IN VITRO REGENERATION, VIRUS ELIMINATION AND TRANSFORMATION OF SELECTED CASSAVA LANDRACES IN TANZANIA

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In Vitro Regeneration, Virus Elimination and Transformation of Selected Cassava Landraces in Tanzania

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DECLARATION

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

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DEDICATION

This work is dedicated to my parents, Mr. Amazulu Kidulile and Mrs. Enely Kidulile, my brothers and sisters, my dearest husband Benjamin Ngoso and my children Ian and Melvin for being with me throughout the period of this study

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF APPENDICES

LIST OF ACRONYMS AND ABBREVIATIONS

2, 4-D	2, 4- Dichlorophenoxyacetic acid
ANOVA	Analysis of Variance
BAP	6- Benzylaminopurine
	purine
CBSD	Cassava Brown Streak Disease
CBSV	Cassava brown streak virus
CEM	Cost-effective Medium
CM	Conventional Medium
CRD	Completely Randomized Design
CMD	Cassava Mosaic Disease
DNA	De-oxy ribo Nucleic Acid
dNTPS	Dinucleotide TrisPhoshate
EACMV	East African Cassava Mosaic Virus
EDTA	Ethylene Diamine Tetra Acetic Acid
FAO	Food and Agriculture Organization
FAOSTAT	Food and Agriculture Organization Statistics
FEC	Friable Embryogenic Callus
FPCL	Farmers' preferred cassava landraces
GDP	Gresshof and Doy with
	Picloram
GDPT	Gresshof and Doy with Picloram

	and Tyrosine
GUS	β- gluculonidase
MARI	Mikocheni Agricultural Research Institute
MS	Murashige and Skoog
OD	Optical Density
PCR	Polymerase Chain Reaction
TC	Tissue Culture

ABSTRACT

Cassava is among the most produced and consumed crops in Africa with an annual production of 176 million metric tons. Lack of clean planting materials of farmer-preferred cassava varieties is one of the main constraints to increased cassava productivity. This study aimed at evaluating *in vitro* regeneration, virus elimination and transformation protocols for high quality farmer preferred cassava landraces in Tanzania. Cassava cuttings, Kibandameno and Paja la mzee collected from Coast area of Tanzania were grown in two media, conventional Murashige and Skoog medium and an alternative medium for six weeks. Four parameters were measured namely; plant height, number of leaves, number of nodes and number of roots. The cost of production was calculated for both media and compared. The use of the alternative medium led to a cost reduction of 93% over the conventional Murashige and Skoog medium. The efficacy of chemotherapy and thermotherapy in viral elimination was also determined, where cassava plants infected with East African cassava mosaic virus were subjected to chemical treatment in medium supplemented with Ribavirin and Salicylic acid at different concentrations. In another separate experiment, infected plants were subjected to thermotherapy. DNA was extracted from plant leaves after 42 days of treatment and Polymerase chain reaction amplification was performed using East African Cassava Mosaic Virus (EACMV) specific primers. The most effective concentrations were 20 mg/l of ribavirin, and 30 mg/l of salicylic acid which resulted in 85.0% and 88.9% virus-free plantlets, respectively. Thermotherapy at 35°C resulted in 71.4% virus-free plantlets. The regenerable cassava varieties were assessed for transformability using *Agrobacterium tumefaciens* with the reporter gene. The established FEC from two cassava varieties TMS60444 and Albert were infected with Agrobacterium LBA4404 harboring the vector Pc062 having the reporter gene β-glucuronidase (GUS). Transformants were selected and taken through regeneration. Transformability was confirmed by GUS histochemical assay and later by

PCR using specific primers. The highest transformation efficiency of 86% and 83% was observed for TMS60444 and Albert, respectively. The high-throughput cost-effective platform for mass production of cassava plantlets, viral cleaning and genetic modification protocols evaluated will greatly enhance availability of clean cassava planting materials to farmers at an affordable cost and conservation of valuable germplasm.

CHAPTER ONE

INTRODUCTION

1.1Background

Increasing agricultural productivity is one way of poverty alleviation and improving food security in Africa. However, high population growth and subdivision of land in high potential regions, have led to increased land scarcity (FAO, 2001). Moreover, drought has played a significant role in reducing food yields as a result, farmers are being encouraged to grow crops such as cassava *(Manihot esculenta Crantz)* which are relatively drought tolerant and take a shorter time to mature. Cassava is among the most produced crops in Africa with annual production of 176 million metric tons (FAOSTAT, 2014).

There has been a reduction in cassava productivity especially in sub-Saharan Africa despite its potential role in alleviating food insecurity (Nweke *et al.*, 2002). This is mainly due to factors such as virus diseases namely cassava mosaic disease (CMD) and cassava brown streak disease (CBSD) (Bull *et al.* 2011). One way in which these viruses spread is through the use of cuttings obtained from infected plants. Subsistence farmers obtain planting materials from previous plants and this accelerates virus accumulation and dissemination. In addition, cuttings also are highly perishable as they dry out quickly and also involve high transport and handling costs (Escobar *et al.*, 2006).

Lack of affordable and healthy farmer-preferred cassava planting materials is one of the major constraints to the expansion of cassava

production in Tanzania. Biotechnological approaches such as tissue culture (TC) for the production of high quality planting materials provide an important solution to this problem (Staden *et al.,* 2008). Tissue culture technology largely uses culture medium containing macronutrients, micronutrients, vitamins, growth regulators, carbon source and gelling agent such as agar (Murashige and Skoog, 1962). The tissue culture techniques are critical in improving cassava productivity in sub- Saharan Africa and globally (Acedo, 2006) as adoption of the technology in disease-free seedling production will improve cassava production. However, to optimize its application in cassava propagation, there is a need to identify and implement strategies that will contribute towards reducing the cost of plantlet production so that farmers can afford them. Reduction in production cost in cassava tissue culture can be approached in a number of ways. For instance, the expensive and sophisticated equipment required for tissue culture can be substituted with cheaper alternatives. In previous studies, the autoclave has been replaced with a pressure cooker, with no detectable contaminations (Gitonga *et al.*, 2010).

The use of alternative nutrient sources that are available locally at an affordable cost for example fertilizers can be explored. Tissue culture has also proven to produce planting materials in bulk thus enables the application of chemotherapy and thermotherapy to produce virus-free plants. Further, available cassava genotypes do not have resistance genes against these viruses coupled with high heterozygosity, low fertility and

unsynchronized flowering all make cassava improvement through conventional breeding is a time consuming (Rudi *et al.,* 2010). Due to these limitations cassava genetic transformation has emerged as an important tool in the improvement process (Liu *et al.*, 2011).

Particle bombardment and *Agrobacterium* mediated transformation have been used on cassava cotyledons or embryogenic cultures as target tissues for transformation (Liu *et al.,* 2011). The most favored method in transformation however has been the use of friable embryogenic calli (FEC) in combination with *A. tumefaciens* mediated transformation due to its higher efficiency compared to the cotyledon-based protocol (Koehorst *et al.,* 2012). Even with these technologies, cassava remains difficult to transform as a result of low transformation and regeneration frequencies (Bull *et al.,* 2009). Furthermore, instability of Agrobacterium-FEC system produces variable numbers of transgenic events (Koehorst *et al.,* 2012). Consequently, cassava requires well trained tissue culture specialists, substantial amounts of plant material and repeated transformation cycles to generate a sufficient number of independent transgenic lines.

It was reported that much work has been done on model cultivar T M S 60444 (Nyaboga *et al.,* 2013) but transformation of local cassava cultivars is essential for adoption of transgenic cassava. It is important to evaluate genetic engineering technology in different cassava genotypes for various field environments. Farmers continue to cultivate locally

available genotypes due to their desirable traits. Improving local genotypes will increase adoption of the transgenic plants by farmers.

1.2 Problem Statement and Justification

Scarcity of clean cassava planting materials and use of infected cuttings are the main cause of the drastic decline in cassava production in the recent past (Escobar *et al.,* 2006). Due to limited flowering and production of few seeds whose germination is irregular multiplication of cassava is mainly through cuttings. (Santana *et al.,* 2009). Cuttings also are highly perishable as they dry out quickly in addition to high transport and handling costs (Escobar *et al.,* 2006). The bulkiness of the cassava cuttings makes the rate of multiplication low as compared to other crops propagated by seeds.

Since farmers in many areas are not ready to adopt improved varieties, they rely on their local varieties due to the good traits they possess (Elibarik *et al.,* 2013). Most of these farmer preferred cassava landraces are infected with viruses as the result of using infected cuttings. Moreover, high cost of producing clean planting materials through biotechnological approaches makes it difficult for farmers to afford clean planting materials. Furthermore, the need to genetically improve farmer preferred cassava landraces against biotic and abiotic stresses is hindered by the absence of regeneration protocols that will enable mass production of the clean and improved farmer cassava landraces.

The use of tissue culture or *in vitro* propagation has helped overcome challenges involved in propagating vegetatively propagated crops as it avails disease free planting materials in large scale (Staden *et al.,* 2008). Tissue culture also requires less space as compared with conventional means of producing seedlings (Aladele and Kuta, 2008). The sterile operational nature of TC procedures excludes fungi, bacteria and pests from the production system resulting in production of large quantities of disease free, superior quality planting materials in form of plantlets which season independent as opposed to use of cuttings. Besides, tissue culture permits rapid dissemination of healthy and improved plants within countries since the material is easily certified as pathogens free (FAO, 2003).

Prakash *et al.* (2004) reports that sub-Saharan African countries have found it difficult to embrace tissue culture due to the high costs involved to purchase chemicals. This has limited the technology to a few institutions and rich farmers while locking out the resource-challenged subsistence farmers. Despite all these challenges, tissue culture is vital in improving productivity of cassava in Africa. This therefore calls for the development of new strategies aimed at reducing the cost of producing healthy plantlets and availing them to resource poor farmers affordably. Since transformation and cleaning of plants through different ways like chemotherapy and thermotherapy depend on tissue culture, development of cost effective regeneration protocols will enable mass production of cleaned and genetically improved farmer preferred cassava landraces,

which farmers will be willing to adopt.

1.3 Objectives

1. 3.1 General objective

To develop *in vitro* cost-effective regeneration, virus elimination and transformation protocols for selected cassava landraces in Tanzania.

1.4.2 Specific objectives

1. To evaluate the potential of using alternative commercially available nutrients for the *in vitro* mass production of farmer-preferred cassava landraces (FPC) in Tanzania
2. To evaluate the potential of chemotherapy and thermotherapy eliminate EACMV from infected Tanzanian cassava landraces
3. To determine the transformability of selected regenerable cassava landraces

1.5 Null hypotheses

1. It is not possible to regenerate FPC landraces using locally available nutrients
2. Chemotherapy and thermotherapy are not effective in the production virus-free cassava planting materials
3. It is not possible to transform FPC landraces with a binary vector carrying a reporter gene.

CHAPTER TWO

LITERATURE REVIEW

2.1 Cassava (Manihot esculenta Crantz) origin, distribution and utilization

Cassava (*Manihot esculenta Crantz*) has its genetic, geographical and agricultural origin in Latin America. Its domestication began 5000 – 7000 years BC in the Amazon, Brazil (Leotard *et al.,* 2009) and it was distributed by Europeans to the rest of the world. Cassava was taken from Brazil to the West coast of Africa by Portuguese navigators in the 16th century (Zeder *et al.,* 2006). It was brought to East Africa in the 18th century by the Portuguese from Cape Verde and into Mozambique from Zanzibar Island (Hillocks, 2002).

