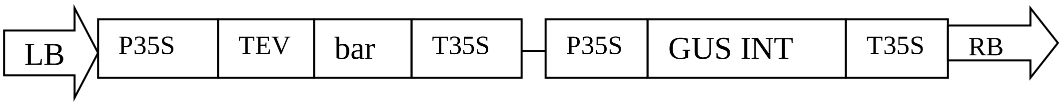

Figure 2.2: A schematic presentation of the transfer DNA in the gene construct; LB, Left Border; 35SP, CaMV35S promoter; GUS INT, β-Glucuronidase reporter gene; T35s, CaMV35S terminator; TEV, Tobacco Etch Virus (enhances transcription); b*ar*, Phosphoinothrycin acetyltransferase gene; RB, Right border.

3.4.2 *Agrobacterium tumefaciens* preparation

Agrobacterium tumefaciens strain (EHA 101) used for transformation was maintained on Yeast Extract Peptone (YEP) media (Yeast extract 15 mg/l, peptone 10 mg/l, sodium chloride 5 mg/l and bacterial agar 10 mg/l. After autoclaving, ultrafilter sterilized kanamycin 50 mg/l and spectromycin 50 mg/l were added. These were refreshed every 2 weeks for the rejuvenation of the bacterial cultures.

Liquid cultures were initiated from the solid cultures by inoculating 15 ml YEP medium containing 100 mg/l streptomycin and 50 mg/l kanamycin with a single colony of *Agrobacterium* harboring the plasmid and grown at 28 ^{o}C on a rotary shaker (250 rpm) overnight. This was followed by centrifugation at 4000 rpm for ten minutes, the supernatant was discarded. The sediments were suspended in 15 ml of infection media (liquid MS) (Murashige and Skoog, 1962) supplemented with 2 % (w/v) sucrose and 200 µM acetosyringone then grown at 28 ^{o}C on a rotary shaker for

2-5 h (Li *et al.,* 1996). The optical density of the suspension was adjusted to OD 0.6 using a spectrophotometer set at a wavelength of 260 nm.

3.4.3 Explant infection, co-cultivation and resting

Immature leaf lobes were excised from *in-vitro* cultures, placed onto a sterile filter paper and poked using a sterile needle. The poked leaves were then placed on co-cultivation medium (MS basal salts supplemented with 2 % (w/v) sucrose, B5 vitamins, 50 mg/l casein hydrolysate, 0.5 mg/l $CuSO_4$ and 10 mg/l picloram supplemented with 200 µM acetosyringone). The bacterial suspension (20 µl) in infection medium was applied to the explant tissue, which was then incubated in the dark for 5 minutes. Using a pippete, the excess infection medium was sucked and the plates containing the explants in co-cultivation medium were incubated in the dark for 3 days at 28 °C for co-culturing.

Following co-cultivation with *A. tumefaciens*, explants were transferred to resting media (MS basal salts supplemented with 2 % (w/v) sucrose, B5 vitamins, 50 mg/l casein hydrolysate, 0.5 mg/l $CuSO_4$ and 10 mg/l picloram supplemented with 250 mg/l of carbenicillin) for 2 days to avoid *Agrobacterium* recurrence. Copper II Sulphate ($CuSO_4$) was added to enhance embryo formation and visualization. For negative control, a separate set of explants was not co-cultivated with *A. tumefaciens* but were transferred into the resting media.

3.4.4 Selection of putatively transformed callus

To select for the putatively transformed callus, the callus were transferred to selection medium (MS basal salts supplemented with 2 % (w/v) sucrose, B5 vitamins, 50 mg/l casein hydrolysate, 0.5 mg/l $CuSO_4$ and 10 mg/l picloram supplemented with bialaphos (1.5 mg/l) and carbenicillin (250 mg/l). After 2 weeks of culture, proliferating callus was subdivided into smaller sizes to ensure firm contact with the media and then transferred onto fresh selection medium. Carbenicillin was added to the media to prevent recurrence of *Agrobacterium* while bialaphos was added to eliminate the escapes (non-transformed callus). The callus was maintained in the selection medium for 4 weeks.

3.4.5 Regeneration of plants from putative transformed callus and plant recovery

The selected callus was transferred onto maturation medium M4 supplemented with bialaphos (1.5 mg/l) and carbenicillin (250 mg/l) for embryo maturation. For the induction of rooting and shoot elongation, each green cotyledonary embryo was transferred to cassava micropropagation media: MS basal salts with vitamins (Murashige and Skoog, 1962) supplemented with 3 % sucrose, 0.3 % Gelrite (pH 5.8) supplemented with bialaphos (0.75 mg/l) and carbenicillin (250 mg/l). The putative transgenic regenerants were maintained on this medium with a two week subculture regime at 16/8 hours light/dark at 28 ^oC.

3.5 Histochemical *Gus* Assay

β -Glucuronidase (*GUS*) enzyme activity was detected histochemically on sections of putatively transformed callus and *in vitro* regenerated plants. On the 3rd day of co-cultivation, callus was assessed for transient *gus* activity using the procedure described by Jefferson *et al.* (1987) and modified by Bull *et al.* (2009). Randomly selected callus and *in vitro* regenerated plants were immersed in a *GUS* reaction buffer and incubated overnight at 37 °C after which the *gus* buffer was removed. Blue staining of the callus was visualized and scored for transient *gus* gene expression. Transient *gus* expression frequencies were calculated by expressing the number of callus showing blue staining due to *gus* activity as a proportion (%) of the total number of inoculated callus.

3.6 Molecular analysis of putative transformants

3.6.1 Extraction of plant genomic DNA

Total genomic DNA was isolated from fresh leaves of transformed and untransformed (control) plants using DNAeasy plant mini kit (Qiagen, GmbH, Germany) according to manufacturer's instructions. The plants used as control were non transformed regenerated plantlets.

3.6.2 DNA quality and quantity estimation

The plant genomic DNA (100 mg) concentration was estimated by running on a 0.8 % (w/v) agarose gel. A volume of 2 µl of the extracted DNA was mixed with 5 µl of loading dye (New England Bio Labs Ipswich, USA) and electrophoresed on 1% (w/v) agarose gel alongside 5 µl of 1 kb plus molecular marker (Fermentas). The gel was run at 100 volts for 30 minutes and visualized under UV light on a transilluminator. The amount of the DNA was determined by comparing the auto fluorescence of the isolated DNA bands with that of the 1 kb plus ladder standard. Determination of the quality of the DNA was also carried out using a Nanodrop spectrophotometer (Wilmington, USA).

3.6.3 Polymerase Chain Reaction (PCR) analysis of putative transgenic lines

Presence of the *gus* gene was confirmed by PCR amplification of the *gus* gene using gene specific primers (Sigma) – 528 bp. The GUS primer sequences were: Forward 5′-TTT AAC TAT GCC GGG ATC CAT CGC-3′ and reverse 5′-CCA GTC GAG CAT CTC TTC AGC GTA-3′. Each PCR reaction was performed in 10 µl (total volume) of reaction mixture. The PCR amplification was run with the following conditions: 10 minutes at 95 ^{o}C, 30 seconds at 94 ^{o}C, 1 minute at 60 ^{o}C, 1 minute at 72 ^{o}C and a final 10-min extension at 72 ^{o}C. A negative control consisting of 2.5 µl of DNA sample extracted from a non-transformed plant and a positive control consisting of 2.5 µl of plasmid DNA were run alongside the DNA from the putatively transformed plants. A volume of 10 µl of amplified DNA fragments was mixed with 2 µl of the loading dye and electrophoresed at 100 volts on a 1.5 % agarose gel containing 5 µg/ml of gel red The gel was visualized under UV light and the molecular weight of the amplified DNA fragments was estimated using a 1 kb plus molecular marker (Fermentas).

3.6.4 Reverse transcriptase Polymerase Chain Reaction (PCR) analysis of putative transgenic lines

Reverse transcriptase-PCR (RT-PCR) analysis was carried out to examine transgene expression in the putative transgenic cassava lines. Total RNA was isolated from 100 mg leaf tissue of *in vitro* putative transgenic lines and non-transgenic control plant using a Qiagen Plant RNA Extraction Kit (Qiagen, GmbH, Hilden, Germany). RNA quantity and purity were determined using a NanoDrop ND-2000 spectrophotometer (NanoDrop products, Wilmington, USA). The extract was treated with 1 unit DNase (Invitrogen, Carlsbad, CA) for 15 minutes at room temperature to eliminate DNA contamination. The DNase was inactivated according to the manufacturer’s instructions to prevent digestion of newly synthesized cDNA. The first strand cDNA synthesis was carried out using 2 µg of total RNA and reverse transcriptase of the Maxima H Minus First Strand cDNA synthesis kit with oligo (dT) 18 primers (Thermo scientific, Waltham, MA) according to the manufacturer's instructions. The synthesized cDNA was amplified by PCR using 1X PCR buffer, 1.5 mM $MgCl_2$, 0.1 mM dNTP, 2.5 units of Taq polymerase, 0.4 µM of each primer specific for *gus*A gene (primer sequences: Forward 5′-TTT AAC TAT GCC GGG ATC CAT CGC-3′ and reverse 5′-CCA GTC GAG CAT CTC TTC AGC GTA-3′) – 528 bps. To check the quality of synthesized cDNA, Protein phosphatase 2A (PP2A) amplification was used as an internal control. The primer sequences were: forward 5′-TGC AAG GCT CAC ACT TTC ATC-3′ and reverse 5′ -CTG AGC GTA AAG CAG GGA ACG-3′ - 220 bps. The RT-PCR products were run on 1 % (w/v) agarose gel at 100 V for 30 minutes and visualized under UV light.

3.6.5 Data management and statistical analysis

Frequencies of callus induction, somatic embryogenesis, shoot formation and transformability between genotypes were analyzed using One-Way ANOVA. Means were separated using Duncan's multiple-range test (DMRT) at a 95 % confidence level ($p < 0.05$). All quantitative data values were expressed as Mean ± SEM ($n = 60$). The frequency of somatic embryogenesis was expressed from the number of callus forming somatic embryos as a percentage of the number of explants cultured in callus induction media. Transformation efficiency was calculated as the percentage of PCR positive events as a fraction of the total number of tested events. Qualitative data was presented in form of photographs of *in vitro* cultures, histochemical *GUS* assays and gels.

CHAPTER FOUR

RESULTS

4.1 Effect of explant source on callogenesis and somatic embryogenesis in cassava

Embryogenesis was induced in all cassava genotypes using either 2, 4-D or Picloram in the callus induction medium using immature leaf lobes and meristemic stem segments. Formation of callus and their development into organized embryogenic structures were successfully achieved in all the tested auxin concentrations in the five genotypes. There were no significant differences in the frequencies of callus induction between the two explant sources in picloram but significant differences ($P \leq 0.05$) were observed in media containing 2,4-D in genotypes KME 1 and 08/274 (Tables 4.1 and 4.2). Callus formation was faster in leaf explants compared to stem explants. It took 10 days to initiate callus from leaf explants while the stem explants took an average of 15 days.

