REGENERATION AND RNAi-MEDIATED DOWNREGULATION OF CYANO-GLYCOSIDE BIOSYNTHESIS IN CASSAVA (*Manihot esculenta,* Crantz)

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DECLARATION

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

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DEDICATION

This thesis is dedicated to;

Joan Murugi Njagi-Ngugi (My Lovely Wife)

She is several deviations beyond the mean and, therefore, an indispensable partner in this sojourn!

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ABBREVIATIONS AND ACRONYMS

2, 4-D	2, 4-Dichlorophenoxy acetic acid
ANOVA	Analysis of Variance
BA	Benzyladenine
BAP	Benzylaminopurine
β-CAS	β-cyanoalanine synthase
cDNA	Complementary DNA
CGIAR	Consultative Group on International Agricultural Research
CIAT	Centro Internacional de Agricultura Tropica
CIM	Callus induction media
CN	Cyanide
COSCA	Collaborative Study of Cassava in Africa
CYPD1/D2	Cytochrome P_{450} genes
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside Triphosphate
FEC	Friable embryogenic calli
FHL	Formamide hydrolyase
G3PDH	Glyceraldehyde 3-phosphate dehydrogenase
GM	Germination medium
GUS	β-Glucuronidase
GA_3	Gibberelic Acid
HCN	Hydrogen cyanide/Hydrocyanic acid
HNL	Hydroxynitrile lyase
IBA	Indole butyric acid
IFAD	International Fund for Agricultural Development
IITA	International Institute on Tropical Agriculture
KARI	Kenya Agricultural Research Institute
LAC	Latic America and Caribbean
MS	Murashige and Skoog
PCR	Polymerase chain reaction
Picloram	4-Amino-3,5,6-trichloro-2-pyridinecarboxylic acid
RE	Regeneration efficiency
RF	Regeneration frequency
RNA	Ribonucleic acid
RNAi	RNA interference
RT-PCR	Reverse transcriptase-PCR
SCN	Thiocyanate
TAN	Tropical Ataxic Neuropathy
T-DNA	Transfer DNA
TF	Transformation frequency
UDPG	Uridine 5'-diphosphoglucose
WHO	World Health Organization

ABSTRACT

Cassava (*Manihot esculenta,* Crantz) is an important perennial tropical crop for millions of people around the world particularly in Sub-Saharan Africa. It is preferred due to its agronomical attributes such as ability to grow in poor soils and drought resistance. Cassava storage roots are also a good source of starch. In addition, cassava leaves and tender shoots are eaten as vegetables in many parts of Africa, Kenya included and are an excellent source of vitamins, minerals and protein. Although most cassava is used for food, it is also used in the production of ethanol for fuel, for animal feed, and as a raw material for the starch industry. Cassava has high photosynthetic rates and its roots can persist in the soil for 8-24 months without decaying, thereby making it an ideal food security crop. In Kenya cassava is a major source of subsistence and cash income to farmers in agro-climatically-disadvantaged regions and high potential areas of Coast, Central and Western regions of Kenya. Sadly, cassava leaves and roots contain potentially toxic levels of cyanogenic glycosides, a demerit that generated interest in this study. Cassava is largely propagated clonally making it an ideal plant for improvement through genetic engineering. The objective of this study was to develop and optimize regeneration protocol for Kenyan cassava varieties and produce transgenic acyanogenic cassava plants in which the expression of the cytochrome P_{450} genes (*CYP79D1/D2*) is knocked down through RNAi. Three Kenyan cassava genotypes; *Adhiambo lera, Kibanda meno* and *Serere* along with an exotic model cultivar *TMS 60444* were used. As a prerequisite for transformation, a reproducible *in vitro* regeneration protocol was optimized for Kenyan cassava lines using immature leaf lobes as explants. The transformable lines were then taken through *Agrobacterium*-mediated transformation with an RNAi cassette harbouring cytochrome P_{450} genes (*CYP92D1*) to down regulate production of cyanoglycosides. Molecular analysis by PCR and RT-PCR confirmed transformation of the putative transformants. Analysis of cyanide content of the transgenic Kenyan cassava lines corroborated with the molecular analysis data that transformation had indeed occurred. From this study, an optimized and reproducible transformation protocol for Kenyan cassava varieties has been developed. In addition, transgenic cassava lines with cyanide content three folds less than the cyanide content of the wild type relatives were produced. The results of this study disapproves the view that African cassava genotypes are recalcitrant to *in vitro* manipulations. Production of transgenic lines with greatly reduced cyanide contents will further add value to cassava utilization. This, therefore, is an impetus for further genetic manipulations on Kenyan cassava cultivars to mitigate the various genetic demerits associated with cassava in order to ultimately maximise on the numerous benefits of cassava.

CHAPTER ONE

GENERAL INTRODUCTION

1.1 Background

Cassava (*Manihot esculenta,* Crantz, Euphorbiaceae, 2n=36) is one of the most important food crops in the world, especially in the tropics (Zhang, 2000). Its roots contain up to 85% of their dry weight as starch. It is a woody perennial shrub that serves as a basic staple food for over 500 million people worldwide, most of whom live in developing regions of the world (Cock , 1982). Cassava is native to tropical South America and its evolutionary center of origin is thought to be along the southern border of the Amazon River basin in Brazil (Olsen and Schaal, 1999). Casava was initially introduced into Africa in the 16th century by the Portuguese and then to Asia by Spanish traders during the 17th century (Jones, 1959; Leone, 1977). At present, cassava is grown in over 60 countries in Africa, Asia and Latin America (Siritunga, 2002).

Cassava is the fourth most important crop in the developing countries surpassed only by maize, rice and sugarcane as a source of calories (Bradbury, 1988). In sub-Saharan Africa cassava provides up to 60% of the daily calorie uptake (Cock, 1985; CIAT, 1994) and more than 80% of the harvest is used as food. In certain regions the leaves, which contain appreciable quantities of protein and vitamins, are used as a major component of the diet to provide supplementary protein, vitamins and minerals to complement the carbohydrate rich staple (Lancaster and

Brooks, 1983). Cassava is a vital food security crop that helps the fight against hunger and poverty in developing countries and especially in Africa, where the food security problems are the most severe in the world (FAO, 2000).

In Africa, cassava is recognized as a famine reserve crop due to its drought tolerance, ability to grow on infertile soils and its ability to recover from disease and pest attacks (Mburu *et al.,* 2012). In Kenya the crop is grown for food, livestock feeds and industrial use making it an important source of subsistence and income to farmers in agro-climatically-disadvantaged regions and high potential areas of Coast, Central and Western regions of Kenya (Githunguri *et al.,* 2007). In 2007, FAO estimated that the land area in Kenya under cassava cultivation is approximately 77,502 ha with an annual output of 841,196 tons grown for both food and income (FAO, 2007). There are over 5000 known phenotypically distinct cassava cultivars (Best and Hangrove , 1993).

By introducing high yielding varieties of cassava, Ghana managed to reduce malnourishment more rapidly than any other country in the world between 1980 and 1996 (FAO, 2000). In many countries, cassava chips and flour are important animal feeds. Cassava is propagated vegetatively from stem cuttings and planting usually starts at the beginning of the rainy season. A unique advantage of cassava over other crops is its flexible harvesting time that makes it an excellent famine foodstuff. The starchy roots of cassava produce more calories per unit of land than

any other crop except sugarcane (Henry *et al.,* 1995; Scott *et al.,* 2000). Therefore cassava is one of the most reliable and cheapest sources of food. Cassava is also known as a 'food-bank' because it is amenable to partial harvest where the subsistence farmer can harvest only the required amount of roots and restore to rest back in the soil for later harvest.

With all the above merits however, cassava has its shortcomings as a food crop. The tuberous roots though a good source of dietary carbohydrate, have extremely low protein content (1-2%) and have limited amounts of sulphur-containing amino acids. This means together with cassava, additional food sources are required to ensure a diet balanced in protein, vitamins and minerals (Omole, 1977; Cock, 1985). During the long cultivation period (up to 18 months) of cassava, repeated attacks by various insect pests and viral diseases can cause 20-50% yield losses worldwide, and locally they can lead to total crop failures (Thresh *et al.,* 1994; Belloti *et al.,* 1999). In addition, cassava suffers from postharvest physiological deterioration during transport, storage and marketing (Wenham, 1995). Although the roots can remain in the ground for many months, once they are harvested they deteriorate rapidly and within 48 hours they are unmarketable.

Besides, all parts of the plant contain toxic levels of cyanogenic glycosides, which have to be removed by laborious processing before cassava can be safely consumed (Akintowa *et al.,* 1993). Apparently, the presence of cyanogenic

glycosides protects the plant from herbivores and residual cyanogens may cause health problems for human consumers (Siritunga, 2002). Biotechnology holds the key to overcoming these limitations and producing cassava plants that are more nutritious, resistant to pests and viruses, more profitable and safer (Siritunga, 2002).

1.2 Problem statement and justification

Attempts to address the shortcomings of cassava through conventional breeding have not been successful due to a lack of flowering, its consequent fruiting problems and heterosis, as well as the long duration for generating new clones. Unfortunately, several research laboratories in South America have been undertaking cassava regeneration and transformation systems with large focus on South American varieties (Ihemere, 2003). Less effort has been placed on developing regeneration and transformation systems for African cassava varieties (Taylor and Henshaw, 1993; Konan *et al.,* 1994a) despite the fact that cassava is one of the major sources of energy for the sub-Saharan African peoples.

Environmental and biological aspects in which African cassava varieties thrive are different from those in South America. Besides, human demands for African cassava varieties are different from those for South American varieties. It is, therefore, expected that African cassava varieties have genetically diverged from their South American progenitors (Kawano, 2003).

To achieve discernible success in exploiting the desirable traits of African cassava varieties as well as to increase the genetic diversity of transformable cultivars and produce disease free varieties, it is imperative to identify and develop strategies for regenerating and transforming African cassava varieties. This is important in cognizance that the traditional breeding methods have experienced difficulties in developing cassava varieties with improved traits.

Irrespective of the good qualities harboured by cassava, it contains cyanogenic glycosides which when not effectively removed may cause several health problems in consumers. Genetic engineering holds the key to overcoming these limitations and producing cassava plants that are more nutritious, resistant to pests and diseases, more profitable and safer.

1.3 Hypotheses

This study is based on two null hypotheses. These are;

- **i.** It is not possible to regenerate cassava *in vitro* through somatic embryogenesis.
- **ii.** It is not possible to genetically transform cassava *in vitro* using immature leaf lobes as explants.

1.4 Objectives

1.4.1 Broad Objective

The overall goal of this study was to develop and optimize regeneration of Kenyan cassava varieties and produce transgenic acyanogenic cassava plants in which the expression of the cytochrome P_{450} genes (*CYP79D1*) is knocked down through RNAi.

1.4.2 Specific Objectives

i. To optimise *in vitro* regeneration protocol for Kenyan cassava genotypes.

ii. To genetically transform the Kenyan cassava genotypes with RNAi transformation cassette harbouring *CYP79D1* gene.

iii. To determine the molecular profile of the transformed cassava by use of PCR, RT-PCR and southern blotting.

iv. To compare the amounts of cyanogenic glycosides in wild type and transgenic cassava lines using biochemical assays.

CHAPTER TWO

LITERATURE REVIEW

2.1 Cassava (*Manihot esculenta,* Crantz)

2.1.1 The biology of cassava

Cassava (Cerqueira, YM) is a member of dicotyledonous family Euphorbiaceae (Alves, 2002). Reportedly, the genus *Manihot* has about 100 species of which *M.esculenta* is the only agronomically important species. Cassava is a shrub that is 1-4 meters tall in height with sympodial branching. The main stem is typically divided into two – four branches that may have branches of their own. Branching is followed shortly by flowering. This developmental series has been termed reproductive flowering. However, in some cases, the flower buds are aborted before maturity, giving the impression that there might be cassava stem branching without the accompaniment of flowering (Alves, 2002).

Cassava leaves are lobed with palmate veins. The number of leaf lobes range from five to seven (Siritunga, 2002). Leaves produced at the time of flowering are reduced in size and lobe number. The leaves closest to the inflorescence are commonly simple and have no lobes. The leaves have an alternate arrangement and a phyllotaxy of 2/5 (Ihemere, 2003). The mature leaves are glaborous and leaves are surrounded by two stipules. The adaxial leaf surface is characterized by a waxy epidermis that gives it a shiny appearance (Ihemere, 2003). The stomata are located mainly on the lower leaf surface (Cerqueira, 1989). Only 2% of the

1500 cultivars studied had stomata on their adaxial surface (El-Sharkawy and Cock, 1990). Stomata are functional on both sides of the leaf, but those on the upper surface of the leaf are larger (Cerqueira, 1989).

Cassava is photosynthetically a C3 plant (Ueno and Agarie, 1997). Its maximum photosynthetic rates range from 13 to 24 μmol CO_2 m^{-2} s^{-2} under greenhouse or growth chamber conditions and from 20 to 35 μmol CO_2 m^{-2} s^{-2} in the field (El-Sharkawy and Cock, 1990). Cassava has a high CO_2 compensation point. The optimal temperature for photosynthesis for field grown cassava is 35^{o}C but the range for optimal photosynthesis is 25 to 45^{o}C (El-Sharkawy and Cock, 1990). Therefore, cassava is adapted to the tropical environment.

Cassava is propagated by stem cuttings. Plants propagated by stem cuttings lack the typical dicotyledonous taproot system. Instead adventitious roots arise from the base of the cut surface or from subterranean buds. Some of the adventitious roots transform to storage roots while others remain fibrous and serve to supply water and nutrients to the plant (Ihemere, 2003). Anatomically, cassava storage root is not a tuber or stem due to the organization of the vascular tissue (Wheatley and Chuzel, 1993) The fully developed cassava storage root comprises three sections: the periderm (bark), cortex (peel) and the parenchyma. The parenchyma consists mainly of starch and makes up about 85% of the total root mass. It is interspersed with xylary tissues (Wheatley and Chuzel, 1993). The cortex is made up of

sclerenchyma, cortical parenchyma and phloem and accounts for 11-20% of the root weight (Barrios and Bressani, 1967). The periderm comprises about 3% of the total root mass and is a thin layer that can be readily removed from the exterior of the root. The size and shape of roots is a function of the cultivar and environmental factors (Wheatley and Chuzel, 1993).

Cassava is a monoecious plant whose inflorescence is located in the juncture between branches. The staminate flower is located on the lower part of the branches opposed to the pistillate flower on the upper part of the branch. The pistillate flowers are more numerous than the staminate flowers (Ihemere, 2003). The female flowers open 1-2 weeks before the male flowers on the same inflorescence. The plants are therefore prone to cross-pollination which might explain the high level of heterozygozity in cassava (Alves, 2002). The flowers lack corolla or calyx but have a perianth that has five tepals varying in color (yellow, red, purple) depending on cultivar. The male flower is about half the size of the female. The male flower has ten stamens (Alves, 2002).

Very little is known about flowering of cassava plants (Alves, 2002). Flowering can start six weeks after planting but the time it takes to flower is a function of cultivar and environment. Moderate temperatures (approximately 24^{o}C) are known to be conducive for flower initiation. Photoperiod also is known to affect flowering. Flower initiation occurs best at photoperiods >13.5 hours (Keating *et*

al., 1982). Cassava pollen is yellow or orange with the pollen grains ranging in size from 122 – 148 μm (Ghosh *et al.,* 1988). The female flower has a ten-lobed basal disk. The ovary is tricarpellary with six ridges. The three locules each contain one ovule which matures into carunculate seeds. The seed coat is smooth and dark brown mottled with grey (Ihemere, 2003).

All cassava organs apart from seeds contain cyanogenic glycosides. Cultivars with less than 100 mg CN equivalents kg^{-1} fresh weight in their roots are considered 'sweet' while those with 100 – 500 mg CN equivalents kg^{-1} are considered 'bitter' (Wheatley *et al.,* 1993). The most abundant cyanogenic glycoside is linamarin (95%) with lesser amounts of lotaustralin. The total amount of cyanogenic glycoside depends on cultivar, cultural practice, environmental conditions and plant age (McMahon *et al.,* 1995).

2.1.2 Taxonomy and etymology of cassava

The origin of cassava has been obscure and has been disputed for many years. However, there is botanical, genetic and archeological evidence supporting origin of cassava from South American, particularly pointing to the Amazon region as the center of cassava domestication (Olsen and Schaal, 1999; Hillocks, 2002). Attempts have been made to determine the geographical and evolutionary origin of cassava by taking advantage of the high DNA sequence variation in the non-coding regions (introns) of the glyceraldehyde 3-phosphate dehydrogenase

(G3PDH) gene. Using this technique, Olsen and Schaal, (1999) demonstrated that cassava was domesticated from wild *M. esculenta* populations along the southern border of the Amazon basin.

In the 16th century, Portuguese navigators took cassava with them from Brazil to West Africa (Jones, 1959) then later to East Africa (Jennings, 1976). Although cassava was grown in Fernando Po in the Gulf of Benin and around the Congo River in the 16th century, cassava's dispersal into West Africa did not take place until the 20 th century (Hillocks, 2002). Cassava was initially grown mainly in the coastal areas of Africa. The inland spread of cassava cultivation was by African traders who were drawn to cassava for its fabled characteristic of providing security against famine. Cassava is now grown in most African countries especially south of the Sahara desert (Ihemere , 2003). Realizing the importance of cassava, the International Institute of Tropical Agriculture (IITA) was established with its headquarters in Ibadan, Nigeria in 1972 under the guidance of the Consultative Group on International Agricultural Research (CGIAR) to oversee the development of the crop in Africa. Together with Centro Internacional de Agricultura Tropica (CIAT) in Colombia, IITA has the global mandate for cassava improvement (Hillocks, 2002).

The *Manihot esculenta* ssp *esculenta,* Crantz sub-species does not exist as a wild plant and it has been proposed to be the product of human domestication, being

closely related to the wild sub-species *M. esculenta* ssp. *flabellifolia* and *M. esculenta* ssp. *peruviana* within the Euphorbiaceae family (Allem, 2002). Differences between cassava and its wild relatives rely mainly on: the ability of the crop to develop secondary roots from stem cuttings at a faster rate, the formation of tuberous roots, being less toxic for food and animal feeding, and having shorter cultivation periods (Allem, 2002).

2.1.3 Beneficial aspects of cassava

Cassava is characterized by its ability to develop secondary roots of high starch storing capacity. These tuberous roots develop during the first months of the plant as fibrous roots, which initially grow longitudinally, providing root length and anchorage. The roots start radial growth and thickening later due to starch accumulation, after which they develop into tuberous roots (Ceballos and De la Cruz, 2002; Degró, 2009). Root varies in length between cultivars, reaching up to 2.5 m in length, depending on consumer and farmer preferences. The economical value of cassava roots lies in its ability to accumulate carbohydrates. Cassava roots comprise 90% of their dry weight as starch, stored mainly as granules in the amyloplasts of root parenchyma (Buitrago, 1990).

Cassava also shows the capability of growing in marginal conditions, such as drought and poor and acidic soils (Degró, 2009). Its high drought tolerance is due to stomata closing, which leads to a low stomatal conductance that impedes water

loss under drought conditions (Mejía de Tafur, 2002). Several studies have reported that cassava root yield is only slightly affected at drought conditions (Mejía de Tafur, 2002). However, even though cassava is known for being a low input crop, previous studies show that irrigation practices increase yield up to 60% (López, 2002).

Cassava has developed natural barriers against herbivory. Physical barriers, such as the presence of foliar pilosity, insect induced calli (phloem cicatrisation) and the cell wall, sabotage establishment of aphids in developing leaf meristems (Calatayud *et al.,* 1996). Chemical barriers against arthropods include secondary metabolites, such as cyanogenic glycosides that provide resistance to root eating insects (Belloti *et al.,* 1999), and glycosilated flavonoids such as rutin, which together with the aforementioned foliar pilli, constitute the main constraining agents against cassava mealy bug (*Phenacoccus manihoti*) establishment (Calatayud and Múnera, 2002)

Furthermore, cassava can persist in the soil for 8-24 months without decaying thereby ensuring food security during drought periods. This makes it a good source of staple food to subsistence farmers (Nweke *et al.,* 2002).

2.1.4 Demerits of cassava

Cassava is not a perfect crop and has its share of demerits. Though a good source of carbohydrates the roots lack appreciable amounts of protein. Due to the low protein content (1-2%) additional food sources are required to ensure a diet balanced with proteins (Cock, 1985). Cassava has the lowest amount of protein content among major crops at 0.9 grams per 100 grams of edible portion. Due to the low protein content additional food sources are required to ensure a diet balanced with proteins (Cock, 1985). During the lengthy period in the soil attack by insect pests and virus diseases can lead to 20-50% yield losses worldwide resulting in complete crop failure in some instances (Thresh *et al.,* 1994; Belloti *et al.,* 1999).

Post harvest deterioration during transport, storage and marketing of cassava is another problem (Wenham, 1995). Even though roots can stay underground for extended periods of time, once harvested the roots deteriorate very rapidly and are unmarketable within 48 hours. To extend the shelf life of fresh roots cassava can be peeled, precooked and vacuum packed prior to reaching the market or in the case of export to the USA and Europe coated with paraffin (Siritunga, 2002).

Another problem with cassava is the presence of cyanogenic glycosides, linamarin and lotaustralin, in all parts of the plants with exception of seeds (Conn, 1994). Linamarin accounts for 95% of the cyanogenic glycosides present in intact cassava

tissue (Balagopalan *et al.,* 1985). These cyanogenic glycosides can be broken down to produce cyanide. It is thought that the presence of cyanogenic glycosides protects the plant from herbivores, however, residual cyanogens may cause health problems for human consumers (Siritunga, 2002).

Efforts to tackle these problems using conventional breeding are severely frustrated by the high heterozygosity of the allopolyploid plants with low natural fertility, long growing season as well as by the lack of disease tolerant genes in the sexually compatible germplasm. Although some new cassava varieties have already been introduced by CGIAR, traditional breeding is difficult, time consuming and laborious. Biotechnology has been identified as a powerful tool to overcome these limitations because it not only provides an alternative approach but also complements the efforts in traditional breeding (Puonti-Kaerlas *et al.,* 1999).

2.1.5 Global cassava production

Cassava is cultivated in tropical America, Asia and more extensively in certain African regions, extending from Madagascar in the Southeast to Senegal in the Northwest, forming what has been sometimes called the cassava belt (Bokanga, 1994). Ghana, Nigeria, the Democratic Republic of Congo and Tanzania are countries that contribute to main outputs in cassava harvesting (FAO/IFAD, 2000).

Annually, approximately 165 million tonnes of cassava roots are harvested worldwide, being forth among the main sources of calories in the tropics, after rice, wheat and maize (Ceballos, 2002). According to statistics published by the Food and Agriculture Organization (FAO) of the United Nations, cassava production increased by a rate of about 2.5% per year during the period from 1965 to 1995 (FAO/IFAD, 2000; Ceballos, 2002), probably mainly due to an increase in its extensive cultivation in Africa. These values compare to trends in other crops such as wheat (4.32%), potato, maize (3.94%), yams (3.9%), rice (2.85%) and sweet potato (1.07%). In addition, 60% of cassava cultivated lands worldwide belong to this continent, providing 50% of total cassava production (Ceballos, 2002). At 142 trillion kilocalories per year, cassava ranks first in edible energy among major root and tuber crops (Siritunga, 2002).

According to UNCTAD (2012), global cassava production was around 250 million tonnes (Mt) a year. After 15 years of uninterrupted growth and an increase of 13% between 2006 and 2009, it fell to 249 Mt in 2010 following a poor harvest in Thailand due to diseases and drought. Africa contributes to more than half of global supply, with Nigeria on top, representing more than a third of African production alone (around 45 Mt). It is also the largest world producer of cassava.

Although cassava is native to the Amazon region, Africa now produces more cassava than the rest of the world combined. Africa's largest producers of cassava

are Nigeria (37,504,100 tonnes), Democratic Republic of Congo (15,049,500 tonnes), Ghana (13,504,100 tonnes), Angola (12,866,500 tonnes) and Mozambique (5.7 million tonnes). In Kenya, cassava production is estimated at 323,389 tonnes tonnes (FAOSTAT, 2012). Kenya is globally ranked 31st in terms of annual cassava production quantity in tonnes, 34th in terms of harvesting area in hactares and 47th in terms of cassava yield in hectogram per hectare (FAOSTAT, 2011).

World cassava output is expected to increase vigorously in 2012 and may continue to do so in 2013, sustained by growth in Africa, where cassava remains a strategic crop for both food security and poverty alleviation. Prospects are more uncertain in Asia, where the sector expansion next year will largely depend the competiveness of cassava in the production of ethanol relative to other feedstocks. In 2012, world trade in cassava products is set to undergo a marked increase, entirely sustained by industrial demand (FAO, 2012).

World cassava output in 2012 is expected to reach 282 million tonnes, an increase of 7 percent from the level of 2011, and the fourteenth annual rise in succession. The expansion, which has been particularly prominent in recent years, is being driven by increasing industrial applications of cassava in East and Southeast Asia, especially for ethanol, and rising demand for food in the African continent.

Further, FAO (2012) indicated that world trade in cassava products, is set to undergo a marked expansion by 2025. This is the result of the price competitiveness that cassava has gained over maize thanks, by and large, to policies in Thailand, the world's leading international supplier of cassava products.

The outlook for 2013 points to a continued expansion of production in Africa, where cassava remains a strategic crop for both food security and poverty alleviation. In Asia, prospects remain far from certain, depending on how the price relation between maize and cassava evolves and on the competiveness of cassava in the production of ethanol relative to other feedstocks (UNCTAD, 2012).

These outcomes will be heavily influenced by Thailand's "price pledging scheme" and in particular, the degree of price discounting in sales from official stockpiles. The region's uncertainty has been compounded by the recent weakness of domestic root prices in major producing countries that do not administer domestic price supports. The extent of these price falls cast doubt on the degree of market incentive for producers to plant cassava for the new season (FAO, 2012).

2.1.6 Industrial use of cassava

Cassava is an important industrial commodity mainly due to its starch which is applied in the production of various items. Cassava starch is used in the production of adhesives like gums (Cock, 1985; Balagopalan, 2002). Cassava starch is used

for paste production because of its cohesiveness and clarity. Cassava starch is preferred for food packaging over other types of starch because of its bland taste (Cock, 1985; Balagopalan, 2002)

Cassava starch is also used in production of dextrins. An aqueous solution of dextrin is used for bonding similar and dissimilar surfaces. Dextrins are not as strong adhesives as starch films but are preferred to starch since they can be used at higher concentrations than starch and accelerate drying. Dextrins are used as envelope gums, bottle-labeling adhesives, postage stamps adhesives, in making cardboard boxes and photographic mounting materials (Cock, 1985; Trim *et al.,* 1996; Balagopalan, 2002).

Glucose and dextrose are liquefied into sugar-syrups, which can be used in making confection and in the pharmaceutical industry. Cassava starch is also used in making fructose syrup and fructose crystals which are used in the substitution of sucrose, glucose and synthetic sweeteners. Fructose is 1.7 times sweeter than sucrose and four times sweeter than glucose (Abraham, 1996). Cassava starch is also used in the manufacture of maltodextrin which substitutes for glucose as a sweetener. It is also used as a thickening agent (Balagopalan *et al.,* 1988). Cassava starch oxidized with hypochlorite or chlorine is useful in the paper industry because of its low viscosity, film strength and clarity in making glossy papers (Ihemere, 2003).

These starches are also used in the textile industry for sizing warps of cotton and spun rayons and for laundry finishes (Trim *et al.,* 1996). Cationic starches made by treating starch with amino, imino, ammonium, sulphonium or phosphonium groups are used in the paper industry to provide glaze and strength to paper (Cock, 1985; Abraham, 1996; Trim *et al.,* 1996). Cassava starch is used in blending synthetic polymers to give it biodegradable characteristics (Ren, 1996).

Cassava starch, due to its low swelling and gelatinization temperature, is easily saccharified to simple sugars. These simple sugars are used in the production of sugar alcohols such as sorbitol, mannitol and maltol. (Ren, 1996).

2.1.7 Cassava as food

Cassava serves as the main staple food for more than 500 million people in the tropical and subtropical regions of the world (Balagopalan, 2002). It also contributes significantly to the livelihood of these people. After removing the skin, cassava is eaten raw in Africa and other parts of the world. However, the cultivars that are eaten raw are the ‘sweet cultivars’ which have low cyanogenic glycoside content. The cultivars that have high cyanogenic glycoside content are processed and cooked before consumption (Balagopalan, 2002).