Cassava can grow in low-nutrient soils where cereals and other crops do not grow well (El-Sharkawy, 2004). It grows well in sandy to light soils where the storage roots can develop easily. Cassava can tolerate soils with low Ph; the crop yields up to 25-35% starch providing food for over 500 million people for subsistence farmers in developing countries (Ceballos *et al.,* 2004). The crop has different uses that include famine reserve crop, rural food staple, cash crop and urban food staple, industrial raw material and livestock feed (Nweke, 2004). In different parts of Africa roots are boiled, roasted or eaten raw. (Nweke *et al.,* 2002). Cassava leaves are also edible and a convenient food than even roots as they have similar nutritive

value as other dark green leaves valuable sources for vitamin A, C, Iron and calcium (Nweke *et al.,* 2002). This crops also supplies income to poor households since their leaves and roots are available all year round (Chavez *et al.,* 2005).

Cassava roots cannot be stored for long due to physiological deterioration hence are mostly processed in order to increase storage life and reduce the level of toxic cyanide. There has been a recent interest in production of ethanol from feed stocks expected to increase demand for cassava and help African countries reduce trade imbalance through exports (Acedo and Labana, 2008). The utilization of cassava for food accounts for 88% while animal feed takes 12% (Nweke, 2004).

2.2 Cassava production

Cassava is widely grown in sub-Saharan Africa and it supplies calories to more than 250 million people in sub-Saharan Africa and 250 million people worldwide (Kumah, 2007). It is considered a poor man's crop in most countries and can tolerate adverse conditions like infertile soils and drought, tolerance to low rainfall (Jaramillo *et al.,* 2005). In 2016 the world produced over 277 million tons of cassava of which 157 million tons were form Africa (FAOSTAT, 2016). Cassava is the second most significant food crop after maize providing half of the dietary calories for over half of both rural and urban populations (Perez *et al.,* 2011). Tanzania is the fourth producer of cassava in Africa and annual root production is estimated at 5,500,000 tons from 761,100 hectares. Main producing areas in Tanzania include: the coastal strip along the Indian

Ocean, around Lake Victoria, Lake Tanganyika and along the shores of Lake Nyasa. About 48.8% of total production is produced in the coastal strip along the Indian Ocean, 23.7% comes around Lake Victoria, 13.7% from Lake Nyasa areas and 7.9% from Lake Tanganyika areas. In these areas, cassava is regarded as the first or second staple food (Mtunda *et al.*, 2002).

Before the introduction of cassava in Africa the crops of interest were sorghum, rice, yam and millet. However, with introduction of cassava the situation has changed with introduction of cassava because it has filled the hungry gap when other crops are not available especially in times of drought hence its increased production in SSA (Baratt *et al.*, 2007). The crop is popular to the poor due to its ease in cultivation, low input requirements and ease of propagation. In areas with high population growth like Malawi, cassava is replacing maize as the primary food crop (FAO, 2010) partly due to declining soil fertility and climate change. Nweke *et al.* (2002) reports that increased cassava production in the recent past has been due to increased breeding research and good agronomic practices.

2.3 Constraints to cassava production

There has been a reduction in cassava production especially in sub-Saharan Africa despite its potential to alleviate food insecurity (Nweke *et al.*, 2002). The average production in African countries is 20 tons/ha much less than potential yields of 80 tons/ha. Common constraints include pests

and diseases, poor agronomic practices, low yielding varieties, high cyanide levels, lack of clean planting materials and long maturity periods (Herrera et al., 2011). Pests and diseases are the most economically important constraints to cassava production. Pests infesting cassava include mealy bugs (*Phenacoccus manihot*), green spider mite (*Mononychellus tanajoa*) cassava green mite (*Mononychellus tanajoa*), cassava hornworm (*Erinnyis ello*), scales, thrips and whitely (*Bemisia tabaci)*

Diseases of cassava include cassava mosaic disease (CMD), cassava brown streak disease (CBSD), cassava bacterial blight, cassava anthracnose, cassava bud necrosis, and root rots (Calvert and Thresh, 2002). Virus diseases, particularly CMD and CBSD, are the most important economic constraints to cassava production in East and Southern Africa (Patil and Fauquet, 2009). The two diseases are thought to have risen from infection of cassava by viruses already present in the indigenous African flora (Legg and Hillocks, 2003). These viral diseases spread due to the use of cuttings obtained from previous crops, thereby enhancing spread of the diseases. Viruses have been shown to cause yield loss of up to 100% when susceptible genotypes are used (Varma and Malathi, 2003). Growth of cassava for long seasons exposes the crop to a host of pests and pathogens in almost all the growing seasons in Africa. Subsistence farmers obtain planting materials from neighbours, during travel hence accelerating disease accumulation and dissemination. Cuttings also are highly perishable as they dry out quickly and also involve high transport and

handling costs (Escobar *et al.,* 2006).

In crops like cassava where seeds are rarely used for propagation, tissue culture is important in setting seed systems in Africa as it can also eliminate viruses and other systemic diseases from cassava vegetative materials. Due to the bulkiness of the cassava cuttings the rate of multiplication is low as compared to other crops propagated by seeds. Besides, tissue culture permits rapid production of healthy and improved plants within and even between countries since the material is easily certified as pathogens free (FAO, 2003). These materials also grow uniformly hence are highly marketable.

2.4 Tanzanian cassava landraces

Tanzanian farmers have their own preferred cassava landraces (Appendix1) that are well adapted to their local environmental conditions and some have developed naturally through traditional breeding. Preferences to these landraces are due to characteristics such as high yield, good taste, resistance to diseases and pests, early maturity and good ground storability. Farmers recognize those cassava landraces using morphological characters and each community has its own local name (s) for each (Elibarik *et al.*, 2013).

2.5 Propagation of cassava

2.5.1 Conventional propagation methods

There is absence of flowering in some varieties and in many varieties special conditions are required to stimulate flowering. In addition, there

is low production of seeds often associated with irregular germination (Mussio *et al.,* 1998). Therefore, lignified stem cuttings are used in traditional cassava multiplication systems. Lignification occurs after the plant has matured, which usually takes place after about 5-8 months of growth. In this way, up to four hundred, cuttings are produced in one year from one plant. This traditional technique has been refined into two effective rapid propagation techniques namely; multiple shoot technique and auxiliary bud proliferation technique (Mynie, 2009).

In the multiple shoot technique, lignified cuttings with two nodes are cut from a healthy mature plant and grown in sterilized soil. Sprouts are formed within three weeks. When they have a length of 5-10 cm, the upper part is cut off and rooted in liquid medium after which they are transferred to the field. The auxiliary buds of the lower part of the shoot of the lignified cutting will sprout again and the procedure can be repeated. In the auxiliary bud proliferation technique, stem pieces with a leaf and the accompanying bud are grown in the soil. At least 100 single cuttings can be obtained from one plant. Five months later, these plants can be used to repeat this process. Mynie, (2009) also reported that conventional means of propagation of cassava results in low yield which is 30 plants per year/mother plant which is very low.

Multiplication by traditional methods is often a slow process compared to seed multiplication in grain crops (Santana *et al.,* 2009). Diseases such as cassava mosaic disease and cassava brown streak disease among others also accumulate in the stem cuttings resulting in infected plants and low

yields. Small-scale farmers acquire planting materials from neighbours or as volunteer plants left in fallow (Mutegi, 2009). This contributes to pest and disease accumulation and dissemination. Other challenges with the use of cuttings include high perishability since they dry up within a few days, high handling and transport costs and bulk of the material (Escobar *et al.*, 2006). The feasibility of overcoming these problems by classical plant breeding is hampered by the high degree of heterozygosity, low fertility, poor seed set and low rates of seed germination.

2.5.2 Cassava tissue culture

The invention of tissue culture or *in vitro* propagation has helped overcome challenges involved in propagating vegetative crops as it avails disease free planting materials in large scale (Acedo, 2006). Besides this, tissue culture requires less space as compared to conventional means of producing seedlings (Aladele and Kuta., 2008). Plant tissue culture technology has been used extensively to propagate cassava in many parts of the world (Staden *et al.*, 2008). This is because it has been found to be the best method of producing a large number of disease-free cassava planting materials, hybridization, variety improvement, germplasm conservation and rapid clonal propagation (Staden *et al.*, 2008).

Through tissue culture, challenges such as diseases often associated with conventional practices can be overcome (Santana *et al.*, 2009). This

makes tissue culture an important technology in setting up cassava seed systems. The major components of tissue culture include nutrients/media chemicals (plant growth hormones, vitamins and mineral nutrients (Gitonga *et al.,* 2010).

Tissue culture has effectively been applied in the elimination of viruses and other systemic diseases from cassava vegetative materials allowing exchange and conservation of rejuvenated propagation materials with higher yields (Danci *et al.,* 2011). Prakash *et al.* (2004) reports that sub-Saharan African countries have found it difficult in embracing tissue culture due to high costs involved to purchase chemicals and highly skilled manpower. The technology has majorly been restricted to people with the financial capacity. However, the cost of *in vitro* plant production is an obstacle to its access by smallholder farmers. This therefore calls for the development of new strategies aimed at reducing the cost of plantlet production thereby availing to resource poor farmers healthy planting materials affordably. This can be enhanced by replacement of the costly and high-tech equipment with cheaper alternatives (Gitonga *et al.,* 2010). Use of alternative nutrient sources is also a possible cost reduction intervention in tissue culture, like in the past, a pressure cooker has replaced the auto-clave without detectable contaminations (Ogero *et al.,* 2012a).

2.6 Transformation of cassava

The lack of resistance genes to diseases in the available cassava germplasm, low fertility and unsynchronized flowering makes cassava

improvement by conventional breeding a tedious and long process (Rudi *et al.*, 2010). Due to these limitations genetic engineering has emerged as an important tool in the improvement of cassava (Liu *et al.*, 2011). The important issue that will guide adoption of transgenic cassava plants by farmers in Africa is the development of capacity aimed at integrating transgenes of agronomic significance into appropriate germplasm. There is also need to genetically engineer the most critical landraces, improved varieties and breeding lines for major cassava growing region in Africa (Taylor *et al.*, 2004). Screening vital African genotypes for their transformability represents technical challenges for cassava biotechnologists. There have been several protocols that have been reported such as using either cotyledons or embryogenic cultures as target tissues and particle bombardment (Bull *et al.*, 2009) or *Agrobacterium* mediated transformation (Liu *et al.*, 2011). The most favored method in transformation however has been the use of friable embryogenic callusin combination with agrobacterium mediated transformation due to its higher efficiency compared to the cotyledon-based protocol (Bull et *al.*, 2009; Koehorst *et al.*, 2012).

Moreover, instability of *Agrobacterium*-FEC system produces variable numbers of transgenic events (Koehorst *et al.*, 2012). Much work has been done on model cultivar TMS 60444 (Liu *et al.*, 2011; Taylor *et al.*, 2012) but transformation of farmer and industry preferred cassava cultivars is essential for adoption of transgenic cassava (Bull *et al.*, 2011). It is important to evaluate genetic engineering in different cassava

genotypes adapted to various field environments. Farmers continue to utilize locally available genotypes due to particular desirable traits. Improving local genotypes will increase adaption of the transgenic plants by farmers

2.8 Elimination of viruses from infected plants

Tissue culture is very important technique for the production of clean planting materials in many laboratories. There are many virus elimination methods to date which include thermotherapy, chemotherapy, somatic embryogenesis and meristematic tip culture (Qiaochun and Jari, 2009).