Additionally, the two explant types showed significant differences in the ability to form organized embryogenic structures ($P \leq 0.05$). The leaf explants gave much higher frequencies of embryogenesis compared to the stem explants in both picloram and 2,4-D (Tables 4.3 and 4.4). The frequencies of somatic embryogenesis in leaf explants ranged from 23.6% obtained in genotype KME 1 at 4 mg/l of 2,4-D to 90.8% obtained in genotype 08/354 at 10mg/l 2,4-D. In stem explants, the highest frequency of somatic embryogenesis was 54.5 % obtained in genotype 08/354 at 10 mg/l of 2,4-D while the lowest frequency was 8 % attained in genotype KME 1 at 4 mg/l of 2,4-D

In MS media supplemented with 4 mg/l 2, 4-D, stem and leaf explants of genotypes KME 1 and 08/274 differentiated to form roots leading to significantly lower frequencies of callus induction and somatic embryogenesis in these genotypes (Tables 4.3 and 4.4). While callus from meristematic stem segments were mainly loose, friable, non-embryogenic and white in colour (Figure 4.1 A), the callus from immature leaf lobes were mainly translucent, gelatinous and highly embryogenic (Figure 4.1 B).

Table 4.1: Effect of explant type on the frequency (%) of cassava callus induction in MS media supplemented with picloram in the dark

	Picloram concentration							
	4 mg/l		**6 mg/l**		**8 mg/l**		**10 mg/l**	
Genotype	**Leaf**	**Stem**	**Leaf**	**Stem**	**Leaf**	**Stem**	**Leaf**	**Stem**
08/354	77.5±6.3^{a}	94.8±2.1^{a}	80.8±4.1^{a}	96.5±1.3^{a}	76.3±4.3^{a}	96.6±2.3^{a}	84.9±3.4^{a}	92.8±2.1^{a}
KME1	77.6±5.9^{a}	93.0±2.3^{a}	77.9±4.8^{a}	95.7±1.4^{a}	78.3±4.6^{a}	94.8±3.2^{a}	89.7±2.4^{a}	96.5±1.4^{a}
08/274	77.7±2.1^{a}	95.6±1.5^{a}	75.7±1.3^{a}	97.1±1.4^{a}	83.5±2.3^{a}	95.7±1.5^{a}	87.1±2.1^{a}	96.6±2.3^{a}
08/080	86.6±3.5^{a}	97.3±0.9^{a}	78.6±4.7^{a}	98.1±1.0^{a}	85.1±3.5^{a}	92.2±2.6^{a}	85.9±2.3^{a}	95.5±1.5^{a}
TMS60444	81.2±1.8^{a}	95.0±2.1^{a}	82.9±3.6^{a}	94.1±1.5^{a}	84.5±2.4^{a}	94.1±2.0^{a}	82.0±3.3^{a}	95.8±1.5^{a}

Values are means (±standard error). Values followed by different superscripts in the same row are significantly different at $P \leq 0.05$ according to Duncan's multiple range test.

Table 4.2: Effect of explant type on the frequency (%) of cassava callus induction in MS media supplemented with 2,4D in the dark

	2,4-D concentration							
	4 mg/l		**6 mg/l**		**8 mg/l**		**10 mg/l**	
Genotype	**Leaf**	**Stem**	**Leaf**	**Stem**	**Leaf**	**Stem**	**Leaf**	**Stem**
08/354	95.7±0.9^{a}	72.5±1.2^{b}	96.3±2.3^{a}	64.1±2.2^{a}	91.6±2.3^{a}	78.7±5.0^{a}	98.3±0.9^{a}	64.5±1.9^{bc}
KME1	34.0±4.2^{b}	15.8±7.8^{e}	82.2±6.4^{ab}	63.0±1.3^{a}	90.6±2.1^{a}	50.0±1.0^{b}	85.9±5.1^{b}	84.1±4.8^{a}
08/274	40.1±1.4^{b}	34.3±1.7^{d}	70.5±1.4^{b}	54.9±1.6^{a}	91.6±0.6^{a}	77.1±1.4^{a}	98.0±1.1^{a}	76.5±1.4^{ab}
08/080	86.5±1.6^{a}	51.6±2.0^{c}	92.4±1.5^{a}	61.0±3.8^{a}	90.9±1.9^{a}	73.8±3.4^{a}	90.0±2.4^{ab}	58.9±5.2^{c}
TMS60444	88.3±0.9^{a}	95.0±2.1^{a}	90.0±1.3^{a}	54.5±3.0^{a}	95.8±2.0^{a}	75.4±5.2^{a}	95.8±1.5^{a}	72.9±4.9^{a}

Values are means (±standard error). Values followed by different superscripts in the same row are significantly different at $P \leq 0.05$ according to Duncan's multiple range test.

Table 4.3: Effect of explant type on the frequency (%) of cassava somatic embryogenesis in MS media supplemented with picloram in the dark

	Picloram concentration							
	4 mg/l		6 mg/l		8 mg/l		10 mg/l	
Genotype	**Leaf**	**Stem**	**Leaf**	**Stem**	**Leaf**	**Stem**	**Leaf**	**Stem**
08/354	66.4±5.5^{a}	51.0±3.1^{a}	75.1±5.6^{a}	46.4±2.4^{ab}	72.3±2.4^{a}	41.2±1.9^{b}	81.4±3.1^{a}	48.5±0.8^{ab}
KME1	66.8±3.0^{b}	31.5±2.7^{a}	64.5±3.4^{b}	32.2±1.2^{a}	71.2±1.9^{ab}	32.2±2.3^{a}	78.8±2.2^{a}	37.5±2.4^{a}
08/274	51.0±3.1^{b}	33.8±2.1^{a}	46.4±2.4^{b}	30.0±1.1^{a}	41.2±1.9^{b}	39.7±2.0^{a}	48.5±0.8^{a}	34.4±4.3^{a}
08/080	55.7±1.1^{b}	28.6±3.1^{a}	59.2±2.8^{b}	30.4±3.3^{a}	76.5±2.1^{a}	29.7±3.7^{a}	83.0±2.9^{a}	29.4±2.5^{a}
TMS60444	70.0±2.4^{a}	38.3±3.1^{a}	78.3±2.7^{a}	42.5±1.5^{a}	80.8±3.6^{a}	43.3±2.3^{a}	80.4±3.2^{a}	44.1±2.8^{a}

Values are means (±standard error). Values followed by different superscripts in the same row are significantly different at P≤0.05 according to Duncan's multiple range test.

Table 4.4 Effect of explant type on the frequency (%) of cassava somatic embryogenesis in MS media supplemented with 2,4D in the dark

	2,4-D concentration							
	4 mg/l		6 mg/l		8 mg/l		10 mg/l	
Genotype	**Leaf**	**Stem**	**Leaf**	**Stem**	**Leaf**	**Stem**	**Leaf**	**Stem**
08/354	90.8±2.1^{a}	50.2±5.8^{a}	88.0±4.4^{a}	39.4±2.9^{a}	90.0±4.5^{a}	52.2±2.7^{a}	90.8±1.6^{a}	54.5±2.9^{a}
KME1	23.5±8.7^{b}	8.0±1.7^{c}	46.7±2.8^{d}	28.0±2.3^{b}	70.5±3.5^{b}	32.7±2.6^{c}	93.5±2.5^{a}	46.1±5.0^{a}
08/274	39.2±2.3^{b}	22.2±1.5^{c}	57.2±2.5^{bc}	32.0±1.5^{ab}	88.7±2.1^{a}	46.8±3.4^{abc}	86.9±2.5^{a}	37.3±2.6^{ab}
08/080	71.2±1.5^{a}	31.7±3.0^{bc}	70.7±2.0^{ab}	34.7±1.6^{ab}	76.8±2.3^{ab}	36.3±2.8^{bc}	86.1±4.7^{a}	48.3±3.4^{a}
TMS60444	74.1±5.5^{a}	31.6±3.1^{ab}	79.5±9.0^{a}	39.1±3.4^{a}	83.7±5.2^{ab}	50.0±4.9^{ab}	85.4±4.4^{a}	46.6±5.6^{a}

Values are means (±standard error). Values followed by different superscripts in the same row are significantly different at P≤0.05 according to Duncan's multiple range test.

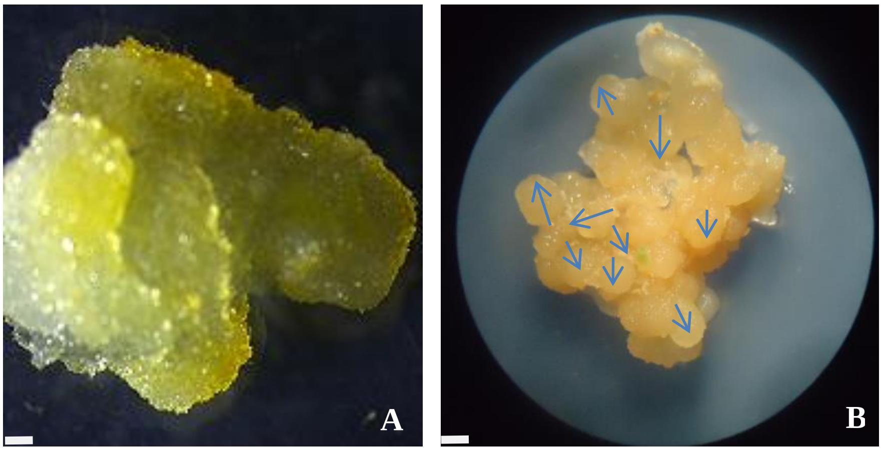

Figure 4.1: Morphology of callus formed from different explant types; A: Loose non-embryogenic friable white callus from a stem explant; B: Translucent gelatinous embryogenic callus from a leaf explant (arrows indicate embryos); Scale- 0.5mm

4.2 Effect of light on callogenesis and embryogenesis in cassava

All genotypes formed callus when the explants were cultured in the dark and when exposed to 16 h light a day. Significant differences ($P \leq 0.05$) were observed in the ability of explants to form callus (Tables 4.5 and 4.6) and on the frequency of embryogenesis in the two photoperiods in both picloram and 2,4-D (Tables 4.7 and 4.8). Exposing the explants to light affected the process of embryogenesis with the light-exposed explants forming more non-embryogenic callus after four weeks of culture (Tables 4.7 and 4.8). While some of the callus cultured in light formed hard non-embryogenic structures (Figure 4.2 A) or rhizogenic structures (Figure 4.2 B), other callus formed highly embryogenic structures (Figure 4.2 C) that formed shoots (Figure 4.2 D).

Table 4.5: Effect of light on the frequency of cassava callogenesis in MS media supplemented with picloram

	Auxin Concentration							
	4 mg/l		6 mg/l		8 mg/l		10 mg/l	
Genotype	**8/16**	**0/24**	**8/16**	**0/24**	**8/16**	**0/24**	**8/16**	**0/24**
08/354	84.8±7.2^{a}	87.6±6.4^{a}	87.9±5.5^{a}	89.4±5.4^{a}	84.1±6.6^{a}	88.8±6.7^{a}	88.8±4.8^{a}	89.0±1.9^{a}
KME1	84.4±7.0^{a}	86.3±5±5.4^{a}	83.9±6.7^{a}	89.7±5.3^{a}	86.5±5.1^{a}	86.6±7.1^{a}	90.5±2.8^{a}	95.7±1.7^{a}
08/274	85.3±6.6^{a}	87.9±7.3^{a}	88.1±4.7^{a}	84.8±9.4^{a}	88.4±3.7^{a}	90.8±3.6^{a}	89.4±3.7^{a}	94.3±2.4^{a}
08/080	94.1±1.2^{a}	89.8±5.2^{a}	86.0±6.9^{a}	90.7±5.9^{a}	86.1±3.7^{a}	91.1±3.0^{a}	88.7±3.5^{a}	92.6±2.9^{a}
TMS60444	86.2±4.3^{a}	90.0±4.3^{a}	84.5±4.4^{a}	92.5±2.5^{a}	86.2±3.3^{a}	92.5±2.8^{a}	86.2±4.2^{a}	91.6±4.7^{a}

Values are means (±standard error). Values followed by different superscripts in the same row are significantly different at P≤0.05 Duncan's multiple range test.