The increase in cassava production worldwide can be associated to an increase in its use for food where it serves as a food reserve when other basic crops fail. In

Africa, cassava is traditionally cultivated by small farmers, who mainly cultivate it in small farms and backyards. This practice provides the crop to benefit from residual fertilizer, but could probably account for low yields (FAO/IFAD, 2000).

Cassava is used as food in various ways across the world (Ihemere, 2003). Processed cassava roots are eaten while boiled, baked, and fried and as flour. In Western Africa, cassava roots are commonly eaten as *gari*, a cassava flour paste made by peeling and grating the roots until a mash is made, followed by fermentation for 1-2 days in bags to make it lose water and drying by pressing the bags. Further heating allows complete drying and roasting, leading to formation of the granular *gari*, which can be further ground to fine flour. (Ihemere, 2003). Still in West Africa, cassava roots are fermented in a pot for 4-7 days, boiled and pounded into *fufu* and eaten with vegetable soup supplemented with fish and meat (Lancaster *et al.,* 1982).

In Liberia, cassava is made into *dumby* which is prepared by placing boiled cassava roots in mortar followed by pounding. Then, the *dumby* is cut into pieces and put in soup supplemented with vegetables or meat, after which it is swallowed whole (Omole, 1977). Cassava is also made into *farina* in South America and West Indies (Omole, 1977). *Farina* is prepared by removing the skin of the cassava root and grating. The mash is then depulped, sieved and roasted in a slow fire. It could then be stored for several months and can be eaten as a cereal and in

combination with other foods (Omole, 1977). In Eastern Africa countries, cassava is mainly fried. Some African cultures also consume cassava leaves, which provide a good source of protein and vitamins (Bokanga, 1994).

Cassava macaroni is prepared by blending cassava flour, groundnut flour and wheat semolina in the ratio 60:12:15. It is enriched with 12% protein. The food is used to feed children because of its high protein content. Cassava is also used in preparing fast foods such as wafers, fried chips, cakes and doughnuts (Balagopalan, 2002).

Cassava is also sometimes used as animal feeds. Various parts of the plant such as leaves, stem and roots are used to feed animals. The high energy value of cassava makes it a good source of carbohydrate in animal diets (Omole, 1977). However, roots have low protein content. This short-coming is often overcome by supplementing it with soya which is rich in protein. Fresh roots are used in feeding farm animals such as cattle, goats and sheep in the developing countries (Ihemere, 2003). Sometimes, the roots are boiled before feeding animals. Unfortunately, feeding livestock with fresh roots might cause cyanide toxicity depending on the level of cyanogenic glycosides in the roots. Replacement of cereals with cassava up to 50 – 100 % did not affect the milk quantity and quality of dairy animals (Mathur *et al.,* 1969). In some cases higher milk yield has been reported up to 19.5% as a result of increased energy from cassava (Balagopalan, 2002).

Cassava leaves are used as forage in many developing countries especially in the dry season when other feeds are scarce. However, there is some opposition to the use of cassava leaves as forage, for fear of cyanogenic poisoning (Balagopalan, 2002).

2.1.8 Cyanogenic Glycosides in Cassava

2.1.8.1 Biosynthesis

Cyanogenic glycosides are usually D-glucose joined by a β-linkage to an acetone cyanohydrin derivative (Jørgensen *et al.,* 2005). They are synthesized by the plant to protect it from predation, as hydrogen cyanide, which is released from these compounds when they are digested, is poisonous to virtually all known eukaryote life forms (Singh *et al.,* 1986). Their synthesis (cyanogenesis) can also occur in plants due to cyanolipid hydrolysis and as a byproduct of ethylene synthesis (Poulton, 1990), but its release due to cyanogenic glycoside breakdown generally provide toxic quantities for human consumption in cassava (Degró, 2009).

Cassava is known to accumulate cyanogenic glycosides in all of its tissues except seeds. Linamarin is the most abundant cyanogenic glycoside in cassava, accounting for 95% of total cyanogens in the plant (Cock, 1985). Extensive research has been done in unraveling what are the biosynthesis pathways involved in linamarin synthesis, which is known to be synthesized from valine, in reactions that occur in plant cell vacuoles (Koch *et al.,* 1992; McMahon *et al.,* 1995).

Initially, two sequential hydroxylation reactions of valine occur, catalyzed by the enzymes *CYP79D1* and *CYP79D2*, which are cytochromes P-450 enzymes encoded by the genes *CYP79D1/D2* (Andersen *et al.,* 2000) that allow the formation of the two subsequent intermediates *N*-hydroxy valine and 2-methyl-propanal oxime. Since the two genes are present in *M. esculenta,* the plant is allopolyploid. Further addition of oxygen by a putative *CYP71E* takes place, producing the non-stable intermediate acetone cyanohydrin. The *CYP71E* gene has been proposed to be present in cassava by Andersen *et al*. (2000) in homology to dhurrin synthesis in sorghum. Acetone cyanohydrin is finally glycosylated by a vacuolar uridine 5'-diphosphoglucose (UDPG)-glucosyl transferase, forming linamarin (McMahon *et al.,* 1995) (Figure 2.1).

The vacuolar localization of these reactions has been suggested by evidence showing *CYP79D1/D2* to be targeted to the tonoplast membrane (Koch *et al.,* 1992). In addition, the quantities of linamarin in isolated vacuolar fractions account for that present in intact cells (White *et al.,* 1998). Besides, the localization of cyanogenic glycosides in vacuoles has also been suggested in other plants such as sorghum and rubber tree (McMahon *et al.,* 1995).

Linamarin is synthesized in leaves and transported to roots by mechanisms that to date are not clearly understood (McMahon *et al.,* 1995). The two linamarin metabolizing enzymes linamarase and hydroxynitrile lyase, which trigger cyanide

release, are located in the cell wall, requiring a symplastic transport of linamarin, or a partial modification of linamarin that prevents its undesired metabolization, if an apoplastic pathway for its translocation is preferred. Various works have suggested linustatin, the glycosylated form of linamarin, as the translocable form, since its presence in other cyanogenic plants has been proved (Selmar *et al.,* 1988; Koch *et al.,* 1992; Selmar, 1994;). Apparently, however, the levels of linustatin in cassava tissues are too low to account for the only source of linamarin in roots. Moreover, certain studies have documented that roots are capable of certain levels of linamarin synthesis (White *et al.,* 1994; McMahon *et al.,* 1995).

Transgenic approaches demonstrated the leaf specific silencing of the genes encoding *CYP79D1* and *CYP79D2* involved in the first steps of linamarin synthesis, resulting in a significant reduction in leaf and root linamarin content, while a root specific silencing meant no change in linamarin accumulation in any tissue (Siritunga and Sayre, 2003, 2004). This information clarifies that linamarin is at least mainly synthesized in the leaves, which serve as a main source of linamarin to other tissues.

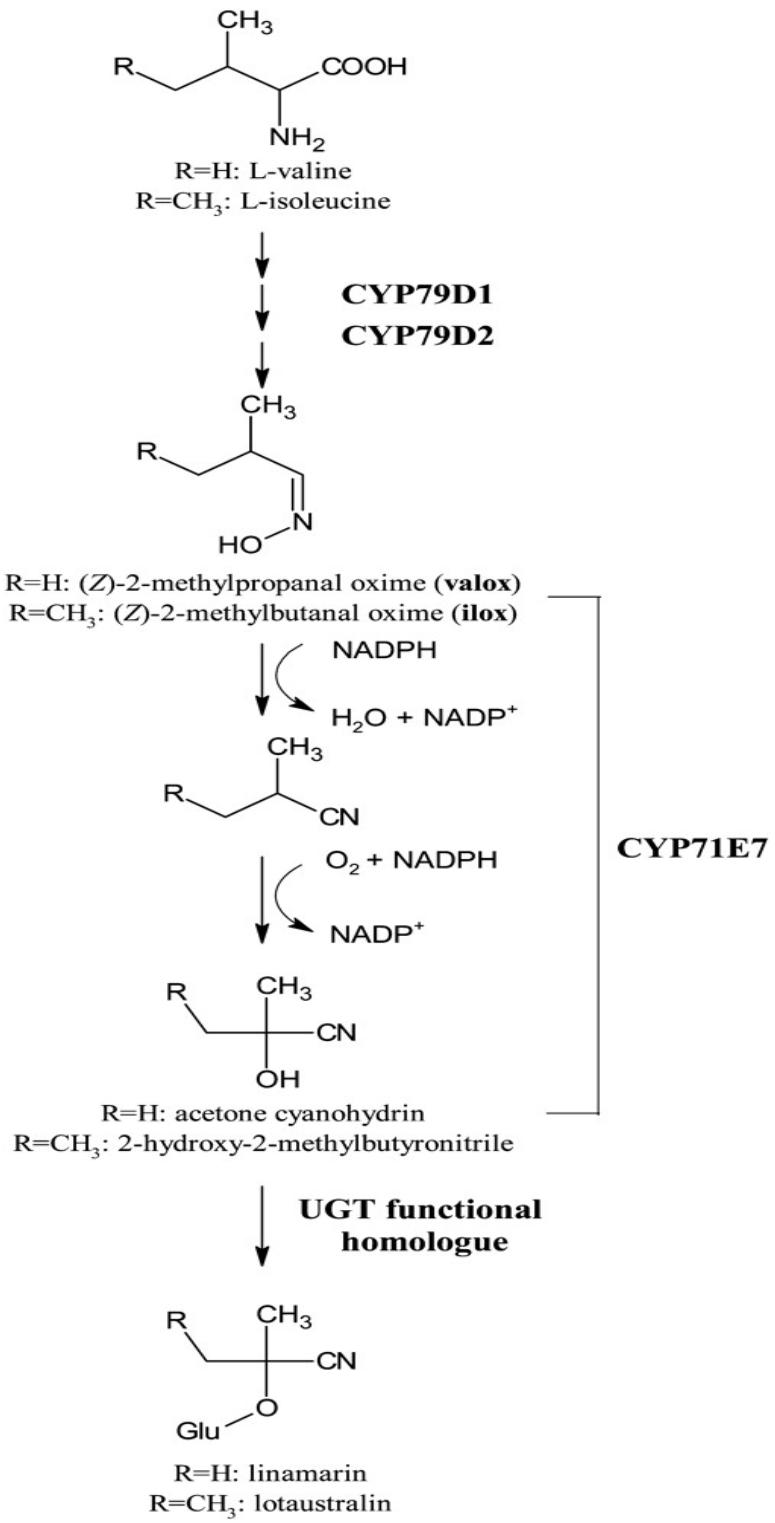

Figure 2.1: Cyanogenic glycoside biosynthetic pathway (Jørgensen *et al.*, 2005)

2.1.8.2 Cyanogenesis

Cyanogenesis involves a series of reactions that yield free cyanide. The first reaction is the deglycosilation of linamarin to produce acetone cyanohydrin and glucose by linamarase (Mkpong *et al.,* 1990), a very stable β-glycosidase enzyme. This step is broadly accepted to be limiting for cyanogenesis to occur, since the produced aglycone acetone cyanohydrin can be subjected to spontaneous decomposition to cyanide and acetone at pH > 5.0 and/or temperatures greater than 35ºC (White *et al.,* 1998) (Figure 2.2). Linamarase has a narrow range of substrate specificity compared to other β-glycosidase, as it is capable of hydrolyzing linamarin and other non-physiological monoglycosidic substrates, but not the diglycosides amygdalin and linustatin (McMahon *et al.,* 1995), being one of the proofs of linustatin serving as the linamarin translocated form.

Cyanide can also be released enzymatically from acetone cyanohydrin by hydroxynitrile lyase (HNL). This is the critical step that releases cyanide, acetone being an additional product. The HNL enzyme, like linamarase, is very stable, showing no reduction in its activity after its storing for one month at 4ºC (McMahon *et al.,* 1995), a feature particularly common to extracellular enzymes. Moreover, the highest HNL activity levels have been found in protein extracts prepared from the apoplast, suggesting a localization of the enzyme at the outer inter-cellular space (White *et al.,* 1994), similar to linamarase (Selmar *et al.,* 1988; McMahon *et al.,* 1995). This information has driven the commonly accepted

statement that these two enzymes are localized in the cell wall (McMahon *et al.*, 1995).

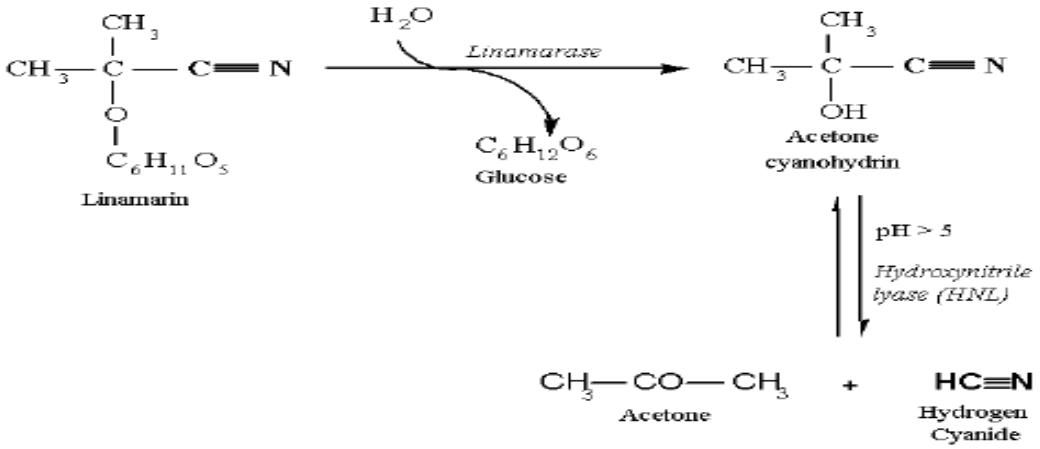

Figure 2.2: Catabolism of linamarin to produce hydrogen cyanide (Mburu *et al.*, 2012)

Linamarase is localized in the cassava cell wall and is also abundant in laticifers (Mkpong *et al.*, 1990). Therefore, release of cyanide occurs only after tissue damage when linamarin comes in contact with linamarase, as is the case during herbivore attack or during food processing (Balagopalan *et al.*,1985; Cock, 1985; Belloti and Arias, 1992). The compartmentalization of linamarin in the vacuole and linamarase and HNL in the cell wall prevents the formation of toxic cyanide in undamaged cells (Figure 2.3). The breakdown of the physical barriers between substrates and the enzymes, following tissue damage, initiates cyanogenesis (Poulton, 1990).

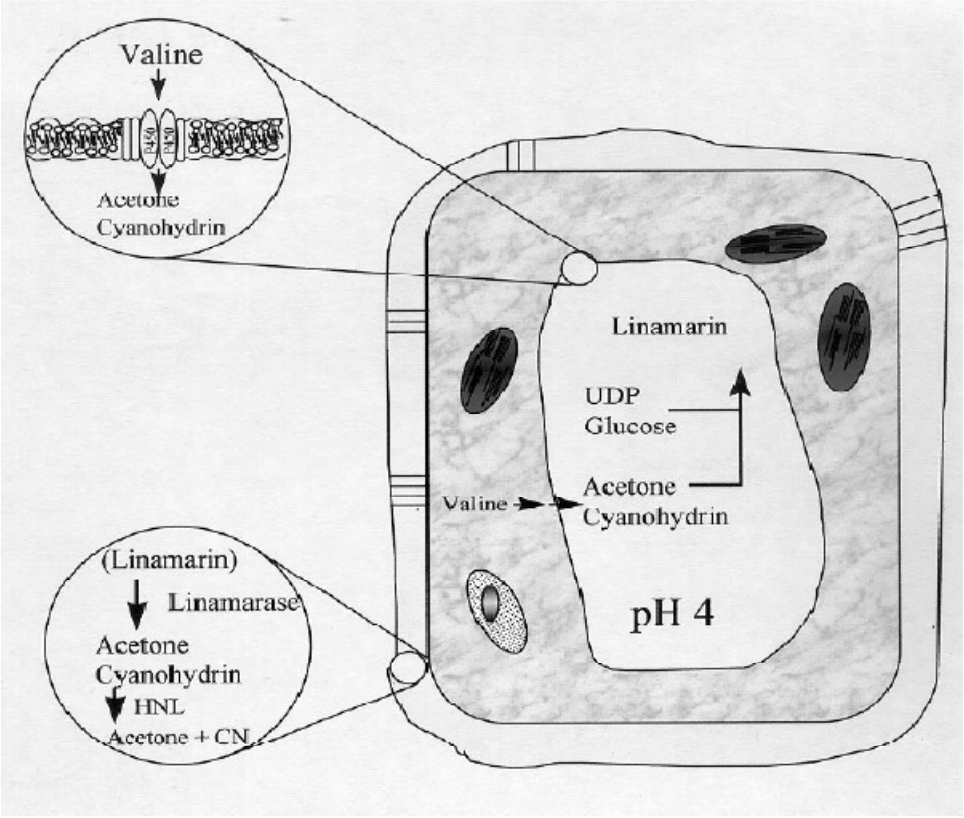

Figure 2.3: Sub-cellular distribution of linamarin and the enzymes involved in its synthesis and cyanogenesis (Siritunga, 2002)

2.1.8.3 Physiological roles of cyano-glycosides in cassava

Cyanogenic glycosides are thought to function as chemical defense molecules against herbivores, and as transportable and storage forms of reduced nitrogen (Belloti and Arias, 1992; Hickel *et al.,* 1996; Degró, 2009). Studies have confirmed that certain insect pests that prey upon cassava show preference for low cyanogenic varieties over high cyanogenic varieties (Arias and Bellotti, 1984).

Evidence that cyano-glycosides act as transportable and storage forms of reduced nitrogen was availed when it was reported that two week old cassava seedlings, when fed with ^{14}C-radiolabeled cyanide, accumulated 49% of the label in asparagine (Nartey, 1969).

Since a direct reaction leading to the formation of asparagine from cyanide and serine or cysteine seemed unlikely, it was proposed that asparagine formation involves formation of β-cyanoalanine as a transient intermediate that is rapidly converted to asparagine (Dunhill and Fowden, 1965; Floss *et al.,* 1965; Nartey, 1969). Formation of asparagine from β-cyanoalanine was later confirmed to occur in a reaction catalyzed by β-cyanoalanine hydrolase (Castric *et al.,* 1973).

Asparagine, is not only a protein precursor, but also a key compound in nitrogen storage and transport, due to its stability and high nitrogen/carbon ratio (Taiz and Zeiger, 2002). Asparagine is further metabolized to aspartate and ammonia by asparaginase. Ammonia is utilized in amino acid synthesis, being incorporated into the glutamine and glutamate biosynthesis pathways (Degró, 2009).

Nartey, (1969) confirmed that a significant part of the label (6%) was also found in aspartate, confirming the possible role of cyanogens as alternate sources of reduced nitrogen, other than nitrate and nitrite reduction to ammonia. On the other hand, asparaginase activity levels were found to be lower in cassava roots than in

leaves, in contrast to the other enzymes involved in asparagine synthesis, which showed higher activity in roots, suggesting that asparagine metabolism occurs mainly in leaves (Elias *et al.,* 1997). Furthermore, Nartey (1969) also observed that the levels of asparagine increased after seed germination.

The accumulation of asparagine was apparently accompanied by a decrease in linamarin content in the endosperm and an increase in linustatin content. At the same time, activities of the linustatin diglycosidase and β-cyanoalanine synthase increased in seedlings (Degró, 2009). This is an indicating that linamarin was somehow converted to linustatin and translocated from the maternal endosperm to the germinating embryo, following its metabolism to asparagine (Degró, 2009). It was, later on, found that the utilization of linamarin in cassava as an alternate nitrogen source was plausible (Poulton, 1990).

There is further evidence indicating that cyanogenic glycosides are mobile nitrogen storage compounds in cassava. Labelling studies of cassava with ^{14}C-Valine showed that the primary site of linamarin synthesis is the leaf, while the roots and lower stem had minimal ability to incorporate labeled valine (Bediako *et al.,*1981). Later, a 13-fold decrease in root cyanogens occurred when girdling was performed on the cassava stem (Ramanujam and Indira, 1984). A series of grafting experiments performed between roots and shoots of low- and high-cyanogenic cultivars of cassava showed that, at least in part, cyanogen accumulation in the

roots was due to the transportation of cyanogens from the leaves (Makame *et al.*, 1987).

Recent experiments aimed at decreasing cyanogen content in cassava tissues using transgenic approaches provided further evidence of their role in nitrogen transport and supply (Siritunga and Sayre, 2004; Siritunga and Sayre, 2007). Transgenic cassava plants developed to specifically silence the *CYP79D1/D2* gene expression showed no accumulation of linamarin and these plants apparently failed to produce roots in medium devoid of ammonium (Degró, 2009).

2.1.8.4 Cyanide detoxification

Three possible cyanide detoxification pathways exist in living organisms. These pathways are catalysed by the enzymes rhodanase, formamide hydrolyase (FHL) and β-cyanoalanine synthase (β-CAS). Rhodanase is more likely to be present in insects and at a very low level in higher plants but is not present in cassava (Miller and Conn, 1980; Poulton, 1990). Formamide hydrolyase is absent in any cyanide metabolism pathway in plants (Miller and Conn, 1980). The most common enzyme involved in cyanide detoxification in plants is β-CAS, which catalyzes the incorporation of cyanide into L-Serine or L-cysteine to form β-cyanoalanine, releasing sulphide (Figure 2.4).

The β–cyanoalanine intermediate is then hydrolyzed to asparagine by β-cyanoalanine hydrolase (Siritunga, 2002). The presence of both these enzymes in all cassava tissues indicates the importance of this pathway to detoxify cyanide (Nambisa and Sundaresan, 1994). The reaction was determined to occur at a faster rate when cysteine and cyanide were used as substrates and not serine as originally suggested (Degró, 2009). The detoxification pathway catalysed by rhodanase entails reaction of hydrogen cyanide with thiosulfate to yield thiocyanate and sulfate (Figure 2.4).

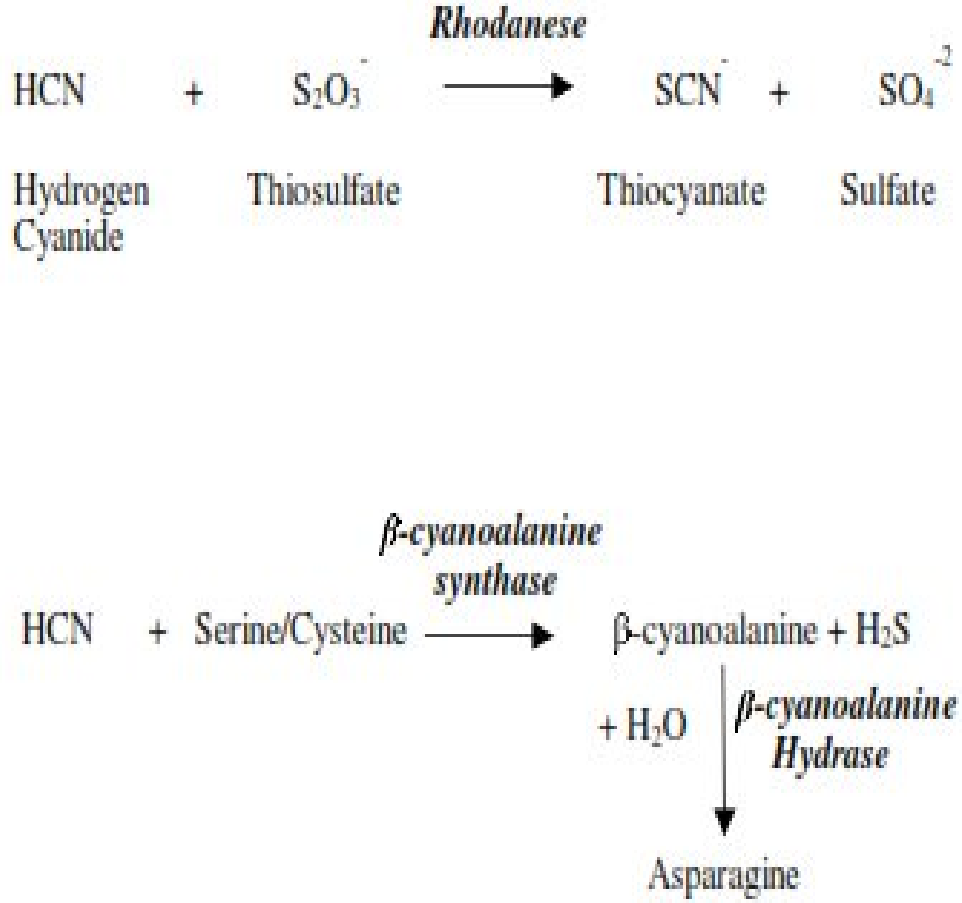

Figure 2.4: Hydrogen cyanide metabolism in plants (Siritunga, 2002)

2.1.8.5 Cyanide-related health complications

Cyanide is toxic to most living organisms mainly by the inhibition of cytochrome oxidase (complex IV), a mitochondrial enzyme involved in oxygen reduction in the respiratory electron transport chain. Cyanide can also inhibit catalase. Besides, it inhibits plastocyanin reduction in photosynthesis. Upon entry into the body, linamarin metabolism to acetone cyanohydrin and cyanide mainly by bacteria in the intestine causes toxicity (Degró, 2009). Additionally, linamarin can reach the brain through a glucose transporter. An *in vitro* study using neural cell lines showed that linamarin considerably causes cell death (Sreeja *et al.*, 2003).

Poorly processed cassava has been associated with certain health disorders and even death in Africa (Aregheore and Agunbiade, 1991). The level of harm caused by these disorders depends on the level and frequency of cyanogens exposure and the state of nutrition of the consumer. Symptoms are presented at a higher level in nutritionally compromised individuals who have a low protein intake in their diets, especially of sulphur containing amino acids, which are key molecules involved in cyanide assimilation into thiocyanate, which allows a certain level of cyanide detoxification (Tylleskar *et al.,* 1992).

Western African cultures show a high incidence of tropical ataxic neuropathy (TAN), caused by long-term consumption of cassava-based diets with relatively low cyanogen content (Nhassico *et al.,* 2008). Nigerian communities consume

cassava daily, but the processing of cassava to *gari* allows the release of most of the cyanide, thus not resulting in chronic cyanide exposure (Oluwole *et al.,* 2000, 2002; Siritunga, 2002; Nhassico *et al.,* 2008). The prevalence of TAN, a progressive disease, causing unsteady walking, loss of vision and sensation in hands, deafness and weakness, is higher amongst Western African seniors of 60 years or older, and also more frequent in women, possibly because of their cultural role in cassava culturing and processing, thus being more prone to cyanide poisoning (Oluwole *et al.,* 2000; Nhassico *et al.,* 2008). In diseased persons, high plasma thiocyanate levels were associated with high cyanide intake (Siritunga, 2002).

A low dietary protein intake, resulting in a deficiency of sulphur, needed for cyanide detoxification, was proposed as a contributing factor in cyanide poisoning (Osuntokun *et al.,* 1968; Osuntokun, 1981). The occurrence of TAN has decreased during the last decade which may be explained by a change in the diet by populations at risk (Siritunga, 2002).

Epidemic spastic paraparesis (Konzo) is an acute disease that rapidly and permanently cripples the victim by damaging nerve tracts in the spinal cord that transmit signals for movement, causing a spastic paralysis of both legs (Howlett *et al.,* 1990). Paraparesis refers to the paralysis of both legs and spastic implies that muscles of the legs are not flaccid. The legs can usually support affected persons

sufficiently to allow them to stand, especially if supported by a stick, but the affected persons will have their knees crossed. Attempts to walk often result in uncontrolled muscle jerks. In the most extreme case the arms are also affected similar to the legs and poisoned individuals may also have diminished vision and difficulties in speaking (Siritunga, 2002).

The occurrences of all reported incidence of epidemic spastic paraparesis are during seasons when populations experience food shortages (Howlett *et al.,* 1990; Mlingi *et al.,* 1991; Tylleskar *et al.,* 1991). Cassava was the only food available in quantity in these areas experiencing food shortages and thus famine was avoided, but the roots were consumed without sufficient processing. The disease has not been reported from any population not consuming cassava or from cassava consuming populations having balanced diets (Siritunga, 2002).