Thermotherapy is the use of high temperatures from 30^{o}C to 40^{o}C to eliminate viruses. The success of this method depends on the target virus's inability to multiply and move readily within the plant being exposed to high temperatures (Panattoni *et al.,* 2013). Chemotherapy is the use of chemicals to eliminate pathogens. Most of the chemicals that have been reported to be potent in viral elimination are salicylic acid, ribavirin and thiouracil (Panattoni *et al.,* 2013). Meristem tip cultures involve the use of apical dome or shoot tip as ositive. Application of meristem tip culture is considered a reference tool for virus eradication (Panattoni *et al.,* 2013). According to Qiaochun and Jari (2009), the size of the meristem tip is critical for virus elimination. The smaller the tip, the better the chance for virus exclusion. Some of these methods can be combined to increase the efficiency of eliminating viruses (Panattoni *et al.*, 2013).

Somatic embryogenesis, usually adopted to regenerate plantlets in breeding programs has been used to eliminate viruses from plants. Explants such as anthers, ovaries or leaves were cultured on a callus induction medium, and the kind of infected tissue used interfered with elimination rates (Popescu *et al.,* 2010).

Cryotherapy is a novel application of plant cryopreservation techniques that allows pathogen eradication at a high frequency (Wang e*t al.,* 2006). It eliminates plant pathogens such as viruses; phytoplasmas and bacteria by briefly treating shoot tips in liquid nitrogen. In cryotherapy, infected cells are eliminated by the lethal effects of the ultra-low temperature. The number of regenerated shoot tips might be lower after cryotherapy than shoot tip culture but the difference is compensated by the easier excision of larger shoot tips (Wang *et al.,* 2006). Cryotherapy has the potential to replace more traditional methods like meristem culture (Qiaochun and Jari, 2009). Besides, its traditional use for the conservation of genetic resources, it has been proven that cryotherapy can be successfully applied to eradicate viruses from plum, banana and grape (Wang *et al.,* 2008).

CHAPTER THREE

EVALUATION OF LOCALLY AVAILABLE NUTRIENTS FOR MASS PROPAGATION OF TANZANIAN CASSAVA LANDRACES

3.1 Abstract

Cassava (*Manihot esculenta* Crantz) is a staple food for over 800 million people in the tropics. However, its production is constrained by an inadequate supply of clean planting materials. Tissue culture carried out in laboratories is one established method for the mass production of clean planting materials. However, the cost of conventional tissue culture is high and the cassava industry would benefit from an alternative means of propagation In the current study, we evaluated a cost effective protocol for micropropagation of the farmer-preferred cassava landraces 'Kibandameno' and 'Paja la mzee' in Tanzania was evaluated. Ammonium fertilizer, potassium fertilizer, osit salt, monopotassium phosphate and calcinit were used as an alternative source to conventional Murashige and Skoog (MS) macronutrients while Stanes Iodized Microfood® was used as alternative to MS micronutrients. Nodal cuttings of the 2 cultivars were initiated in either conventional MS or cost-effective medium supplemented with 20 g/l table sugar, and 3 g/l agar. Conventional MS was used as the control in this study. Four parameters namely plant height, number of leaves, number of nodes and number of roots were recorded from the two media and the differences were determined. For all 4 parameters, both cultivars performed better in the cost-effective medium compared to conventional MS. More than 75% of plantlets acclimatized to greenhouse conditions from both types of media survived. The cost of production of cassava plantlets in both types of media was then calculated and compared. The use of the cost effective medium led to a cost reduction of 93% over conventional MS medium, which makes it a feasible and attractive alternative for growers.

3.2 Introduction

Cassava (*Manihot esculenta* Crantz) is a food crop for millions of people in East Africa and rural areas throughout the tropics and Asia; it is a second staple food after maize (Perez *et al*., 2011). It is a shrub plant with a starchy, tuberous root it is easy to cultivate once established, it can be transformed into different products, and can be stored for several years (Nassar *et al*., 2009). Moreover, it is a valuable source of calories, especially in countries where malnutrition is widespread (Ceballos *et al*., 2004). Because of these qualities, intensive efforts have been made to breed better cultivars. However, the most common method of propagation, the use of cuttings from old plants, makes cassava multiplication tedious and slow (Santana *et al*., 2009), leading to insufficient planting materials which may restrict productivity.

Tissue culture is a technique with the potential to produce a massive number of healthy plantlets regardless of the growing season, thereby ensuring availability of planting material throughout the year. Nevertheless, the adoption of tissue culture techniques is hindered by the high cost of production (Prakash *et al*., 2004). Establishing a tissue culture facility is highly expensive and hence limits the application of this method in many institutions.

To optimize the use of tissue culture, there is a need to develop cost-effective technology in terms of equipment, chemicals, protocols, and required skills so that a reduction in the unit cost of production can take

place without compromising plant quality (Gitonga *et al*., 2010). The aim of this study was to evaluate a cost-effective micropagation protocol for Tanzanian cassava landraces by replacing the macronutrients and micronutrients used in Murashige and Skoog (1962) MS medium with locally available nutrients, particularly fertilizers, for the mass production of farmer-preferred cassava landraces.

3.3 Materials and methods

3.3.1 Study area and collection of plant materials

Two farmer-preferred cassava landraces namely 'Kibandameno' and 'Paja la mzee' were obtained from coastal Tanzania. The cuttings from these landraces were planted in 4L buckets containing loamy soil and allowed to sprout in a greenhouse for 3 weeks at an average temperature of 28ºC to 30ºC.

3.3.2 Preparation of culture media

Two types of culture media were prepared. Conventional Murashige and Skoog (MS) and an alternative media. Conventional medium was supplemented with sucrose at 20 g/l, and phytagel at 2 g/l; this was used as the control (Table 3.1). The alternative MS medium was prepared using locally available materials where the MS macronutrients and micronutrients listed in Table 3.2 were substituted with local fertilizers from Yale industry Ltd; all other nutrients remained the same. The Ph of the 2 types of media were measured, they were dispensed into culture bottles, and sterilized at 121°C and 15 psi for 15 minutes.

3.3.3 Preparation and sterilization of explants

Nodal cuttings of each variety were collected and put into bottles with detergent (Tarmol®) and 3 drops of Tween-20 and washed vigorously with tap water to remove soil particles. The explants were there after taken to a safety clean bench and surface sterilized in 70% ethanol for 5 minutes. They were then rinsed three times in sterile distilled water, followed by 100% JIK® with 2 drops of Tween-20 for 2 minutes and rinsed in sterile distilled water until no foam remained.

3.3.4 Initiation and multiplication

Using a sterile sharp scalpel blade, the damaged parts of each ositive were cut and cultured onto one of the 2 types of media. The cultures were transferred to the growth room and incubated at $28 \pm 2°C$ with a photoperiod of 16 hours light and 8 hours dark for 3 weeks and then subcultured onto fresh media for shoot multiplication.

3.4 Acclimatization

Regenerated plantlets were acclimatized in sterile soil with coconut husks in a ratio of 4:1. Plantlets were washed with sterile water to remove media in order to avoid fungal growth. The plantlets were there after transferred into small pots containing sand, coconut husk and starter fertilizer. The pots were covered with polythene transparent sheets to ensure optimum humidity to protect the plantlets from withering. After 2 weeks, the plantlets were removed from the small pots and transferred into larger pots with soil

and finisher fertilizer. The number of plantlets that survived was recorded after 4 weeks.

3.5 Cost analysis

The price of alternative sources of nutrients were obtained and analysis of each nutrients used in the media was calculated for both conventional and alternative medium. The cost differences between the two media were determined depending on the amount used in the media using the following formula;

$$\text{Cost} = \frac{\text{Amount of nutrient used in medium (g/l) x price of the amount of nutrient bought}}{\text{Amount of nutrient bought (g)}}$$

Table 3.1 Composition of tissue culture medium (MS) used in the micropropagation of cassava

MS Macronutrients	**Concentration in stock solution (g/l)**	**Concentration in a litre of medium (g/l)**	**Amount of stock solution used per litre of medium (ml/l)**
Ammonium nitrate	41.25	1.65	
Potassium nitrate	47.50	1.90	
Magnesium sulfate heptahydrate	9.25	0.37	40
Potassium phosphate monobasic	4.25	0.17	
Calcium chloride dihydrate	11.00	0.44	
MS Micronutrients			
Boric acid	0.62	0.0031	
Manganese sulfate monohydrate	1.69	0.0085	5
Zinc sulfate heptahydrate	0.86	0.0043	
Potassium iodide	83.00	0.0830	1
Sodium molybdate dehydrate*	2.50	0.0025	1
Copper sulfate pentahydrate*	2.50	0.0025	1

Cobalt chloride hexahydrate*	2.50	0.0025	1
Vitamis			
Myo inositol	10.00	0.0100	
Glycine	4.00	0.0002	
Nicotinic acid	0.10	0.0001	1
Pyridoxine HCl	0.10	0.0001	
Thiamine HCl	0.20	0.0010	
Iron source			
Ferrous sulphate heptahydrate	5.56	0.0278	5
Disodium EDTA dihydrate	7.44	0.0372	
Carbon source			
Sugar	-	20	-
Gelling agent			
Phytagel	-	2	-
Ph	5.7-5.8		

*MS = Murashige and Skoog

Table 3.2 Composition of alternative medium used in the micropropagation of cassava (with substitution of macronutrients and micronutrients)

Component	Weight per litre of stock solution(g/l)	Weight per litre of medium (g/l)	Amount of stock solution used/litre of medium (ml/l)
Macronutrients			
Ammonium nitrate	41.25	1.65	
Potassium fertilizer	47.50	1.90	
Epsom salt	9.25	0.37	40
Monopotassium phosphate	4.25	0.17	
Calcinit	11.00	0.44	
Micronutrients			
Microfood®		0.2*	
Carbon source			
Table sugar		20*	
Gelling agent			
Phytagel		2*	
Ph	5.7-5.8		

*****Ingredients that were added during culture media preparation

3.6 Research design and Data analysis

There were four replicates for each variety in the 2 types of media and the plants were arranged in a completely randomized design within the growing area. *In vitro* plantlets were observed for any bacterial and fungal contamination and growth on a daily basis and results were recorded. Comparison between the two types of media was recorded based on four parameters, plant height, number of leaves, number of nodes and number of roots were recorded at 14 days intervals for 6 weeks. Data collected were subjected to T- test using SPSS computer software 16.4 Version using a 5% level of significance.

3.7 Results

3.7.1 Cost efficiency

In total, a 96.06% and 90.27% saving were achieved in the cost of alternative macronutrients and micronutrients, respectively. This translated to a significant price reduction for plantlet production with an overall cost saving of 93.12% per liter of medium (Table 3.3)

Table 3.3 Cost efficiency of conventional Murashige and Skoog (MS) medium compared to alternative tissue culture medium

Conventional MS medium	Alternative medium	Cost in one litre of the medium (Tshs) Conventional medium	Cost effective medium	Cost reduction (%)
Macronutrients				
NH_4NO_3	Ammonium fertilizer	5791	82.5	98.58
KNO_3	Potassium fertilizer	555.75	10.175	98.17
$MgSO_4$	Epsom salt	765.9	22	97.13
KH_2PO_4	Monopotassium phosphate	478.13	62.37	86.96
$CaCl_2$	Calcinit	842.6	4.08	99.51
Sub-total				96.06
Micronutrients				
H_3BO_3		39.6		
$MnSO_4.4H_2O$		1.434		
$ZnSO_4.7H_2O$		3.24		
KI	Stanes iodized microfood®	0.171	37.5	
$Na_2MoO_4.2H_2O$		50.22		
$CuSO_4.5H_2O$		200.772		
$CoCl_2.6H_2O$		74.304		
Ferrous sulphate		15.6		
Disodium EDTA		1.56		
Sub-total				90.27
Total				93.12

3.7.2 Regeneration of cassava plants during multiplication

Regeneration of the two cassava varieties Kibandameno and Paja la mzee in two medium (Conventional MS and alternative meadia) was successful as shown in Plate 3.1 and Plate 3.2.