Table 4.6: Effect of light on the Frequency of cassava callogenesis in MS media supplemented with 2,4-D

	Auxin Concentration							
	4mg/l		6mg/l		8mg/l		10mg/l	
Genotype	**8/16**	**0/24**	**8/16**	**0/24**	**8/16**	**0/24**	**8/16**	**0/24**
08/354	79.3±4.9^{a}	84.0±5.8^{a}	71.5±5.5^{a}	80.6±8.6^{a}	76.2±3.5^{a}	92.5±3.2^{a}	76.2±7.6^{a}	79.1±7.8^{a}
KME1	5.6±1.9^{b}	35.0±4.0^{b}	66.0±3.1^{a}	79.2±8.1^{a}	68.1±11.2^{a}	72.5±12.2^{a}	76.4±0.7^{a}	93.5±1.0^{a}
08/274	35.9±1.8^{c}	37.7±3.0^{a}	53.2±1.1^{b}	59.0±1.8^{a}	80.2±3.1^{a}	85.6±3.7^{a}	79.3±2.4^{a}	84.1±4.1^{a}
08/080	61.1±5.0^{a}	61.8±6.6^{a}	62.7±4.2^{a}	69.0±3.0^{a}	72.5±1.6^{a}	78.2±3.3^{a}	70.9±7.6^{a}	74.1±10.5^{a}
TMS 60444	55.8±5.3^{a}	70.8±7.3^{a}	57.9±5.5^{a}	76.2±0.1^{a}	70.8±3.0^{a}	88.3±2.4^{a}	71.6±4.1^{a}	86.6±3.9^{a}

Values are means (±standard error). Values followed by different superscripts in the same row are significantly different at P≤0.05 according to Duncan's multiple range test.

Table 4.7: Effect of light on the frequency of cassava somatic embryogenesis in in MS media supplemented with picloram

	Auxin Concentration							
	4 mg/l		**6 mg/l**		**8 mg/l**		**10 mg/l**	
Genotype	**8/16**	**0/24**	**8/16**	**0/24**	**8/16**	**0/24**	**8/16**	**0/24**
08/354	59.2±6.9^{a}	58.2±5.7^{a}	55.6±7.1^{a}	65.9±10.3^{a}	55.9±8.7^{a}	57.6±9.7^{a}	63.4±8.7^{a}	66.6±10.6^{a}
KME1	44.8±10.3^{a}	53.5±10.2^{a}	46.7±9.6^{a}	50.0±9.6^{a}	50.2±12.7^{a}	53.3±10.0^{a}	56.2±12.6^{a}	60.0±11.5^{a}
08/274	50.1±10.9^{a}	51.0±8.5^{a}	48.7±11.1^{a}	46.3±9.2^{a}	53.8±9.9^{a}	58.0±9.3^{a}	56.3±15.9^{a}	62.2±13.0^{a}
08/080	39.8±9.4^{a}	44.6±4^{a}	42.9±10.2^{a}	46.7±7.1^{a}	51.4±15.6^{a}	54.8±11.6^{a}	53.6±15.8^{a}	58.8±15.4^{a}
TMS60444	52.0±9.4^{a}	56.2±9.5^{a}	58.3±9.7^{a}	62.5±11.2^{a}	57.5±10.2^{a}	66.6±11.5^{a}	59.1±10.3^{a}	65.4±11.1^{a}

Values are means (±standard error). Values followed by different superscripts in the same row are significantly different at P≤0.05 according to Duncan's multiple range test.

Table 4.8: Effect of light on the frequency of cassava somatic embryogenesis in in MS media supplemented with 2,4-D in cassava

	Auxin Concentration							
	4 mg/l		**6 mg/l**		**8 mg/l**		**10 mg/l**	
Genotype	**8/16**	**0/24**	**8/16**	**0/24**	**8/16**	**0/24**	**8/16**	**0/24**
08/354	68.2±16.0^{a}	77.8±10.3^{a}	64.7±17.5^{a}	71.0±15.4^{a}	69.3±12.6^{a}	76.1±11.2^{a}	74.1±14.0^{a}	78.6±11.3^{a}
KME1	16.6±6.1^{b}	24.1±8.5^{b}	33.3±5.5^{ab}	41.4±5.7^{ab}	46.8±10.2^{ab}	56.5±11.7^{ab}	65.5±14.9^{a}	74.1±13.0^{a}
08/274	29.4±4.8^{a}	32.9±5.7^{c}	49.1±11.3^{a}	53.4±10.9^{b}	66.4±14.2^{a}	72.0±11.8^{a}	65.6±18.6^{a}	69.7±16.5^{a}
08/080	57.0±16.9^{a}	61.2±14.9^{a}	62.5±16.9^{a}	64.7±16.5^{a}	60.4±16.7^{a}	66.8±14.9^{a}	64.5±12.3^{a}	73.7±11.8^{a}
TMS 60444	57.5±17.8^{a}	62.5±14.9^{a}	62.5±16.0^{a}	66.6±13.6^{a}	68.3±14.7^{a}	77.5±12.1^{a}	68.3±15.7^{a}	74.1±13.4^{a}

Values are means (±standard error). Values followed by different superscripts in the same row are significantly different at P≤0.05 according to Duncan's multiple range test.

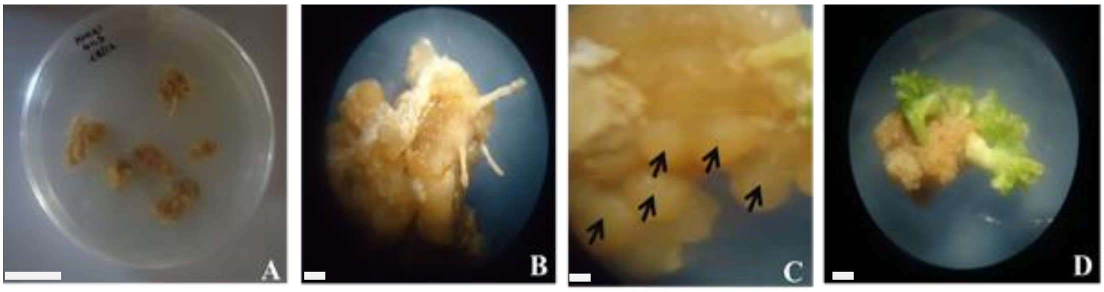

Figure 4.2: Morphology of calli formed when explants are exposed to light; A: Hard non-embryogenic callus; B: Callus with rhizogenic structures; C: Highly embryogenic callus with arrows indicating embryos; D: Shoots formed from callus cultured in light; scale - 0.5 mm.

4.3 Somatic embryos maturation and shoot induction

Callus proliferated to develop nodular structures that subsequently formed embryo like structures with globular, heart and torpedo shapes (Fig 4.3) in combinations of plant growth regulators that were tested viz: M (3 mg/l BAP, 0.02 mg/l NAA 0.5 mg/l GA_3), M1 (3 mg/l BAP, 0.01 mg/l NAA, 1 mg/l GA_3), M2 (2 mg/l BAP, 0.03 mg/l NAA 0.5 mg/l GA_3), M3 (2 mg/l BAP, 0.01 mg/l NAA, 1.5 mg/l GA_3), M4 (1 mg/l BAP, 0.02 mg/l NAA, 1.5 mg/l GA_3), There were no significant differences (P=0.05) in the ability of the five hormone formulations to initiate maturation of somatic embryos in the individual genotypes (Table 4.9). The embryos observed in the maturation media either had two distinct cotyledons, one cotyledon or fused cotyledons. The callus cultured on M1 (3 mg/l BAP, 0.01 mg/l NAA, 1 mg/l GA_3) and M (3 mg/l BAP, 0.02 mg/l NAA 0.5 mg/l GA_3), formed cotyledonary embryos, but most of the shoots had stunted growth which led to low plant recovery rates from the two combinations. Most of the callus from these two combinations produced 0 - 7 shoots each (Figures 4.3 A and B). In the medium supplemented with 1 mg/l BAP, 0.02 mg/l NAA, 1.5 mg/l GA_3 (M4) and 2 mg/l BAP, 0.01 mg/l NAA, 1.5 mg/l GA_3 (M3), the callus produced many cotyledons (6 - 22) each (Figures 4.3 C, D, E) giving high shoot formation frequencies (Figure 4.4). The callus obtained from 2,4-D concentration of 4 mg/l formed small sized globular embryos that turned green on maturation but did not differentiate to distinct root and shoot apices (Figure 4.3 F).

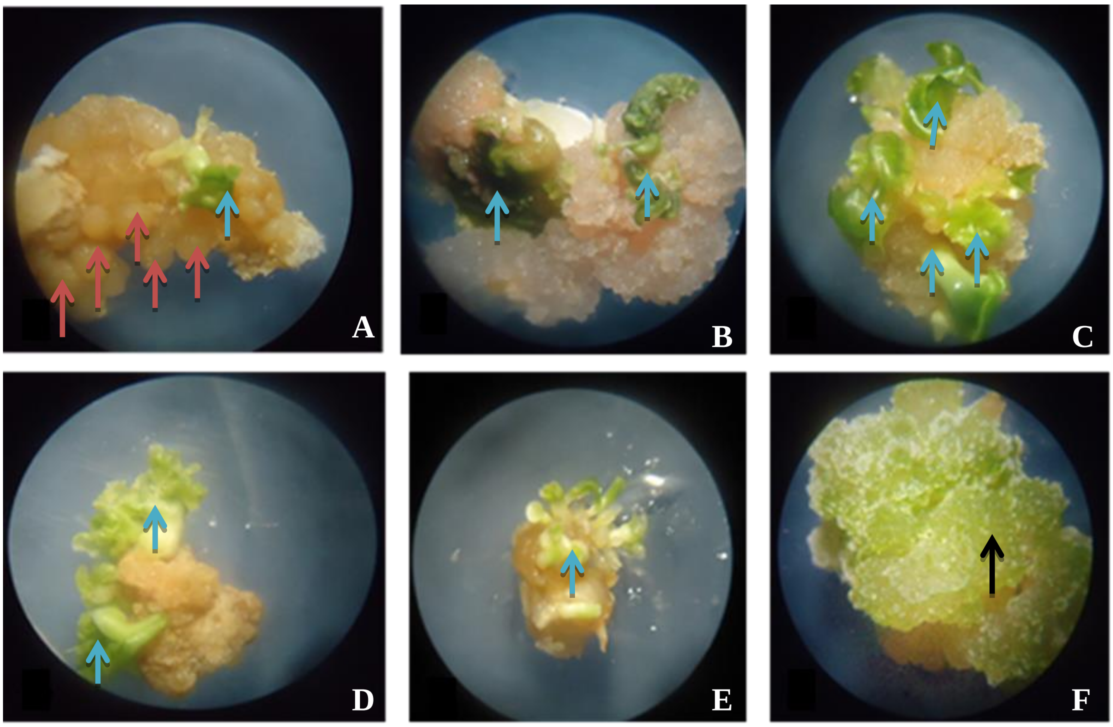

Figure 4.3: Morphology of embryogenic callus; A and B: Embryos matured on M (3 mg/l BAP, 0.02 mg/l NAA 0.5 mg/l GA_3) and M2 (2 mg/l BAP, 0.03 mg/l NAA 0.5 mg/l GA_3); C, D and E: Embryos matured on M1 (3 mg/l BAP, 0.01 mg/l NAA, 1 mg/l GA_3), M3 (2 mg/l BAP, 0.01 mg/l NAA, 1.5 mg/l GA_3) and M4 (1 mg/l BAP, 0.02 mg/l NAA, 1.5 mg/l GA_3); F: Morphology of globular embryos obtained from 4 mg/l 2, 4-D. Red arrows indicate single globular stage embryos, blue arrows indicate mature cotyledonary stage embryos, black arrow indicates many small sized globular stage embryos.