Another major cyanide health-related disorder is goitre. When the dietary intake of iodine is insufficient the thyroid gland starts to enlarge to enable a maximal extraction of iodine from the blood. The occurrence of such enlargement is known as goiter. The most severe public health effect of goitre is that the children born to iodine-deficient mothers will suffer from cretinism, a condition characterized by mental retardation and stunted growth (Delange and Ahluwalia, 1983).

Extensive studies have established that goitre and cretinism due to iodine deficiency can be considerably aggravated by a continuous dietary cyanide exposure from insufficiently processed cassava. This effect is caused by thiocyanate (Siritunga, 2002). Thiocyanate has a similar size to the iodine molecule and interferes with the iodine intake in the thyroid gland (Bourdoux *et al.,* 1978). Populations in northern Congo with very low iodine intake and having high thiocyanate levels resulting from consumption of inadequately processed cassava, suffered from very severe endemic goiter with a high prevalence of cretinism. When the population was given iodine supplementation by injection of iodized oil, the goitre problem decreased considerably in spite of continued consumption of insufficiently processed cassava (Ermans *et al.,* 1982).

2.1.8.6 Conventional removal of cyano-glycosides from cassava

To prevent cyanide poisoning, linamarin and lotaustralin have to be removed from cassava foods. The most efficient processing procedures include peeling, soaking, fermenting, chopping, grating, drying, and cooking.

Many methods of processing cassava roots commence with the peeling of the roots, which reduces the cyanogenic glycoside content by at least 50% (Siritunga, 2002). This is probably because the outer cortical part of the cassava root, which contains high levels of cyanogenic glycosides is removed during peeling.

Grating takes place after peeling and is sometimes applied to whole roots. Grating of the whole root ensures homogeneous distribution of the cyanide in the product, and will also make the nutrients contained in the peel available for use. In the grated product, the residual concentration of cyanogens will depend on the time during which linamarin and linamarase interact (Ihemere, 2003).

Soaking of cassava roots is normally done prior to cooking. It extracts the soluble cyanide into the water. Although this process removes about 20% of the free cyanide within 4 hours, a significant reduction of total cyanide is achieved only after 4-5 days; helped by routine changing of the soaking water. It has been reported that peeled cassava roots soaked for 4-5 days followed by sun drying showed a reduction of cyanide of about 98.6% of the initial content in the roots (Ihemere, 2003).

Similar to soaking, there is a rapid reduction in the free cyanide from boiling cassava roots. Boiling of cassava pieces to remove cyanide was studied by Nambisan and Sundaresan (1985). In a classical study, samples of different size (A: 6x3x3 cm, B: 3x2x1 cm, C: 1x0.5x0.5 cm) were boiled in water for 30 minutes. Reduction was highest for the smallest size C, 69-75% of the initial level, followed by size B and size A, where reductions were 50% and 25%, respectively. Most of the cyanide removed was recovered in the water, indicating that cyanide dissolves in water (Nambisan and Sundaresan, 1985).

Further, the effects of different water volumes were studied by extracting cyanide from cassava pieces at ratios of 1:0, 1:1, 1:2, and 1:5 cassava weight to water volume. An increase in cyanide reduction was observed from 30% (1:1) to 64% (1:2) and 76% (1:5). An increase of water volume to 1:0 did not further decrease cyanide content in cassava pieces (Nambisan and Sundaresan, 1985).

About 90% of free cyanide is removed within 15 minutes in boiling water, whereas 55% of the cyanogenic glycoside is lost after 25 minutes (Cooke and Maduagwu, 1985).

Proper sun drying is achieved in 1-3 days in the dry season and up to 8 days in the rainy season. Gomez *et al.* (1984) indicated that more than 86% of HCN present in cassava roots was lost during sun drying. Cyanide removal was studied after an incident of acute intoxication in 1988 in Tanzania during preparation of makopa, a Tanzanian cassava meal (Mlingi *et al.,* 1992; Mlingi *et al.,* 1995). In this study, fresh roots and cassava pieces, sun-dried for 8 and 17 days, were analyzed. Cyanide levels, initially 1090 mg/kg dwt, remained high. Only 46% and 63% were eliminated after sun-drying for 8 and 17 days, respectively. The final cyanide level after 17 days was 401 mg/kg dwt, mostly in the form of cyanogenic glycosides (96%). In a second batch using roots with a lower cyanide content (493 mg/kg dwt), the elimination was 71% after 8 days and 73% after 17 days, resulting in 132 mg/kg dwt in the final product. Products from the market showed similar amounts

of cyanides (145 mg/kg dwt) (Mlingi *et al.,* 1995). Therefore, this shows that longer drying periods achieved more reductions in cyanide levels in cassava. However, sun drying alone does not mitigate the toxic effect of cyanogenic glycosides in cassava in the long run.

From multiple stage sampling, it was concluded that enzymatic hydrolysis ceases whenever the moisture content reaches 13%, which is the border level for enzymatic hydrolysis. If roots are cut into smaller pieces, the drying will be faster, resulting in even higher levels of cyanogenic glycosides (Mlingi *et al.,* 1992). Since cyanohydrin and HCN are removed when the product is completely dry, the residues in the final product will be cyanogenic glycosides. The conversion of cyanogenic glycosides to cyanohydrin is therefore the limiting step, determining the cyanide content of the final product (Mlingi and Bainbridge, 1994).

Fermentation is one of the most common practices in cassava processing, about 75% of harvested roots in Africa are processed to fermented products (Westby, 1991), leading to improvements in shelf life, taste and flavour. It is generally accepted that fermentation plays an important role in the reduction of cyanides.

Most marketed cassava products like "*gari*", "*fufu*", "pupuru", "apu" etc., in Africa are obtained through fermentation. The importance of fermentation in cassava processing is based on its ability to reduce the cyanogenic glycosides to

relatively insignificant levels. Unlike alcoholic fermentation, the biochemistry and microbiology of cassava fermentation is only superficially understood, but it is believed that some cyanidrophilic/cyanide tolerant microorganisms are involved in the breakdown of the cyanogenic glycoside. Higher retention of starch in grated cassava leads to more efficient detoxification of cyanogens and cyanide by the bacteria. This could be due to the fermentative substrate provided by the starch (Siritunga, 2002).

2.2 *In vitro* cassava regeneration

Despite being an important crop in the tropics, cassava is not easily amenable to breeding via classical genetics (Ihemere, 2003). Through conventional breeding, cassava takes ten years to generate a new cultivar (Alves, 2002), largely because of inconsistent flowering along with poor seed set (Ihemere, 2003). Cassava is also heterozygous and clonally propagated (Jennings and Iglesias, 2002).

This shortcoming has invoked realization that genetic engineering holds the key to improvement of many crop plants including cassava. A prerequisite to any plant genetic transformation strategy is an efficient and reproducible *in vitro* regeneration system. Roca, (1984) indicates that nearly all parts of cassava plant have been used to establish *in vitro* culture. The following is an exploration of various techniques that have been used for *in vitro* cassava regeneration.

2.2.1 Meristems culture

Meristems on apical or axillary buds can be induced by cytokinin containing media to produce shoots (Kartha and Gamborg, 1975; Ng *et al.,* 1990). This could provide an alternative source of explants for transformation studies, but it is difficult to obtain the number of meristems adequate for transformation experiments (Schopke *et al.,* 1993). Apart from serving as explants for cassava transformation, meristem culture serves a more important role in providing disease-free plants, especially for plants infected with viruses as meristems are generally devoid of viruses (Schopke *et al.,* 1993). In this regard, meristems serve as a means for viral decontamination and in multiplication of cassava (Ihemere, 2003).

2.2.2 Protoplasts culture

In vitro cassava regeneration from protoplasts has not been very successful (Siritunga, 2002). In spite of the use of various media, protoplasts only regenerated into green callus or adventitious roots (McDonnell and Gray, 1997; Sofiari *et al.,* 1997). But more recently protoplasts isolated from FECs were found to divide and develop readily into callus and eventually lead to plantlets (Sofiari *et al.,* 1998). Currently, the most reliable method for cassava regeneration is through the young leaf lobes and through suspension cultures which is more time consuming and requires more dexterity. Somatic embryos have been obtained from a number of cultivars, but there is still the need for optimization of the regeneration procedures

(Ihemere, 2003). Field test results of cassava plants derived from embryogenesis showed that somaclonal variation is not a problem in cassava (Schopke *et al.,* 1993). This makes plant regeneration via somatic embryogenesis a reliable tool for genetic improvement of cassava (Ihemere, 2003).

2.2.3 Somatic embryogenesis

This is the best method for *in vitro* regeneration of cassava. Somatic embryos have been induced from cassava young leaf lobes and cotyledons (Ihemere, 2003). A somatic embryo is an independent bipolar structure that is not attached vascularly to the tissue of origin (Ammirato, 1987). The embryos can then develop to zygotic embryos, and subsequently to plantlets. Primary embryos can be induced from different cassava tissues: shoot apical and axillary meristems (Stamp and Henshaw, 1987; Szabados *et al.,* 1987; Puonti-Kaerlas *et al.,* 1998), immature leaves (Stamp and Henshaw, 1987; Szabados *et al.,* 1987; Raemakers *et al.,* 1993; Li *et al.,* 1995), and cotyledons of zygotic embryos (Konan *et al.,* 1994b). Even though it is now possible to regenerate cassava plants from a limited number of cultivars, the efficiency of regeneration achieved so far is low (Schopke *et al.,* 1993). Primary embryos will develop to become mature embryos with green cotyledons and eventually produce shoots. Cyclic systems of somatic embryo production can be maintained through continuous culture of somatic embryos in auxin-supplemented medium (Siritunga, 2002).

2.2.4 Organogenesis

This entails the emergence of adventitious organs (shoot or roots) directly from the explants without an intervening callus phase (Siritunga, 2002). Adventitious shoots have been produced from cotyledons of cassava somatic embryos using medium supplemented with cytokinins (BAP and IBA). These shoots are successfully transplanted to soil after being rooted in hormone-free medium (Li *et al.,* 1996, 1998). Cassava organogenesis is independent of callus formation and auxin treatment. Unlike most plants, there is no intermediary of callus required for organogenesis. Instead shoot primordia are induced directly from cotyledons of somatic embryos and young leaf lobes on MS (Murashige and Skoog, 1962) medium supplemented with 6-benzyladenine (Li *et al.,* 1996; Mussio *et al.,* 1998).

2.2.5 Friable embryogenic callus (FEC)

A type of less organized embryogenic tissue, FECs, can be produced from organized mature somatic embryos of cassava (Taylor *et al.,*1996). Friable embryogenic calli can be maintained for over two years as suspension cultures under a three-week subculture regime. In the presence of maturation medium the suspension develops into mature embryos and germinates into plants after desiccation treatment (Raemakers *et al.,*1996; Taylor *et al.,*1996).

2.3 *In vitro* cassava genetic transformation

In the 20th century, plant breeding has had immense contribution to major increases in crop yield and productivity. Cassava genetic improvement started later than in other crops of agronomical importance such as wheat and maize, whose economical exploitation depends mainly on first world countries where the private sector of the seed industry finds an attractive market (Degró, 2009). In the second half of the 1970's, the "Centro Internacional de Agricultura Tropica" (CIAT) in Cali, Colombia and the International Institute of Tropical Agriculture (IITA) in Ibadan, Nigeria developed the first scientific programs for gathering and improving cassava germplasm (Ceballos *et al.,* 2004).

These initiatives were invoked by the need to ameliorate hunger in the developing countries. Productivity and stability of production are among the main priorities in cassava breeding. An increase in starch and dry matter quantity is important for the industrial exploitation of cassava (Ceballos *et al.,* 2004; Degró, 2009).

Conversely, the improvement of cassava lines for human consumption requires the modification of starch composition and other physiological and morphological traits involved in food quality, such as cyanogen, carotene (pro-vitamin A) and protein content, as well as post-harvest physiological deterioration. It appears that the last two traits exhibit limited genetic variability within the known cassava germplasm, thereby requiring the employment of close-related species and

subspecies within the *Manihot* genus in order to attempt the introgression of desired alleles (Jennings and Iglesias, 2002; Ceballos *et al.,* 2004).

Stability of production involves the improvement of cassava to resist certain biotic and abiotic stresses. Among the major biotic agents constraining cassava production are viral (cassava mosaic disease mainly in Africa and Asia), bacterial (cassava bacterial blight in LAC), fungal and insect pests (Ceballos *et al.,* 2004; Degró, 2009). Certain abiotic factors affecting productivity such as drought and low fertility soils have driven breeding programs to suggest certain characteristics of the plant involved in its adaptation to these environments. These characteristics include leaf longevity, optimum leaf area index and ideal plant architecture (Ihemere, 2003; Ceballos *et al.,* 2004).

Classical cassava breeding is, laborious, difficult and time consuming, largely due to challenges in synchronization of flowering between parental lines, high frequency of self pollination, scarcity of flowers in the inflorescence due to farmer selection and time required for the seed to mature (Schopke *et al.,* 1996; Ceballos *et al.,* 2004). Further, cassava is characterized by high levels of heterozygosity within populations that makes it difficult to obtain pure homozygous individuals for the desired characters (Degró, 2009). In light of all these and more inherent constraints, genetic engineering approaches have been suggested as additional

alternatives for the introgression of genes into parental lines of agronomical importance (Taylor *et al.*, 2004a,b).

Genetic transformation offers the advantage of the incorporation of a single gene of particular interest into cultivars of agronomical importance, without the problems of gene linkage and whole genome sharing that occur when traditional breeding is employed in crop genetic improvement (Degró, 2009). The use of these technologies depends mainly on a method for the delivery and integration of exogenous DNA molecules into the plant genome and a method for the *de novo* regeneration of plants in *in vitro* conditions. DNA transfer technologies require the establishment of successful protocols, but apparently this is not the limiting factor in plant transgenesis (Degró, 2009). It is postulated that the limiting factor in plant transgenesis is reproducible regeneration protocol for the plant in question. On the other hand, not only the development of a regeneration method, but also its compatibility with a selection system has been a matter of intense research among scientists attempting the implementation of these technologies in plants, although it sometimes remains unachievable (Ihemere, 2003; Siritunga and Sayre, 2003; Degró, 2009).

The most common strategies for plant cells transformation with foreign genes are *Agrobacterium*-mediated gene transfer and particle bombardment (Siritunga, 2002). *Agrobacterium*-mediated transformation is favored over particle

bombardment because of the greater stability of transformants. *Agrobacterium*-mediated transformation usually results in the transfer of 1-3 copies of the transgene to plants compared to the particle gun which has been known to deliver up to 12 copies of a transgene (Ihemere, 2003). Importantly, the introduction of multiple transgene copies can lead to gene silencing (Alien *et al.*, 1993; Matzke and Matzke, 1995; Meyer and Saedler, 1996).

Agrobacterium-mediated transformation is currently routinely used to transform cassava. *Agrobacterium* has been successfully used to transfer genes to all the cassava tissue that has the ability to regenerate into plantlets (Siritunga, 2002; Sarria *et al.,* 1995, 2000) Wild-type strains of *Agrobacterium tumefaciens* have been shown to transfer to cassava somatic embryos a T-DNA carrying *nptII*, *bar* and *uidA* genes (Sarria *et al.,* 1995, 2000). A transient expression of GUS was shown in meristems, meristem-derived somatic embryos after co cultivation with *Agrobacterium* (Konan *et al.,* 1994b; Puonti-Kaerlas *et al.,* 1997). Li *et al.* (1996) used *Agrobacterium*-mediated transformation to make transgenic cassava plants via shoot organogenesis from somatic cotyledons (Li *et al.,* 1996). A system was developed for transformation of cassava FECs using *Agrobacterium*. The *nptII* gene and *uidA* gene was used as the selectable marker and visible marker, respectively (González *et al.,* 1998).

The biolistic transformation technique is mediated by microprojectile bombardment (Klein *et al.,* 1987; Siritunga, 2002). DNA-coated microprojectiles are accelerated by explosive material or a high-pressure burst of gas to penetrate the plant cell wall and deliver the DNA for expression and /or integration into the plant genome (Siritunga, 2002). The technique is widely used to deliver foreign DNA to regenerable cells without the troubles of *Agrobacterium* related host specificity limitations. Schopke *et al.* (1996) and Raemakers *et al.* (1996) were the first to report the use of biolistics to transform cassava. In both instances somatic embryogenesis was utilized as the regeneration method. Recently Zhang *et al* (2000a) reported the successful use of particle bombardment to produce stably transformed cassava.

Other less common strategies (due to their low transformation efficiencies) include; electroporation (Fromm *et al.,* 1986; Lurquin, 1997), silicon carbide whisker-mediated DNA transfer (Thompson *et al.,* 1995), protoplast transformation (Shillito, 1999) and microinjecting DNA into cells or zygotes (Leduc *et al.,* 1996).

Cassava has been transformed using different types of explants as starting material (Ihemere, 2003) including; somatic embryo cotyledons (White *et al.,*1998; Zhang, 2000; Siritunga and Sayre, 2003), young leaf lobes (White *et al.,*1998), and friable embryogenic callus (Raemakers *et al.,* 1996; González *et al.,* 1998; Zhang *et al.,*

2000b). Embryogenesis has been reportedly observed from friable embryogenic calli, somatic embryo cotyledons and young leaf lobes. Direct organogenesis has been reported with somatic embryo cotyledons and young leaf lobes (Zhang *et al.,* 2000b).

In June 2003, the efficiency of transformation of the different explants and methods was scored by scientists in the Advanced Cassava Transformation Group who met at CIAT in Palmira, Colombia. The somatic embryogenesis method using embryo cotyledons as explants was chosen to be the best method based on efficiency of regeneration, incidence of chimeras, speed of regeneration of transformed plants, and simplicity of transformation technique. The different methods were scored 1-5, with 5 being the best score (Ihemere, 2003).

2.3.1 Shortcomings of cassava transformation

Although discernible gains have been made in cassava genetic transformation, the process is still bedeviled by a number of shortcomings. Firstly, cassava has a low transformation efficiency compared to other crops (Ihemere, 2003). The best recorded transformation efficiency for cassava is in the range of 3-5%. Most other crops have transformation efficiencies greater than 10 % (Raemakers *et al.,* 1997). Further, it is seldom possible to characterize transformed cassava plants molecularly. Southern blot analysis of cassava has proved to be very difficult partly because the cassava genome is large (Ihemere, 2003). Siritunga, (2002)

reported that gene insertions from independently transformed cassava plants may have similar restriction patterns making it difficult to identify independently transformed line.

Scientists working on cassava transformation have tried a variety of tissues for cassava transformation, the most prolific being the friable embryogenic callus (FEC) which has been reported to generate more transformed cassava (Raemakers *et al.,* 1996; González *et al.,* 1998). However, the challenge is that production of FEC requires that the cassava embryogenic tissues stay on high auxin (50µg/L) media for up to six months compared to the one month requirement for somatic embryogenesis. This long exposure to high auxin concentrations leads to somaclonal variation in cassava. The severity of the somaclonal variation has led to production of stunted cassava plants and those with variegated leaves (González *et al.,* 1998; Ihemere, 2003).

It is equally speculated that cassava transgenes are prone to deletion. Sarria *et al.* (2000) imparted herbicide resistance to cassava through genetic engineering. Proof of transformation events included positive GUS stains, RT-PCR and Southern blotting. However, some of their positive transformants lost the transgenes with time.

CHAPTER THREE

OPTIMIZATION OF *IN VITRO* REGENERATION OF KENYAN CASSAVA GENOTYPES

3.1 INTRODUCTION

One of the requirements for the generation of transgenic cassava is an efficient and reproducible plant regeneration system. Somatic embryos have been induced from cassava young leaf lobes and cotyledons leading to plant regeneration (Stamp and Henshaw, 1987; Szabados *et al.,* 1987; Taylor and Henshaw, 1993; Konan *et al.,* 1994a; Ihemere, 2003). Unfortunately, focus of most cassava tissue culture regeneration efforts have been on cassava varieties from South America. Little if any efforts have been placed on developing regeneration and transformation systems for African cassava varieties (Ihemere, 2003).

Reportedly, African cassava varieties respond differently in culture from South American varieties. These disparities may have led to genetic divergence of African cassava from their South American progenitors potentially accounting for the different responses in culture (Ihemere, 2003). This chapter demonstrates successful development of an optimized regeneration protocol for Kenyan cassava varieties.

3.2 MATERIALS AND METHODS

3.2.1 Plant materials

Four cassava varieties viz; *TMS 60444, Serere, Adhiambo lera* and *Kibanda meno* were sourced from Kenya Agricultural Research Institute (KARI), Kakamega. *TMS 60444* is an exotic cassava genotype native to Columbia. It is a nodel cultivar in cassava regeneration and transformation. Many regeneration and transformation protocols have been developed and optimized using *TMS 60444* (Ihemere, 2003). *Serere* is native to Uganda but it has been domesticated in Busia, Kenya. Researchers at KARI have evaluated the levels of tolerance of *Serere* genotype to Cassava Mosaic Virus (CMV) (AIC, 2002). *Adhiambo lera* is native to Kisumu and Busia counties of Kenya. It is credited for its large starch biomass, early maturity and resistance to post harvest deterioration (Agricultural Information Centre, 2002). *Kibanda meno* is native to Mombasa and Kilifi counties of Kenya. It is sweet and hard to chew. Earlier research on this genotype has shown that it has short shelf life (NRI Ltd, 2004).

The plantlets were grown *in vitro* on Murashige and Skoog media (1962) supplemented with 20g/L sucrose, MS Vitamins (Duchefa, Germany) and 8g/L of noble agar. All media used for *in vitro* propagation of cassava was sterilized through autoclaving. The growth chamber conditions were set at a temperature of 28˚C and a 12 hr day/12 hr night cycle.

3.2.2 Callus induction and somatic embryogenesis

Meristematic leaf lobes (2-6 mm long) from *in vitro*-grown plants were cultured on MS basal medium supplemented with 2% (w/v) sucrose, B5 vitamins (Gamborg *et al.,*1968), 50 mg/L casein hydrolysate, 0.5 mg/L $CuSO_4$ (Schopke *et al.,*1992) and 4-16 mg/L 2,4-dichlorophenoxyacetic acid (2, 4-D). The same set of meristematic leaf lobes was put in the same media with 2, 4-D being substituted with Picloram. Four concentrations of 2, 4-D and Picloram (4, 8, 12 and 16 mg/L) were tested for their ability to induce calli and somatic embryogenesis. The media pH was adjusted to 5.7 and it was solidified with 0.8 % (w/v) noble plant agar. Factors affecting embryogenesis were studied with 0 - 8% sucrose, 0-1.0 mg/L additional $CuSO_4$ and keeping the embryogenesis cultures in darkness or on a 12 hr day/12 hr night cycle. The cultures were maintained at a temperature of 28 ˚C. The explants were left in the induction medium for 4-6 weeks. The type of calli was observed at each step and the frequency of embryogenic calli formation was recorded after four weeks of culture on callus induction medium (CIM). The genotype *TMS 60444* was used as a control in regeneration experiments.

3.2.3 Maturation of somatic embryos

This entailed the development of globular stage embryos into green cotyledonary embryos with defined shoot and root axes (Mathews *et al.,* 1993). The globular stage somatic embryos were subcultured on maturation media composed of MS salts (Murashige and Skoog, 1962) supplemented with 2 % (w/v) sucrose, 1 mg/L

thiamine-HCl, 100 mg/L myo-inositol, 0.01 mg/L 2,4-D, 1.0 mg/L BAP and 0.5 mg/L GA_3; MS medium supplemented with 2 % (w/v) sucrose, 0.01 mg/L NAA, 0.1 mg/L BAP and 0.1 mg/L GA_3; or MS basal medium supplemented with 2 % (w/v) sucrose. The media pH was adjusted to 5.7 and it was solidified with 0.8 % (w/v) noble plant agar. The embryos were maintained in the maturation medium in the dark for 4 weeks.

3.2.4 Germination and plant recovery

Mature somatic embryos were transferred to basal MS medium supplemented with 2 % (w/v) sucrose; MS medium supplemented with 2% (w/v) sucrose, 1 mg/L thiamine-HCl, 100 mg/L myo-inositol, 0.01 mg/L 2,4-D, 1.0 mg/L BAP and 0.5 mg/L GA_3; MS basal salt supplemented with 2 % (w/v) sucrose and 0.8 % (w/v) activated charcoal. All the media were solidified with 0.8% (w/v) noble agar for germination and plant recovery. Germination and conversion rates in terms of germination and regeneration frequencies were recorded after four weeks in culture. The cultures were exposed to a daily photoperiod of 12 hours. All cultures were kept at 28^{o}C. Some regenerated plantlets were maintained in growth room ready for transformation experiments while others were hardened in the glasshouse.

3.2.5 Data analysis

Data on effects of different auxins on callus induction and somatic embryogenesis, effect of light on somatic embryogenesis, effect Copper Sulphate on somatic embryogenesis, effect of sucrose on somatic embryogenesis, effect of different cytokiinins on maturation of somatic embryos, and germination and regeneration frequencies was analyzed by ANOVA. Statistically significant variables were analysed by Tukey's Pairwise comparison at a confidence level of 95% ($P<0.05$). Minitab statistical software (version 2012) was used for data analysis.

3.3 RESULTS

3.3.1 Effects of 2, 4-D and Picloram on callus induction and somatic embryogenesis

It was possible to induce calli in all the cassava varieties at all the concentrations of 2, 4-D and Picloram. The different auxin treatments were used to determine which concentration was best for calli and somatic embryo induction of the cassava cultivars. All the treatments produced calli and somatic embryos.

For both callus induction and somatic embryogenesis, the best auxin concentration was 8mg/L of both 2, 4-D and Picloram, although 2,4-D had better response than Picloram (Table 3.1 and 3.2). The 12mg/L concentration of both 2, 4-D and Picloram also induced calli and embryogenesis appreciably compared to 4mg/L and 16mg/L, which induced the least responses. *Adhiambo lera* and *Serere* were the best responding varieties with regards to callus induction and somatic embryogenesis (Table 3.1 and 3.2).

For the genotypes *Adhiambo lera* and *Kibanda meno*, there was no significant difference in the frequency of callus induction and somatic embryogenesis at all auxin concentrations ($P>0.05$). For the genotypes *TMS 60444* and *Serere*, callogenesis and somatic embryogenesis were significantly different between 2, 4-D and Picloram at the concentration of 16mg/L ($P<0.05$; Table 3.1 and 3.2) (Appendices 1 and 2). At 4mg/L auxin levels, the ferequency somatic

embryogenesis was significantly different among the two phytohormones ($P<0.05$; Table 2) (Appendices 3 and 4). Generally, formation of embryogenic calli was consistent with the frequency of callus induction in all the cassava varieties.

Embryogenesis began as swollen regions at the cut ends and mid-veins on the adaxial surface of the immature leaf lobes. The swollen segments then gave rise to two kinds of calli viz; a loose non-embryogenic friable white callus and a translucent gelatinous embryogenic callus that formed globular stage embryos (Figure 3.1).

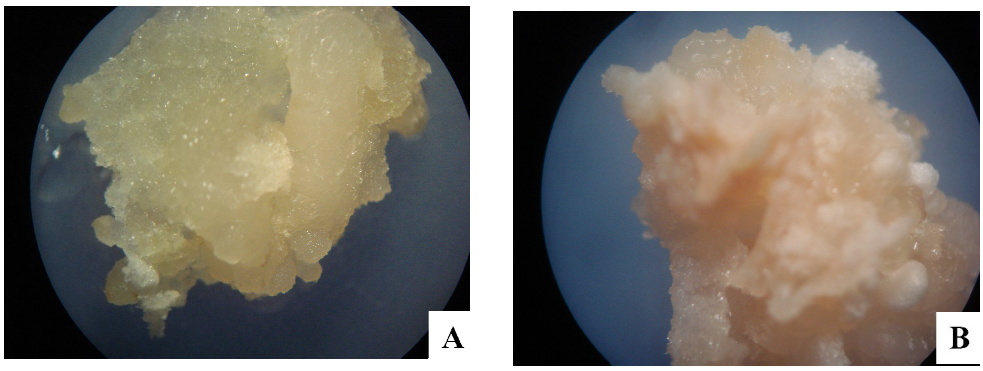

Figure 3.1: Tissue culture profile of somatic embryogenesis of cassava lines.