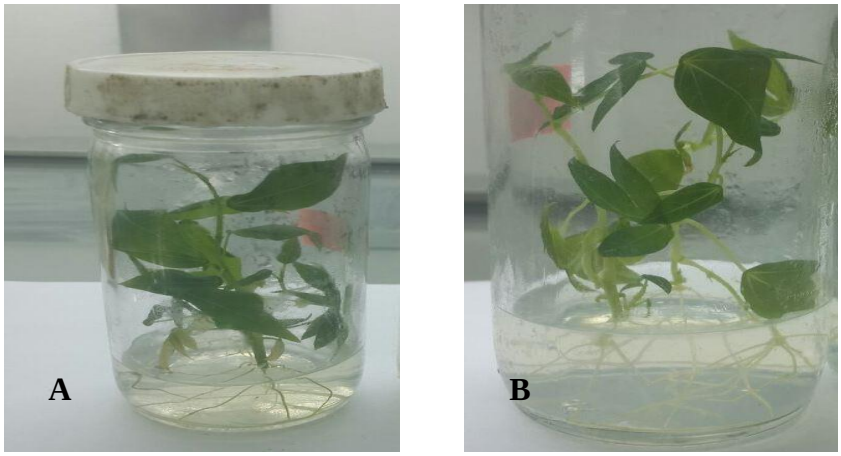

Plate 3.1 Six weeks old cassava plantlets of Paja la mzee (A) and Kibandameno (B) regenerated on alternative medium

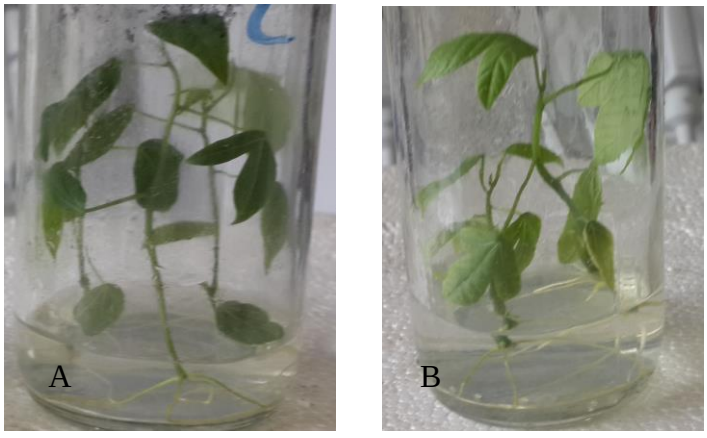

Plate 3.2 Six weeks old cassava plantlets of Paja la mzee (A) and Kibandameno (B) regenerated on conventional media.

Plant height

Cassava plants cultured on cost- effective medium medium were significantly different than those on MS medium for both cultivars during the post-multiplication stage (Table 3.4). The height of 'Paja la mzee' plantlets was significantly taller ($p \leq 0.05$) than that of 'Kibandameno' on alternative medium at sixth week (means of 3.26cm and 3.35cm, respectively), whereas there was no significant difference ($p \geq 0.05$) between the heights of the 2 cassava cultivars on MS medium.

Table 3.4 Plant height produced by the 2 cassava cultivars on alternative medium and conventional medium

Medium	**Variety**					
	Kibandameno			**Paja la mzee**		
	2 weeks	4 weeks	6 weeks	2 weeks	4 weeks	6 weeks
CM	1.65^{b}	2.19^{b}	2.63^{b}	1.26^{b}	1.86^{b}	2.37^{b}
AM	1.53^{b}	2.58^{a}	3.26^{a}	1.6^{a}	2.74^{a}	3.35^{a}

*****Mean ± standard error of plant height. Means having same letters are not significantly different at 5% level. ^{a} and ^{b} comparison within columns.

Number of leaves

There were significant differences ($p \leq 0.05=0.00$) in the number leaves produced by the 2 cultivars on the 2 types of media at 4 weeks; 'Paja la mzee' had a significantly ($p \leq 0.05$) higher mean number of leaves on alternative medium on week 4 (4.25) compared to MS medium (1.83). However, no significant difference ($p \geq 0.05$) in number of leaves of Kibandameno was noted on either medium during the first 2 weeks (Table 3. 5). On average, 'Kibandameno' and 'Paja la mzee' produced the highest number of leaves on alternative medium followed by MS medium.

Table 3.5 Number of leaves produced by the 2 cassava cultivars on alternative medium and conventional medium

Medium	**Variety**					
	Kibandameno			**Paja la mzee**		
	2 weeks	4 weeks	6 weeks	2 weeks	4 weeks	6 weeks
CM	1.08^{b}	2.00^{b}	2.50^{b}	0.42^{b}	1.83^{b}	2.50^{b}
AM	1.25^{b}	3.50^{a}	4.58^{a}	1.58^{a}	4.25^{a}	6.17^{a}

*****Mean ± standard error of leaves. Means having same letters are not significantly different at 5% level. ^{a} and ^{b} comparison within columns.

Number of Roots

Kibandameno had a higher mean number of roots on alternative medium compared to conventional medium at every recording during subculture

(Table 3.6). There was no significant difference ($p \leq 0.05$) in the number of roots produced by Paja la mzee and Kibandameno in at week 2 (Appendix III). Paja la mzee had a higher number of roots on cost-effective medium than conventional medium (Table 3.6). Generally Paja la mzee had a higher mean number compared to Kibandameno on alternative and conventional medium

Table 3.6 Number of roots produced by the 2 cassava cultivars on alternative medium and conventional medium

Medium	**Variety**					
	Kibandameno			**Paja la mzee**		
	2 weeks	4 weeks	6 weeks	2 weeks	4 weeks	6 weeks
CM	1.83^{b}	2.50^{b}	3.33^{b}	1.83^{b}	2.58^{b}	3.17^{b}
AM	2.33^{b}	2.92^{b}	4.65^{a}	1.58^{a}	3.83^{a}	4.92^{a}

*****Mean ± standard error of roots. Means having same letters are not significantly different at 5% level. ^{a} and ^{b} comparison within columns

Number of Nodes

'Paja la mzee' had the highest number of nodes produced on alternative medium followed by MS medium from week 2 to week 6. The mean number of nodes produced by 'Kibandameno' was increasing in every two weeks in both alternative medium and MS medium (Table 3.7).

'Kibandameno' produced a significantly higher number ($p \leq 0.05$) of nodes in alternative medium compared to 'Paja la mzee' on all occasions. There was a significant difference ($p \leq 0.05$) in the number of nodes produced with the 2 cultivars in both media at 6 weeks; however, in the second week no significant differences were observed between the 2 cultivars. For both cultivars, the highest number of nodes was recorded in alternative medium.

Table 3.7 Number of nodes produced by the 2 cassava cultivars on alternative and conventional medium

Medium	**Variety**					
	Kibandameno			**Paja la mzee**		
	2 weeks	4 weeks	6 weeks	2 weeks	4 weeks	6 weeks
CM	1.42^{b}	2.17^{b}	3.08^{b}	1.00^{a}	1.58^{b}	2.50^{b}
AM	1.58^{b}	3.42^{a}	4.17^{a}	0.75^{b}	2.58^{a}	3.75^{a}

*****Mean ± standard error of nodes. Means having same letters are not significantly different at 5% level. ^{a} and ^{b} comparison within columns.

3.7.3 Survival of cassava during the acclimatization stage

Plantlets of the 2 cassava cultivars showed different survival rates when transplanted into small pots containing sterile soil/coconut husk (Plate 3.3). Plantlets from alternative medium showed a higher survival rate than those from CM medium. On CE medium, survival rates were 85% and 79% for

two verities ‘Kibandameno’ and ‘Paja la mzee’, respectively (Fig 3. 1). However, on MS medium, survival rates were 81% and 75% for Kibandameno and Paja la mzee, respectively (Fig 3. 1).

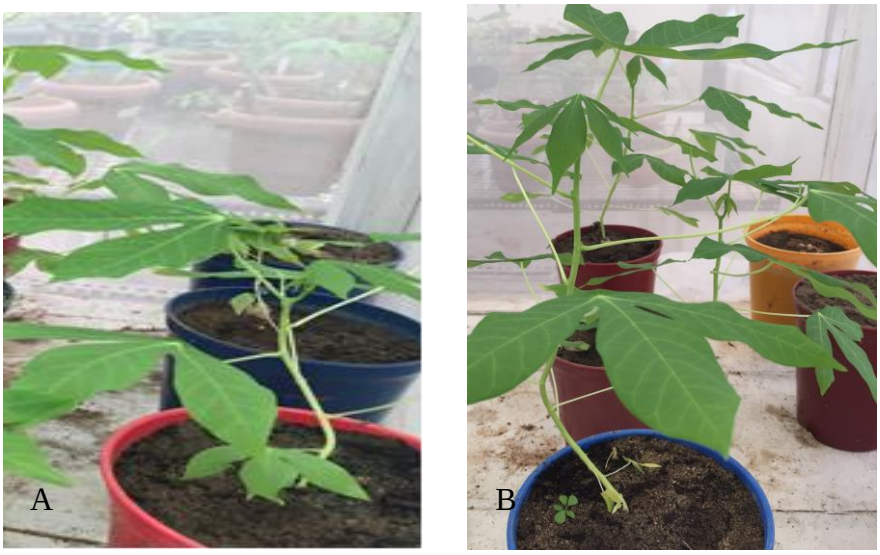

Plate 3.3 Acclimatized cassava plants Paja la mzee (A) and Kibandameno (B)

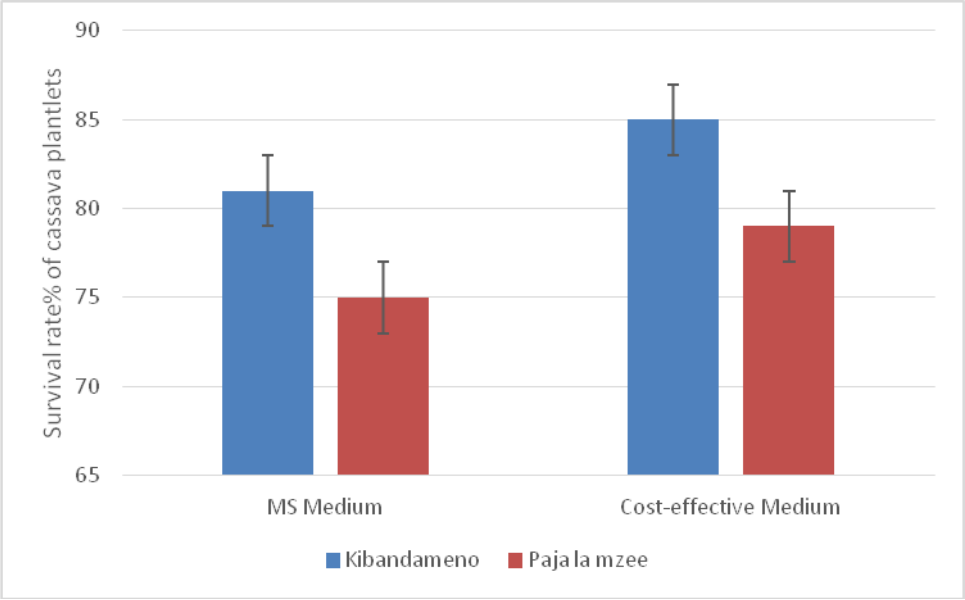

Figure 3.1 Survival (%) of cassava plantlets of the varieties Kibandameno and Paja la mzee, acclimatized from cuttings propagated in MS medium and alternative media.