Table 4.9: Effect of different concentrations of BAP, NAA and GA3 on maturation frequencies of cassava somatic embryos

	Maturation media				
Genotypes	**M**	**M1**	**M2**	**M3**	**M4**
08/354	76.15±2.60^a	73.11±2.15^a	77.94±3.89^a	70.83±7.22^a	73.60±1.53^a
08/274	58.01±1.27^a	55.18±6.95^a	52.61±5.76^a	68.67±1.79^a	57.73±8.80^a
TMS60444	67.71±8.28^a	68.19±2.67^a	63.96±9.81^a	67.75±0.98^a	69.25±3.58^a
08/080	76.75±6.67^a	72.22±4.81^a	67.72±5.17^a	59.03±8.62^a	63.97±5.60^a
KME 1	61.31±7.11^a	60.00±4.04^a	61.11±9.61^a	63.91±6.72^a	63.65±3.38^a

Values are means (±standard error). Values followed by different superscripts in the same column are significantly different at $P \leq 0.05$ according to Duncan's multiple range test.

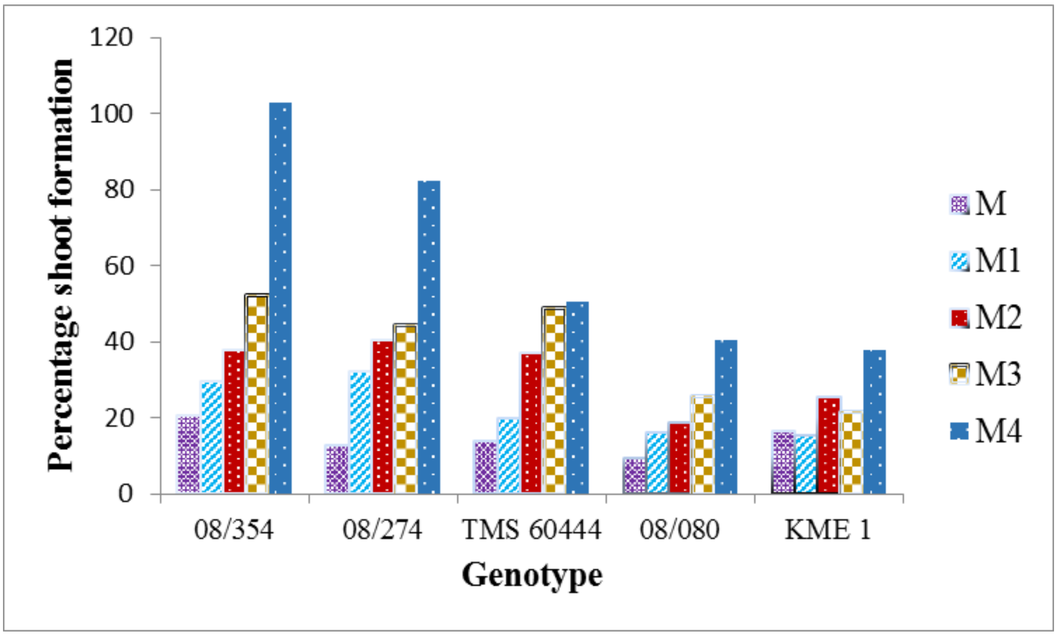

Figure 4.4: Percentage shoot formation frequency on varying formulations of maturation media; M (3 mg/l BAP, 0.02 mg/l NAA 0.5 mg/l GA_3), M1 (3 mg/l BAP, 0.01 mg/l NAA, 1 mg/l GA_3), M2 (2 mg/l BAP, 0.03 mg/l NAA 0.5 mg/l GA_3), M3 (2 mg/l BAP, 0.01 mg/l NAA, 1.5 mg/l GA_3), M4 (1 mg/l BAP, 0.02 mg/l NAA, 1.5 mg/l GA_3).

4.4 Rooting and plantlet recovery

Cotyledonary embryos were transferred to hormone free MS media containing activated charcoal for the absorption of phenolics and for establishment of darkened environment (Figure 4.5 A). When cotyledons with distinct shoots were transferred to hormone free cassava micro propagation media rooting was successfully achieved (Figure 4.5 B and C). Genotype 08/354 gave the highest rooting frequency of 74.3 % while genotype 08/080 gave the lowest rooting frequency of 32.8 %. Genotypes KME 1 and TMS 60444 gave the highest regeneration frequencies of 31.9 % and 31.2 % respectively. The regeneration frequency varied between 21 % and 31 % with genotypes KME 1 and 08/080 recording highest and lowest frequencies respectively (Table 4.10). The rooted shoots were gradually transplanted into the soil in the greenhouse (Figure 4.6 A, B, C, D and E).

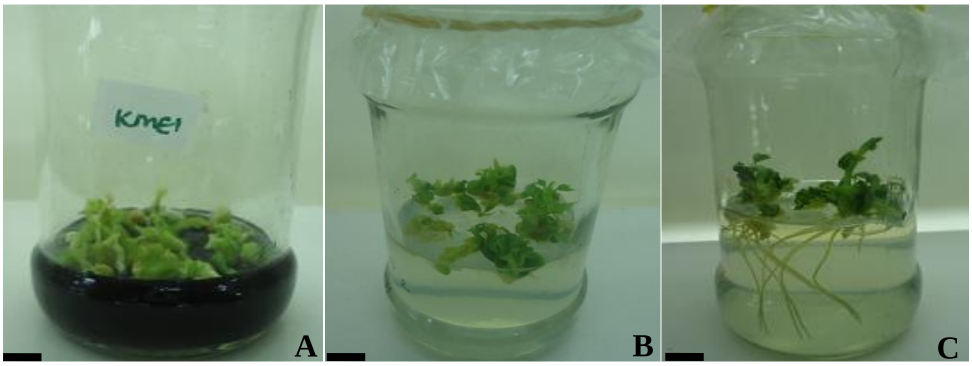

Figure 4.5: Germination and recovery of cassava shoots; A: Desiccation of shoots in activated charcoal; B: Rooting in hormone free MS media; C: After 6 days in rooting media: 1 cm.

Table 4.10: Cassava rooting and regeneration frequencies

Genotype	Rooting frequency	Regeneration frequency
08/354	32.8±2.4 ^{cd}	28.9±5.3 ^{a}
08/274	47.6±3.8 ^{bc}	27.4±5 ^{a}
08/080	74.3±1.2 ^{a}	21.6±3.4 ^{a}
KME 1	43.5±3.5 ^{cd}	31.9±3.4 ^{a}
TMS 60444	59.3±3.5 ^{b}	31.2±3.9 ^{a}

Values are means (±standard error). Values followed by different superscripts in the same column are significantly different at P≤0.05 according to Duncan’s multiple range test.

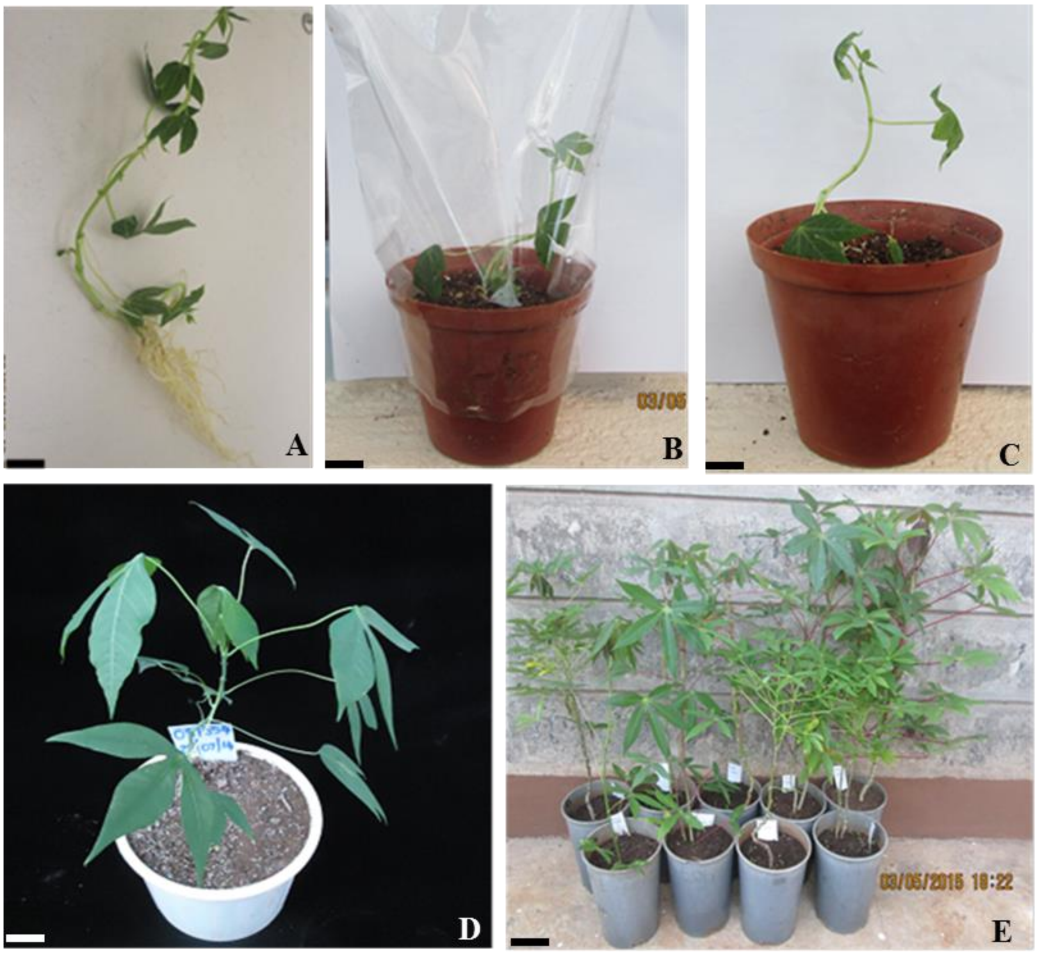

Figure 4.6: Different stages of hardening of in vitro cassava plantlets; A: Washed *in vitro* plantlet ready for hardening; B: Regenerated plantlets in plastic cups covered with polythene bags; C: Regenerated plantlet 2 weeks after hardening; D: Regenerated plantlet 3 months after hardening; E: Regenerated plantlets 6 months after hardening. Scale: 0.5 cm.