A: A loose non-embryogenic friable white callus
B: Translucent gelatinous embryogenic callus

Table 3.1: Effects of the different concentrations of 2,4-D and Picloram on callus induction

	4mg/L		8mg/L		12mg/L		16mg/L	
	2,4-D	**Picloram**	**2,4-D**	**Picloram**	**2,4-D**	**Picloram**	**2,4-D**	**Picloram**
TMS 60444	64.3 ± 0.9[a]	60.3±0.9[a]	85.0 ±0.6[a]	81.0±0.6[a]	74.3 ±0.9[a]	70.0±0.6[a]	68.2 ± 2.4[a]	58.0±1.2[b]
Adhiambo lera	75.0 ± 1.5[a]	71.7±1.2[a]	93.0 ±1.8[a]	90.0 ± 1.2[a]	82.7 ±0.9[a]	78.7±1.3[a]	68.3 ± 0.9[a]	65.0 ±2.3[a]
Kibanda meno	58.3 ± 1.2[a]	55.3 ± 0.9[a]	74.7 ±2.4[a]	71.0 ± 0.6[a]	67.7 ±1.5[a]	63.0±1.5[a]	57.0 ± 1.7[a]	54.3 ±2.3[a]
Serere	66.3 ± 0.9[a]	61.3±0.9[a]	78.7 ±0.9[a]	75.3 ±1.8[a]	68.7±1.9[a]	65.7±2.9[a]	63.3± 0.9[a]	57.1±2.1[b]

The values indicate the average numbers of calli per explant cultured in media supplemented with different 2,4-D and picloram concentrations. Values followed by different letters are significantly different (P<0.05).

Table 3.2: Effects of the different concentrations of 2,4-D and Picloram on somatic embryogenesis

	4mg/L		8mg/L		12mg/L		16mg/L	
	2,4-D	**Picloram**	**2,4-D**	**Picloram**	**2,4-D**	**Picloram**	**2,4-D**	**Picloram**
TMS 60444	20.7±1.2[a]	20.5 ± 3.4[a]	33.3±2.0[a]	29.7 ± 3.0[a]	26.7±0.9[a]	27.7 ± 1.5[a]	21.0±0.6[a]	19.3 ± 1.8[a]
Adhiambo lera	27.0±2.7[a]	26.3 ± 2.3[a]	40.7±0.9[a]	39.4 ± 3.2[a]	37.3±1.2[a]	33.8 ± 1.9[a]	26.7±0.9[a]	27.7 ± 1.5[a]
Kibanda meno	22.0±2.3[a]	23.7 ± 3.6[a]	34.7±0.9[a]	33.6 ± 2.6[a]	33.0± 0.6[a]	29.8 ± 2.5[a]	22.7±0.3[a]	18.7 ± 1.9[a]
Serere	27.0±0.6[a]	22.2 ± 2.4[b]	37.7±1.5[a]	42.4 ± 3.1[b]	33.3±1.7[a]	30.5 ± 2.3[a]	31.3±0.9[a]	19.8 ± 1.6[b]

The values indicate the average numbers of embryogenic calli per explant cultured in media supplemented with different 2,4-D and picloram concentrations. Values followed by different letters are significantly different (P<0.05).

3.3.2 Effect of light on somatic embryogenesis

Light was observed to affect the process of somatic embryogenesis. Following four weeks in culture, the light-exposed explants formed more non-embryogenic calli than those kept in the dark (Table 3.3). *Adhiambo lera* and *Serere* showed the best responses with 36.6% and 33.8% of light-exposed explants developing somatic embryos respectively compared to 87.7% and 80.1% of dark incubated explants respectively. The two cassava varieties had the highest mean numbers of embryogenic calli per explants compared to *TMS 60444* and *Kibanda meno*, which had 32.5% and 31.5% of light-exposed explants producing somatic embryos respectively compared to 75.4% and 74.1% of dark incubated explants respectively.

Nevertheless, light is required for germination of embryos (Raemakers *et al.,* 1997). *Adhiambo lera* had the best response with regard to callus induction of explants exposed to darkness compared to other genotypes ($P<0.05$) (Table 3.3) (Appendix 5).

Table 3.3: Effect of light on somatic embryogenesis.

	Total No. of explants		**No. of explants with developed callus**		**Mean No. of embryonic calli /explant**	
	12	**0**	**12**	**0**	**12**	**0**
TMS 60444	124.0±1.7	125.0±1.7	40.3±1.2[a] **(32.5%)**	94.3±2.3[b] **(75.4%)**	12.7±1.8[a]	127.3±2.3[b]
Adhiambo lera	125.7±2.9	121.7±4.9	46.0±2.3[a] **(36.6%)**	106.7±1.8[b] **(87.7%)**	17.7±2.0[a]	161.3±5.8[b]
Kibanda meno	115.3±2.9	123.7±2.7	36.3±1.8[a] **(31.5%)**	91.7±1.2[b] **(74.1%)**	13.0±1.7[a]	130.3±0.9[b]
Serere	125.0±2.5	122.0±1.2	42.3±2.7[a] **(33.8%)**	97.7±2.6[b] **(80.1%)**	25.7±2.2[a]	159.3±1.2[b]

Medium; MS + 12 mg/L 2,4-D, 50mg/L Casein Hydrolysate, 0.5mg/L $CuSO_4$, Gamborg's B5 Vitamins. 12 denotes twelve hours of light; 0 denotes lack of light. Values with the same number are not statistically significant ($P<0.05$).

3.3.3 Effects of Copper Sulphate on somatic embryogenesis

The results of the effect of Copper sulphate on somatic embryogenesis were cultivar-dependent and consistent (Table 3.4). For cultures devoid of additional copper and those with 0.25mg/L, formation of embryogenic calli was not significantly different ($P>0.05$). Overall, 0.5 mg/L $CuSO_4$ produced the best response with regards to the number of embryonic calli per explants, with *Adiambo lera* showing the best response at all $CUSO_4$ concentrations (Table 3.4) (Appendix 7).

Table 3.4: Effect of different $CuSO_4$ concentration on somatic embryogenesis of cassava

	0.0mg/L	0.25mg/L	0.5mg/L	0.75mg/L	1mg/L
TMS 60444	23.7±2.0[a]	25.3±1.2[a]	43.7±1.9[b]	34.0 ±0.6[c]	27.3±3.1[a]
Adhiambo lera	31.0±1.2[a]	33.0±1.7[a]	62.7±1.2[b]	53.0±1.7[c]	44.3± 2.5[d]
Kibanda meno	25.7±2.2[a]	32.0 ±3.2[b]	52.3 ±1.9[c]	50.0 ±0.6[c]	39.0 ±0.6[d]
Serere	29.0±1.2[a]	32.0 ± 1.2[a]	59.0 ±0.6[b]	45.7 ±5.2[c]	40.0 ± 1.2[d]

The values indicate the average numbers of calli per explant cultured in media supplemented with different $CUSO_4$ concentrations. Values with the same letter are not significantly different (P>0.05).

3.3.4 Effect of sucrose on embryogenesis

Callus formation and embryogenesis hardly took place with the exclusion of sucrose from the callus induction medium (Table 3.5). The explants died after four weeks in culture. The number of embryos produced in 2-6 % sucrose media was cultivar dependent. The 2% sucrose concentration promoted formation of healthier embryos than the others. Explants grown on 6 % sucrose were all cream-colored and most turned brown after 4 weeks in culture. This is a sign of cell death caused by formation of phenolic compounds in the cultures (Ihemere, 2003). For *TMS 60444* and *Adhiambo lera* genotypes, there was no significant difference in the degree of somatic embryogenesis between the media supplemented with 2% and 4% sucrose (P>0.05). On the other hand, for the genotypes *Kibanda meno* and *Serere,* there were significant differences in terms of the mean numbers of embryonic calli among the three media supplemented with 2%, 4% and 6%

sucrose (Table 3.5). However, the response of *Adhiambo lera* significantly differed from that of *Serere* at 4% and 6% sucrose concentrations ($P<0.05$). *Kibanda meno* and the model cultivar *TMS 60444* had comparable responses at 2% and 4% sucrose ($P<0.05$; Table 3.5) (Appendix 6).

Table 3.5: Effects of different sucrose concentrations on somatic embryogenesis.

	Sucrose concentration (percent w/v)			
	0	**2**	**4**	**6**
Cultivar	**Mean Number of Embryogenic Calli**			
TMS 60444	0.0 ± 0.0	84.0 ± 2.3[a]	77.3 ± 1.7[a]	39.3 ± 1.2[b]
Adhiambo lera	0.0 ± 0.0	110.7 ± 5.8[a]	108.0 ± 2.3[a]	52.0 ± 1.2[b]
Kibanda meno	0.0 ± 0.0	89.3 ± 2.9[a]	79.0 ± 1.5[b]	32.0 ± 1.2[c]
Serere	0.0 ± 0.0	106.3 ± 3.6[a]	97.0 ± 1.5[b]	37.3 ± 1.8[c]

Values with the same letter are not significantly different ($P>0.05$).

3.3.5 Maturation of somatic embryos

The early stage somatic embryos were transferred to the maturation medium after 4 weeks of culture with adjoining callus. All the embryos grew to cotyledonary embryos with distinct root and shoot axes in all media compositions but showed differences in frequencies (Table 3.6). Three different maturation media differing in cytokinins content and composition were used in this experiment. Embryos maintained on MS medium supplemented with 2% sucrose, 1 mg/L thiamine-HCl, 100 mg/L myo-inositol, 0.01 mg/L 2,4-D, 1.0 mg/L BA and 0.5 mg/L GA_3 were superior to MS medium supplemented with 2 % sucrose, 0.01 mg/L NAA, 0.1 mg/L BA and 0.1 mg/L GA_3 and MS medium supplemented with 2 % sucrose.

More than 50% percent of embryonic calli produced cotyledonary embryos in the medium containing MS salts supplemented with 2 % (w/v) sucrose, 1 mg/L thiamine-HCl, 100 mg/L myo-inositol, 0.01 mg/L 2,4-D, 1.0 mg/L BAP and 0.5 mg/L GA_3. In the medium supplemented with 0.01 mg/L NAA, 0.1 mg/L BAP and 0.1 mg/L GA_3, more than 70% of embryonic calli hardly produced cotyledonary embryos. The medium supplemented with 2% sucrose promoted the least frequency of maturation (Figure 3.2). Overall the cultivar *Adhiambo lera* had the highest frequency of maturation of somatic embryos followed by *Serere* and *TMS 60444. Kibanda meno* had the least response in all the media compositions. The embryos first of all turned green and then progressively formed distinct shoot and root apices (Figure 3.3) (Appendix 7).

For all the four studied genotypes, there were significant differences in the frequencies of maturation of somatic embryos among the three media compositions, with the best response being observed in the media containing MS salts supplemented with 2% (w/v) sucrose, 1 mg/L thiamine-HCl, 100 mg/L myo-inositol, 0.01 mg/L 2, 4-D, 1.0 mg/L BAP and 0.5 mg/L GA_3 (Table 3.6).

Table 3.6: Effects of different cytokinins on maturation of somatic embryos

Cultivar	Mean No. of Cotyledonary Embryos			
	Initial no. of Embryonic Calli	**Maturation Media 1***	**Maturation Media 2†**	**Maturation Media 3‡**
TMS 60444	98.3±1.5	53.3±0.9[a]	23.9±0.9[b]	4.7±1.2[c]
Adhiambo lera	91.3±0.9	58.3±1.2[a]	33.0±1.2[b]	6.3±1.8[c]
Kibanda meno	90.7±1.2	46.3±1.5[a]	19.3±2.0[b]	3.3±1.2[c]
Serere	90.7±1.8	50.7±1.2[a]	23.0±1.7[b]	4.3±0.9[c]

Values with the same letters are not significantly (P>0.05)
*MS salts supplemented with 2 % (w/v) sucrose, 1 mg/L thiamine-HCl, 100 mg/L myo-inositol, 0.01 mg/L 2,4-D, 1.0 mg/L BAP and 0.5 mg/L GA_3;
†MS salts supplemented with 2 % (w/v) sucrose, 0.01 mg/L NAA, 0.1 mg/L BA and 0.1 mg/L GA_3;
‡MS basal medium supplemented with 2 % (w/v) sucrose.

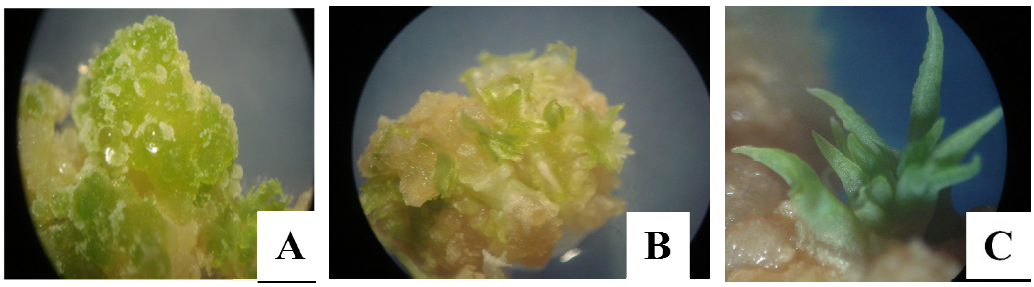

Figure 3.2: Tissue culture profile of maturation of embryogenic somatic embryos.

A: Greening of embryogenic embryos.

B: Initiation of shoots.

C: Formation of distinct shoots.

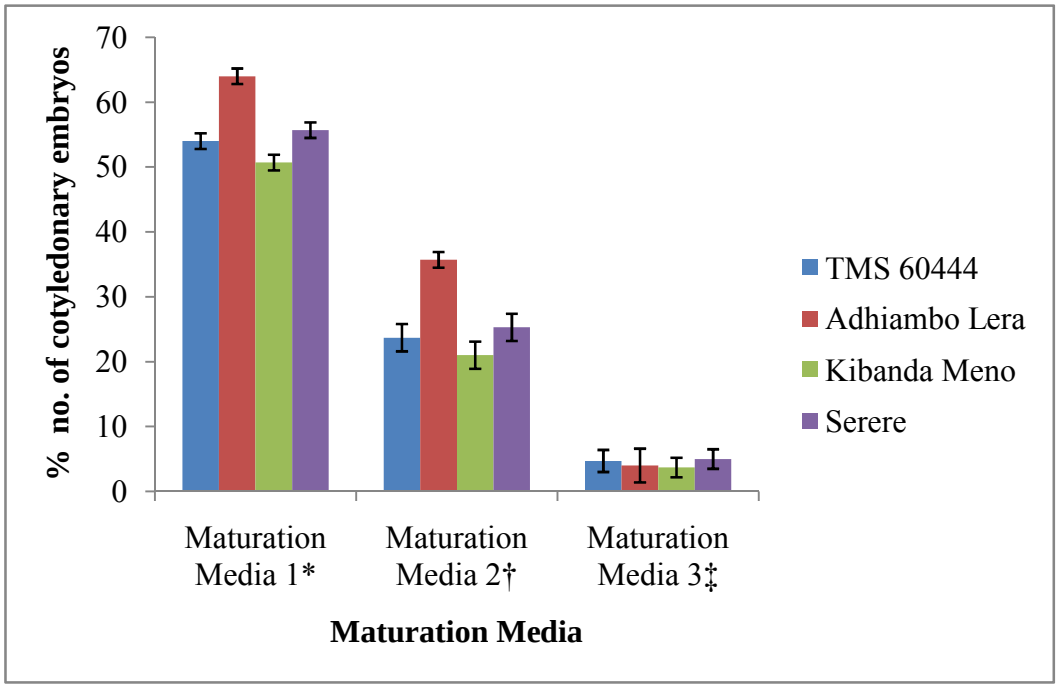

Figure 3.3: Effects of cytokinins on maturation of somatic embryos

*MS salts supplemented with 2 % (w/v) sucrose, 1 mg/L thiamine-HCl, 100 mg/L myo-inositol, 0.01 mg/L 2,4-D, 1.0 mg/L BAP and 0.5 mg/L GA_3
†MS salts supplemented with 2% (w/v) sucrose, 0.01 mg/L NAA, 0.1 mg/L BA and 0.1 mg/L GA_3
‡MS basal medium supplemented with 2% (w/v) sucrose.

3.3.6 Germination of somatic embryos and plant recovery

The germination medium comprising MS salts, B5 vitamins, 2% sucrose, and 0.8% activated charcoal was superior to basal MS medium plus 2% sucrose, and MS supplemented with 2% sucrose, 1 mg/L thiamine-HCl, 100 mg/L myo-inositol, 0.01 mg/L 2,4-D, 1.0 mg/L BA and 0.5 mg/L GA_3 in terms of germination time and frequency (Table 3.7). Single cotyledonary embryos did not always produce single plantlets. In some cases up to five plantlets arose from one embryo. The recovery of plantlets from embryos was genotype-dependent and it

was consistent. Overall, *Adhiambo lera* was the best genotype in terms of both germination time and frequency.

There was significant difference between *Adhiambo lera* and other genotypes in all media compositions in terms of germination frequency (Table 3.7) ($P<0.05$). For *TMS 60444,* there was no significant difference, in terms of germination frequencies of the matured somatic embryos, in the basal MS medium supplemented with B5 vitamins and 2% (w/v) sucrose and MS medium supplemented with 2% (w/v) sucrose, 1 mg/L thiamine-HCl, 100 mg/L myo-inositol, 0.01 mg/L 2, 4-D, 1.0 mg/L BAP and 0.5 mg/L GA_3 ($P>0.05$)..

On the other hand, for the genotypes *Adhiambo lera, Kibanda meno* and *Serere,* there was significant difference in terms of germination frequencies in all the three media compositions ($P<0.05$).

Table 3.7: Germination and Regeneration Frequencies of the four cassava genotypes

Cultivar	Number of Regenerated Plants			
	Initial No. of Cotyledonary Embryos	**Germination Media 1***	**Germination Media 2†**	**Germination Media 3‡**
TMS 60444	77.7±0.6	22.0±1.5[a]	23.7±1.8[a]	63.3±1.5[b]
Adhiambo lera	78.7±1.5	31.7±1.2[a]	37.0±2.5[b]	76.3±2.3[c]
Kibanga Meno	82.3±1.5	18.7±0.9[a]	23.7±0.3[b]	58.3±1.2[c]
Serere	79.3±1.9	23.0±0.6[a]	28.3±0.9[b]	67.7±1.5[c]

Values with the same letters are significantly different ($P<0.05$).
*basal MS medium supplemented with B5 vitamins and 2% (w/v) sucrose;
†MS medium supplemented with 2% (w/v) sucrose, 1 mg/L thiamine-HCl, 100 mg/L myo-inositol, 0.01 mg/L 2,4-D, 1.0 mg/L BAP and 0.5 mg/L GA_3;
‡MS basal salt supplemented with 2% (w/v) sucrose and 0.8% (w/v) activated charcoal

Upon germination, the recovered plants were grown in the growth room for two weeks following which they were hardened in the glasshouse. Growth of the regenerated plantlets is shown in Figure 3.4 below.

Figure 3.4: Recovery of regenerated cassava plantlets.
A: Young cassava plantlets immediately transferred to soil in glasshouse from growth room plantlets.
B: Mature cassava plants in glass house.
C: Mature cassava plantlets in the field outside glasshouse.
Media: MS basal salt supplemented with 2% (w/v) sucrose and 0.8% (w/v) activated charcoal.

3.4 DISCUSSION

In this study, a protocol for somatic embryogenesis and plant recovery for Kenyan cassava varieties has been optimized. Studies on African cassava varieties by Ihemere, (2003) established that 12 mg/L 2, 4-D was better than 12 mg/L Picloram for embryogenesis induction for most of the cultivars. In this study, it was established that 8mg/L 2, 4-D and Picloram produced the best responses in terms of callus induction and somatic embryogenesis in all cultivars. The 2, 4-D concentration of 8mg/L was better with regards to frequency of leaf lobe embryogenesis than Picloram (8mg/L) in all cultivars (Table 3.1 and 3.2). Embryogenesis was induced at all ranges of auxin concentrations (4-16mg/L) in all cultivars. There was no significant difference in the frequency of callus induction and somatic embryogenesis at auxin concentrations of 4mg/L and 16mg/L ($P<0.05$). Formation of embryogenic calli was consistent with the frequency of callus induction in all the genotypes.

Successful somatic embryogenesis in *Glycine max* on medium supplemented with 40 mg/L 2, 4-D has been accomplished in the past (Finer and Nagasawa, 1988). This is in spite of the studies by Konan *et al.* (1994b), who reported an inhibition of embryogenesis in cassava by the supplementation of the induction-medium with more than 12 mg/L of 2, 4-D. The observed disparities in the results of this study can be attributed to use of different explants. This study used young leaf lobes, while Konan's group used the cotyledons of zygotic embryos. This indicates that

young leaf lobes are better explants than cotyledons for cassava regeneration via somatic embryogenesis.

Many plant species regenerated *in vitro* through callus are bedeviled by somaclonal variation (Ihemere, 2003). This is postulated to be due to the high rate of cell division during callus formation induced by high auxin levels in the induction medium. This drawback is reduced in plants recovered through somatic embryogenesis. Konan *et al.,* 1994 observes that the incidence of somaclonal variation in cassava regenerated by somatic embryogenesis with 4 – 16 mg/L 2, 4-D is negligible. As a convention, embryogenesis in most plants starts with callus induction, followed by transfer to another medium for embryo emergence. In cassava, the explant on the induction medium proliferates to globular stage embryos in about two weeks of culture, hence reducing the length of time the regenerative tissues stay on high auxin (8-12 mg/L) medium (Mathews *et al.,* 1993). Perhaps this ameliorates the incidence of somaclonal variation further in cassava plants recovered from somatic embryos. Studies by Baba *et al.* (2008) have reported incidences of somaclonal variation in cassava regeneration systems. They observed abnormal cassava shoot elongation following addition of a combination of BAP and Picloram.

The drive to improve on the efficiency of cassava transformation has led to the development of additional techniques for plant regeneration. A classical example

of such efforts is the use of friable embryogenic callus to regenerate somatic embryos. This technique requires the callus to be on the high auxin medium (50 mg/L) for six or more months resulting in high rates of somaclonal variation (Raemakers *et al.,* 1997; Puonti-Kaerlas *et al.,* 1998). This approach has yielded regeneration results with minimal somaclonal variations in cassava. In their recent studies of regeneration frequency and efficiency of Swiss cassava lines, Beltrán *et al.* (2010) obtained high regeneration frequencies and efficiencies via production of friable embryogenic calli compared to somatic embryogenic calli.

Inclusion of additionl $CuSO_4$ in somatic embryogenesis medium in this study resulted in generation of distinct embryos at the early stages of embryogenesis. This observation corroborates with study results by Ihemere (2003), who observed somatic embryogenesis experiments with media lacking additional copper produced embryos difficult to distinguish from the non-embryogenic callus because they were too small. However, the inclusion of additional copper made the embryos distinct at the early stage of embryogenesis. It was later inferred that Copper is thought to enhance growth of embryos during somatic embryogenesis.

Although earlier studies indicate that callus formation inhibits plant regeneration (Zhang *et al.,* 2001), this study successifully regenerated cassava through somatic embryogenesis. It is assumed that the increased capacity of shoot organogenesis is at least partially related to the inhibition of callus formation (Zhang *et al.,* 2001).

Cassava regeneration via organogenesis involves the induction of shoots from young leaf lobes and embryo cotyledons on MS medium supplemented with cytokinins (23 µM zeatin and 44 µM benzylaminopurine) (Guohua, 1998; Mussio *et al.,* 1998). Earlier studies resulted in regeneration of multiple shoots but the origin of each shoot was doubtful making it a suspect for plant transformation purposes (Ihemere, 2003).

Embryo development achieved in somatic embrygenesis medium containing 2% sucrose, 1 mg/L thiamine-HCl, 100 mg/L my-oinositol, 0.01 mg/L 2, 4-D, 1.0 mg/L BAP and 0.5 mg/L GA_3 was superior to the medium containing 2% sucrose, 0.01 mg/L NAA, 0.1 mg/L BAP and 0.1 mg/L GA_3 and the medium containing 2% sucrose but devoid of phytohormones. This agrees with Mathews *et al*. (1993), where globular stage embryos of cassava were cultured on medium without plant growth regulators and achieved the highest rate of regeneration.

Culturing explants in light at the beginning of the induction process led to reduction in the number of embryos formed. This indicates that light-intensity affects embryogenesis. Embryogenesis in cassava is enhanced by lowering light intensity (Raemakers *et al.,* 1997). Studies on the effect of light on callus growth and somatic embryogenesis from *Lavandula vera,* Mill and *Teucrium chamaedrys,* L showed that the relative length of the incubation period under illumination significantly affected callus growth and somatic embryo induction and

proliferation, although in a species-specific fashion (Spiridon *et al.,* 2002). Lavender callus growth was improved under increased incubation in darkness while somatic embryogenesis was remarkably reduced under the same conditions (Spiridon *et al.,* 2002). The role light in somatic embryogenesis is still obscure (Baba *et al.,* 2008).

Further, somatic embryogenesis from some species, such as cucumber, squash, melon and gardenia is promoted under initial culture incubation in darkness (Cade *et al.,* 1988; Kintzios *et al.,* 1998). It is, thus, thought that lowering the light intensity of the cultures could have accounted for the high rates of embryogenesis in all the cultivars studied in this research. Successful somatic embryogenesis and plant recovery in the African cassava cultivars provides a method for the cassava transformation as a gateway to improvement of agronomic and nutritional quality of cassava.

In this study, it was observed that germination frequencies of somatic embryos in the four genotypes under study were highest in the medium supplemented with activated charcoal. This agrees with the studies by Zhang *et al.* (2000a), who observed similar results while evaluating the role of activated charcoal in cassava regeneration via direct shoot organogenesis. In their study, it was inferred that activated charcoal scavenges phenolic compounds that are inhibitory to shoot formation.

CHAPTER FOUR

GENETIC TRANSFORMATION OF KENYAN CASSAVA GENOTYPES

4.1 INTRODUCTION

Cassava (*Manihot esculenta*) is one of the most important food crops in the tropics and subtropics. Traditional breeding of cassava is difficult due to irregular flowering and low fertility as well as low seed set and germination rates of the plants, and attempts to improve the quality of cassava roots have so far been unsuccessful. Advances in plant genetic engineering now provide an alternative to traditional breeding in improving cassava. The recent development of transformation methods (Bull *et al.,* 2009) will allow the addition of agriculturally valuable traits to cassava for improved root quality. Methods used to introduce DNA into plant species include transformation using *Agrobacterium* and direct gene transfer methods such as protoplast transformation, electroporation, microinjection, co-cultivation and microprojectile bombardment.

Cassava was considered to be a recalcitrant species for genetic engineering (Li *et al.,* 1996; Raemakers *et al.,* 1996; Schopke *et al.,* 1996; González *et al.,* 1998; Zhang, 2000). However, recent studies have optimized successful transformation protocols for a wide variety of cassava genotypes (Bull, *et al.,* 2009). This chapter reports successful *Agrobacterium*-mediated transformation of regenerable Kenyan cassava varieties using immature leaf lobes as explants.

4.2 MATERIALS AND METHODS

4.2.1 Plant material

Sterile *in vitro* cassava plants were initiated from immature stem cuttings obtained from 4 to 6 months-old growth room-grown plants. Stems were excised, and divided into pieces with two to three nodes that were then surface sterilized to arrest contamination. The stem pieces were cultured in MS shoot multiplication medium [Murashige and Skoog media (1962) supplemented with 20g/L sucrose, MS vitamins (Duchefa, Germany) and 8g/L of noble agar]. Cultures were grown at 28°C under a 12-hour light/12-hour dark regime until young actively growing apical leaves emerged in about 4 weeks following culture. The genotype *TMS 60444* was used as a control in transformation experiments.

4.2.2 *Agrobacterium* preparation

Agrobacterium strain LBA4404 harbouring the binary vector plasmid pCAMBIA 2301 with the RNAi construct of *CYP79D1/D2* was streaked and grown for 2 days on solid YEP medium supplemented with 50 mg/L streptomycin and 50 mg/L kanamycin. Cultures were incubated at 28°C. After 2 days, single colonies were used to inoculate 20 mL of liquid YEP medium supplemented with 200 μ*M* acetosyringone, 50 mg/L streptomycin, 50 mg/L kanamycin and incubated at 28°C on a shaker for 2 hours. Kanamycin resistance gene had been inserted in the transformation gene cassette between the left and right borders. Kanamycin serves as a marker for transformation. The bacteria were then pelleted by centrifugation

(10,000*g*) for 10 min, and resuspended in 20 mL of MS basal salt (co-culture) medium devoid of nitrogen, but supplemented with 1% (w/v) of glucose and 1% (w/v) of galactose, and the pH adjusted to 5.5. At this stage the bacteria were ready for inoculating the explants.