3.8 Discussion

Cassava productivity is mainly constrained by either a lack of adequate planting material or disease (viral diseases and pests). Tissue culture technology can be used to produce healthy plantlets without seasonal limitations, hence solving one of the constraints of cassava production. However, tissue culture is generally expensive, making such seedlings affordable to farmers, therefore there is a low adoption of this technique in developing countries. In order to achieve sufficient clean plantlets, this study developed a alternative medium that can be used to propagate cassava landraces from nodal cuttings.

Tissue culture technology can easily use locally available nutrients as sources of micropropagation medium nutrients, leading to a significant cost reduction in the production of cassava plantlets *in vitro*. In this study, the cost of media was reduced by over 90% when alternative macronutrients and micronutrients were used. This agrees with the findings of Ogero *et al.* (2012b), where they reported a cost reduction of 95% when alternative sources of macronutrients and micronutrients were used in the multiplication of cassava plantlets in Kenya.

Gitonga *et al.* (2010) reduced the cost of producing tissue culture banana seedlings by 93.9% while using alternative nutrient sources. Several studies on cost-effective protocols for tissue culture have been reported in different areas. For instance, botanical starch (cassava, wheat, and sweetpotato) has been used as an alternative gelling agent (Madege *et al.,* 2015 Mengesha *et al.*, 2012). Mvuria and Ombori (2014) report the successful regeneration of sweet potato using low cost macronutrients. Low cost micropropagation of pineapple is also described by Be and Debergh (2006). Ogero *et al.* (2012a) report *in vitro* micropropagation of cassava through low cost tissue culture in Kenya.

In the current study, the 2 cassava landraces evaluated exhibited different responses to alternative medium and MS medium, perhaps due to genetic variation between the 2 cultivars. ‘Paja la mzee’ performed better on alternative medium compared with ‘Kibandameno’; it produced a higher number of leaves, nodes, and roots. The 2 cassava cultivars have a good

mean plant height, which is necessary for rapid micropropagation as taller plants have a higher number of nodes. This enables easy splicing of nodes during *in vitro* multiplication. Since the two tested cultivars performed well in the alternative medium we suggest that it can be used effectively for other cultivars. The number of leaves developed during the post-multiplication stage was higher in the alternative medium than in the MS medium. Leaves are the major site of food production for the plant and a well-developed leaf system is important for plant survival during acclimatization (Ogero *et al.,* 2012a).

Using a media without plant growth regulators is one way of reducing costs. Roots are essential for plant growth and development as they facilitate the absorption of nutrients (Xiansong, 2010). The alternative medium in the current study contained no plant growth regulators as Yona *et al.* (2010) had previously stated that cassava cuttings root easily without plant growth regulators. Furthermore, Dhanalakshml and Stephan (2014) they also reported the rooting of banana cuttings on low cost tissue culture medium without any plant growth regulator. In our study, roots grew well in the alternative cost-effective medium. Regenerated plantlets were successfully acclimatized into coconut husks and then transplanted into potted soil in the greenhouse. Survival rates of 82% and 78% in plants cultured in the ositive s medium and the MS medium, respectively, were observed

In conclusion, this research has shown that replacement of conventional macronutrients and micronutrients with cheaper locally available nutrients

reduces the cost of cassava plantlet production *in vitro*. The alternative medium evaluated in this study was found to be cot effective and can be used for the mass production of cassava plantlets and hence increase their availability to resource-challenged farmers. The adoption of such a program could increase cassava productivity in Tanzania and worldwide.

CHAPTER FOUR

EVALUATION OF CHEMOTHERAPY AND THERMOTHERAPY FOR THE ELIMINATION OF CASSAVA MOSAIC GERMINIVIRUSES FROM TANZANIAN CASSAVA LANDRACE

4.1 Abstract

Cassava mosaic disease is caused by cassava mosaic begomoviruses (CMBs) and can result in crop losses up to 100% in cassava (*Manihot esculenta*) in Tanzania. The efficacy of chemotherapy and thermotherapy for elimination of *East African cassava mosaic virus* (EACMV) of Tanzanian cassava landrace. *In vitro* plantlets from EACMV infected plants obtained from coastal Tanzania were established in the greenhouse. Leaves were sampled from the plants and tested to confirm the presence of EACMV. Plantlets of plants positive for EACMV were initiated in Murashinge and Skoog (MS) medium. On the second subculture they were subjected into chemical treatment in the medium containing salicylic acid (0, 10, 20, 30, and 40 mg/l) and ribavirin (0, 5, 10, 15, and 20 mg/l). In the second experiment, EACMV infected plantlets were subjected to temperatures between 35°C and 40°C with 28°C as the control. After 42 days of growth DNA was extracted from plant leaves and PCR amplification was performed using EACMV specific primers. It was found that plant survival decreased with increasing levels of both salicylic acid and ribavirin concentrations. In general, plants treated with salicylic acid exhibited a lower plant survival % than those treated with ribavirin. However, the percentage of virus-free plants increased with an increase in the concentration of both ribavirin and salicylic acid. The most effective concentrations were 20 mg/l of ribavirin, and 30 mg/l of salicylic acid these resulted in 85.0% and 88.9% virus-free plantlets respectively. With regard to thermotherapy, 35°C resulted in 79.5% virus-free plantlets compared to 69.5% at 40°C. Based on virus elimination, ribavirin at 20 mg/l, salicylic acid 30 mg/l, and thermotherapy at 35°C are recommended for production of EACMV free cassava plantlets from infected cassava landraces.

4.2 Introduction

There has been a low productivity of cassava per unit area in sub Saharan Africa (Nweke *et al.,* 2002). This has mainly occurred due to the impact of viral diseases such as cassava mosaic disease (CMD) (Bull *et al.,* 2011). CMD is caused by at least nine species of cassava mosaic begomoviruses (CMBs) that can be broadly categorized as African cassava mosaic viruses (ACMVs) and East African cassava mosaic viruses (EACMVs). These viruses are transmitted by whiteflies (*Bemisia tabaci* Gennadius), and their existence can cause yield losses of up to 100% on susceptible genotypes (Varma and Malathi, 2003).

Virus elimination is a technique used for the production of virus-free planting materials. There are several virus elimination methods, including thermotherapy, chemotherapy, electrotherapy, somatic embryogenesis and meristem tip culture (Panattoni *et al.*, 2013).

There have been reports of successful elimination of viruses from infected plants through chemotherapy and thermotherapy, for example Hu *et al.* (2015). In another study, chemotherapy produced 100% virus-free grapevine plants with simple and mixed infection (Guta *et al.,* 2014). Furthermore the elimination of Cassava brown streak virus from cassava was achieved by combining chemotherapy and thermotherapy in Kenya (Mwagangi *et al.,* 2014). However, currently efficacy of chemotherapy and thermotherapy in eliminating viruses causing CMD from Tanzanian landraces is unknown. The aim of this study was to evaluate the

effectiveness of chemotherapy and thermotherapy in eliminating EACMV in widely-grown, farmer- preferred cassava landraces in Tanzania.

4.3 Materials and methods

4.3.1 Study area, source of plant screening for the presence of virus

Cassava stem cuttings of 4-month old Kibandameno variety showing cassava mosaic disease symptoms which are green to yellow mosaic leaves, twisted, narrow and malformed leaf. In condition of severe infections affected plant has small and fewer tubers, were collected from coast area of Tanzania.

Twenty stem cuttings of the cassava cultivar Kibandameno showing CMD symptoms were collected from Tanga region in Tanzania, established and maintained in a greenhouse at Mikocheni Agricultural Research Institute. Ten days after sprouting, leaves were collected and used for DNA extraction using the cetyl trimethly ammonium bromide (CTAB) method (Appendix…) (Lodhi *et al.,* 1994; Xu *et al.*. 2010).

The extracted DNA was subjected to polymerase chain reaction using the primer pair EAB555, forward (5'-TACATCGGCCTTTGAGTCGCATGG-3') and reverse (5'- CTTATTAACGCCT ATATAAACACC-3') that amplifies approximately 555 bp nucleotides of the virus. The polymerase chain reaction mix contained 17.3 µl of sterile distilled water, 1 µl dNTPs (2.5 Mm), 2.5 µl of X10 PCR buffer + 20 Mm $MgCl_2$, 0.2 µl Taq polymerase, 1.0 µl of 10µm primer mix and 2 µl of DNA template.

Polymerase chain reaction conditions were as follows; pre-denaturation at 94 °C for 4 minutes, followed by 35 cycles of denaturation at 94 °C for 1 minute, annealing at 56 °C for 1 minute and elongation at 72 °C for 2 minutes and final elongation at 72 °C for 10 minutes. Samples were then stored at 4 °C until used for further analysis. Polymerase chain reaction amplification was checked by loading 5µl of PCR products in 1.5% agarose gel stained with ethidium bromide submerged in 1X TE buffer for 50 minutes at 180 V. The PCR products were visualized on a gel documentation system (BioDoc-It® 210, USA) and photographed.

4.3.3 Multiplication of EACMV positive cassava plants

4.3.3.1 Medium preparation

Murashige and Skoog medium (MS) supplemented with 20 g/l sucrose and 3 g/l plant agar was sterilized at 121°C for 15 minutes before being used for initiation and multiplication of EACMV infected cassava plants.

4.3.3.2 Initiation and multiplication of EACMV positive plants

Nodal cuttings of EACMV-infected cassava plants were taken using a clean surgical blade and samples were washed vigorously with tap water containing detergent (Tarmol®) and three drops of Tween-20 to achieve surface sterilization. The explants were thereafter transferred to a safety cabinet and washed with 70 % ethanol for 5 minutes. They were subsequently rinsed in sterile distilled water three times. The explants were then soaked in sodium hypochlorite containing Tween-20 for 2 min and

then rinsed with sterile distilled water until foam had been removed. The ends of the nodes were cut with a sterile surgical blade, grown on MS medium and incubated in a growth room at 28°C with a photoperiod of 16 hours light and 8 hours dark. Sub-culturing was performed after 5 weeks.

4.3.4 Virus elimination

4.3.4.1 Chemotherapy

Salicylic acid and ribavirin were used as antiviral compounds. They were prepared by weighing the required chemicals; dissolve in ethanol and then top up with sterile distilled water to the required volume and filter-sterilized using a 0.22 µm Millipore filters. Nodal cuttings from infected cassava were grown in MS medium supplemented with salicylic acid at concentrations of 0, 10, 20, 30, and 40 mg/l and ribavirin at concentrations of 0, 5, 10, 15, and 20 mg/l for three weeks at 28°C in a photoperiod of 16 hours light and 8 hours dark. After three weeks the plants were transferred into medium without antiviral compounds. After treatment leaf samples were collected and molecular analysis was performed.