4.5 *Agrobacterium*-mediated transformation of cassava

Explants were co-cultivated with *A. tumefaciens* for 3 days in the dark (Figure 4.7A). Subsequently, the explants were maintained on selection free medium for 10 days (Figure 4.7B) in order to recover from the shock caused by *Agrobacterium* infection. When transferred onto selection media, the non-transformed explants died while the putatively transformed explants survived selection (Figure 4.7C). After desiccation in activated charcoal media (Figure 4.7D), putative transgenic shoots were rooted under selection (Figures 4.7E and F) then hardened in the glasshouse (Figure 4.7G).

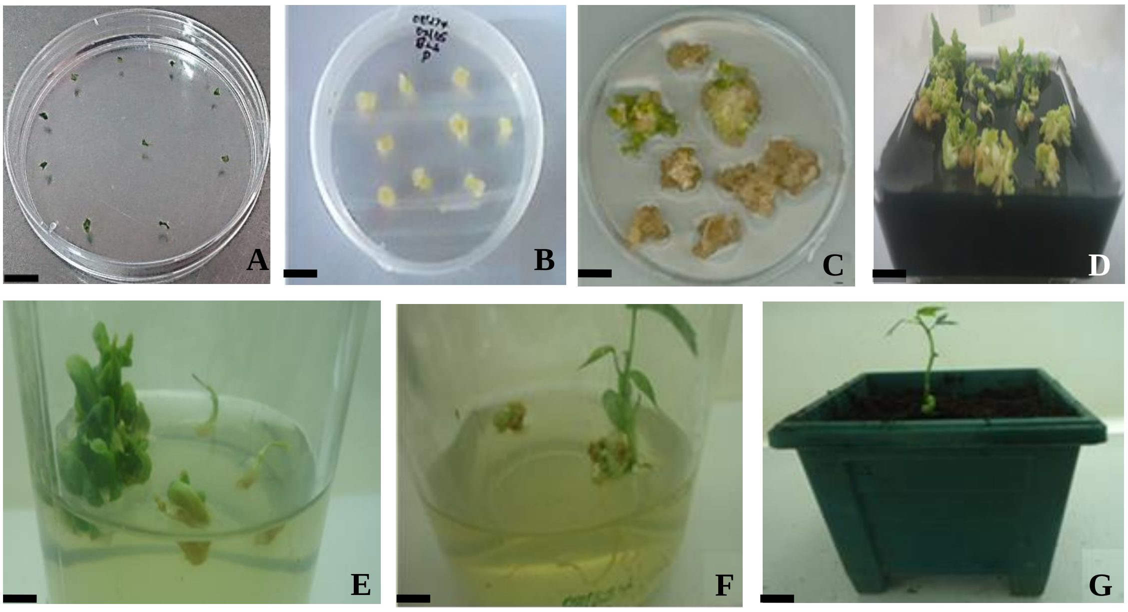

Figure 4.7: Agrobacterium mediated transformation in cassava; A: Co-cultivation of immature leaf lobes with *Agrobacterium*; B: Resting of explants in selection free medium; C: Maturation of callus under selection; D: Desiccation of shoots in activated charcoal media; E: Rooting of shoots under selection; F: Elongation of putative cassava transgenic shoots under selection; G: Putative transgenic plant hardened in the glasshouse. Scale: 1.5 cm.

4.6 Assessment of transformability in selected cassava genotypes

Transformability of the cassava genotypes was assessed through transient *GUS* expression assay 3 days after co-cultivation of callus. A uniform blue coloration was observed in all GUS positive callus (Figure 4.8A) while GUS negative callus did not have the blue colouration (Figure 4.8B). Histochemical staining of the shoots was used to confirm expression of the *gus*A gene throughout the plant. While shoots from putative transgenic lines gave a blue colouration (Figure 4.8C), shoots from non-transformed plants did not stain blue (Figure 4.8D).

Model genotype TMS 60444 and genotype 08/274 gave the highest frequencies of transient *gus* expression of (73.33 %) and 63.33% respectively (Table 4.11). A total of eight transgenic plants were regenerated and the overall regeneration efficiency was

0.34 %. Model genotype TMS 60444 gave the highest transformation efficiency of 0.5 % while genotype 08/274 gave the lowest transformation efficiency of 0.23 % (Table 4.12).

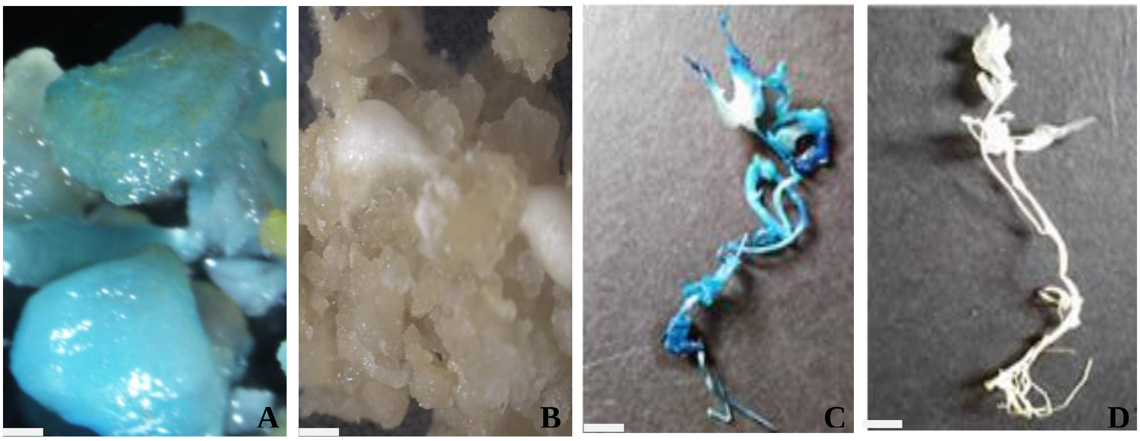

Figure 4.8: Morphology of cassava callus and plantlets after GUS staining; A: Cassava callus positive for GUS; B: Cassava callus negative for GUS; C: Putative transgenic shoot turned blue following GUS staining; D: Non-transgenic control shoot lacking blue colouration following GUS staining. Scale – 0.5 cm.

Table 4.11: Frequency (%) of transient *gus* expression produced on selective medium

Genotype	**Frequency (%) of transient *gus* expression on callus**
08/354	56.67±2.89^{a}
08/274	68.33±7.64^{a}
TMS 60444	73.33±7.64^{a}
08/080	53.33±7.64^{a}
KME 1	55.00±10.00^{a}

Values are means (±standard error). Values followed by different superscripts are significantly different at P≤0.05 according to Duncan's multiple range test.

Table 4.12: Cassava transformation efficiency

Genotype	No. of explants used	No of PCR positive plantlets	Transformation efficiency (%)
08/354	526	2	0.38
08/274	442	1	0.23
TMS 60444	393	2	0.51
08/080	394	1	0.25
KME 1	598	2	0.33
Total	2353	8	0.34

4.7 Polymerase Chain Reaction (PCR) analysis for the T-DNA integration

A PCR analysis on the genomic DNA of bialaphos resistant regenerated putative transgenic plants was used to confirm the presence of transgenes. The expected band size of 528 bp of *gus*A was successfully amplified from all the transgenic lines confirming integration of the gene in the genome of the plant. There was no amplification in the untransformed wild-type plants (Figure 4.9).

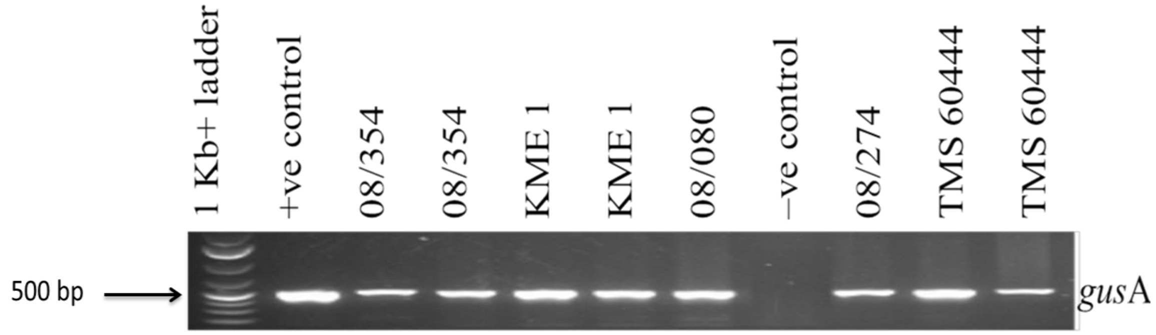

Figure 4.9: PCR analysis of putative cassava transgenic lines; +ve control, Plasmid DNA; -ve control: Non-transformed regenerated plant.

4.8 Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) analysis

When the RT-PCR products were separated in an electrophoretic gel, the expected band sizes of 528 bp (Figure 4.10A) and 220 bps (Figure 4.10B) for *gus*A and PP2A genes respectively were obtained in putative transgenic lines. Amplification primers for the PP2A genes successfully amplified the target sequence in cDNA derived from wild-type plant. The cDNA from the wild-type plant was however not amplified by the *gus*A gene (Figure 4.10).

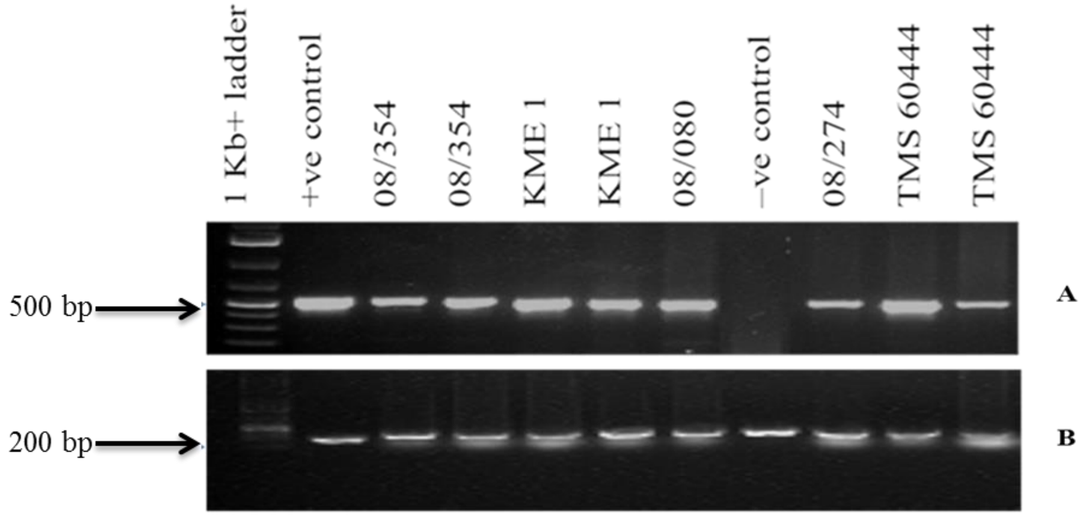

Figure 4.10: RT-PCR analysis of putative cassava transgenic lines; **A**: RT-PCR analysis using *gus*A specific primers; **B**: RT-PCR analysis using *pp*2A specific primers. +ve control, Plasmid DNA; -ve control: Non-transformed regenerated plant.