4.2.3 Explant inoculation and co-cultivation

Young actively growing apical leaves were excised from clean cultures, placed onto a sterile filter paper and wounded using a sterile needle. Wounded apical leaves were then placed with the adaxial surface on co-cultivation medium [MS basal medium supplemented with 2 % (w/v) sucrose, B5 vitamins, 50 mg/L casein hydrolysate, 0.5 mg/L $CuSO_4$ and 8 mg/L 2, 4-dichlorophenoxyacetic acid supplemented with 200 μ*M* acetosyringone]. Two drops of the bacterial suspension in liquid MS [to which was added two drops of Tween-20] were applied to the explant tissue, which was then incubated in the dark for 2 days at 28°C for co-culture. Subsequently, the explants were transferred to a new co-cultivation medium for another 3 days of co-cultivation. Following co-cultivation with *A. tumefaciens*, explants were transferred to selection media [MS basal medium supplemented with 2 % (w/v) sucrose, B5 vitamins, 50 mg/L casein hydrolysate, 0.5 mg/L $CuSO_4$ and 8 mg/L 2,4-dichlorophenoxyacetic acid supplemented with 50mg/L kanamycin and 500 mg/L of carbenicillin) to induce embryogenesis. For negative control, a separate set of explants was not co-cultivated with *A. tumefaciens* but was transferred into the selection media. The cultures were

incubated on a 12hr day/12hr night cycle for 4 weeks. The cultures were maintained on selection medium for 4 weeks.

4.2.4 Selection and regeneration of putative transformants

Somatic embryos produced on selection media were transferred to selection shoot regeneration medium (MS basal medium with 1 mg/L thiamine, 100 mg/L myo-inositol, 2% (w/v) sucrose, 1.0 mg/L BAP and 0.5 mg/L GA_3 at pH 5.7 supplemented with 50mg/L kanamycin and 500 mg/L of carbenicillin) to induce the development of cotyledonary leaves. Survival of regenerants in the medium containing kanamycin is indicative of transgenicity because the transformation gene construct contained kanamycin resistance gene. Once the individual somatic embryos germinated and formed shoots they were transferred to cassava micropropagation medium (MS salts, 2% (w/v) sucrose and activated charcoal, pH 5.7) for plant recovery for four weeks. All the cultures were incubated at 28°C under a 12-hour light/ 12-hour dark regime.

Regenerated putative transgenic plantlets were transferred to sterile peatmoss and grown in confinement within the glasshouse to adhere to biosafety rules under natural lighting conditions and a temperature range of 22–30°C. The transferred plants were covered with transparent plastic bags to maintain high humidity. The bags were removed after 2 weeks and the plants were transferred to normal soil to

expose them to normal environment. The transformed plants were given the same water treatment as the wild-type cassava plants.

4.2.5 Molecular analysis of putative transgenic cassava lines

4.2.5.1 PCR analysis

Genomic DNA was isolated from leaves of 3 months old grown wild-type plants and kanamycin-resistant putative transformants. Wild-type plants used were not transformed but grown in tissue culture media (without selection) similar to the putative transformants. The presence of the *CYP79D1/D2* gene was detected by PCR amplification. Polymerase Chain Reaction (PCR) reactions were performed in a total volume of 25 µL containing: 1X PCR buffer, 20-100 ng of leaf DNA, 0.1 mM each dNTP, 1 unit Taq polymerase, 0.4 µM each primer. The *CYP79D1/D2* gene was amplified using forward (5'-TGGCCATGAACGTCTCCACC-3') and reverse (5'-TGGAGCCATTTGTGTCTTGC-3') primers. The PCR amplification profile was as follows; 3 minutes at 94^{o}C, 30 seconds at 94^{o}C, and 45seconds at 58^{o}C and 30 seconds at 72^{o}C for 30 cycles. The PCR product was run on 1% (w/v) agarose gel at 70V in 0.5X TBE buffer. Control experiments were carried out with DNA from the respective wild type cultivars.

4.2.5.2 Reverse transcriptase-PCR (RT-PCR) analysis

This was done to determine the abundance of transgene insertion in the putative transgenics. Total RNA was extracted from 100 mg of *in vitro* putative

transformed root material using a Qiagen Plant RNA Extraction Kit (Qiagen Inc., Valencia, CA). 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 manufacturer's instructions. The first strand cDNA synthesis was carried out with 10 µg of total RNA using 1X reverse transcription buffer (50 mM Tris-HCl pH 8.0, 75 mM KCl, 3 mM $MgCl_2$) 0.3 mM dNTP, 0.5 µg oligodT(12-18) primers, and 200 units of SuperScript II reverse transcriptase (Life Technologies, Rockville, MD). The mixture was incubated at 65^{o}C for 5 minutes without reverse transcriptase followed by incubation at 42^{o}C for 1 hour with reverse transcriptase. The synthesized cDNA was amplified by PCR using 1X PCR buffer, 1.5mM $MgCl_2$, 0.1 mM dNTP, 2.5 units of Taq polymerase, 0.4 µM of each primer specific for *CYP79D1* gene. The forward primer sequence is 5'-TGGCCATGAACGTCTCCACC-3' and the reverse primer sequence is 5'-TGGAGCCATTTGTGTCTTGC-3'. The PCR conditions were as follows: 3 minutes at 94^{o}C, 30 seconds at 94^{o}C, 45 seconds at 58^{o}C and 30 seconds at 72^{o}C for 30 cycles. The PCR product was run on 1% (w/v) agarose gel at 70V in 0.5X TBE buffer. Control experiments were carried out without cDNA (negative control) and by amplification of the *CYP79D1* gene. A separate PCR was conducted using primers specific to cassava starch branching enzyme- II (SBE-II) gene to serve as internal control.

4.2.5.3 Southern blot analysis of the transgenic plants

Genomic DNA was isolated from leaves of 3 months old grown wild-type plants and kanamycin-resistant putative transgenics using a protocol adapted from Soni and Murray, (1994). Upon DNA isolation, Southern blotting was undertaken following a standard protocol adapted from Sambrook *et al.* (1989).

Approximately 10 µg of genomic DNA was digested with *Kpn1* followed by fractionation on a 0.8% agarose gel. The fractionating gel was then soaked in HCl (250 mM) for 12 minutes, denaturation buffer comprising 1.5 M NaCl and 0.5 M NaOH for 50 minutes and neutralization buffer comprising 1 M Tris-HCl and 1.5 M NaCl for 35 minutes. Constant gentle agitation accompanied each soaking stage following, there was a short-lived rinsing with distilled water. The DNA was then transferred to nitrocellulose membrane for 20 hours. Transferred DNA was cross-linked to the membrane by UV light exposure for 40 seconds. Membrane prehybridization was done for a minimum of 1hour, 45 minutes at 40^0C in CHURCH buffer (0.5 M $NaHPO_4$ (pH 7.2), 1 mM EDTA, 1% (w/v) BSA, 7% (w/v) SDS). Hybridization of the membrane was conducted at 40^0C with Biotin-labeled *CYP79D1* probe for 20 hours. The probe was made using PCR primers specific to *CYP79D1* and the PCR reaction was performed using 1X PCR buffer, 1.5mM $MgCl_2$, 0.1 mM dNTP, 2.5 units of Taq polymerase, 0.4 µM of each primer specific for *CYP79D1* gene.

The biotin-labeled probe was then cleaned using Qiagen PCR cleaning kit (Qiagen Inc, Valencia, CA) and boiled for 5 minutes prior to addition to the membrane. Following hybridization, the membrane was washed with 1X Saline-Sodium Citrate (SSC) containing 0.1% (w/v) SDS at 45^0C for 20 minutes followed by two washes with 0.1X SSC buffer containing 0.1% (w/v) SDS each at 45^0C for 20 minutes. The membrane was then exposed to an imager overnight.

4.2.6 Data analysis

The data on regeneration of the putative transformants was analysed in terms of regeneration frequency, regeneration efficiency and transformation frequency. Regeneration frequency was calculated as the number of germinated shoots divided by the total number of recovered calli events in germination medium (GM). Regeneration efficiency was calculated as the total number of regenerated shoots divided by the total number of infected explants. Transformation frequency was calculated as the total number of recovered calli events on GM divided by the total number of infected explants. Means of the number of explants, live calli on selection media, live calli on germination media and regenerated shoots were also computed. Minitab statistical software (version 2012) was used for data analysis.

4.3 RESULTS

4.3.1 *Agrobacterium*-mediated transformation and regeneration of cassava genotypes

Four cassava cultivars were transformed via *Agrobacterium*-mediated gene transfer. Transformed cells underwent somatic embryogenesis in 4-6 weeks on embryogenesis medium supplemented with 50 mg/L kanamycin and 500 mg/L carbenicillin. Some embryogenic calli kanamycin survived selection, although some portions of embryogenic calli were dying (Figure 4.1). Kanamycin resistance indicates that most tissues that regenerate are transgenic. Kanamycin-resistant embryos converted to plantlets in 1-2 months. Plantlets were then transferred to individual test culture bottles after 1 month. The individual plants in culture bottles were grown for 2 weeks and were then transferred to the greenhouse and later to the glass house (Figure 4.2). The negative control explants that were not *Agro*-co-cultivated but were transferred to selection media, did not survive and they all died.

The trend of transformation of the regenerable cassava cultivars was genotype dependent and consistent with regeneration data. *Adhiambo lera* showed the best response in terms of transformation frequency, regeneration frequency and regeneration efficiency, followed by *Serere, TMS 60444* and *Kibanda meno* (Table 4.1). There was regeneration of the transformed cultivars for all varieties.

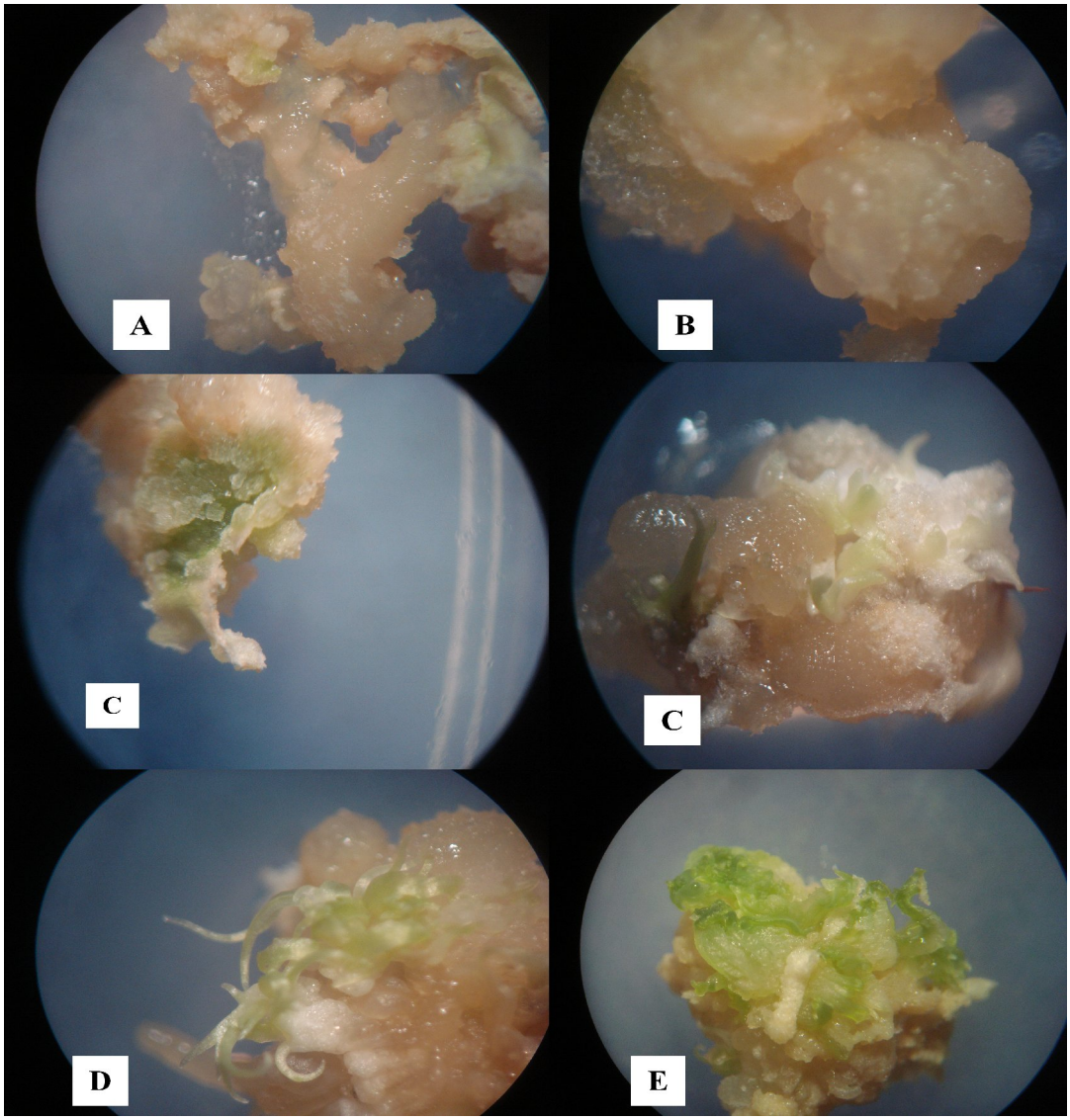

Figure 4.1: Tissue culture profile of transformed cassava lines. (Mag. X1000)

A: Initiation of callogenesis in surviving infected explants.
B: Embryogenic calli from the surviving explants.
C: Matured embryos from the surviving embryogenic calli (Greening).
D: Germination of mature embryos into cotyledonary embryos.
E: Continued germination of cotyledonary embryos surviving selection with distinct shoots.

Table 4.1: Transformation, callus recovery and regeneration of cassava lines

Cultivar	No. of Explants	SM Live Calli	GM Calli	Shoots	RF%	TF%	RE%
TMS 60444	126	67	38	26	38.81	30.16	2.06
Mean ± SEM	29.9±1.4	19.4±1.1	5.8±1.3	5.2±0.5			
Adhiambo lera	119	80	53	39	48.75	44.54	3.28
Mean ± SEM	33.2±1.1	23.4±1.6	12.3±0.6	8.7±0.3			
Kibanda meno	121	62	34	29	46.77	28.10	2.40
Mean ± SEM	24.2±1.7	15.3±1.4	6.7±1.1	4.0±0.7			
Serere	124	71	45	32	45.07	36.29	2.68
Mean ± SEM	44.6±1.4	18.3±1.2	9.4±0.8	4.2±0.4			

RF (%), Regeneration Frequency = Number of shoots germinated over total number of recovered calli events in germination medium (GM)
TF (%), Transformation Frequency = Total number of recovered calli events on GM over total of infected explants
RE (%), Regeneration Efficiency = Total number of shoots regenerated over total number of explants infected

Figure 4.2: Transgenic cassava lines at different stages of growth.

A: Young transgenic cassava plantlet growing in soil in glasshouse immediately after transfer from culture bottles.
B: Mature transgenic cassava plants continuing growth in glasshouse.
C: Fully mature transgenic cassava plants in bio-containment field.
Mature refers cassava plants growing but not to the level of producing tubers.
Fully mature refers to cassava plants grown to the level of producing tubers.

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

The kanamycin-resistant putants were screened by PCR for the *CYP79D1* gene. Each putant was obtained from a unique explant and not from a secondary somatic embryo. Similarly, untransformed wild-type plants used in all analyses were generated from independent explants via the same tissue culture procedures used to generate putative transformants. Twelve PCR-screened putants were positive for the *CYP79D1/D2* genes (Figure 4.3).

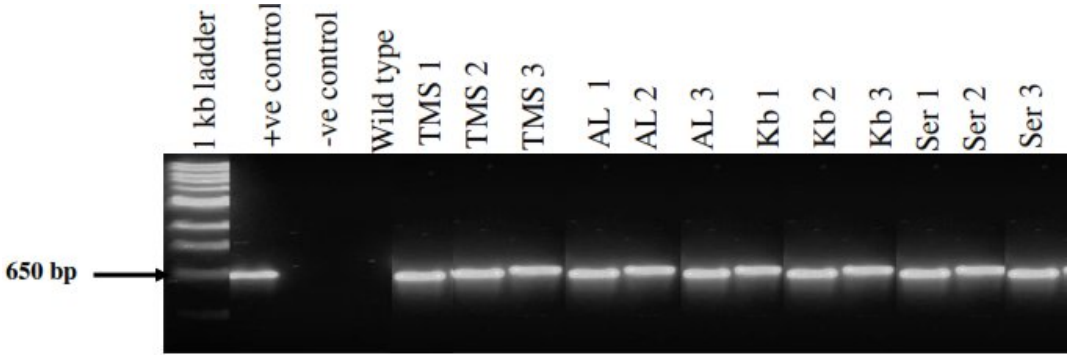

Figure 4.3: PCR analysis of putative cassava transformants

Given below are three independent transgenic events for:
TMS 1, TMS 2, TMS 3*-TMS 60444*
AL 1, AL 2, AL 3*-Adhiambo Lera*;
Kb 1, Kb 2, Kb 3 *-Kibanda Meno;*
Ser 1, Ser 2, Ser 3 *-Serere.*

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

The cDNA from transformed plants had negative and reduced amplification of a *CYP79D1/D2* gene reverse transcriptase product compared with the wild-types. Three transgenic plants (*Kb 2, Ser 2* and *Ser 3*) had reduced levels of the *CYP79D1/D2* transcripts. Conversely, nine transgenics (*TMS 1, TMS 2, TMS 3, AL*

1, AL 2, AL 3, Kb 1, Kb 3 and Ser 1) seldom had any detectable *CYP79D1/D2* transcripts. Polymerase chain reaction (PCR) of the internal control was run in parallel (Figure 4.4).

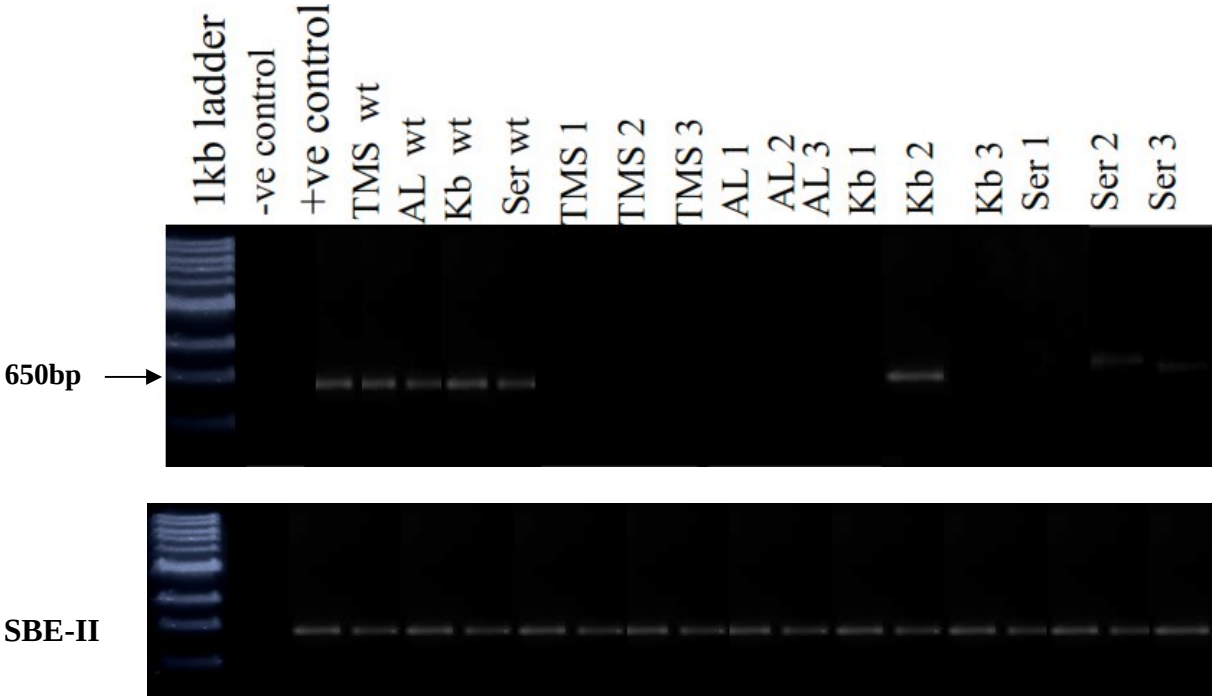

Figure 4.4: RT-PCR analysis of putative cassava transformants leaves

TMS wt **=** *TMS 60444* wild type; ***AL wt*** = *Adhiambo lera* wild type***; Kb wt*** **=** *Kibanda meno* wild type***; Ser wt*** **=** *Serere* wild type. The other labels indicate three independent transgenic events for *TMS 60444*, *Adhiambo lera*, *Kibanda meno* and *Serere* respectively

4.3.4 Southern blot analysis

Southern blot analysis was undertaken to confirm the PCR and RT-PCR analysis results for transgene integration. Genomic DNA of twelve putative transgenics along with one one wild type cultivar were restricted with *Kpn1* and probed with

biotin-labeled *CYP79D1/D2*. As figure 4.5 shows, the copy numbers of the inserted transgene ranged from one to three. *TMS 2* and *Ser 3* had one copy number of transgene insertion. *TMS 1, TMS 3, AL 1, AL 2, Kb 2, Ser 1* and *Ser 2* had two copy numbers of transgene insertion. Three copy numbers of transgene insertion occurred in *AL* 3, *Kb* 1 and *Kb* 3 (Figure 4.5).

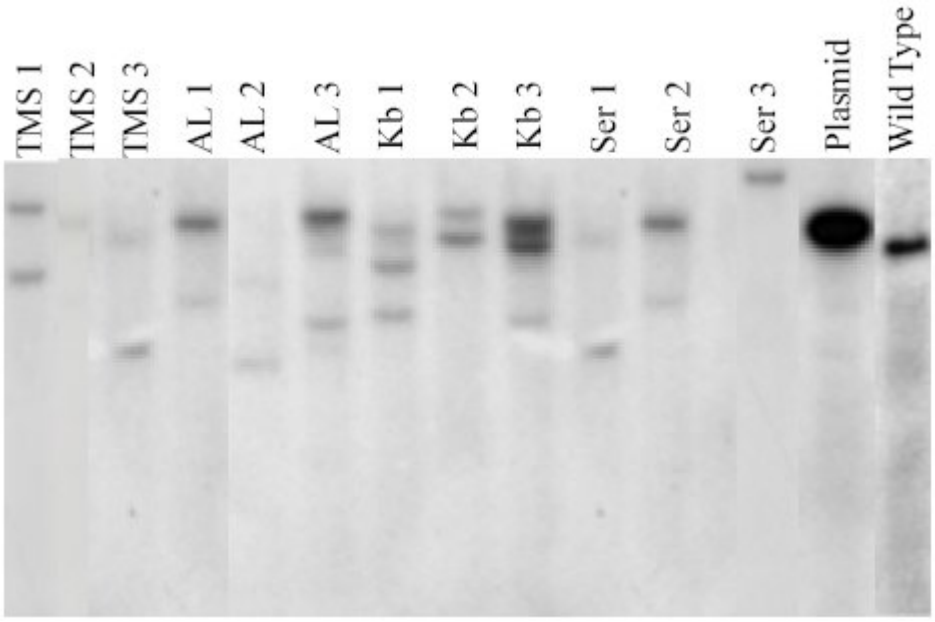

Figure 4.5: Southern blot analysis of putative cassava transgenics

TMS 1, TMS 2, TMS 3 = Three independent transgenic events for *TMS 60444*;
AL 1, AL 2, AL 3 = Three independent transgenic events for *Adhiambo lera*;
Kb 1, Kb 2, Kb 3 = Three independent transgenic events for *Kibanda meno;*
Ser 1, Ser 2, Ser 3 = Three independent transgenic events for *Serere*.

4.4 DISCUSSION

Successful transformation and regeneration of four cassava lines and a model line, was demonstrated in this study. The genotype *TMS 60444* was used as model cultivar while the others were Kenyan cassava lines. The study results indicate that immature leaf lobes are also good explants for transformation of cassava. Friable embryonic calli (FEC) and germinated somatic embryos have previously been used as explants in cassava transformation (Taylor *et al.,* 2001; Siritunga, 2002; Ihemere, 2003; Hankoua *et al.,* 2006). The transformation and regeneration frequencies of the transformed cassava lines were genotype dependent and consistent. Similar findings were observed by by Hankoua *et al.* (2006) who reported genotypic related transformation and regeneration frequencies and efficiencies of cassava genotypes *TME 13, TME 127, TME 8, TME 1, TMS I 91/02327* and *TMS 60444.*

Agrobacterium strain LBA4404 harbouring pCAMBIA 2301 was used for transformation cassava in this study. This has previously been found to be more efficient in T-DNA transfer than other strains. Previous reports describing cassava transformation have shown that the *Agrobacterium* strain LBA4404 was suitable for the transformation of the cassava genotype *MCol 22* (Li *et al.,* 1996) due to the presence of duplicated Vir D region on the co-integrated plasmid pTOK233 of LBA4404 that enhanced the virulence of such *Agrobacterium* strains in rice (Hiei *et al.,* 1994) and cassava (Li *et al.,* 1996). This may account for the slightly higher

transformation frequencies and efficiencies observed in this study.

In embryogenic callus of cotton, LBA4404 was also shown to be more efficient in producing transformants than C58C3 (Jin *et al.,* 2005). In this study, LBA4404 harbors pCambia2301 instead of pDgusbin19 in the work of Jin *et al.* (2005). This, therefore, demonstrates that the superiority of LBA4404 strain in infecting both cassava and cotton tissues are independent of the binary plasmid type, but it is rather associated with the nature of the LBA4404 genome.

Agrobacterium strain EHA105, a derivative of A281, has previously been shown to be unsuitable for cassava transformation (Li *et al.,* 1996). However, it was surprisingly found to be highly virulent with the cassava genotypes *TME 13, TME 127, TME 8, TME 1, TMS I 91/02327* and *60444* (Hankoua *et al.,* 2006). These observations clearly demonstrate that in cassava, the virulence of *Agrobacterium* strains varies based on the *Agrobacterium* strain, cassava genotype, tissue type, and the T-DNA transfer ability of the binary or co-integrated resident plasmids present in the disarmed *Agrobacterium* vectors as previously demonstrated in *Chrysanthemum moriflolium,* Ramat (Bush and Pueppke, 1991).

Although the results of transformation frequencies and efficiencies of the transformed cassava in this study were slightly higher than other studies, it is clear that the transformation frequencies and efficiencies achieved in this study were

still low (Table 4.1). The reason for the low efficiency might be attributed to the selection pressure of transformed tissues. The antibiotic typically used in selection of transformed tissues in this study is kanamycin, which, together with hygromycin, are considered very toxic to cassava tissues. This can possible be ameliorated by use of the kanamycin analogue, paromomycin, in selecting the transformed cassava tissues (Shopke *et al.,* 1996).

Polymerase chain reaction (PCR) and RT-PCR positive, transgenic cassava plants have been recovered in this study. This indicates that this transformation system has significant replicable potential for use in genetic transformation programmes at least for the apparently recalcitrant African cassava genotypes.

Results of southern blot analyses show that there was minimal transgene insertion in the transgenic studies. This makes it possible for further segregation analysis on the transgenic varieties generated in this study. This also underscores the advantages of *Agrobacterium*-mediated plant transformation over particle bombardment. Particle bombardment leads to multiple gene insertion, thus making segregation analysis difficult (Sititunga, 2002).

In summary, this study has demonstrated successful and reproducible transformation of three Kenyan cassava landraces and the model cultivar *TMS 60444* using immature leaf lobes as explants.

CHAPTER FIVE

DETERMINATION OF CYANIDE CONTENT IN TRANSGENIC CASSAVA LINES

5.1 INTRODUCTION

Cyanide occurs in cassava in the form of two cyanogenic glycosides, linamarin and lotaustralin. Hydrolytic enzymes which are capable of breaking down these cyanogenic glycosides to free cyanide (hydrocyanic acid, HCN) are also present in the plant (Heuberger, 2005). However, under normal conditions, they are separated from the substrate. Any process that ruptures the cell walls will bring the enzymes into contact with the glycosides and will thus release free cyanide and reduce the glycosides' content of the final product (Heuberger, 2005).