4.3.4.2 Thermotherapy

Infected nodal cuttings from sub-cultured plants were grown in MS media in a tissue culture growth room at 28°C for 2 weeks, and then moved to a heat chamber with 16 hours light and 8 hours dark for 3 weeks. Temperature levels were set at 28°C (control), 30°C, 35°C and 40°C. leaf samples for all the plants treated were collected and molecular analysis was done to confirm the virus elimination.

4.4 Experimental design, data collection and analysis

The chemotherapy experiment had two treatments, composed of salicylic acid and ribavirin at five concentration levels for each. Each level of treatment contained 12 plants, replicated four times making a total of 480 plants for the chemotherapy experiment. The thermotherapy experiment had four levels of temperature, 28°C, 30°C, 35°C and 40°C. At each level of temperature, 12 plants were used, replicated four times. Surviving plants were recorded, virus- tested and EACMV elimination expressed as percentage virus- free plants out of those survived. Data were analyzed by analysis of variance (ANOVA) with a 5% level of significance, using Genstat (Lawes Agricultural Trust, Rothamsted Experimental Station 2006, Version 15).

4.5 Results

4.5.1 Amplification of DNA extracted from test plants

Of the leaf samples tested, 13 gave PCR products using EACMV- specific primers while 2 did not amplify (Plate 4.1).The EACMV positive plants were cultured and used for thermotherapy and chemotherapy treatment.

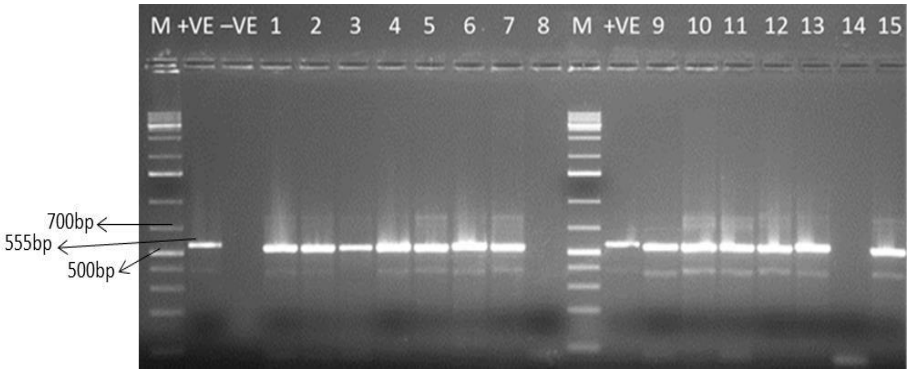

Plate 4.1 Detection of EACMV infected

cassava on 1.5% agarose gel. Lane 1; 1kb molecular marker, lane 2 and 3; are positive and negative control respectively. Lane numbers 1 to 15 correspond to the infected cassava plants that were tested.

4.5.2 Effect of ribavirin on survival and growth of in vitro cassava plants

A total of 123 out of 192 cassava plants survived treatments with ribavirin at different concentrations (Table 4.1). There was a significant difference ($p \leq 0.05$) between different concentrations (Appendix I.i) on survival percentage and virus- free plants. After 42 days of culture some plant leaves and stems at concentration of 20 mg/l became chlorotic (Plate 4.2c) on ribavirin-treated plants and growth was reduced. This was due to the phytotoxic effects of the chemicals such as leaf necrosis and chlorosis, which also led to leaf defoliation. Plants treated with medium concentration (10 mg/l) showed normal growth (Plate 4.2b). Control plants exhibited better growth and remained green (Plate 4.2a). The concentration of antiviral stimulated the number of plants that were regenerated. It was observed that the increase in antiviral concentration decreased the survival percentage of plants as observed in Table 4.1.

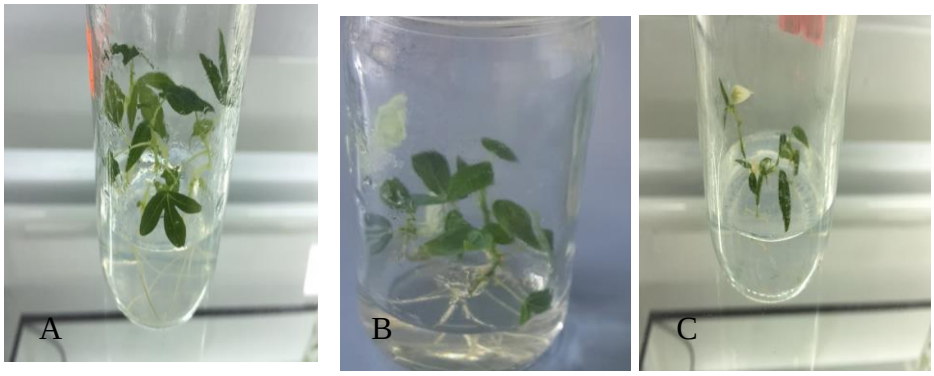

Plate 4.2 Growth of *in vitro* cassava plantlets on Musharige and Skoog (MS) medium after 42 days on ribavirin treatment. Control 0 mg/l (A), 10 mg/l (B), 20 mg/l (C)

Table 4.1 Means of survival and virus elimination in cassava plants (after 42 days) on ribavirin treatment

Ribavirin conc (mg/l)	Plants initiated	Plants survived	Survival (%)	Survival	Negative plants (%)*	Negative means	Positive plants (%)*	Positive
0	48	48	100	3.0[d]	0(0.0)	0.0[a]	48(100.0)	3.0[c]
B 5	48	40	83.3	2.5[cd]	21(52.5)	1.4[b]	19(47.5)	1.1[b]
10	48	35	72.9	2.2[bc]	23(65.7)	1.9[b]	12(34.3)	0.4[a]
15	48	28	58.3	1.8[ab]	20(71.5)	1.5[b]	8(28.6)	0.3[a]
20	48	20	41.6	1.3[a]	17(85.0)	1.1[b]	3(15.0)	0.2a

*Values in parentheses indicate the percentage of plants tested negative and positive. Means followed by a common letter are not significantly different at $p \leq 0.05$.

4.5.3 Effects of salicylic acid on survival and growth of *in vitro* cassava plants

Cassava plants surviving treatments with salicylic acid at different concentrations were 69 out of 192 (Table 4.2**).** Phytotoxic effects like leaf necrosis and chlorosis was observed on plants treated with salicylic acid at 30 mg/l and 40 mg/l after 42 days of culture (Plate. 4.3c) which led to growth reduction of the plantlets, while control plants exhibited normal growth (Plate. 4.3a). There was a significant difference ($p \leq 0.05$) between different concentrations (Appendix I.ii) on survival % and virus- free plants. The number of plants regenerated was proportional to the concentration of

salicylic acid. The number of regenerated plants from the salicylic acid treatment at 10 mg/l and 30 mg/l were 32 and 9 respectively. It was observed that the increase in antiviral concentration decreased the survival % of plants. Plants did not survive the highest concentration of salicylic acid (Table 4.2).

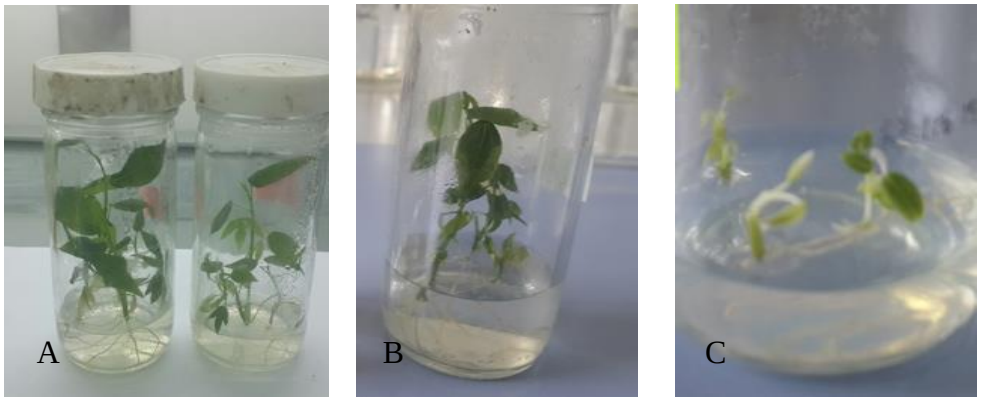

Plate 4.3 Growth of *in vitro* cassava plantlets on Murashige and Skoog (MS) medium after 42 days on salicylic acid treatment. Control 0 mg/l (A), 20 mg/l (B), 30 mg/l (C).

Table 4.2 Survival and virus elimination in cassava plantlets grown on media treated salicylic acid (after 42 days)

Salicylic acid conc (mg/l)	Plants initiated	Plants survived	Survival (%)	Survival	Negative plants (%)*	Negative means	Positive plants (%)*	Positive
0	48	48	100	3.0[c]	0(0.0)	0.0[a]	48(100.0)	3.0[c]
10	48	32	66.7	1.8[b]	22(68.8)	1.1[b]	10(31.2)	0.6[b]
20	48	28	28.3	1.6[b]	21(75.0)	1.2 b	7(25.0)	0.3[ab]
30	48	9	18.8	0.6[a]	8(88.9)	0.6[a] b	1(11.1)	0.0[a]
40	48	0	0	0.0[a]	0(0.0)	0.0[a]	0(0.0)	0.0[a]

*Values in parentheses indicate the percentage of plants tested negative and positive. Means followed by a common letter are not significantly different at $p \leq 0.05$.

4.5.4 Effects of thermotherapy on growth and survival of in vitro cassava plants

Plants that were subjected to different temperature regimes had different responses. Results indicate that there was significant difference ($p \leq 0.05$) between temperature regimes on survival percentage and virus-free plants (Appendix I.iii). All the plants exposed at 28°C grew normally (Plate 4.4a) while all the plants at higher temperatures (35°C- 40°C) exhibited abnormal growth. Plants subjected at 30°C exhibited normal growth as plants

subjected at 28°C. It was observed that at higher temperatures the plant leaves became chlorotic, dried up and dropped off (Plate. 4.4c). Survival percentage among the four temperature regimes was over 47% (Table 4.3). Thermotherapy treated plants at higher temperature resulted in lower survival percentage of 47.9 % at 40°C compared to 93.8% at 30 °C. Survival decreased with increase in temperature **(**Table 4.3).

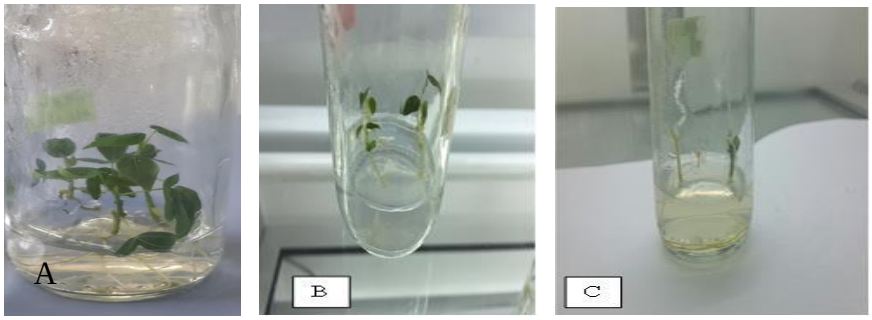

Plate 4.4 Growth of *in vitro* cassava plants after 42 days temperature treatment. Control 28°C (A), 35°C (B), 40°C (C)

Table 4.3 Survival and virus elimination in cassava plants (after 42 days) on thermotherapy

Temperature (°C)	Plants initiated	Plants survived	Survival (%)	Survival	Negative plants (%)*	Negative means	Positive plants (%)*	Positive
28	48	48	100	3.0[c]	0(0.0)	0.0[a]	48(100)	3.0[c]
30	48	45	93.8	2.5[bc]	22(48.9)	0.94[b]	23(51.1)	1.6[b]
35	48	39	81.3	2.3[b]	31(79.5)	1.7[b]	8(20.1)	0.6[a]
40	48	23	47.9	1.3[a]	16(69.5)	1.0[b]	7(30.5)	0.3[a]

*Values in parentheses indicate the percentage of plants tested negative and positive. Means followed by a common letter are not significantly different at $p \leq 0.05$.