CHAPTER FIVE

DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 Discussion

The source and type of the initial explant is an important factor for somatic embryogenesis in cassava. It was established from this study that leaf explants gave significantly higher frequencies of somatic embryogenesis compared to meristematic stem segments. The difference in the response to somatic embryo formation between leaf and stem segments can be attributed to difference in their morphological structure and stages of maturity (Schädel *et al.*, 2010).

Additionally, it was observed that when stem segments were cut and poked, they produced some latex that possibly interfered with the formation of somatic embryos. The round shape of stem segments could also have contributed to the low frequency as compared to the flat shape of the leaf which ensured that a big surface area of the leaf was in contact with the media. According to Twumwasi *et al.* (2009), this can be attributed to the fact that leaves are easily programmed to dedifferentiate into undifferentiated cells and the fact that leaves are less lignified as compared to stem segments making them more responsive to plant regulatory hormones (San-José *et al.*, 2010; Schädel *et al.*, 2010).

The most commonly used explants to induce somatic embryogenesis in cassava are immature leaf lobes (Raemakers *et al.*, 1997; Sofiari *et al.*, 1998; Guohua, 1998; Li *et al.*, 1998; Taylor *et al.*, 2001; Danso and Ford-Lloyd, 2002; Guohua and Qiusheng, 2002; Zhang and Puonti-Kaerlas, 2005; Atehnkeng *et al.*, 2006; Hankoua *et al.*, 2006, Ngugi *et al.*, 2015), followed by shoot apical meristems (Raemakers *et al.*, 1997;

Hankoua *et al.,* 2005; Atehnkeng *et al.,* 2006; Hankoua *et al.,* 2006). The variability in the ability of apical meristems and immature leaf lobes to form somatic embryos in cassava was also reported by Taylor *et al.* (1996a).

Somatic embryogenesis begins with the induction of primary embryos from the explants by culturing them on MS medium supplemented with an auxin such as 2,4-D, Dicamba, NAA or picloram. The formation of embryogenic structures must be optimized for each individual cultivar and not all genotypes are prone to embryo formation, regeneration, or transformation (Rossin and Rey, 2011).

In this study, all tested genotypes showed a positive response in primary callus induction and somatic embryos were successfully obtained for all the cassava genotypes in the media types tested. When explants were cultured on embryo induction medium containing 2,4-D, concentration 8 mg/l was the best for the induction of embryos in 08/354, 08/274, 08/080 and TMS 60444 genotypes. In KME *1,* however, 10 mg/l 2, 4-D gave the highest frequency of somatic embryogenesis. This study determined that higher levels of 2,4-D were the best inducers of somatic embryos. The results of this study agreed with the previous study by Fletcher *et al.* (2011) who observed 8 mg/l of 2,4-D to be the best concentration for callus induction in four Ghanaian cassava cultivars. Similarly, Ihemere. (2003) determined higher number of cassava somatic embryos produced under higher concentrations of 2,4-D and Ngugi (2013) determined that 8mg/l 2,4-D was the best for somatic embryo formation in a range of African cassava genotypes. This is consistent with previous reports on other genotypes (Hankoua *et al.,* 2005; Atehnkeng *et al.*, 2006; Kone *et al.,* 2015) this study found somatic embryo formation to be genotype dependent.

Occurrence of dedifferentiation enables acquisition of embryogenic competence and allows erasing or alteration of the existing developmental information to make the cells responsive for new signals. The developmental switch from a differentiated and resting cell state to a dedifferentiated, dividing, embryogenic state involves the general reorganization of chromatin structure, overall reprogramming of gene expression, as well as cellular metabolism (Dudits *et al.,* 1991; 1995).

Many *in vitro* somatic embryogenesis systems rely on the use of exogenous 2,4-D as an inducer. In carrot, one of the most extensively studied systems; it was proven that single cultured cells require 2,4-D to initiate embryo development (Nomura and Komamine, 1995). The continuous presence of this artificial auxin, however, blocks further development and results in the accumulation of the already determined pro-embryogenic cell mass in the cultures (De Vries *et al.,* 1988). However, 2,4-D may also act as an herbicide, and it may induce stress responses in plant cells (Grossmann, 2000). Early phases of somatic embryogenesis are characterized by the induction of many stress-related genes, which leads to the hypothesis that somatic embryogenesis is an extreme stress response of cultured plant cells (Dudits *et al.*, 1991, 1995).

Although light is known to enhance somatic embryogenesis and germination of embryos (Raemakers *et al.,* 1997), significantly more embryos formed on explants cultured in darkness while very few somatic embryos developed on explants cultured in the light. Raemakers *et al.* (1993) also reported enhancement of embryogenesis in cassava by lowering the light intensity. The results of this study concur with the results of studies in other species (Gingas and Lineberger, 1989; Michler, 1995).

Light is one of the most important environmental signals, known to affect somatic embryogenesis through its effect on induction (Verhagen *et al.*, 1989) and on some morphological characteristics of differentiated somatic embryos (Halperin, 1966; Ammirato and Steward, 1971). The intensity, spectrum and duration of the light supplied for *in vitro* cultures can influence morphogenic response and its efficiency. The influence of light on *in vitro* plant morphogenesis can be related with a stimulatory or inhibitory effect of light on different endogenous substances, including plant growth regulators (Zelena, 2000).

According to Uzelac *et al.* (2007), low frequency of somatic embryo maturation and germination is a limiting factor for the application of somatic embryogenesis in the improvement of a number of species. It is for this reason that the maturation media for cassava somatic embryos was validated in this study. Culturing of somatic embryos in MS media supplemented with activated charcoal was found to increase plant recovery rates. This could be attributed to the absorption of auxins that had been used in the callus induction media along with polyphenols produced by the callus.

Charcoal may affect the activity or the stability of plant growth regulators by reducing/ excluding light in *in vitro* cultures. The excessive production of polyphenols during initial stages of culture has been shown to cause browning and eventual death of the tissues possibly by triggering of defense reactions. Polyphenols are, therefore, inhibitory substances that should be avoided or eliminated from *in vitro* environments. Similar results have been obtained with other plant species: activated charcoal improved potato protoplast culture by reducing browning (Carlberg *et al.*, 1983). Activated charcoal was found to reduce browning of palm explants and culture media

(Yam *et al.,* 1990) thereby increasing explant survival and organogenesis. Pretreatment of embryogenic callus with activated charcoal also increases the level of germination of somatic embryos in red fescue (Zaghmout and Torello, 1988) due to the adsorption of 2,4-D along with other inhibitors. In other studies carried out with *Strelitzia reginae* and *Anemone oronaria*, activated charcoal was shown to control browning and stimulate shoot growth (Mensuali-Sodi *et al.,* 1993) and in European orchid media its incorporation into the media stimulated growth of the species which exhibited browning (Van, 1987).

Mature somatic embryos were regenerated into cotyledons by placing callus on MS media supplemented with various formulations of BAP, NAA and GA_3. High auxin concentrations stimulate callus formation, but inhibit shoot regeneration. In most studies, a high auxin/cytokinin ratio is usually used for inducing callus formation. Thereafter, the auxin level must be reduced while increasing the cytokinin level for stimulating shoot formation (Skoog and Miller, 1957, Dexit *et al.,* 2016)). Based on this theory, the effect of a stepwise increase in BAP concentration and a decrease in NAA concentration was investigated.

Differences in plant recovery rates were observed between the various photohormones in this study. These results on influence of shoot formation rates by varying auxin-cytokinin ratios concur with previous reports by Skoog and Miller (1957) who reported that alterations in auxin and cytokinin ratios which was sufficient to control morphogenesis in tobacco. In *Pinus caribaea*, it was found necessary to have a 1:2 NAA/BAP ratio to regenerate plants (Akaneme and Eneobong, 2008). Peres and

Kerbauy (1999) found that alteration in endogenous auxin/cytokinin ratio, favoring cytokinins, strongly influenced plant development under *in vitro* conditions.

The use of *in vitro* systems based on somatic embryogenesis for plant regeneration is determined not only by a high efficiency of somatic embryo formation, but also depends on the capacity of the embryos for plant development. The absence and low rate of conversion of the somatic embryos into plantlets have hindered the application of this biotechnological techniques in a number of crops such as *Acrocomia aculeate* (Moura *et al.,* 2009) and *Accasello wiana* (Pescador *et al.,* 2008). The process of plant recovery involves the formation of primary roots, a shoot meristem with a leaf primordium and greening of hypocotyls and cotyledons (Redenbaugh *et al.,* 1986).

The problem of lack of or a low frequency of embryo conversion into plantlets has been observed in numerous systems in spite of the high number of somatic embryos produced. In such instances, Giberellic acid (GA_3) is frequently added in the media to stimulate embryo conversion, and to improve the efficiency of plant regeneration (Eapen and George, 1994; Garcia and Martinez, 1995; Binzel *et al.,* 1996; Kim *et al.,* 1997). In this study, an increase in plant recovery rates was observed with increase in the concentration of GA_3 used. Significant stimulatory effects of this phytohormone in cultures of *Sesamum indicum* were reported by Xu *et al.* (1997) and in *P. ginseng* by Yang and Choi (2000). Rapid development of somatic embryos was achieved in cultures of *A. thaliana* (Gaj, 2001), *Cichorium endivia* leaf-explants (Bellettre *et al.,* 1999), and in *Brassica napus* immature cotyledons (Turgut *et al.,* 1998).

The results of this study on genotypic differences in the number of plants regenerated per callus in different genotypes concur with the study outcomes by Hankoua *et al.* (2005) and Feitosa *et al.* (2007) who reported variation of regeneration potentials of various cassava genotypes.

The development of an efficient transformation system is an important tool for gene manipulation. In this study, transgenic cassava plants for the five tested cassava genotypes were produced by transformation of immature leaf lobes using *Agrobacterium tumefaciens* strain EHA 101. The cassava genotype TMS 60444 was used as a model genotype (Bull *et al.,* 2009; Ngugi *et al.,* 2015) while the other four lines were Kenyan genotypes. Assessment of transformability using callus and putative transgenic lines using *GUS* histochemical assay showed that all the genotypes tested were amenable to *Agrobacterium* mediated transformation. Expression of the *gus*A-intron gene is a reliable indicator of plant transformation, since the gene can only express efficiently in plant cells but not in *Agrobacterium* (Vancanneyt *et al.,* 1990).

The presence and integration of the *gus*A gene was confirmed by Polymerase Chain Reaction (PCR) and Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) analysis of transgenic plants confirmed the expression of *gus*A gene. The results of this study indicate that immature leaf lobes are good targets for transformation in cassava. Other explants that have successfully been used as targets for the transformation of cassava are friable embryonic callus (FEC) and germinated somatic embryos (Taylor *et al.,* 2001; Siritunga, 2002; Ihemere, 2003; Hankoua *et al.,* 2006; Nyaboga *et al.,* 2013; 2015).