Cassava toxicity in humans is a well-documented problem. Cassava tubers vary widely in their cyanogen content, although most varieties contain 15 to 400 mg HCN per kg fresh weight (Padmaja, 1995). Cyanide doses of 50 to 100mg are reportedly lethal to adults (Halstrom and Moller, 1945). Several diseases are associated with the consumption of inadequately processed cassava roots, such as tropical ataxic neuropathy, endemic goiter and spastic paraparesis (Konzo) (Adewusi and Akintonwa, 1994; Siritunga, 2002; Heuberger, 2005). Konzo is mainly a disease of women and children. It is an acute disease, rapidly and permanently crippling the victim by damaging nerve tracts in the spinal cord that

transmit signals for movement, causing a spastic paralysis of both legs (Howlett *et al.,* 1990).

The different techniques of processing cassava roots aim to reduce the levels of cyanogenic compounds in order to obtain a safe food. The traditional methods usually include chipping, soaking, fermentation, cooking, steaming, drying and roasting. They all permit the enzyme linamarase to interact with the cyanogenic compounds to release HCN. The HCN then either dissolves in water or escapes into the air. However, it is often impossible to remove all the cyanogenic compounds through conventional processing (Heuberger, 2005). Genetic engineering approach offers an alternative method to reduce cyanogenic compounds in cassava.

Different methods are available for the quantitative determination of cyanogenic compounds (linamarin, cyanohydrin and free cyanide). The majority require three steps. The first step, extraction of cyanogens, is normally carried out in dilute acid (Bradbury *et al.,* 1994) to stop the degradation of cyanogenic compounds. The second step involves degradation of linamarin to cyanohydrin and glucose and, subsequently, to HCN. This can be achieved either by autolysis, which relies on the endogeneous linamarase (Cooke and de la Cruz, 1982), by enzymatic hydrolysis by adding exogenous linamarase (Rao and Hahn, 1984) or by alkalinic hydrolysis by addition of NaOH.

For the third step, determination of HCN, various methods have been developed, such as titration with $AgNO_3$ (AOAC, 1990), reaction with alkaline picrate (Egan *et al.,* 1998), and, most widely used, the photometric method based on the König reaction (Cooke, 1978; O'Brian *et al.,* 1991; Essers *et al.,* 1993).

Several other methods are available and have been reviewed by Bradbury *et al.* (1994). The method based on the König reaction is suitable for a laboratory with limited equipment and for field analysis, and hence it was chosen for this study.

This chapter aims at determining the cyanide levels in both wild type and transgenic cassava lines used in this study. This is in an effort to corroborate the molecular analysis results of this study with biochemical analysis.

5.2 MATERIALS AND METHODS

5.2.1 Determination of cyanogenic compounds

5.2.1.1 Sample preparation

Kanamycin-resistant transgenic cassava lines and the wild type cassava were analyzed for cyanogenic compounds content. Cassava roots were harvested, peeled, cut to pieces and washed three times with cold water. Since cyanide content varies both longitudinally and radially (Bradbury *et al.,* 1991), a homogeneous sample was obtained by removing both the stem end and the distal end of the tuber and cutting the root into cubes. They were dried at room temperature (25^{o}C) and were then ground to powder. Leaves were also harvested from both transgenic and wild type cassava, washed once with cold water and dried at room temperature after which they were ground to powder. For determination of distribution of total cyanide content in roots, powder from different root parts was prepared separately for separate analysis. The different parts of the root prepared were central disc, distal root tip, apical root tip, outer radial part and root cortex (Figure 5.1).

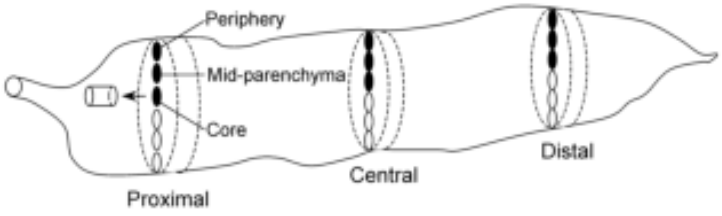

Figure 5.1: Longitudinal section of a cassava root (Chavez *et al.,* 2008)

5.2.1.2 Extraction of cyanogenic compounds

Samples of 10 g were homogenized in 30 ml of acid extraction medium (Polytron). The amount of sample varied, but the ratio of sample to extraction medium was always approximately 1:3. The homogenized samples were left to stand for 10 min and then centrifuged at 10,000*g* for 10 min (Beckman, J-25i, Fullerton, USA). The supernatant was stored at 4°C until assayed for cyanogenic compounds.

5.2.1.3 Enzymatic procedure

The reagent solutions used in enzymatic asays were prepared as follows; Phosphate buffer pH 7.0, 6.0 and 4.0 were prepared from 0.1 M H_3PO_4 and 0.1 M Na_3P0_4. Linamarase from BDH was dissolved in phosphate buffer pH 6.0 to give an activity of 5 enzyme units (EU)/ml (hydrolysis of 5 μmol of linamarin per min at 30°C in phosphate buffer, pH 6.0). Chloramin T reagent was prepared by dissolving 0.5 g of chloramin T in 100 ml water. The isonicotinic acid/barbituric acid reagent was prepared by dissolving 3.5 g barbituric acid and 2.85 g isonicotinic acid in 0.5 M NaOH solution. The pH of this reagent was adjusted between 7 and 8 with 2 M HCl or NaOH, respectively. Acetone cyanohydrin, used to calibrate the samples, was prepared as follows: A stock solution of 628 mg cyanohydrin per litre in 0.1 M phosphoric acid (corresponding to 200 mg HCN/L) was diluted in 0.1 M phosphoric acid so that the standard solutions contained 3.1, 9.4, 15.7, 25.1, 31.4, 47.1 and 62.8 mg/l cyanohydrin (corresponding to 1, 3, 5, 8, 10, 15 and 20 mg HCN/L).

The assay procedures were done as follows;

i. Total cyanide (cyanogenic glycosides + cyanohydrin + HCN)

In a stoppered 1.5 ml tube 0.1 ml extract and 0.05 ml linamarase were added to 0.45 ml phosphate buffer pH 7.0. After incubation at 37°C for 30 min, the mixture was transferred to a 15 ml tube containing 0.6 ml 0.2 M NaOH. After 5 min, the sample was diluted with additional 2.8 ml phosphate buffer (pH 6.0) and analyzed in the spectrophotometric procedure (Figure 5.2).

ii. Free cyanide (cyanohydrin + HCN)

An amount of 0.1 ml extract was mixed with 0.4 ml phosphate buffer pH 4.0 in a 15 ml tube, and 0.6 ml 0.2 M NaOH was added. After 5 min, 2.9 ml phosphate buffer (pH 4.0) was added and the mixture was analyzed spectrophotometrically (Figure 5.2).

iii. HCN

In a 15 ml tube, 0.1 ml extract and 3.9 ml phosphate buffer (pH 4.0) were mixed and analyzed spectrophotometrically (Figure 5.2).

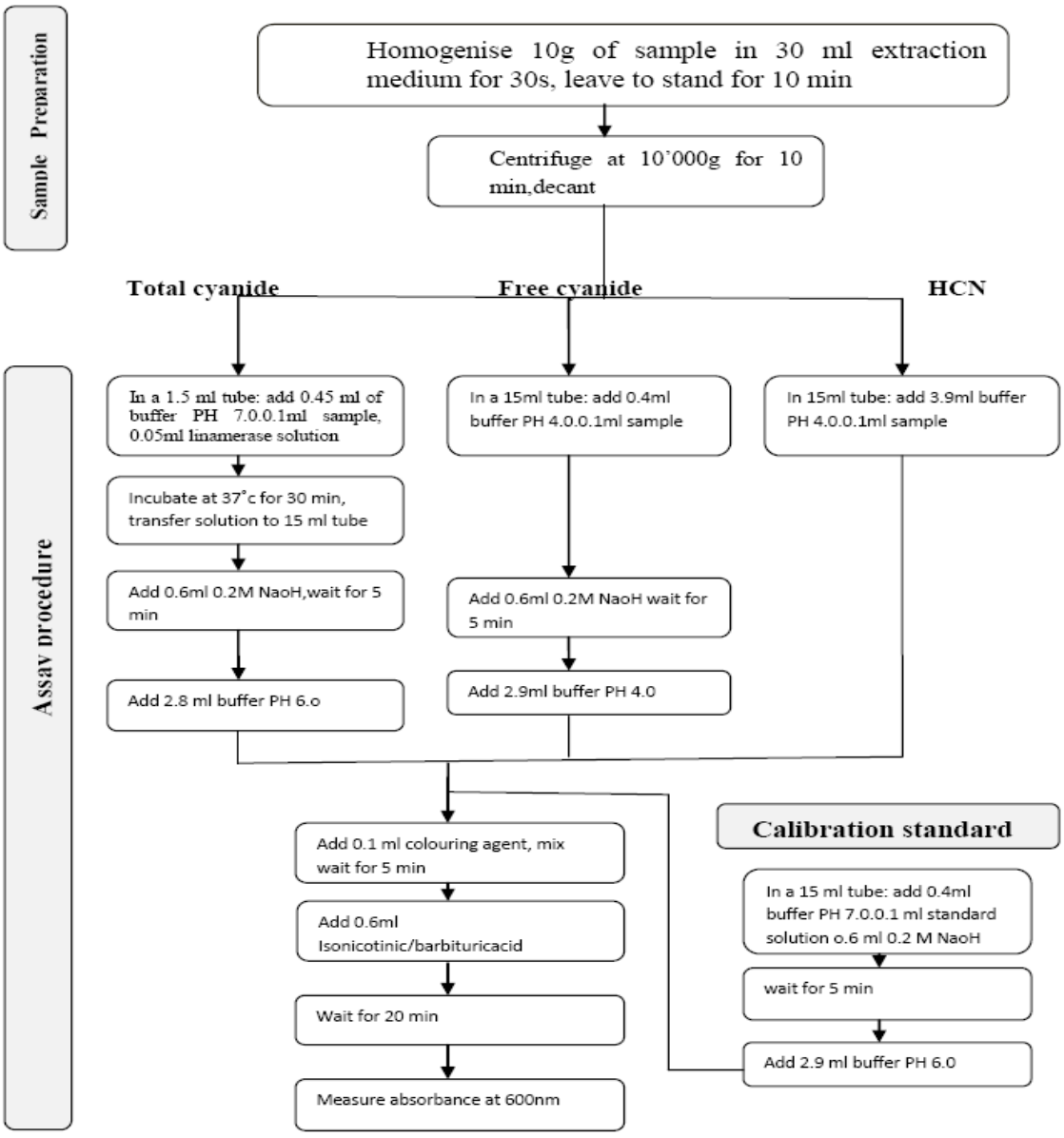

Figure 5.2: Flow sheet of the assay procedure to determine cyanogenic compounds. Courtesy of O'Brian *et al.* (1991) and Essers *et al.* (1993).

5.2.1.4 Calibration standards

Standard solutions were assayed as described in IITA (Essers *et al.*, 1993). The calibration curve was established at least once every day. To the samples, 0.1 ml

chloramin T reagent was added and mixed on a shaker. After 5 min, 0.6 ml colour reagent (isonicotinic acid /barbituric acid reagent) was added and mixed well. The absorbance was measured spectrophotometrically after 20 min at 600 nm. Duplicate analyses for samples and standard solutions were performed (Figure 5.3).

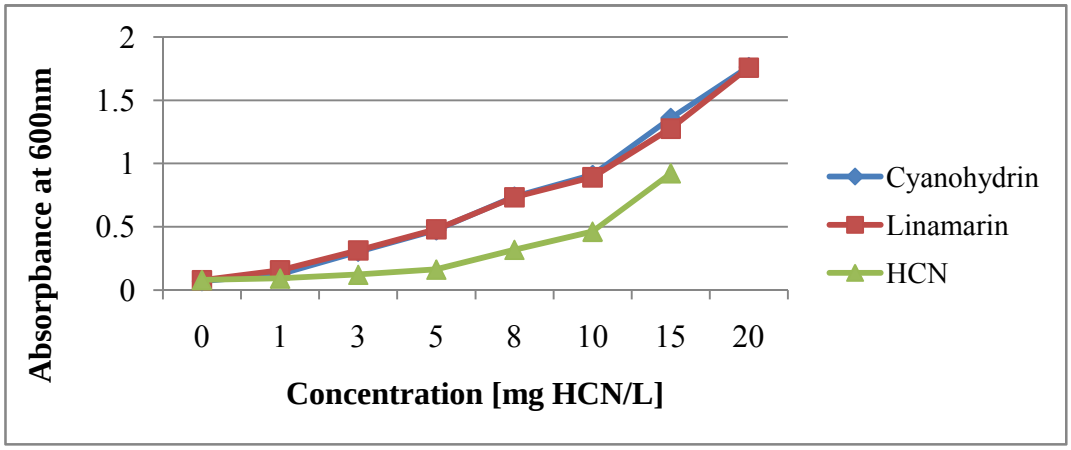

Figure 5.3: Calibration curves using linamarin. (n=2), cyanohydrin (n=6) and HCN (n=4).

5.2.1.5 Calculation of the cyanide content

Total cyanide, free cyanide and HCN contents of the samples were calculated as mg HCN equivalent/kg dwt using the formulae below (Essers *et al.*, 1993)

Extraction factor = $\frac{(\quad)\qquad\qquad(\quad)}{(\quad)}$

Cyanide content$_{\text{sample solution}}$ (mg/l) =

Cyanide content $_{(\text{mg/kg fwt})}$ = dilution factor $_{\text{sample}}$ x extraction factor x cyanide content $_{\text{sample solution}}$

Cyanide content (mg/kg dwt) = $\frac{(\quad / \quad)\times}{}$

Cyanogenic glycosides were calculated as (total cyanide minus free cyanide) and cyanohydrin as (free cyanide minus HCN).

5.2.2 Data analysis

Data on cyanogenic glycoside levels was determined, calculated and recorded in mg/kg fresh weight. The computed levels of cyanogenic glycosides in the leaves, stems and different parts of the roots of both wild type and transgenic cassava were analysed by unpaired student's t-test at a confidence level of 95% ($P<0.05$). Minitab statistical software (version 2012) was used for data analysis.

5.3 RESULTS

5.3.1 Determination of cyanogenic compounds content in cassava roots and leaves

Both wild type and transgenic Kenyan cassava lines were analyzed for content of the cyanogenic compounds linamarin, cyanohydrin and hydrocyanic acid (HCN). In all the transgenic lines, there was remarkable reduction in the levels of cyanogenic compounds compared to their wild type counterparts. The wild type of *Kibanda meno* had the highest level of linamarin and hydrocyanic acid, while the wild type of *Serere* had the highest levels of cyanohydrins. The wild type of the model cultivar *TMS 60444* had the lowest levels of all the cyanogenic compounds determined in this study (Figures 5.4, 5.5 and 5.6).

In all the cassava genotypes used in this study, the levels of cyanogenic compounds in the transgenic lines were significantly different from the levels in their wild type counterparts ($P<0.05$). Since cyanohydrin and hydrocyanic acid are both derived from enzymatic cleavage of linamarin, reduction of their levels in transgenic lines was consistent with reduction of linamarin levels. Linamarin levels in wild type genotypes were higher than 10mg/kg fwt, which is the maximum recommended levels of linamarin by FAO. In transgenic genotypes, the linamarin levels were reduced to levels below the maximum FAO recommended of linamarin (Appendix 8).

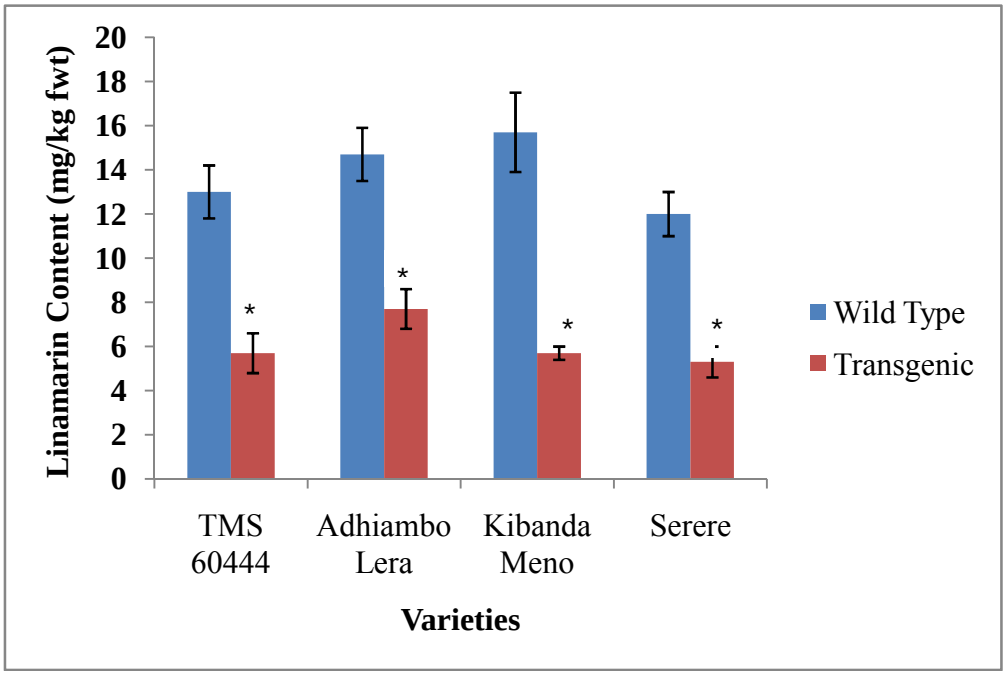

Figure 5.4: Linamarin content of phosphoric acid extracts of freshly ground cassava roots. *P<0.05 for transgenic versus wild type
Maximum recommended levels of linamarin is 10mg/Kg fwt

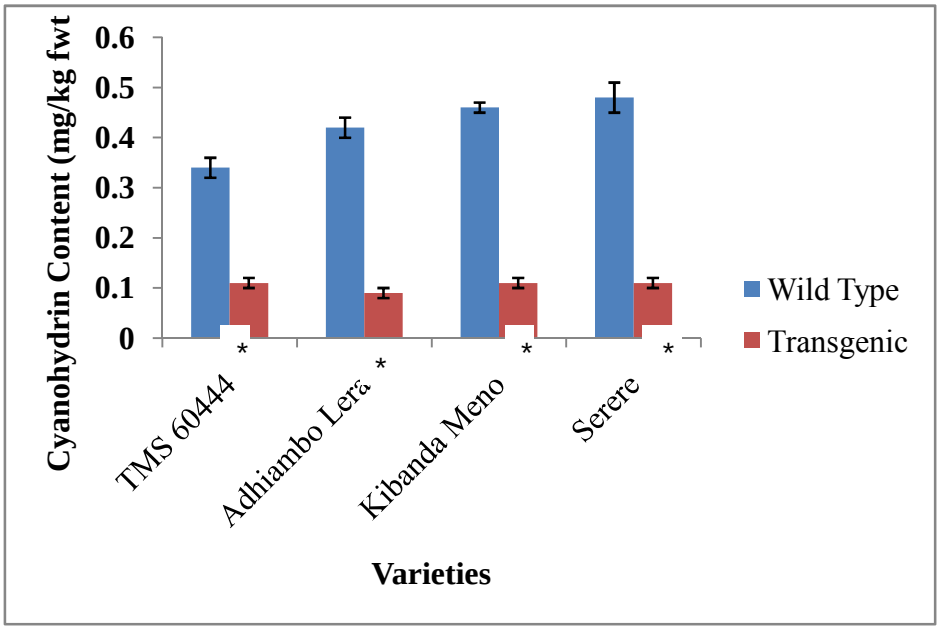

Figure 5.5: Cyanohydrin content of phosphoric acid extracts of freshly ground cassava roots. *P<0.05 for transgenic versus wild type

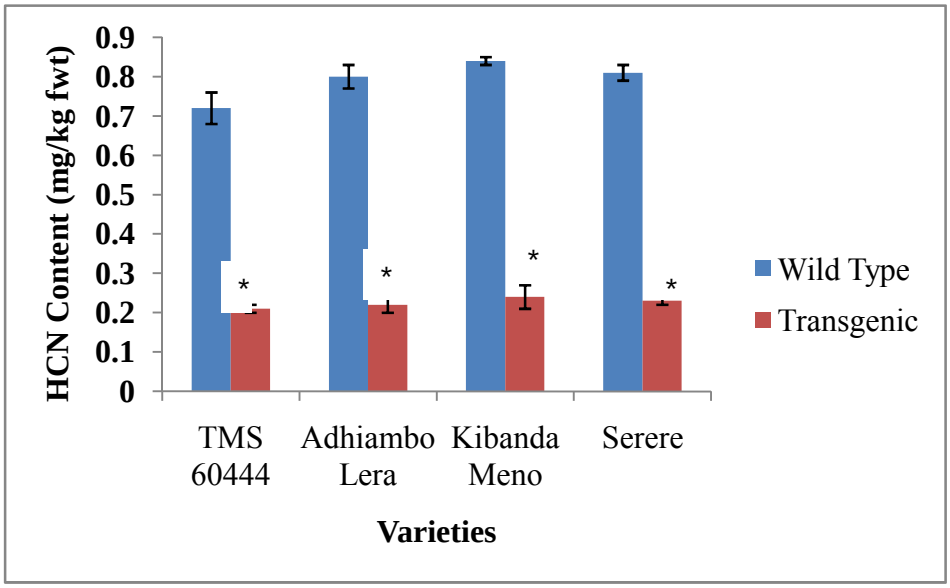

Figure 5.6: HCN content of phosphoric acid extracts of freshly ground cassava roots. *P<0.05 for transgenic versus wild type

The levels of cyanogenic compounds in leaves of the wild type cassava were higher in leaves than in roots by between three and four folds. The genotype *Kibanda meno* had the highest levels of cyanogenic compounds compared to the wild types of the other genotypes used in this study. Conversely, the wild type of the model cultivar *TMS 60444* had the lowest levels of the cyanogenic compounds (Figures 5.7, 5.8 and 5.9). In all the genotypes used in this study, the levels of cyanogenic compounds in transgenic lines were significantly lower than the levels in their wild type relatives (P<0.05) (Appendix 9).

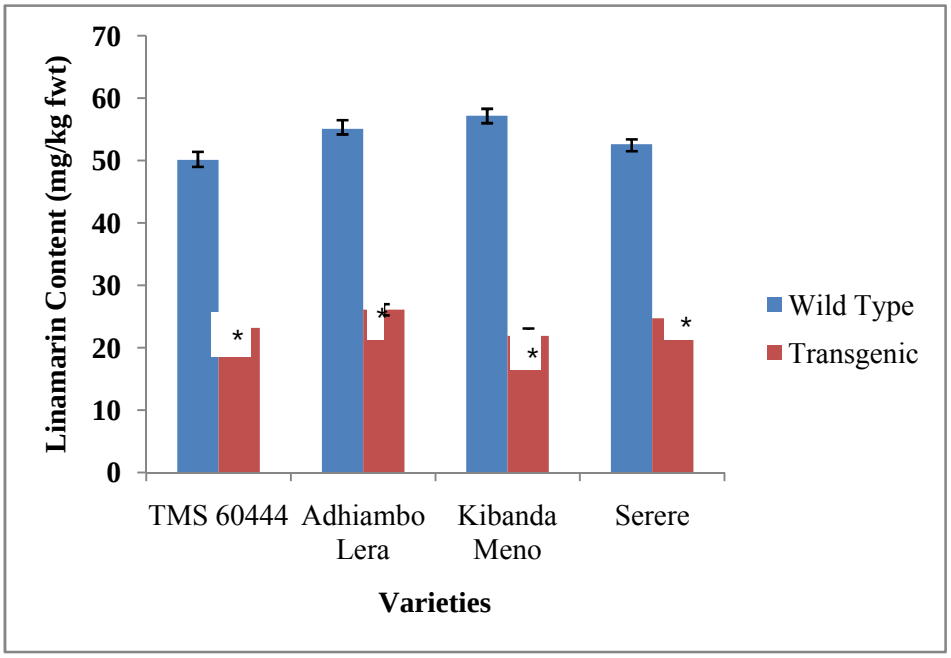

Figure 5.7: Linamarin content of phosphoric acid extracts of freshly ground cassava leaves. *P<0.05 for transgenic versus wild type

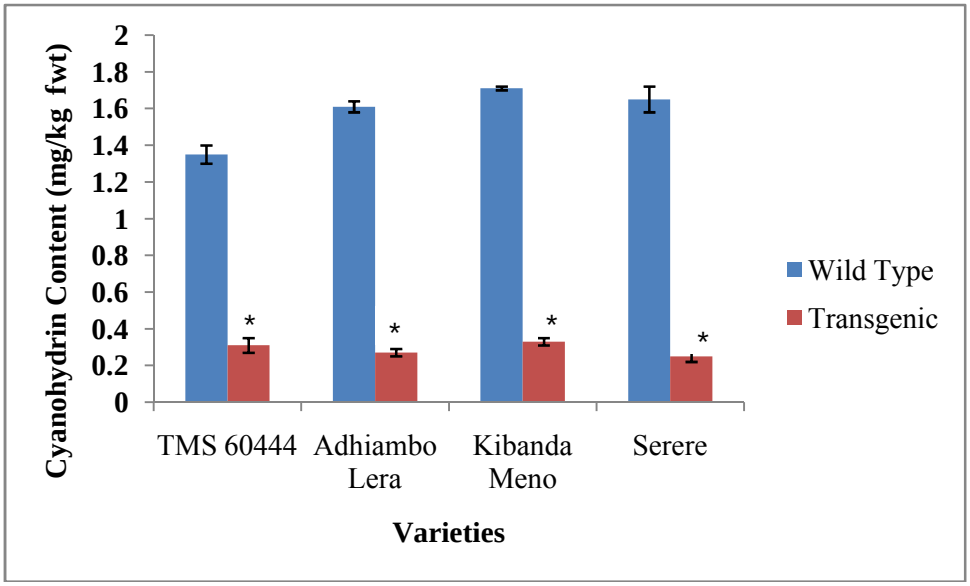

Figure 5.8: Cyanohydrin content of phosphoric acid extracts of freshly ground cassava leaves. *P<0.05 for transgenic versus wild type

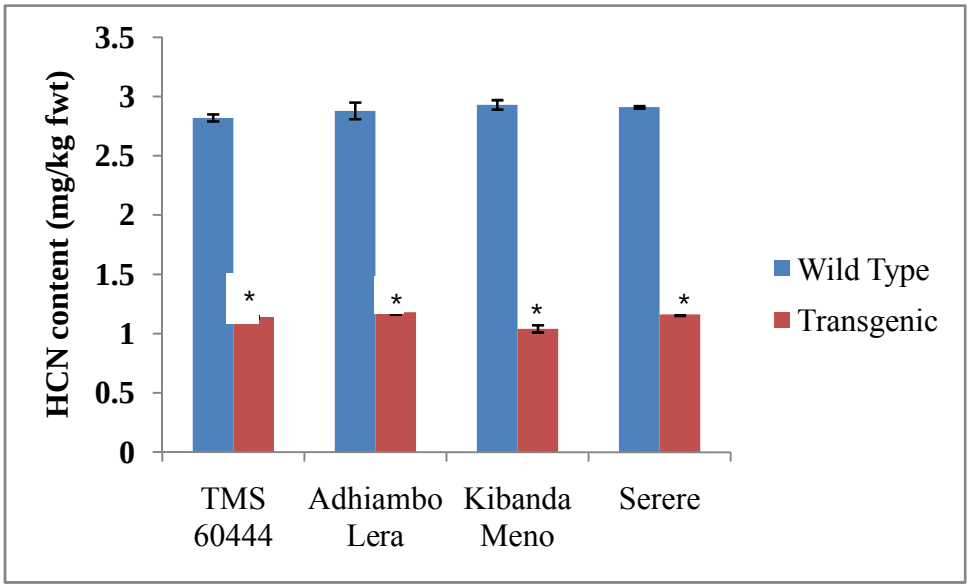

Figure 5.9: HCN content of phosphoric acid extracts of freshly ground cassava leaves. *P<0.05 for transgenic versus wild type

5.3.2 Determination of distribution of total cyanide content in cassava roots

Generally, parts closer to the root cortex and closer to the basal root tip contained more cyanides than parts near the centre of the root. For the wild type of the model cultivar *TMS 60444*, the total cyanide content ranged from 73mg/kg fwt for the central disc part of the root to 286mg/kg fwt for the root cortex. On the other hand for the transgenic line of *TMS 60444*, the total cyanide content ranged from 14mg/kg fwt for the basal root tip to 55mg/kg fwt for the cortical region of the root (Figure 5.10) (Apendix 10). The roots of the wild type of *Adhiambo lera* had the total cyanide levels ranging from 69mg/kg fwt for the central disc of the root to 289mg/kg fwt for the root cortex. The total cyanide levels in the roots of its transgenic relative ranged from 17mg/kg fwt for the basal root tip to 62mg/kg fwt

for the root cortex (Figure 5.11). There was clear significant difference in total cyanide levels between the transgenic and wild type line ($P<0.05$) (Appendix 11).