4.5.5 Efficiency of chemotherapy and thermotherapy in elimination of EACMV from infected cassava plants

The results obtained showed that virus elimination efficiency relied on chemical concentration and temperature regime. Higher concentrations and higher temperatures resulted in greater levels of virus elimination. In the 20 mg/l ribavirin treatment, 85% of plantlets were virus-free, while at 5 mg/l, this figure was 52% (Table 4.1). For salicylic acid at 30 mg/l, 88.9% plantlets were virus-free, compared to concentration of 10 mg/l (68% virus-free) (Tables 4.2). Thermotherapy resulted in more than 50% virus- free plants with 35°C showing the highest virus elimination of 79.5%. At 40°C the number of surviving plantlets was observed to be low but the efficiency

of virus elimination was high (69.5%) compared to 30°C which showed 48.9% (Table 4.3).

4.6 Discussion

The effectiveness of different *in vitro* techniques for elimination of EACMV from infected cassava plants was assessed. Salicylic acid and ribavirin had different effects on survival and growth of cassava plants. It was observed that plant growth was attenuated at higher concentrations of the antiviral compounds and the leaves turned yellowish and eventually dropped off. This observation is consistent with the study of Hu *et al.* (2015) who observed slower growth of apple plants treated with ribavirin. However plants subjected to low concentrations of chemicals in the current study grew better and no defoliation was observed.

The efficiency of virus elimination following chemical treatment was influenced by concentration levels. The rate of virus elimination increased at higher concentrations of both chemicals. At higher concentrations of both antiviral compounds phytotoxic effects were manifested by the low survival percentage on both experimental variants, where by high mortality of the shoots and defoliation was observed. It was reported by Cieslinka, (2007) that while increased concentration of ribavirin increases the effectiveness of virus elimination, it increases phytotoxicity and slows plantlet growth. In the current study, the above findings were observed at 20 mg/l ribavirin treatment. Therefore, a higher number of regenerated clean plants as well as low survival percentage were found for both ribavirin and salicylic acid

treated plants at high concentrations (Table 4.1and 4.2). At this point viral RNA mutation exceeds a critical threshold and leads to infectivity and/or extinction of the virus population (Panattoni *et al.,* 2013). On the other hand, a high plant regeneration rate occurred at low concentrations since these levels were less toxic and furthermore, over 50% were virus-free. These results showed that even at low concentrations virus replication was hindered as in the cases of ribavirin at 5 mg/l and salicylic acid 10 mg/l (Table 4.1 and 4.2). Similar results were obtained by Seker *et al*. (2015) in eliminating Plum pox potyvirus (PPV) from infected apricot shoots using chemotherapy. Even though salicylic acid treated plants had a lower survival percentage at higher concentration of the chemical, treatment resulted in a greater percentage of virus-free plants. This result is consistent with that of Mwagangi *et al.* (2014) where they note a similar effect after chemical treatment.

Regarding temperature, different regimes affected the efficiency of virus elimination from infected cassava plants. Thermotherapy has been widely used to eliminate viruses from trees, herbaceous plants and other vegetative plants (Valero *et al.,* 2003; Tan *et al.,* 2010; Panattoni *et al.,* 2013). Plant-virus interaction is affected by temperature and higher temperatures are associated with minimal virus levels in infected plants (Qu *et al.,* 2005). Wang *et al.* (2008) reported that exposure of plants to heat stress reduces movement of virus particles into the apical meristem by inhibiting viral RNA synthesis. In this study, cassava plantlets showed a varying heat treatment response, which ranged from vitrification to total death of the

plantlet due to heat stress. The growth rate of temperature treated plants decreased with increasing temperature. At higher temperatures, the rate of virus elimination was greater than at low temperature. However, low plantlet survival was observed at high temperatures (40°C) and higher survivalpercentage at low temperatures. Optimal survival was observed with incubation at 35°C for 4 weeks which achieved 79.5% virus-free plants. This agrees with the findings of Delgado and Rojas (1992) and Acheremu *et al.* (2015). The effect of temperature was due to the ability of heat to inhibit virus multiplication (Manangaris *et al.,* 2003). The study observed that thermotherapy and chemotherapy eliminated EACMV, with virus- free plants most optimally obtained at10 mg/l ribavirin (72.9% survival, 65.7% virus- free), 10 mg/l salicylic acid (66.7% survival, 68.8% virus- free) and 35°C (81.2% survival, 79.5% virus- free).

In conclusion, this study demonstrated that virus-free plantlets can be produced *in vitro* from diseased plants thereby enhancing clean seed multiplication and conservation of valuable germplasm. Based on virus-free percentage, ribavirin at 20 mg/l, salicylic acid 30 mg/l, and thermotherapy at 35°C are recommended for production of EACMV free cassava plantlets from infected cassava landraces. . Further studies should be conducted to evaluate the combined effects of chemotherapy and thermotherapy on EACMV elimination.

CHAPTER FIVE

DETERMINATION OF THE TRANSFORMABILITY OF TANZANIAN CASSAVA LANDRACES

5.1 Abstract

Genetic engineering is an important approach for cassava improvement. A lot of research has been done on the model cultivar TMS60444 and the protocols have been established. Less is known for farmer-preferred landraces in Tanzania. The aim of this study was to determine the transformability of a selected Tanzanian cassava landrace using the β-glucuronidase (GUS) as a reporter gene. Somatic embryo cultures were established from two cultivars TMS60444 and Albert in MS medium supplemented with copper sulphate and picloram then transferred into Gresshof and Doy with Picloram and Gresshof and Doy with Picloram and Tyrosine for TMS6044 and Albert, respectively for friable embryogenic callus production. The produced friable embryogenic calli were sub-cultured in Gresshof and Doy with Picloram after every 21 days for three cycles. Friable Embryogenic Calli were infected with *Agrobacterium tumefaciens* strain LBA4404 harboring the Pco62 vector having a GUS reporter gene. The transformability was confirmed by the GUS histochemical assay and later by PCR using GUS specific primers. The transformed friable embryogenic calli were regenerated into somatic embryos on Murashige and Skoog medium supplemented with 1-naphthaleneacetic acid. 55% and 44% of FEC formed somatic embryos. The highest (86%) transformation efficiency was observed in TMS60444 and 83% for Albert. It was concluded that Tanzanian cassava landraces are transformable using *Agrobacterium tumefasciens.*

5.2 Introduction

Cassava is one of the most significant food crops produced in the developing countries as a major source of carbohydrates and income generation (FAO, 2012). The global demand for cassava is rapidly growing because of its increasing use by industries and for biofuel production (Jansson *et al.,* 2009). Despite its importance, there is a need to develop cultivars with increased nutrient content, improved processing quality and with tolerance against biotic and abiotic stresses (Bull *et al*., 2009). Cassava improvement through traditional breeding is difficult due to its heterozygosity nature, thus the use of genetic engineering technologies to incorporate desired traits is vital. (Bull *et al.*, 2009).

Agrobacterium- mediated transformation using friable embryogenic callus (FEC) has emerged as one of the most effective methods of transformation as it has more advantages than other transformation systems such as microprojectile and particle bombardment which are high transformation efficiency, transfer of large pair of DNA, reduced risk of generating chimeric plants and results in fewer non transformed plants being regenerated. (Bull *et al.,* 2009). However, establishing embryogenic tissues is cultivar dependent and requires optimization for each cultivar (Nyaboga *et al.,* 2015).

Many transformation protocols using FEC for model cultivar TMS 60444 have been reported from different laboratories in developing countries, but few transformation reports for local cultivars especially in East Africa are available (Zainnudin *et al.,* 2012). This is due to lack of technology uptake caused by limited a number of well-equipped laboratories and difficulty in adapting protocols developed for model cultivar TMS60444 (Bull *et al.,* 2011; Nyaboga *et al.,* 2013). Improved *Agrobacterium*-mediated transformation protocols for local cassava landraces has been reported in Uganda (Nyaboga *et al.,* 2015). In this study, the transformability of a Tanzania landrace and a model cultivar was determined.

5.3 Material and methods

5.3.1 Plant material

Two varieties were used in this study; one Tanzanian farmer preferred variety Albert and one model variety TMS60444 (control). Stem cuttings of the two varieties were maintained in green house at DSMZ before transferred into a growth chamber.

5.3.2 Bacterial strain

The culture of *Agrobacterium tumefaciens* strain LBA444 harboring vector Pc062 having the reporter GUS gene maintained in the Laboratory at DSMZ was used for the transformation.

5.3.3 Media and reagents preparation

Murashige and Skoog medium, Gresshoff and Doy medium, Yeast extract broth medium, other reagents (Naphthalene acetic acid, 6-

Benzylaminopurine(BAP), Picloram,copper sulphate, Acetosyringone, tyrosine, carbenicilin) were prepared as detailed by Bull *et al.* (2009); Nyaboga *et al*. (2013). Prepared media and reagents were sterilized by autoclaving at 121°C for 15 minutes and stored at 4°C.

5.3.4 Initiation of nodal cuttings into tissue culture

Stem cuttings from the greenhouse were excised using a surgical blade and placed in a bottle with water and Tween-20. The cuttings were washed vigorously with clean tap water to remove all debris. In a hood, the explants were washed with 100% JIK® and 3 drops of Tween-20 for 20 minutes. They were rinsed with sterile distilled water three times. The damaged ends of each node were cut off, the nodes dried on sterile filter paper before placing them on MS medium. Cleaned nodes were grown on MS medium in tubes supplemented with sucrose 20 g/l and 2 g phytagel as solidifying agent. The plantlets were incubated in the growth room at 28°C with photoperiod of 16 hours light/ 8 hours dark. The plantlets were subcultured after every 3 weeks to have enough materials for induction of embryogenic tissues. On the last subculture the plantlets were placed on medium in petri dishes for easy harvesting of immature leaf-lobes.

5.3.5 Induction of somatic embryos

Immature leaf lobes from 5 to 7 days old Albert and TMS6044 *in vitro* plantlets were excised using a sterile needle and the ositive was placed on the adaxial side on plates with MS medium supplemented with 2 µM copper sulphate and 12 mg/l picloram (MSCP). TMS60444 had 12 plates

each with 10 explants and Albert had 10 plates with each plate having 10 explants. They were incubated at 28°C for 14-16 days dark for formation of embryogenic structures. After 16 days, immature embryos were formed and they were cleaned by removing the non-embryogenic calli around the embryos and transferred onto fresh MSCP medium. The formed embryos were repeatedly sub-cultured in the dark after every 10-14 days in order to mature more and later moved onto Greshof and Doy (GD) medium for production of FEC. Using large amount of starting materials allowed the production of large quantities of somatic embryos which are starting materials for FEC production.