5.2 Conclusion

i. The present study has demonstrated that the source of explant greatly influences response to hormone treatments with the leaf explants being a better source compared to stem explants. It was also found that leaf explants responded more readily to callogenesis, taking a shorter time to form callus compared to the meristematic stem segments.

ii. It has been shown that various genotypes respond differently to the induction of somatic embryos depending on the type of auxin used. Maintaining of callus induction cultures in dark was found to give higher frequencies of somatic embryogenesis compared to when the cultures were exposed to 8 hours of light each day. Synergisms between BAP (1.0 mg/l) and NAA (0.02 mg/l) along with GA_3 (1.5 mg/l) was found to be the best phytohormone combination for somatic embryo maturation.

iii. The results of this study indicate that *Agrobacterium*-mediated transformation can be used to integrate genes of interest into all the five genotypes used. This is because the five cultivars were able to pick up the *gus* gene and express it effectively.

iv. A regeneration and transformation protocol for the five selected genotypes was validated.

5.3 Recommendations

- ✓ Regeneration of cassava by dark incubation of immature leaf lobes on either 8 mg/l 2,4-D or 10 mg/l picloram depending on the genotype, maturation of somatic embryos in M4 (1 mg/l BAP, 0.02 mg/l NAA, 1.5 mg/l GA_3), desiccation in 8% activated charcoal media and rooting of shoots in hormone-free cassava micro propagation media.
- ✓ Use of the optimized regeneration protocol reported here to integrate desirable traits in the tested genotypes using *Agrobacterium* mediated transformation.

5.4 Suggestions for further studies

i. Optimization of the appropriate time period of resting the explants to increase transformability.
ii. Optimization of the appropriate concentration of bialaphos for plant selection.
iii. Validation of the regeneration protocol optimized in this study to regenerate other African farmer preferred genotypes.
iv. Testing of the transformability of other *agrobacterium* strains such as LBA 4404 on African cassava genotypes.
v. Transformation of African cassava genotypes using friable embryogenic callus as target tissue should be undertaken.
vi. Further titration of picloram at concentrations 15, 20 and 25mg/l to ascertain the best concentration for somatic embryogenesis in cassava.
vii. Further titration of maturation media with media supplemented with a each phytohormone singly or in pairs to ascertain the effect of phytohormone combination on embryo maturation.

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APPENDICES

Appendix 1: Plant hormone preparation

2, 4-dichlorophenoxyacetic acid (2, 4-D)

A stock solution of 1 mg/l of 2,4-D was prepared by weighing 0.05 g of 2,4-D (Duchefa company) dissolving it in 1ml of 70 % ethanol in a falcon tube and sufficient amount of sterile distilled water added to make 10 ml of stock solution. The solution was then filter sterilized using a syringe and 0.2 µm filter, aliquoted in 1.5 ml eppendorf tubes and stored at 4 °C.

Picloram

A stock solution of 1 mg/l of picloram was prepared by weighing 0.05 g of Picloram (Duchefa company) dissolving it in 1ml of 70 % ethanol in a falcon tube and sufficient amount of sterile distilled water added to make 10 ml of stock solution. The solution was then filter sterilized using a syringe and 0.2 µm filter, aliquoted in 1.5 ml eppendorf tubes and stored at 4 °C.

α -Naphthaleneacetic acid (NAA)

A stock solution of 1 mg/l of NAA was prepared by weighing 0.05 g of NAA (Duchefa Company) which was dissolved in 1 ml of 1N sodium hydroxide (NaOH) and topped up to 50 ml with distilled water. The stock solution was aliquoted into sterile 1.5 ml eppendorf tubes and stored at 4 °C.

Benzyl aminopurine (BAP)

A 1 mg/l stock solution of Benzyl aminopurine (BAP) was prepared by weighing 0.05 g of BAP (Duchefa company) which was dissolved in 1 ml of 1N sodium hydroxide (NaOH) and topped up to 50 ml with distilled water. The stock solution was aliquoted into sterile 1.5 ml eppendorf tubes and stored at 4 °C.

Gibberellic acid (GA_3)

A stock solution of 1 mg/l GA_3 was prepared by weighing 0.05 g of GA_3 (Duchefa company) dissolved in 2 ml of 70 % ethanol, made up to 50 ml with distilled water and filter sterilized using a syringe and a 0.2 µm ministart filter (Sartorius company) into a sterile falcon tube before aliquoting into 1.5 ml sterile eppendorf tubes and stored at 4 °C.

Appendix 2: Praparation of antibiotics

Kanamycin

A stock solution of kanamycin (50 mg/l) was prepared by weighing 0.5 g of kanamycin (Duchefa Company) that was dissolved in sterile distilled water to make 10 ml in falcon tube. The solution was filter sterilized using a syringe and 0.2 µm filter into a sterile falcon tube before aliquoting into sterile falcon tube before aliquoting into sterile 1.5ml eppendorf tubes and stored at 4 °C.

Spectinomycin

A stock of spectinomycin (50 mg/l) was prepared by weighing 0.5 g of spectinomycin (Duchefa company) that was dissolved in 1ml of dimethylsulfonate (DMSO) and made up to 10 ml with sterile water in a falcon tube. The solution was then filter sterilized using a syringe and 0.2 µm filter into a sterile falcon tube before aliquoting into sterile 1.5 ml eppendorf tubes and stored at 4 °C.

Bialaphos (1 mg/l)

to prepare 1mg/l bialaphos, 100 mg of bialaphos was dissolved in 100 ml ddH_2O. The stock solution was filtersterilized, aliquoted into sterile 1.5 ml eppendorf tubes and stored at 4 °C.

Carbenicillin

A stock solution of carbenicillin (250 mg/l) was prepared by weighing 2.5 g of Carbenicillin (Duchefa Company) and dissolving it in distilled water to a final volume of 10 ml in a sterile 50 ml falcon tube. The solution was then filter sterilized using a syringe and 0.2 µm filter into a sterile falcon tube before aliquoting into sterile 1.5 ml eppendorf tubes and stored 4 °C.

Acetosyringone (100 mM)

100 mM acetosyringone was prepared by dissolving 196 mg of 3’5’dimethoxy-4’-hydroxy-acetophenone in 1ml of 70 % EtOH. The volume was topped upto 10ml with dd H_2O. The solution was then filter sterilized using a syringe and 0.2 µm filter into a sterile falcon tube before aliquoting into sterile 1.5 ml eppendorf tubes and stored 4 °C.

Appendix 3: Media preparation and culture conditions

Micropropagation media

Micropropagation media was prepared containing MS salts with vitamins (Duchefa) Murashige and Skoog, 1962, with 3 % (w/v) sucrose and the pH adjusted to 5.8 before adding 0.3 % (w/v) Gelrite. The media was autoclaved at 121 °C for 20 minutes, cooled and about 50 ml poured in sterile culture bottles under aseptic conditions.

Callus induction media

Callus initiation media was prepared containing MS salts, Gamborg B5 vitamins, 100 mg/l myo-inositol, 0.2 % sucrose, 0.5 mg/l $CuSO_4$, 50 mg/l casein hydrolysate supplemented with eitherb2, 4-dichlorophenoxyacetic acid or picloram and the pH adjusted to 5.8 before adding 0.3 % (w/v) Gelrite. The media was autoclaved at 121 °C for 20 minutes, cooled and about 25 ml poured in sterile 90×10 mm sterile Petri plates under asceptic conditions.

Maturation media

Maturation media was prepared containing MS salts, Gamborg B5 vitamins 0.2 % sucrose, various formulations of NAA (α Naphthaleneacetic acid), BAP (6-Benzylaminopurine) and the pH adjusted to 5.8 before adding 0.3 % (w/v) Gelrite. The media was autoclaved at 121 °C for 20 minutes, cooled and Gibberellic Acid (GA_3) was added. About 25 ml was poured in sterile test tubes under aseptic conditions.

Activated charcoal media

Activated charcoal media (0.8 %) was prepared containing 2 % sucrose, MS salts and B5 vitamins and the pH adjusted to 5.8 before adding 0.8 % (w/v) Difco-Bacto agar. The media was autoclaved at 121 °C for 20 minutes, cooled and about 50 ml was poured in sterile culture bottles under aseptic conditions.

Yeast Extract Peptone (YEP)

A litre of solid Yeast Extract Peptone (YEP) medium was prepared containing 10 g yeast extract, 10 g bacto peptone, 5 g NaCl and 15 g of bacto agar. The pH was adjusted to 7.0 and the media was autoclaved at 121 °C for 20 minutes, cooled then about 25 ml was poured in sterile petriplates under aseptic conditions. Bacto agar was not added into liquid media.

Appendix 4: Preparation of *GUS* assay chemicals

Tris.Hydrochloride (Tris.HCl), NaCl

A stock solution of 10 mM Tris.HCl (pH7.2), 50 mM NaCl was prepared by adding 1.21 g Tris and 2.92 g NaCl to 750 ml of sterile distilled water. The pH was then adjusted to 7.2, the volume made up to 1000 ml and stored at room temperature.

Triton X 100 (10 %)

To prepare a solution of 10 % Triton X-100, 5 ml of Triton X-100 was added to 45 ml of sterile distilled water and gently mixed. The solution was stored at room temperature.

5-Bromo-4-chloro-3-indoly-D-glucoronic acid (*X*-Gluc)

A stock solution of 10mg/l 5-Bromo-4-chloro-3-indoly-D-glucoronic acid (X-Gluc, Mr = 521.8) was prepared by dissolving 10 mg of X-Gluc in Dimethyl Sulfoxide (DMSO). This was aliquoted into 1.5 ml tubes, the tubes wrapped using aluminium foil to protect them from light and stored at 4 °C.

Appendix 5: Effect of leaf explants on somatic embryogenesis in cassava

One-way ANOVA: Picloram 4 mg/l leaves somatic embryogenesis

```
Alpha                                  0.05
Error Degrees of Freedom               15
Error Mean Square                      0.00401
Critical Value of Studentized Range    4.36699
Minimum Significant Difference         0.1383

Tukey Grouping      Mean     N   Cultivar

        A        0.70000     3   TMS 60444
        A
     B  A        0.67407     3   08-274
     B  A
     B  A        0.66853     3   KME 1
     B  A
     B  A        0.66472     3   08-354
     B
     B           0.55792     3   08-080
```

One-way ANOVA: Picloram 6 mg/l leaves somatic embryogenesis

```
Alpha                                  0.05
Error Degrees of Freedom               15
Error Mean Square                      0.005211
Critical Value of Studentized Range    4.36699
Minimum Significant Difference         0.1576

Tukey Grouping      Mean     N   Cultivar

        A        0.78333     3   TMS 60444
        A
        A        0.75153     3   08-354
        A
     B A         0.65048     3   08-274
     B A
     B A         0.64583     3   KME 1
     B
     B           0.59286     3   08-080
```

One-way ANOVA: Picloram 8 mg/l leaves somatic embryogenesis

```
                    Alpha                                      0.05
                    Error Degrees of Freedom                  15
                    Error Mean Square                          0.003121
                    Critical Value of Studentized Range  4.36699
                    Minimum Significant Difference       0.122

Tukey Grouping         Mean      N    Cultivar

                      A      0.80833      3    TMS 60444
                      A
                      A      0.76506      3    08-080
                      A
                      A      0.72361      3    08-354
                      A
                      A      0.72083      3    08-274
                      A
                      A      0.71273      3    KME 1
```