There was a similar trend of reduction in total cyanide levels in *Kibanda meno* and *Serere.* In *Kibanda meno,* roots of the wild type line had total cyanide content ranging from 70mg/kg fwt for the basal root tip to 298mg/kg fwt for the cortical part of the roots. In the roots of transgenic line, the total cyanide content was in the range of between 19mg/kg fwt for the basal root tip and 59mg/kg fwt for the root cortex (Figure 5.12) (Appendix 12). As seen earlier, *Kibanda meno* had the highest levels of cyanogenic compounds in both wild type and transgenic cassava roots and leaves. This trend was also observed in the total cyanide levels. The reduction in cyanide content in this genotype was significant ($P<0.05$).

For the *Serere* genotype, there was also a remarkable significant reduction of total cyanide in different parts of the transgenic cassava roots ($P<0.05$). The cortical root region of the transgenic *Serere* lines had total cyanide content of 62mg/kg fwt in comparison to the wild type line, which total cyanide content of 73mg/kg fwt in the root cortex (Figure 5.13). In the roots of both the wild type and transgenic *Serere* lines, the basal root tip part had the lowest total cyanide content ranging from 66mg/kg fwt to 12mg/kg fwt respectively. The degree of reduction of total cyanide content in transgenic *Serere* lines in all root parts was significant

compared to total cyanide levels in the wild type relatives ((P<0.05) (Figure 5.13) (Appendix 13).

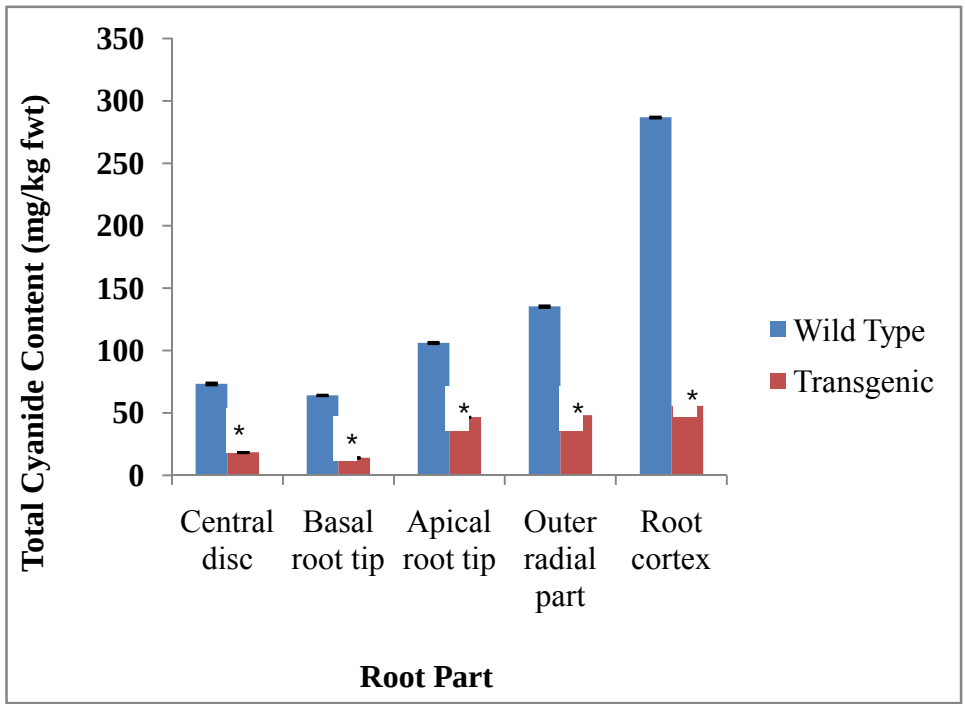

Figure 5.10: Distribution of total cyanide content in *TMS 60444* roots
*P<0.05 for transgenic versus wild type

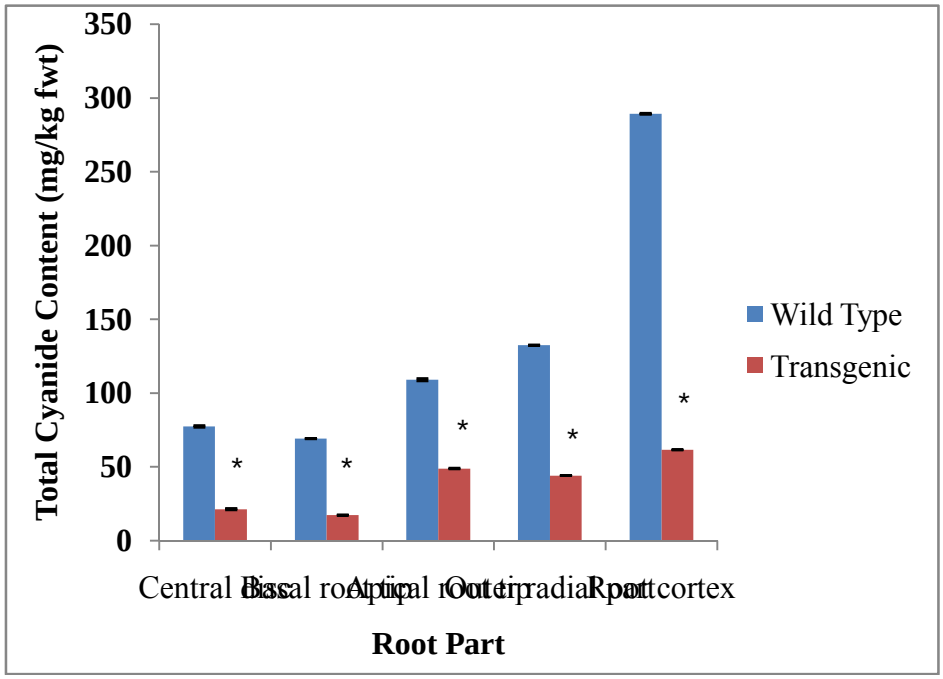

Figure 5.11: Distribution of total cyanide content in *Adhiambo lera* roots

*P<0.05 for transgenic versus wild type

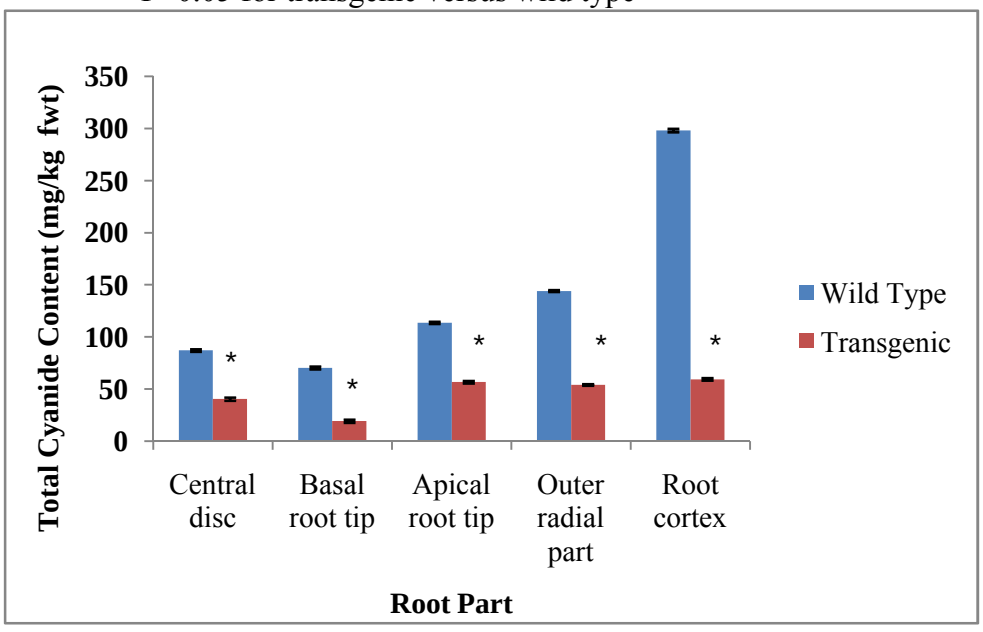

Figure 5.12: Distribution of total cyanide content in *Kibanda meno* roots

*P<0.05 for transgenic versus wild type

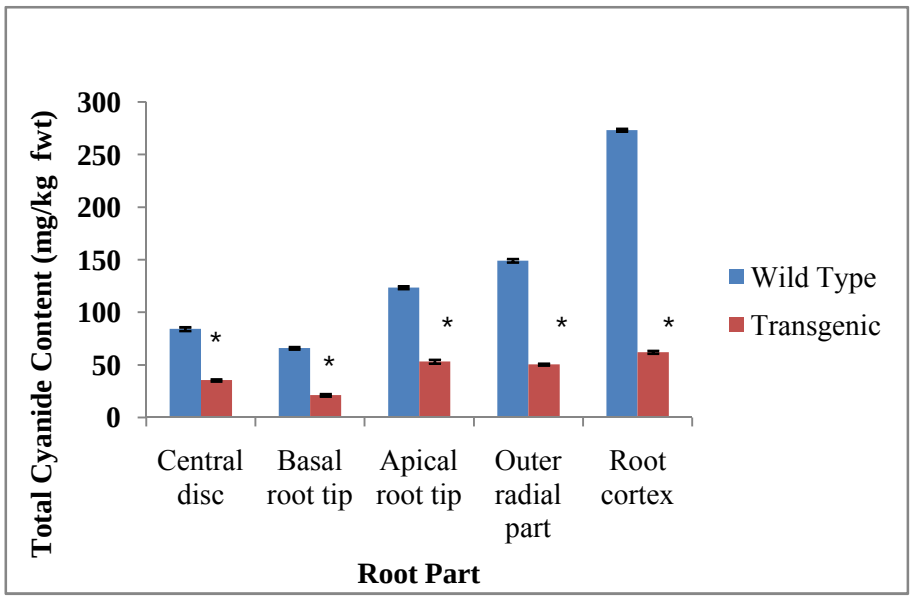

Figure 5.13: Distribution of total cyanide content in *Serere* roots

*P<0.05 for transgenic versus wild type

5.4 DISCUSSION

The wild type lines showed higher levels of cyanide content than the accepted levels by WHO (FAO/WHO, 1991; Cumbana *et al.,* 2007). Results of this study corroborate with previous findings that cyano-glycosides levels in cassava root vary widely between cassava cultivars of same variety, different tissues of the same plant, roots of the same plant and even within the root parenchyma (De Brujin, 1971; Cardoso *et al.,* 2005; Mburu *et al.,* 2012).

In this study, cassava leaves had more Cyano-glycoside levels than roots. This agrees with study results by Hidayat *et al.* (2002), who observed that cyanogenic potential in cassava leaves was three fold higher than in roots. In their study, Fukuba *et al.* (1984) observed that cassava leaves had cyanogenic potential 2-27 times higher than the cyanogenic potential in roots. This can be expected because biosynthesis of cyano-glycosides occurs in cassava levels before their translocation into the roots (Degro, 2009).

Cyano-glycoside levels in cassava also vary according to location, age, method of analysis and environmental conditions (Githunguri *et al.,* 1998; Mburu *et al.,* 2012). These parameters, had no bearing in the results of this study because the cassava genotypes used in this study were sourced from the same locations, they were equally aged, they were subjected to the same analytical methods and they were grown in the same environmental conditions.

Previous research by Wheatley *et al.* (1993) indicate that cassava cultivars with <100mgHCN/kg fwt are considered sweet while those >100mgHCN/kg fwt are considered bitter. By this indication, the cassava cultivars used in this study can well be considered sweet. The transgenic cassava lines developed in this study had cyanide levels below 100mgHCN/kg fwt. In this study, wild type and transgenic cassava plants used for cyanide content determination were three months-old.

The cyanogenic levels of known cassava cultivars range from less than 10mg/kg fwt to more than 500mg/kg fwt (O'Brien *et al.*, 1994). The total cyanide content in the roots of the wild type transgenic cassava lines used in this study did not surpass this ceiling. World Health Organization recommends 10mg/kg body weight as the maximum safe cyanide level (FAO/WHO, 1991) for fresh cassava intake (Bradbury *et al.*, 1991; FAO, 2007).

Linamarin levels were higher than the other cyanogenic compounds in all the wild type and transgenic cassava lines studied. This is perhaps attributable to the fact that linamarin is the predominant cyano-glycoside in cassava and that it is the metabolic precursor of cyanohydrin and hydrogen cyanide (HCN) (Siritunga, 2002). Further, findings from other studies indicate that linamarin is more stable than other cyanogenic compounds even at varying storage temperatures (Heuberger, 2005) . Cyanohydrin and HCN levels were very low in all the wild type and transgenic cassava genotypes used in this study. Nevertheless, that there

were detectable cyanohydrin and HCN levels in the cassava genotypes evaluated is an indication that linamarase enzyme activity on linamarin was pronounced and that cyanohydrin was cleaved to HCN either spontaneously or via endogenous enzymatic cleavage by hydroxynitrile lyase

In all the cassava lines evaluated, the cyanide content of the apical root tip was higher than in the distal root tip. This is in agreement with previous results, which indicated that the apical root end contains higher cyanide content than the distal end (Bradbury *et al.*,1991). The radial gradient in cyanide content was considerably high, total cyanide increased from the centre of the root to the peripheral part of the root parenchyma. These results are in tandem with those obtained by Cooke (1978) who reported a sharp decrease in the radial cyanide gradient towards the centre of the root. However, the variation of cyanide contents both longitudinally and radially may show misleading results of the mean cyanide content of a cassava root (Heuberger, 2005). Probably this can be accounted for by previous findings intimating that cyanide content of the roots varies along the length of the tuber (Bokanga and Otoo, 1994).

Variations in concentrations of cyanogenic compounds in the different cassava genotypes evaluated can be explained by the study results by Siritunga (2002), who observed that cyanide content in cassava is genotype dependent. Some cassava genotypes are considered as highly cyanogenic while others are less

cyanogenic. Further, the variation in the content of cyanogenic compounds in the different parts of cassava roots can be explained by differential translocation of cyanogenic glycosides and their metabolizing enzymes in the different cellular compartments.

CHAPTER SIX
SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

6.1 SUMMARY

In this study, the first objective aimed to fulfill the three prerequisites for using plant transformation. Many protocols for *in vitro* cassava regeneration have been developed in the past using all possible explants. The challenge has been replicability of such approaches of African cassava genotypes, which are generally considered recalcitrant to *in vitro* growth condition. A workable *in vitro* regeneration protocol compatible with Kenyan cassava genotypes was established and optimized in this study. Three Kenyan cassava cultivars viz; *Adhiambo lera*, *Kibanda meno* and *Serere* along with an exotic model cultivar *TMS 60444* were regenerated via somatic embryogenesis. In this protocol, the model cultivar *TMS 60444* was inferior to some Kenyan genotypes in terms of somatic embryogenesis, germination and plant recovery. This provides a major breakthrough and impetus to researchers who have encountered a bottleneck with regards to *in vitro* regeneration of African cassava genotypes.

Many transgene transfer systems, such as protoplast transformation, microinjecting DNA into cells or zygotes, silicon carbide whisker-mediated DNA transfer or electroporation, particle bombardment and *Agrobacterium*-mediated gene transfer have been developed in plant transformation systems. Of these, particle bombardment and A*grobacterium*-mediated gene transfer are the most commonly

used systems to transfer foreign genes into plant cells. Others techniques, however, are bedeviled by low transformation efficiencies. Currently, *Agrobacterium*-mediated gene delivery is routinely applied in important dicotyledonous crops such as sunflower (Everett *et al.,* 1987), cotton (Firoozabady *et al.,* 1987), tomato (Fillatti *et al.,* 1987), potato (Sheerman and Bevan, 1988), soybean (Hinchee *et al.,* 1988), sugarbeet (Lindsey and Gallois, 1990), papaya (Fitch *et al.,* 1993) and cassava (Li *et al.,* 1996; Schopke *et al.,* 1996).

Against this background, the regenerated cassava genotypes in this study were taken through *Agrobacterium*-mediated transformation with an aim of posttranslational silencing of cyano-glycoside biosynthesis in cassava using RNA interference technology (Fillatti *et al.,* 1987). In contrast to the routine use of embryogenic calli as explants for transformation, immature leaf lobes were used as explants for transformation in this study. The infected leaf lobes were taken through the optimized regeneration protocol successfully, suggesting that the regeneration protocol is reproducible.

Positive PCR analysis results showing transgene insertion in the transformed cassava genotypes imply that the Kenyan cassava genotypes used in this study are not recalcitrant to *Agrobacterium*-mediated transgene transfer. Positive RT-PCR analysis results of the transformed cassava genotypes imply successful transformation and transgene transcription. This explains why there was

remarkable reduction in cyanide content in transgenic cassava versus their wild type relatives.

The total cyanide distribution in cassava roots evaluated in this study were different among the studied genotypes. This can be explained by the fact that expression levels of *CYP79D1/D2* genes varies among cassava genotypes. This implies that some cassava genotypes are more cyanogenic than others.

The RNAi technique has rapidly gained favor as a "reverse genetics" tool to knock down the expression of targeted genes in plants, as in other species, due to certain advantages that RNAi technology holds over gene disruptions caused by transposon or T-DNA insertion. The ability to target multiple gene family members with a single RNAi-inducing transgene is one such advantage. Another is that gene knockdowns due to RNAi are dominant, whereas insertional or other loss-of-function mutations are recessive. The dominant aspect of RNAi allows the knock down of genes in polyploid genomes that contain four or more orthologs and are thus refractive to traditional mutagenesis (Preuss and Pikaard, 2003; Lawrence and Pikaard, in press).

Likewise, orthologs can be knocked down in F1 hybrids in which the RNAi-inducing transgene is introduced through only one of the parents. Finally, the dominance of RNAi allows one to save time by eliminating the additional

generations needed to identify individuals that are homozygous for recessive loss-of-function alleles (Preuss and Pikaard, 2003). Against this background, it can be inferred from this study that it is possible that cyanide content was not reduced to zero value because RNAi is not a knock out strategy but rather, a knock down strategy. That gene knockdowns due to RNAi are dominant, is a welcome phenomenon because it implies that down regulation is expected to dominate even in subsequent generations of the transgenic cassava lines produced in this study. The targeted gene for knock down in this study, *CYP79D1* gene, encodes cytochromes P_{450} oxidative enzymes that catalyze the committed step in biosynthesis of cyano-glycosides. Knock down of this gene sporadically reduces expression levels of this enzyme and therefore reduction of cyanide levels downstream.

In a nutshell, three transgenic Kenyan cassava genotypes with notably reduced cyanide levels have been produced in this study.

6.2 CONCLUSIONS

This study has optimized a reproducible *in vitro* regeneration protocol of three popular Kenyan genotypes viz; *Adhiambo lera, Kibanda meno* and *Serere* along with an exotic model cultivar *TMS 60444* using immature leaf lobes as explants for somatic embryogenesis. It avails an opportunity for further studies on cassava with

varying research interests to mitigate the various genetic and phenotypic drawbacks associated with cassava.

Further, this study has demonstrated ease of transformation of Kenyan cassava genotypes through *Agrobacterium*-mediated transgene transfer using immature leaf lobes as explants as opposed to the commonly used embryogenic calli. Transgenic cassava lines with downregulated expression of cytochrome P_{450} genes were produced. This unequivocally demonstrated that the biotechnological approach used to downregulate produce of cyano-glycosides in Kenyan cassava genotypes indeed worked.

Analysis of cyanide content in roots and leaves of both wild type and transgenic lines showed that cyanide content was largely reduced by three folds in the transgenic lines. This was confirmation of down regulation of expression of cytochrome P_{450} genes responsible for biosynthesis of cyano-glycosides. The success of the transformation of Kenyan cassava genotypes opens avenues for further improvement of the African cassava.

Therefore, the two null hypotheses set in this study have been rejected in favour of the corresponding alternative hypotheses.

6.3 RECOMMENDATIONS

From this study, the following recommendations can be made:

1. The *in vitro* regeneration protocol optimized and used in this study to regenerate the cassava lines should be replicated on other African cassava lines probably even using other explants like stems, axillary bud and nodes.
2. Studies on the performance of the explants under extended light exposure of 16 hours, the photoperiods of many plants, should be studied.
3. Testing of various combinations (ratios) of 2,4-D and picloram concentarions for their capacity to induce calli and promote somatic embryogenesis is recommended. In addition other auxins like Dicamba should also be evaluated for their capacity to induce calli and promote somatic embryogenesis in cassava.
4. It would be desirable to compare vulnerability of wild type and transgenic cassava lines to herbivores.
5. Evaluation of the influences of parameters storage temperatures, soil, age and method of processing on cyanide content and distribution in transgenic cassava lines should be done.
6. Adaptation of tissue culture conditions to the specific needs of other cassava genotypes should be done. This will result in the much needed breakthroughs and allow the genetic transformation of a wider range of cassava genotypes.

7. Bioscreening of transgenic cassava for macro and micronutrient composition should be done to establish the effect of downregulation of cyanoglycoside biosynthesis on their expression in cassava.
8. Further characterization of transgenic cassava over several generations should be done to establish stability of transgenicity.
9. Evaluating the levels of cyanogenic compounds among the transgenic cassava lines used in this study is recommended.

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APPENDICES

Appendix 1: Effect of 2,4-D on callus induction

One-way ANOVA: 4mg/L versus Variety

```
Analysis of Variance for 4mg/L
Source     DF        SS        MS        F        P
Variety     3    428.00    142.67    35.67    0.000
Error       8     32.00      4.00
Total      11    460.00

                                    Individual 95% CIs For Mean
                                    Based on Pooled StDev
Level       N      Mean     StDev  --------+---------+---------+--------
Adhiambo    3    75.000     2.646                                (---*---)
Kibanga     3    58.333     2.082   (---*----)
Serere      3    66.333     1.528               (----*---)
TMS 6044    3    64.333     1.528            (---*----)
                                    --------+---------+---------+--------
Pooled StDev =    2.000                60.0      66.0      72.0
Tukey's pairwise comparisons
    Family error rate = 0.0500
Individual error rate = 0.0126
Critical value = 4.53
Intervals for (column level mean) - (row level mean)
          Adhiambo    Kibanga     Serere
Kibanga       11.436
              21.897
Serere         3.436     -13.231
              13.897      -2.769
TMS 6044       5.436     -11.231      -3.231
              15.897      -0.769       7.231
```

One-way ANOVA: 8mg/L versus Variety

```
Analysis of Variance for 8mg/L
Source     DF        SS        MS        F        P
Variety     3    576.33    192.11    31.15    0.000
Error       8     49.33      6.17
Total      11    625.67

                                    Individual 95% CIs For Mean
                                    Based on Pooled StDev
Level       N      Mean     StDev  ---------+---------+---------+-------
Adhiambo    3    93.000     2.000                              (----*----)
Kibanga     3    74.667     4.163   (----*---)
Serere      3    78.667     1.528         (---*----)
TMS 6044    3    85.000     1.000                  (---*----)
                                    ---------+---------+---------+-------
Pooled StDev =    2.483                77.0      84.0      91.0
Tukey's pairwise comparisons
    Family error rate = 0.0500
Individual error rate = 0.0126
Critical value = 4.53
Intervals for (column level mean) - (row level mean)
          Adhiambo    Kibanga     Serere
Kibanga       11.839
```

```
             24.828
Serere        7.839     -10.495
             20.828       2.495
TMS 6044      1.505     -16.828     -12.828
             14.495      -3.839       0.161
```

One-way ANOVA: 8mg/L versus Variety

```
Analysis of Variance for 8mg/L
Source     DF        SS        MS        F        P
Variety     3    576.33    192.11    31.15    0.000
Error       8     49.33      6.17
Total      11    625.67
                                  Individual 95% CIs For Mean
                                  Based on Pooled StDev
Level       N      Mean     StDev  ---------+---------+---------+-------
Adhiambo    3    93.000     2.000                              (----*----)
Kibanga     3    74.667     4.163   (----*---)
Serere      3    78.667     1.528         (---*----)
TMS 6044    3    85.000     1.000                   (---*----)
                                  ---------+---------+---------+-------
Pooled StDev =    2.483                 77.0      84.0      91.0
Tukey's pairwise comparisons
    Family error rate = 0.0500
Individual error rate = 0.0126
Critical value = 4.53
Intervals for (column level mean) - (row level mean)
          Adhiambo    Kibanga     Serere
Kibanga      11.839
             24.828
Serere        7.839     -10.495
             20.828       2.495
TMS 6044      1.505     -16.828     -12.828
             14.495      -3.839       0.161
```

One-way ANOVA: 12mg/L versus Variety

```
Analysis of Variance for 12mg/L
Source     DF        SS        MS        F        P
Variety     3    426.00    142.00    26.62    0.000
Error       8     42.67      5.33
Total      11    468.67
           Individual 95% CIs For Mean
                                  Based on Pooled StDev
Level       N      Mean     StDev  ---+---------+---------+---------+---
Adhiambo    3    82.667     1.528                            (----*----)
Kibanga     3    67.667     2.517   (----*----)
Serere      3    68.667     3.215    (----*-----)
TMS 6044    3    74.333     1.528              (----*----)
                                  ---+---------+---------+---------+---
Pooled StDev =    2.309            66.0      72.0      78.0      84.0
Tukey's pairwise comparisons
    Family error rate = 0.0500
Individual error rate = 0.0126
Critical value = 4.53
Intervals for (column level mean) - (row level mean)
          Adhiambo    Kibanga     Serere
```

```
Kibanga        8.960
              21.040
Serere         7.960      -7.040
              20.040       5.040
TMS 6044       2.293     -12.707     -11.707
              14.373      -0.627       0.373
```

One-way ANOVA: 16mg/L versus Variety

```
Analysis of Variance for 16mg/L
Source     DF        SS        MS        F        P
Variety     3    204.67     68.22    15.16    0.001
Error       8     36.00      4.50
Total      11    240.67

                                  Individual 95% CIs For Mean
                                  Based on Pooled StDev
Level       N      Mean     StDev  --+---------+---------+---------+----
Adhiambo    3    68.333     1.528                        (-----*----)
Kibanga     3    57.000     3.000  (-----*-----)
Serere      3    63.333     1.528             (-----*----)
TMS 6044    3    60.667     2.082         (----*-----)
                                  --+---------+---------+---------+----
Pooled StDev =    2.121           55.0      60.0      65.0      70.0
Tukey's pairwise comparisons
    Family error rate = 0.0500
Individual error rate = 0.0126
Critical value = 4.53
Intervals for (column level mean) - (row level mean)
          Adhiambo    Kibanga     Serere
Kibanga        5.785
              16.881
Serere        -0.548     -11.881
              10.548      -0.785
TMS 6044       2.119      -9.215      -2.881
              13.215     1.881       8.215
```

Appendix 2: Effect of Picloram on callus induction

One-way ANOVA: 4mg/L versus Variety

```
Analysis of Variance for 4mg/L
Source     DF        SS        MS        F        P
Variety     3    423.00    141.00    49.76    0.000
Error       8     22.67      2.83
Total      11    445.67

                                  Individual 95% CIs For Mean
                                  Based on Pooled StDev
Level       N      Mean     StDev  --+---------+---------+---------+----
Adhiambo    3    71.667     2.082                              (--*---)
Kibanga     3    55.333     1.528  (---*---)
Serere      3    61.333     1.528           (---*---)
TMS 6044    3    60.333     1.528          (---*--)
                                  --+---------+---------+---------+----
Pooled StDev =    1.683           54.0      60.0      66.0      72.0
Tukey's pairwise comparisons
    Family error rate = 0.0500
Individual error rate = 0.0126
```

```
Critical value = 4.53
Intervals for (column level mean) - (row level mean)
          Adhiambo    Kibanga     Serere
Kibanga       11.931
              20.736
Serere         5.931     -10.402
              14.736      -1.598
TMS 6044       6.931      -9.402      -3.402
              15.736      -0.598       5.402
```