One-way ANOVA: Picloram 10 mg/l leaves somatic embryogenesis

```
                    Alpha                                       0.05
                    Error Degrees of Freedom                    15
                    Error Mean Square                          0.002799
                    Critical Value of Studentized Range    4.36699
                    Minimum Significant Difference        0.1155

Tukey Grouping         Mean      N    Cultivar

                      A      0.84117      3    08-274
                      A
                      A      0.83014      3    08-080
                      A
                      A      0.81498      3    08-354
                      A
                      A      0.80417      3    TMS 60444
                      A
                      A      0.78823      3    KME 1
```

One-way ANOVA: 2,4-D (4 mg/l) leaves somatic embryogenesis

```
Alpha                                0.05
Error Degrees of Freedom             15
Error Mean Square                    0.009614
Critical Value of Studentized Range  4.36699
Minimum Significant Difference       0.2141

Tukey Grouping       Mean      N    Cultivar

         A      0.90860      3    08-354
         A
         A      0.74167      3    TMS 60444
         A
         A      0.71237      3    08-080

         B      0.39289      3    08-274
         B
         B      0.23584      3    KME 1
```

One-way ANOVA: 2,4-D (6 mg/l) leaves somatic embryogenesis

```
Alpha                                0.05
Error Degrees of Freedom             15
Error Mean Square                    0.00971
Critical Value of Studentized Range  4.36699
Minimum Significant Difference       0.2152

Tukey Grouping       Mean      N    Cultivar

         A      0.88001      4    08-354
         A
         A      0.79583      4    TMS 60444
         A
       B A      0.70700      4    08-080
       B
       B C      0.57285      4    08-274
         C
         C      0.46736      4    KME 1
```

One-way ANOVA: 2,4-D (8 mg/l) leaves somatic embryogenesis

```
        Alpha                                   0.05
        Error Degrees of Freedom               15
        Error Mean Square                       0.005728
        Critical Value of Studentized Range     4.36699
        Minimum Significant Difference          0.1652

Tukey Grouping       Mean     N   Cultivar

          A       0.90035     3   08-354
          A
          A       0.88732     3   08-274
          A
       B  A       0.83750     3   TMS 60444
       B  A
       B  A       0.76814     3   08-080
       B
       B          0.70599     3   KME 1
```

One-way ANOVA: 2,4-D (10 mg/l) leaves Somatic embryogenesis

```
        Alpha                                   0.05
        Error Degrees of Freedom                15
        Error Mean Square                       0.004629
        Critical Value of Studentized Range     4.36699
        Minimum Significant Difference          0.1486
    Tukey Grouping       Mean     N   Cultivar

          A       0.93542     3   KME 1
          A
          A       0.90851     3   08-354
          A
          A       0.86958     3   08-274
          A
          A       0.86154     3   08-080
          A
          A       0.85417     3   TMS 60444
```

Appendix 6: Effect of stem explants on somatic embryogenesis

One-way ANOVA: Picloram (4 mg/l) stem somatic embryogenesis

```
Alpha                               0.05
Error Degrees of Freedom            15
Error Mean Square                   0.003367
Critical Value of Studentized Range 4.36699
Minimum Significant Difference      0.1267

Tukey Grouping      Mean     N   Cultivar

          A       0.51039    3   08-354

          B       0.38333    3   TMS 60444
          B
          B       0.33807    3   08-274
          B
          B       0.31560    3   KME 1
          B
          B       0.28692    3   08-080
```

One-way ANOVA: Picloram (6 mg/l) stem somatic embryogenesis

```
Alpha                               0.05
Error Degrees of Freedom            15
Error Mean Square                   0.001828
Critical Value of Studentized Range 4.36699
Minimum Significant Difference      0.0934


Tukey Grouping      Mean     N   Cultivar

          A       0.46431    3   08-354
          A
          A       0.42500    3   TMS 60444

          B       0.32266    3   KME 1
          B
          B       0.30417    3   08-080
          B
          B       0.30047    3   08-274
```

One-way ANOVA: Picloram (8 mg/l) stem somatic embryogenesis

```
Alpha                                 0.05
Error Degrees of Freedom              15
Error Mean Square                     0.002683
Critical Value of Studentized Range   4.36699
Minimum Significant Difference        0.1131
```

```
Tukey Grouping        Mean     N    Cultivar

      A         0.43333    3    TMS 60444
      A
      A         0.41231    3    08-354
      A
   B  A         0.39790    3    08-274
   B  A
   B  A         0.32262    3    KME 1
   B
   B            0.29722    3    08-080
```

One-way ANOVA: Picloram (10 mg/l) stem somatic embryogenesis

```
Alpha                                 0.05
Error Degrees of Freedom              15
Error Mean Square                     0.003223
Critical Value of Studentized Range   4.36699
Minimum Significant Difference        0.124
```

```
Tukey Grouping        Mean     N    Cultivar

    A           0.48529    3    08-354
    A
 B  A           0.44167    3    TMS 60444
 B  A
 B  A  C        0.37540    3    KME 1
 B     C
 B     C        0.34416    3    08-274
       C
       C        0.29432    3    08-080
```

One-way ANOVA: 2,4-D (4 mg/l) stem somatic embryogenesis

```
               Alpha                                0.05
               Error Degrees of Freedom             15
               Error Mean Square                    0.005007
               Critical Value of Studentized Range  4.36699
               Minimum Significant Difference        0.1545

Tukey Grouping       Mean      N    Cultivar

           A       0.50265     3    08-354
           A
         B A       0.38333     3    TMS 60444
         B
         B C       0.31734     3    08-080
           C
           C       0.22222     3    08-274

           D       0.06253     3    KME 1
```

One-way ANOVA: 2,4-D (6 mg/l) stem somatic embryogenesis

```
               Alpha                                0.05
               Error Degrees of Freedom             15
               Error Mean Square                    0.002468
               Critical Value of Studentized Range  4.36699
               Minimum Significant Difference        0.1085

Tukey Grouping       Mean      N    Cultivar

           A       0.39459     3    08-354
           A
           A       0.39167     3    TMS 60444
           A
         B A       0.34762     3    08-080
         B A
         B A       0.32054     3    08-274
         B
         B         0.28056     3    KME 1
```

One-way ANOVA: 2,4-D (8 mg/l) stem somatic embryogenesis

```
Alpha                                   0.05
Error Degrees of Freedom                15
Error Mean Square                       0.004641
Critical Value of Studentized Range     4.36699
Minimum Significant Difference          0.1487

Tukey Grouping        Mean      N    cultivar

      A             0.52235     3    08-354
      A
    B A             0.50000     3    TMS 60444
    B A
    B A     C       0.46875     3    08-274
    B       C
    B       C       0.36333     3    08-080
            C
            C       0.32734     3    KME 1
```

One-way ANOVA: 2,4-D (10 mg/l) stem somatic embryogenesis

```
Alpha                                   0.05
Error Degrees of Freedom                15
Error Mean Square                       0.006789
Critical Value of Studentized Range     4.36699
Minimum Significant Difference          0.1799

Tukey Grouping        Mean      N    Cultivar

          A         0.54516     3    08-354
          A
          A         0.48333     3    08-080
          A
          A         0.46667     3    TMS 60444
          A
          A         0.46146     3    KME 1
          A
          A         0.37391     3    08-274
```

Appendix 7: Effect of phytohomone combinations on maturation of cassava calli

One way ANOVA test for 08/354 percentage maturation

Alpha	0.05
Error Degrees of Freedom	10
Error Mean Square	405.8756
Critical Value of Studentized Range	4.65429
Minimum Significant Difference	54.136

Tukey Grouping	Mean	N	Conc
A	77.94	3	M2
A			
A	76.15	3	M
A			
A	73.60	3	M4
A			
A	73.11	3	M1
A			
A	70.83	3	M3

One way ANOVA test for 08/274 percentage maturation

Alpha	**0.05**
Error Degrees of Freedom	**10**
Error Mean Square	**171.4819**
Critical Value of Studentized Range	**4.65429**
Minimum Significant Difference	**35.189**

Tukey Grouping	Mean	N	Conc
A	**68.67**	**3**	M3
A			
A	**58.02**	**3**	M
A			
A	**57.73**	**3**	M4
A			
A	**55.18**	**3**	M1
A			
A	**52.61**	**3**	M2

One way ANOVA test for KME1 percentage maturation

Alpha	0.05
Error Degrees of Freedom	10
Error Mean Square	155.501
Critical Value of Studentized Range	4.65429
Minimum Significant Difference	33.509

```
Tukey Grouping         Mean      N    Conc

          A           63.91      3    M3
          A
          A           63.65      3    M4
          A
          A           61.31      3    M
          A
          A           61.11      3    M2
          A
          A           60.00      3    M1
```

One way ANOVA test for 08/080 percentage maturation

Alpha	**0.05**
Error Degrees of Freedom	**10**
Error Mean Square	**94.47179**
Critical Value of Studentized Range	**4.65429**
Minimum Significant Difference	**26.118**

```
Tukey Grouping         Mean      N    Conc

          A          76.752      3    M
          A
          A          72.222      3    M1
          A
          A          67.719      3    M2
          A
          A          63.970      3    M4
          A
          A          59.025      3    M3
```

One way ANOVA test for TMS 60444 percentage maturation

Alpha	0.05
Error Degrees of Freedom	10
Error Mean Square	67.8552
Critical Value of Studentized Range	4.65429
Minimum Significant Difference	22.135

Tukey Grouping	Mean	N	Conc
A	69.247	3	M4
A			
A	68.190	3	M1
A			
A	67.746	3	M3
A			
A	67.712	3	M
A			
A	63.965	3	M2

Means and descriptive statistics

	Mean of 08/354 maturation	Std. Dev. of 08/354 maturation	Mean of 08/274 maturation	Std. Dev. of 08/274 maturation	Mean of KME1 maturation
M	76.1481	2.61980	58.0159	1.2694	61.3050
M1	73.1061	2.15791	55.1796	6.9485	60.0000
M2	77.9412	3.89908	52.6073	5.7620	61.1111
M3	70.8333	2.2169	68.6695	1.7853	63.9104
M4	73.6007	1.5273	57.7302	8.7955	63.6508

	Std. Dev. of KME1 maturation	Mean of 08/080 maturation	Std. Dev. of 08/080 maturation	Mean of TMS6044 maturation	Std. Dev. of TMS6044 maturation
M	7.1078	76.7521	6.6683	67.7124	8.2849
M1	4.0370	72.2222	4.8113	68.1905	2.6703
M2	9.6225	67.7193	5.1687	63.9646	9.8149
M3	6.7231	59.0253	8.6201	67.7460	0.9774
M4	3.3784	63.9696	5.5963	69.2473	3.5792

Appendix 8: Cassava transformation frequency

One-way ANOVA: Percentage of *gus* positive calli

```
Alpha                               0.05
Error Degrees of Freedom            10
Error Mean Square                   56.66667
Critical Value of Studentized Range 4.65429
Minimum Significant Difference      20.22
```

```
Tukey Grouping        Mean     N   Cultivar

          A         73.333     3   TMS 60444
          A
          A         68.333     3   08/274
          A
          A         56.667     3   08/354
          A
          A         55.000     3   KME 1
          A
          A         53.333     3   08/080
```

Cultivar	Mean of *gus* positive	Std. dev. of *gus* positive
08/080	53.3333	7.6376
08/274	68.3333	7.6376
08/354	56.6667	2.8868
KME 1	55.0000	10.0000
TMS60444	73.3333	7.6376