One-way ANOVA: 8mg/L versus Variety

```
Analysis of Variance for 8mg/L
Source     DF        SS        MS        F        P
Variety     3    606.00    202.00    52.70    0.000
Error       8     30.67      3.83
Total      11    636.67

                                    Individual 95% CIs For Mean
                                    Based on Pooled StDev
Level       N      Mean     StDev  ---+---------+---------+---------+---
Adhiambo    3    90.000     2.000                              (---*--)
Kibanga     3    71.000     1.000   (--*---)
Serere      3    75.333     3.055         (---*--)
TMS 6044    3    81.000     1.000                (---*--)
                                    ---+---------+---------+---------+---
Pooled StDev =    1.958             70.0      77.0      84.0      91.0
Tukey's pairwise comparisons
    Family error rate = 0.0500
Individual error rate = 0.0126
Critical value = 4.53
Intervals for (column level mean) - (row level mean)
          Adhiambo    Kibanga     Serere
Kibanga       13.879
              24.121
Serere         9.546      -9.454
              19.787       0.787
TMS 6044       3.879     -15.121     -10.787
              14.121      -4.879      -0.546
```

One-way ANOVA: 12mg/L versus Variety

```
Analysis of Variance for 12mg/L
Source     DF        SS        MS        F        P
Variety     3    423.33    141.11    14.99    0.001
Error       8     75.33      9.42
Total      11    498.67

                                    Individual 95% CIs For Mean
                                    Based on Pooled StDev
Level       N      Mean     StDev  ------+---------+---------+---------+
Adhiambo    3    78.667     2.309                        (----*-----)
Kibanga     3    63.000     2.646  (-----*-----)
Serere      3    65.667     4.933      (-----*-----)
TMS 6044    3    70.000     1.000            (-----*-----)
                                    ------+---------+---------+---------+
Pooled StDev =    3.069               63.0      70.0      77.0      84.0

Tukey's pairwise comparisons
    Family error rate = 0.0500
```

```
Individual error rate = 0.0126
Critical value = 4.53
Intervals for (column level mean) - (row level mean)
          Adhiambo    Kibanga     Serere
Kibanga       7.641
             23.692
Serere        4.974     -10.692
             21.026       5.359
TMS 6044      0.641     -15.026     -12.359
             16.692       1.026       3.692
```

One-way ANOVA: 16mg/L versus Variety

```
Analysis of Variance for 16mg/L
Source     DF        SS        MS        F        P
Variety     3    186.25     62.08     7.23    0.011
Error       8     68.67      8.58
Total      11    254.92
                                    Individual 95% CIs For Mean
                                    Based on Pooled StDev
Level       N      Mean     StDev  ----------+---------+---------+------
Adhiambo    3    65.000     1.000                       (-------*-------
)
Kibanga     3    54.333     4.041   (-------*------)
Serere      3    57.000     3.606        (-------*-------)
TMS 6044    3    58.000     2.000          (-------*-------)
                                    ----------+---------+---------+------
Pooled StDev =    2.930                  55.0      60.0      65.0
Tukey's pairwise comparisons
    Family error rate = 0.0500
Individual error rate = 0.0126
Critical value = 4.53
Intervals for (column level mean) - (row level mean)
          Adhiambo    Kibanga     Serere
Kibanga       3.004
             18.329
Serere        0.338     -10.329
             15.662       4.996
TMS 6044     -0.662     -11.329      -8.662
             14.662       3.996       6.662
```

Appendix 3: Effect of 2,4-D on somatic embryogenesis

One-way ANOVA: 4mg/L versus Variety

```
Analysis of Variance for 4mg/L
Source     DF        SS        MS        F        P
Variety     3      99.0      33.0     3.12    0.088
Error       8      84.7      10.6
Total      11     183.7
                                    Individual 95% CIs For Mean
                                    Based on Pooled StDev
Level       N      Mean     StDev  --------+---------+---------+--------
Adhiambo    3    27.000     4.583              (--------*--------)
Kibanga     3    22.000     4.000    (--------*--------)
Serere      3    27.000     1.000              (--------*--------)
TMS 6044    3    20.667     2.082   (-------*--------)
                                    --------+---------+---------+--------
```

```
Pooled StDev =    3.253                20.0      25.0      30.0
Tukey's pairwise comparisons
    Family error rate = 0.0500
Individual error rate = 0.0126
Critical value = 4.53
Intervals for (column level mean) - (row level mean)
          Adhiambo    Kibanga     Serere
Kibanga       -3.508
              13.508
Serere        -8.508     -13.508
               8.508       3.508
TMS 6044      -2.175      -7.175      -2.175
              14.842       9.842      14.842
```

One-way ANOVA: 8mg/L versus Variety

```
Analysis of Variance for 8mg/L
Source     DF        SS        MS        F        P
Variety     3     96.25     32.08     5.50    0.024
Error       8     46.67      5.83
Total      11    142.92
                                  Individual 95% CIs For Mean
                                  Based on Pooled StDev
Level       N      Mean     StDev  -----+---------+---------+---------+-
Adhiambo    3    40.667     1.528                    (-------*-------)
Kibanga     3    34.667     1.528      (-------*-------)
Serere      3    37.667     2.517              (-------*-------)
TMS 6044    3    33.333     3.512  (-------*-------)
                                  -----+---------+---------+---------+-
Pooled StDev =    2.415              32.0      36.0      40.0      44.0
Tukey's pairwise comparisons
    Family error rate = 0.0500
Individual error rate = 0.0126
Critical value = 4.53
Intervals for (column level mean) - (row level mean)
          Adhiambo    Kibanga     Serere
Kibanga       -0.317
              12.317
Serere        -3.317      -9.317
               9.317       3.317
TMS 6044       1.017      -4.983      -1.983
              13.650       7.650      10.650
```

One-way ANOVA: 12mg/L versus Variety

```
Analysis of Variance for 12mg/L
Source     DF        SS        MS        F        P
Variety     3    354.92    118.31    47.32    0.000
Error       8     20.00      2.50
Total      11    374.92
                                  Individual 95% CIs For Mean
                                  Based on Pooled StDev
Level       N      Mean     StDev  ----------+---------+---------+------
Adhiambo    3    37.333     2.082                    (--*---)
Kibanga     3    33.000     1.000            (---*---)
Serere      3    41.333     1.528                         (---*--)
TMS 6044    3    26.667     1.528   (--*---)
                                  ----------+---------+---------+------
```

```
Pooled StDev =    1.581                   30.0      36.0      42.0
Tukey's pairwise comparisons
    Family error rate = 0.0500
Individual error rate = 0.0126
Critical value = 4.53
Intervals for (column level mean) - (row level mean)
          Adhiambo    Kibanga     Serere
Kibanga        0.198
               8.469
Serere        -8.135     -12.469
               0.135      -4.198
TMS 6044       6.531       2.198      10.531
              14.802      10.469      18.802
```

One-way ANOVA: 16mg/L versus Variety

```
Analysis of Variance for 16mg/L
Source     DF        SS        MS        F        P
Variety     3    190.92     63.64    42.43    0.000
Error       8     12.00      1.50
Total      11    202.92
                                  Individual 95% CIs For Mean
                                  Based on Pooled StDev
Level       N      Mean     StDev  --+---------+---------+---------+----
Adhiambo    3    26.667     1.528               (---*---)
Kibanga     3    22.667     0.577       (---*---)
Serere      3    31.333     1.528                          (---*---)
TMS 6044    3    21.000     1.000  (----*---)
                                  --+---------+---------+---------+----
Pooled StDev =    1.225           20.0      24.0      28.0      32.0
Tukey's pairwise comparisons
    Family error rate = 0.0500
Individual error rate = 0.0126
Critical value = 4.53
Intervals for (column level mean) - (row level mean)
          Adhiambo    Kibanga     Serere
Kibanga        0.797
               7.203
Serere        -7.870     -11.870
              -1.463      -5.463
TMS 6044       2.463      -1.537       7.130
               8.870       4.870      13.537
```

Appendix 4: Effect of Picloram on somatic embryogenesis

One-way ANOVA: 4mg/L versus Variety

```
Analysis of Variance for 4mg/L
Source     DF        SS        MS        F        P
Variety     3     64.67     21.56    14.37    0.001
Error       8     12.00      1.50
Total      11     76.67
                                  Individual 95% CIs For Mean
                                  Based on Pooled StDev
Level       N      Mean     StDev  ------+---------+---------+---------+
Adhiambo    3    26.000     1.000                          (------*------)
Kibanga     3    22.667     1.528            (------*-----)
```

```
Serere      3    20.333     1.528   (-----*------)
TMS 6044    3    20.333     0.577   (-----*------)
                                    ------+--------+---------+---------+
Pooled StDev =    1.225                20.0      22.5      25.0      27.5
Tukey's pairwise comparisons
    Family error rate = 0.0500
Individual error rate = 0.0126
Critical value = 4.53
Intervals for (column level mean) - (row level mean)
          Adhiambo    Kibanga     Serere
Kibanga        0.130
               6.537
Serere         2.463      -0.870
               8.870       5.537
TMS 6044       2.463      -0.870      -3.203
               8.870       5.537       3.203
```

One-way ANOVA: 8mg/L versus Variety

```
Analysis of Variance for 8mg/L
Source     DF        SS        MS        F        P
Variety     3    327.58    109.19    38.54    0.000
Error       8     22.67      2.83
Total      11    350.25
                                    Individual 95% CIs For Mean
                                    Based on Pooled StDev
Level       N      Mean     StDev  --------+---------+---------+--------
Adhiambo    3    38.667     1.528                      (---*----)
Kibanga     3    32.333     2.517          (----*---)
Serere      3    41.667     0.577                               (---*----)
TMS 6044    3    28.333     1.528  (----*---)
                                    --------+---------+---------+--------
Pooled StDev =    1.683                30.0      35.0      40.0
Tukey's pairwise comparisons
    Family error rate = 0.0500
Individual error rate = 0.0126
Critical value = 4.53
Intervals for (column level mean) - (row level mean)
          Adhiambo    Kibanga     Serere
Kibanga        1.931
              10.736
Serere        -7.402     -13.736
               1.402      -4.931
TMS 6044       5.931      -0.402       8.931
              14.736       8.402      17.736
```

One-way ANOVA: 12mg/L versus Variety

```
Analysis of Variance for 12mg/L
Source     DF        SS        MS        F        P
Variety     3     35.00     11.67     4.12    0.049
Error       8     22.67      2.83
Total      11     57.67
                                    Individual 95% CIs For Mean
                                    Based on Pooled StDev
Level       N      Mean     StDev  ------+---------+---------+---------+
Adhiambo    3    32.667     2.517                    (--------*--------)
Kibanga     3    28.667     1.528    (--------*--------)
```

```
Serere      3    29.667     0.577        (--------*--------)
TMS 6044    3    28.333     1.528  (--------*--------)
                                    ------+---------+---------+---------+
Pooled StDev =    1.683                27.5      30.0      32.5      35.0
Tukey's pairwise comparisons
    Family error rate = 0.0500
Individual error rate = 0.0126
Critical value = 4.53
Intervals for (column level mean) - (row level mean)
          Adhiambo    Kibanga     Serere
Kibanga       -0.402
               8.402
Serere        -1.402      -5.402
               7.402       3.402
TMS 6044      -0.069      -4.069      -3.069
               8.736       4.736       5.736
```

One-way ANOVA: 16mg/L versus Variety

```
Analysis of Variance for 16mg/L
Source     DF        SS        MS        F        P
Variety     3    203.58     67.86     9.47    0.005
Error       8     57.33      7.17
Total      11    260.92
                                  Individual 95% CIs For Mean
                                  Based on Pooled StDev
Level       N      Mean     StDev  ----------+---------+---------+------
Adhiambo    3    28.667     4.509                     (------*------)
Kibanga     3    18.667     2.517  (------*------)
Serere      3    20.000     1.000     (------*------)
TMS 6044    3    19.000     1.000   (------*------)
                                    ----------+---------+---------+------
Pooled StDev =    2.677                  20.0      25.0      30.0
Tukey's pairwise comparisons
    Family error rate = 0.0500
Individual error rate = 0.0126
Critical value = 4.53
Intervals for (column level mean) - (row level mean)
          Adhiambo    Kibanga     Serere
Kibanga        2.998
              17.002
Serere         1.665      -8.335
              15.668       5.668
TMS 6044       2.665      -7.335      -6.002
              16.668       6.668       8.002
```

Appendix 5: Effect of Light on somatic embryogenesis

One-way ANOVA: No. of explants developing embryos in light versus Variety

```
Analysis of Variance for No. of e
Source     DF        SS        MS        F        P
Variety     3     147.0      49.0     3.77    0.059
Error       8     104.0      13.0
Total      11     251.0
                                  Individual 95% CIs For Mean
                                  Based on Pooled StDev
```

```
Level       N      Mean     StDev  --------+---------+---------+--------
Adhiambo    3    46.000     4.000                   (-------*-------)
Kibanga     3    36.333     3.055   (-------*-------)
Serere      3    39.333     4.726        (-------*-------)
TMS 6044    3    40.333     2.082         (-------*-------)
                                     --------+---------+---------+--------
Pooled StDev =    3.606                 36.0      42.0      48.0
Tukey's pairwise comparisons
    Family error rate = 0.0500
Individual error rate = 0.0126
Critical value = 4.53
Intervals for (column level mean) - (row level mean)
          Adhiambo    Kibanga     Serere
Kibanga       0.237
             19.097
Serere       -2.763     -12.430
             16.097       6.430
TMS 6044     -3.763     -13.430     -10.430
             15.097       5.430       8.430
```

One-way ANOVA: No. of explants developing embryos in dark versus Variety

```
Analysis of Variance for No. of e
Source     DF        SS        MS        F        P
Variety     3     384.2     128.1    10.18    0.004
Error       8     100.7      12.6
Total      11     484.9

                                    Individual 95% CIs For Mean
                                    Based on Pooled StDev
Level       N      Mean     StDev  ------+---------+---------+---------+
Adhiambo    3    106.67      3.06                         (-----*------)
Kibanga     3     91.67      2.08  (------*------)
Serere      3     97.67      4.51           (------*-----)
TMS 6044    3     94.33      4.04      (------*------)
                                    ------+---------+---------+---------+
Pooled StDev =     3.55               91.0      98.0     105.0     112.0
Tukey's pairwise comparisons
    Family error rate = 0.0500
Individual error rate = 0.0126
Critical value = 4.53
Intervals for (column level mean) - (row level mean)
          Adhiambo    Kibanga     Serere
Kibanga       5.722
             24.278
Serere       -0.278     -15.278
             18.278       3.278
TMS 6044      3.056     -11.944      -5.944
             21.611       6.611      12.611
```

One-way ANOVA: Mean No. of embryonic calli in light versus Variety

```
Analysis of Variance for Mean No.
Source     DF        SS        MS        F        P
Variety     3     330.3     110.1     9.79    0.005
Error       8      90.0      11.3
Total      11     420.3
```

```
                                  Individual 95% CIs For Mean
                                  Based on Pooled StDev
Level       N      Mean     StDev  -------+---------+---------+---------
Adhiambo    3    17.667     3.512          (------*-------)
Kibanga     3    13.000     3.000   (-------*------)
Serere      3    25.667     3.786                         (-------*------)
TMS 6044    3    12.667     3.055   (------*-------)
                                  -------+---------+---------+---------
Pooled StDev =    3.354              12.0      18.0      24.0
Tukey's pairwise comparisons
    Family error rate = 0.0500
Individual error rate = 0.0126
Critical value = 4.53
Intervals for (column level mean) - (row level mean)
          Adhiambo    Kibanga     Serere
Kibanga      -4.106
             13.439
Serere      -16.772     -21.439
              0.772      -3.894
TMS 6044     -3.772      -8.439       4.228
             13.772       9.106      21.772
```

One-way ANOVA: Mean No. of embryonic calli in dark versus Variety

```
Analysis of Variance for Mean No.
Source     DF        SS        MS        F        P
Variety     3    2996.3     998.8    32.13    0.000
Error       8     248.7      31.1
Total      11    3244.9
                                  Individual 95% CIs For Mean
                                  Based on Pooled StDev
Level       N      Mean     StDev  -+---------+---------+---------+-----
Adhiambo    3    161.33     10.07                          (----*----)
Kibanga     3    130.33      1.53     (----*----)
Serere      3    159.33      2.08                        (----*----)
TMS 6044    3    127.33      4.04   (----*----)
                                  -+---------+---------+---------+-----
Pooled StDev =    5.58           120       135       150       165
Tukey's pairwise comparisons
    Family error rate = 0.0500
Individual error rate = 0.0126
Critical value = 4.53
Intervals for (column level mean) - (row level mean)
          Adhiambo    Kibanga     Serere
Kibanga      16.42
             45.58
Serere      -12.58      -43.58
             16.58      -14.42
TMS 6044     19.42      -11.58       17.42
             48.58       17.58       46.58
```

Appendix 6: Effect of different concentrations of sucrose on somatic embryogenesis

One-way ANOVA: 2% versus Variety

```
Analysis of Variance for 2
Source     DF        SS        MS        F        P
```

```
Variety     3    1500.9     500.3    11.44    0.003
Error       8     350.0      43.8
Total      11    1850.9

                                    Individual 95% CIs For Mean
                                    Based on Pooled StDev
Level       N     Mean     StDev  --------+---------+---------+--------
Adhiambo    3   110.67     10.07                        (------*-------
)
Kibanga     3    89.33      5.03       (------*-------)
Serere      3   106.33      5.69                    (-------*------)
TMS 6044    3    84.00      4.00   (------*------)
                                    --------+---------+---------+--------
Pooled StDev =    6.61                    84        96       108
Tukey's pairwise comparisons
    Family error rate = 0.0500
Individual error rate = 0.0126
Critical value = 4.53
Intervals for (column level mean) - (row level mean)
          Adhiambo    Kibanga     Serere
Kibanga       4.03
             38.63
Serere      -12.97      -34.30
             21.63        0.30
TMS 6044      9.37      -11.97       5.03
             43.97       22.63      39.63
```

One-way ANOVA: 4% versus Variety

```
Analysis of Variance for 4
Source     DF        SS        MS        F        P
Variety     3   1962.00    654.00    66.51    0.000
Error       8     78.67      9.83
Total      11   2040.67

                                    Individual 95% CIs For Mean
                                    Based on Pooled StDev
Level       N     Mean     StDev  ----------+---------+---------+------
Adhiambo    3   108.00      4.00                              (--*--)
Kibanga     3    79.00      2.65    (---*--)
Serere      3    97.00      2.65                   (---*--)
TMS 6044    3    77.33      3.06   (--*---)
                                    ----------+---------+---------+------
Pooled StDev =    3.14                    84        96       108
Tukey's pairwise comparisons
    Family error rate = 0.0500
Individual error rate = 0.0126
Critical value = 4.53
Intervals for (column level mean) - (row level mean)
          Adhiambo    Kibanga     Serere
Kibanga     20.799
            37.201
Serere       2.799     -26.201
            19.201      -9.799
TMS 6044    22.465      -6.535      11.465
            38.868       9.868      27.868
```

One-way ANOVA: 6% versus Variety

```
Analysis of Variance for 6
Source     DF        SS        MS        F        P
Variety     3    646.33    215.44    39.77    0.000
Error       8     43.33      5.42
Total      11    689.67
                                    Individual 95% CIs For Mean
                                    Based on Pooled StDev
Level       N      Mean     StDev  ----+---------+---------+---------+--
Adhiambo    3    52.000     2.000                          (---*---)
Kibanga     3    32.000     2.000  (---*---)
Serere      3    37.333     3.055         (---*---)
TMS 6044    3    39.333     2.082           (---*---)
                                 ----+---------+---------+---------+--
Pooled StDev =    2.327             32.0      40.0      48.0      56.0
Tukey's pairwise comparisons
    Family error rate = 0.0500
Individual error rate = 0.0126
Critical value = 4.53
Intervals for (column level mean) - (row level mean)
          Adhiambo    Kibanga     Serere
Kibanga       13.913
              26.087
Serere         8.580     -11.420
              20.754       0.754
TMS 6044       6.580     -13.420      -8.087
              18.754      -1.246       4.087
```

Appendix 7: Effect of different concentrations of $CuSO_4$ on somatic embryogenesis

One-way ANOVA: c1, c2, c3, c4, c5

```
Analysis of Variance
Source     DF        SS        MS        F        P
Factor      4    5889.1    1472.3    34.03    0.000
Error      55    2379.8      43.3
Total      59    8268.9
                                    Individual 95% CIs For Mean
                                    Based on Pooled StDev
Level       N      Mean     StDev  -------+---------+---------+---------
c1         12    27.333     3.892   (--*---)
c2         12    30.583     4.358      (---*--)
c3         12    54.417     7.856                              (--*---)
c4         12    45.667     8.574                     (---*--)
c5         12    37.667     6.853            (---*--)
                                   -------+---------+---------+---------
Pooled StDev =    6.578                 30        40        50
```

One-way ANOVA: Initial No. of Explants versus Variety

```
Analysis of Variance for Initial
Source     DF        SS        MS        F        P
Variety     3      26.9       9.0     0.13    0.937
Error       8     536.0      67.0
Total      11     562.9
```

```
                                  Individual 95% CIs For Mean
                                  Based on Pooled StDev
Level       N      Mean     StDev --------+---------+---------+--------
Adhiambo    3    131.67      3.79   (--------------*--------------)
ibanga M    3    135.33     12.66        (--------------*---------------)
Serere      3    133.00      4.36    (---------------*---------------)
TMS 6044    3    131.67      8.62   (--------------*--------------)
                                  --------+---------+---------+--------
Pooled StDev =     8.19               126.0     133.0     140.0

Tukey's pairwise comparisons

    Family error rate = 0.0500
Individual error rate = 0.0126

Critical value = 4.53

Intervals for (column level mean) - (row level mean)

          Adhiambo    ibanga M    Serere

ibanga M      -25.07
               17.74

Serere        -22.74      -19.07
               20.07       23.74

TMS 6044      -21.41      -17.74      -20.07
               21.41       25.07       22.74
```

One-way ANOVA: No. of calli with germinated embryos versus Variety

```
Analysis of Variance for No. of c
Source     DF        SS        MS        F        P
Variety     3     379.6     126.5     3.88    0.055
Error       8     260.7      32.6
Total      11     640.3
                                  Individual 95% CIs For Mean
                                  Based on Pooled StDev
Level       N      Mean     StDev --------+---------+---------+--------
Adhiambo    3    84.333     3.055                (------*-------)
ibanga M    3    71.667     5.686    (-------*------)
Serere      3    75.333     2.517        (------*-------)
TMS 6044    3    69.667     9.074  (-------*------)
                                  --------+---------+---------+--------
Pooled StDev =    5.708                 70        80        90

Tukey's pairwise comparisons
    Family error rate = 0.0500
Individual error rate = 0.0126
Critical value = 4.53
Intervals for (column level mean) - (row level mean)
          Adhiambo    Kibanga Meno    Serere
Kibanga Meno  -2.262
              27.596
Serere        -5.929     -18.596
              23.929      11.262
TMS 60444     -0.262     -12.929      -9.262
```

```
             29.596      16.929      20.596
```

One-way ANOVA: Regenerated Plants versus Variety

```
Analysis of Variance for Regenera
Source     DF        SS        MS        F        P
Variety     3   1651.33    550.44    82.57    0.000
Error       8     53.33      6.67
Total      11   1704.67

                                    Individual 95% CIs For Mean
                                    Based on Pooled StDev
Level       N      Mean     StDev  --------+---------+---------+--------
Adhiambo    3    73.667     2.082                              (-*--)
ibanga M    3    43.000     3.000   (--*--)
Serere      3    60.000     1.000                (--*--)
TMS 6044    3    48.667     3.512       (--*-)
                                    --------+---------+---------+--------
Pooled StDev =    2.582               48        60        72
Tukey's pairwise comparisons
    Family error rate = 0.0500
Individual error rate = 0.0126
Critical value = 4.53
Intervals for (column level mean) - (row level mean)
          Adhiambo    Kibanga Meno    Serere
Kibanga Meno  23.914
              37.420

Serere         6.914     -23.753
              20.420     -10.247

TMS 6044      18.247     -12.420       4.580
              31.753       1.086      18.086
```

Appendix 8: Cyanogenic compounds of phosphoric acid extracts of freshly ground cassava roots

Variety		Cyanogenic Compounds content (mg/kg fwt) Linamarin	Cyanohydrin	HCN
TMS 60444	Wild Type	13.0 ± 1.2	0.34 ± 0.02	0.72 ± 0.01
	Transgenic	5.7 ± 0.9*	0.11 ± 0.01*	0.21 ± 0.01*
Adhiambo lera	Wild Type	14.7 ± 1.2	0.42 ± 0.02	0.80 ± 0.01
	Transgenic	7.7 ± 0.9*	0.09 ± 0.01*	0.22 ± 0.01*
Kibanda meno	Wild Type	15.7 ± 1.8	0.46 ± 0.01	0.80 ± 0.01
	Transgenic	5.7 ± 0.3*	0.11 ± 0.01*	0.21 ± 0.00*
Serere	Wild Type	12.0 ± 1.0	0.48 ± 0.03	0.81 ± 0.01
	Transgenic	5.3 ± 0.9*	0.11 ± 0.01*	0.21 ± 0.01*

*P<0.05versus wild type

Appendix 9: Cyanogenic compounds of phosphoric acid extracts of freshly ground cassava leaves

		Cyanogenic Compounds content (mg/kg fwt)		
		Linamarin	**Cyanohydrin**	**HCN**
TMS 60444	Wild Type	50.1 ± 0.01	1.35 ± 0.05	2.82 ± 0.03
	Transgenic	23.2 ± 0.05	0.31 ± 0.04	1.14 ± 0.01
Adhiambo lera	Wild Type	55.1 ± 0.04	1.61 ± 0.03	2.88 ± 0.07
	Transgenic	26.1 ± 0.02	0.27± 0.02	1.18 ± 0.02
Serere	Wild Type	52.6 ± 0.03	1.65 ± 0.07	2.91 ± 0.01
	Transgenic	24.7± .01	0.25 ± 0.03	1.16 ± 0.01
Kibanda meno	Wild Type	57.2 ± 0.01	1.71 ± 0.01	2.93 ± 0.04
	Transgenic	21.9 ± 0.03	0.33 ± 0.02	1.04 ± 0.03

*P<0.05versus wild type

Appendix 10: Distribution of cyanogenic compounds in *TMS 60444* roots

	Total Cyanide Content (mg/kg fwt)	
Root Part	**Wild Type**	**Transgenic**
Central disc	73.4 ± 0.8	18.4 ± 0.2*
Root tip basal	64.1 ± 0.3	14.1 ± 0.4*
Root tip apical	106.2 ± 0.6	46.7 ± 0.1*
Outer radial part	135.3 ± 0.7	48.1 ± 0.1*
Root cortex	286.8 ± 0.5	55.6 ± 0.2*

*P<0.05versus wild type

Appendix 11: Distribution of cyanogenic compounds in *Adhiambo lera* roots

	Total Cyanide Content (mg/kg fwt)	
Part	**Wild Type**	**Transgenic**
Central disc	77.5 ± 0.6	21.3 ± 0.5*
Root tip basal	69.2 ± 0.1	17.3 ± 0.2*
Root tip apical	109.1 ± 0.8	48.9 ± 0.3*
Outer radial part	132.4 ± 0.2	44.1 ± 0.1*
Root cortex	289.2 ± 0.4	61.6 ± 0.2*

*P<0.05versus wild type

Appendix 12: Distribution of total cyanide in *Kibanda meno* roots

	Total Cyanide Content (mg/kg fwt)	
Root Part	**Wild Type**	**Transgenic**
Central disc	84.2 ± 1.8	35.5 ± 1.1*
Root tip basal	66.1 ± 1.1	21.2 ± 1.2*
Root tip apical	143.6 ± 1.3	53.3 ± 1.8*
Outer radial part	149.2 ± 1.6	50.5 ± 0.9*
Root cortex	273.3 ± 1.2	62.2 ± 1.4*

*P<0.05versus wild type

Appendix 13: Distribution of total cyanide in *Serere* roots

Root Part	**Wild Type**	**Transgenic**
Central disc	87.1 ± 1.2	40.4 ± 1.6*
Root tip basal	70.3 ± 1.3	19.2 ± 1.4*
Root tip apical	113.6 ± 1.1	56.7 ± 1.2*
Outer radial part	144.1 ± 0.9	54.1 ± 0.8*
Root cortex	298.1 ± 1.5	59.4 ± 1.3*

*P<0.05versus wild type