5.3.6 Production and proliferation of friable embryogenic calli (FEC)

Production of FEC was done according to modified protocol detailed by Bull et al. (2009), where somatic embryos from the two cassava varieties (Albert and TMS60444) were used for FEC production. Somatic embryos from Albert were crushed through metal gauze, washed with sterile distilled water and then GD liquid medium, poured onto a mesh dried briefly on filter paper and placed on GD medium supplemented with 12 mg/l picloram and 750 µM tyrosine (GDPT) for 7 days, in dark and then transferred onto GD medium without tyrosine (GDP). For TMS60444, clusters were transferred on GDP without tyrosine. The plates with clusters were incubated at 28°C in dark for 21 days. Emerging FEC were isolated and transferred onto GDP medium for 21 days in the dark at 28°C. Purification and sub-culturing was done repeatedly for up to 3 cycles in order to

multiply high quality and pure FEC, incubated under 16 hours light and 8 hours dark at 28°C.

5.3.7 Preparation of *Agrobacterium* culture for inoculation

Pre-culturing of *Agrobacterium tumefaciens* strain LBA4404 harboring the plasmid Pc062 with GUS gene and *nptII* marker) was made in 3 ml of yeast extract broth medium (YEB) containing $MgSO_4$ (Yeast extract 1 g/l, beef extract 5 g/l, peptone 5 g/l, and sucrose 5 g/l) with kanamycin as the selective agent overnight. 50 µl of over grown pre-culture was taken and inoculated into 50 ml of YEB medium with kanamycin and 50 µM acetosyringone, incubated with overnight shaking. When the optical density (OD_{600}) reached 0.25 to 0.5, the culture was centrifuged for 15 minutes at 4°C and 5000 rpm. The supernatant was removed and the pellet was re-suspended in GDP broth centrifuged for 10 minutes at 4°C and 5000 rpm. This step was repeated two times to remove all the debris. The pellet was then re-suspended in GDP with acetosyringone 200 µM kept on a shaker at room temperature for 30 minutes and 75 rpm.

5.3.8 Infection of friable embryogenic callus with *Agrobacterium tumefaciens*

Transformation of the two cassava varieties was performed following improved transformation protocols by Bull *et al* (2009); Zainnudin *et al.* (2012); Nyaboga *et al.* (2015). Friable embryogenic callus were co-cultivated with 25 ml Agrobacterium suspension in 50 ml Falcon tubes and incubated for 30 minutes at 40 rpm. Inoculated FEC were transferred onto a

sterile mesh placed on sterile filter paper for 2 minutes to remove excess bacterial suspension. The FEC were co-cultured on GDP medium for 4 days in dark at 22°C. After 4 days, the co-cultured FEC were washed 3 times with GDP containing 500 mg/l carbenicillin by scraping the FEC + *Agrobacterium* from the plate using sterile forceps and placed in 25 ml of the GDP broth. The material was washed by inverting the tube slowly for 1 minute and the supernatant was removed. The washing process was repeated until the supernatant was clear. The FEC was then ositiv onto a sterile mesh and extra fluid was removed. The mesh was placed on GDP with 250 mg/l carbenicillin and incubated for 4 days for TMS60444 and 7 days for Albert with photoperiod of 16/8 hours light / dark at 28°C.

5.3.9 Selection and regeneration of transformed FEC

After 7 days of the recovery phase, the mesh with transformed FEC was transferred onto GDP with 250 mg/l carbenicillin and 20 mg/l geneticin and kept under 16/8 hours photoperiod at 28°C for 7 days. This step was repeated with gradually increasing geneticin concentration (30 mg/l to 50 mg/l) to increase the chance of regeneration. Following selection of FEC, the mesh with FEC was transferred onto regeneration medium, MS supplemented with 1 mg/l NAA, 100 mg/l carbenicillin and 50 mg/l geneticin (MSN) for 14 days with 16/8 hours photoperiod at 28°C. This process was repeated five more times until cotyledons were developed. Cotyledons were isolated and transferred onto new MS medium supplemented with 100 mg/l carbenicillin, 2 µl copper sulphate and 4 mg/l BAP for maturation.

5.3.10 Histochemical Gus assay

Histochemical GUS assay was used as visual representation of transformation success. The GUS buffer was prepared with 50 Mm Na_2HPO_4, 1 Mm Na_2EDTA, 1 Mm Fe/Fe++CN and 0.1% Triton X-100. The Ph was adjusted to 7.0 with NaOH, filter sterilized and kept in dark bottles at 4°C. The stock solution of 25 mg/ml 5-bromo-4-chloro-3 indolyl-ß-D-glucuronic acid (Xgluc) was prepared in dimethyl sulfoxide and was stored at -20°C. The GUS assay buffer was prepared freshly by mixing 1 part of X-Gluc with 49 parts of GUS buffer. The transformed materials (FEC, embryos) were incubated in GUS assay buffer at 37°C overnight. The GUS buffer was removed and the materials were washed using 70% ethanol for 10 minutes. This was repeated 2 times. For the GUS assay, (insert the number of tissues used) was incubated with the assay buffer and the experiment repeated three times. Wild type tissues (not transformed) were also included as a negative control. The number of tissues showing a blue coloration was considered transformed. These were recorded and used to calculate transformation frequency as a percentage of the total transformed tissues.

5.3.11 Molecular analysis of transgenic FEC and embryos

Polymerase chain reaction analysis for GUS gene was done to confirm the presence of the gene in plant genome using gene specific primers. Total genomic DNA was extracted from *in vitro* grown embryos using CTAB method (Appendix II); (Lodhi *et al.*, 1994; Xu *et al.*, 2010). The primer

sequences used were; forward 5'-GGGCATTCAGTCTGGATC-3' and reverse 5'-GTGCGGATTCACCACTTG -3'. Plasmid DNA of Pco62 and non-transformed plant DNA was used as positive and negative controls respectively. The reaction mixture for amplification had the following; The PCR reaction mix contained 17.3 µl of sterile distilled water, 1 µl dNTPs (2.5 Mm), 2.5 µl of X10 PCR buffer + 20 Mm $MgCl_2$, 0.2 µl Taq polymerase (conc), 1.0 µl of primer mix and 2µl of DNA. The plasmid was used as positive control and the DNA from non-transformed embryos was used as negative control. The reaction conditions were 94°C for 2 minutes followed by 30 cycles of 94°C 30 seconds, 56°C for 1 minute, 72°C for 1 minute and final extension of 72°C for 10 minutes. The PCR products were visualized on a gel documentation system (BioDoc-It® 210, USA) and photographed. The number of PCR positive materials was used to determine the transformation efficiency; calculated as number of plants showing presence of the GUS gene after PCR as a percentage of total tissues transformed.

5.4 Results

5.4.1 Induction of somatic embryos

The selected Tanzanian cultivar Albert and model cultivar TMS60444 produced embryogenic structures as explained in section 5.3.5 using leaf lobes after 14 -16 days of culturing. (Fig5.1b). Percent somatic embryos produced from leaf lobes placed on MS medium supplemented with 50 µM was 79 % and 87 % for Albert and TMS60444 respectively (Table 5.1). The

somatic embryos produced were used for production of friable embryogenic callus (FEC) which is target materials for transformation in this study.

Table 5.1 Somatic embryos and FEC induction for two selected cassava cultivars

Variety	Somatic embryo (Clusters)	Percentage of somatic embryos (%)	FEC (Clumps)	Percentage of FEC (%)
TMS60444	70	87	53	76
Albert	58	79	30	52

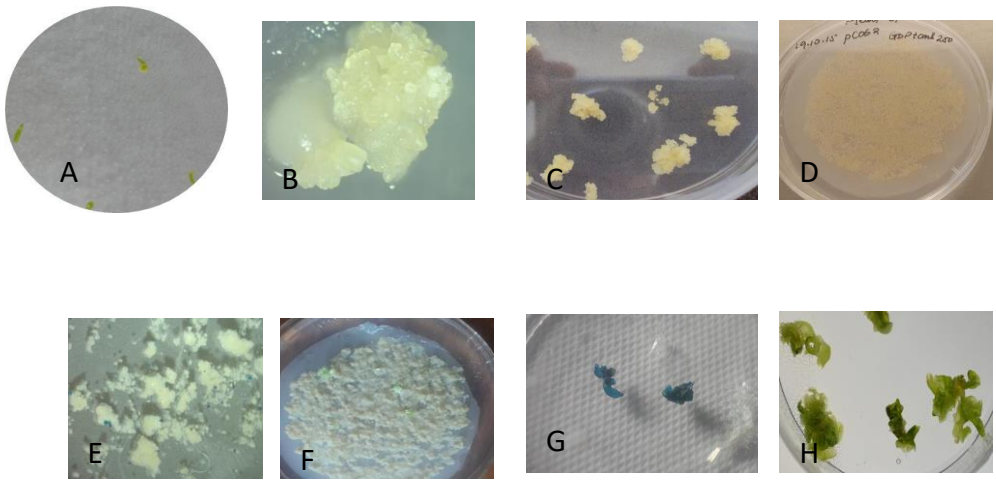

Plate 5.1: Production of transformed plants: Immature leaf lobe (A), primary somatic embryos (B), friable embryogenic callus (C), Agrobacterium-inoculated FEC (D), Gus positive FEC (E), emerging

somatic embryos from FEC F), GUS positive somatic embryos (G), maturing somatic embryos (H).

5.4.2 Production of friable embryogenic callus for selected cultivars

Somatic embryos of the two cultivars Albert and TMS60444 transferred on GD medium supplemented with picloram for production of FEC between 21 days to 30 days, 52% of the transferred clusters of Albert and 76% of TMS60444 developed into FEC (Table 5.1). The potential to generate FEC depends a lot on the quality of somatic embryos produced for both varieties, variety TMS60444 generated high number of FEC than Albert. It was observed that Albert took longer time to produce FEC compared to TMS60444 which took a shorter period.

Table 5.2 FEC and embryo generation percentage, GUS test and transformation efficiency

Variety	FEC on MSN (Clumps)	GUS positive FEC (%)	Percentage that positive embryo	Number of tested embryos	PCR Positive	PCR Negative	Transformation efficiency (%)
TMS60444	11	80	55	8	6	1	86
Albert	9	69	44	6	5	1	83.3

5.4.3 Transformation

Friable embryogenic callus produced form the two cultivars were successful transformed with GUS gene as explained in section 5.3.8. The co-cultured FEC were recovered and transferred into selection medium GDP with 20 mg/l to 30 mg/l Geneticin and carbenicillin to suppress the growth of *A.tumefaciens.* The transformed FEC were proliferated for 6 weeks with medium replenished every week to enhance the chance for regeneration. The transformed FEC from the two cultivars were able to regenerate into cotyledonary embryos. Transformation efficiency was calculated based on the explained formula and the results showed that the efficiency was 83.3% and 86% for Albert and TMS60444 respectively (Table 5.2).

5.4.4 Histochemical GUS assay

Transient GUS expression assay was done in different stages of growth of transformed FEC (callus and cotyledonary–stage embryos) and showed blue coloration which confirms transient expression of the reporter gene in FEC of the two selected cassava cultivars (Plate5.1e and g). The uniform blue color observed in regenerated embryos, confirm stable expression of the GUS gene. There was no colour change on the control materials.

5.4.5 Molecular analysis of transformed embryos from Albert and TMS60444

Polymerase chain reaction done on the transformed embryos confirmed the presence of the introduced gene following the observation of the 400 bp amplicon in tested materials which correspond to the fragment of the GUS

gene (Plate 5.3) same as the positive control. Non-transgenic material (control) did not show any amplification (Plate5.3 lane 2). The embryos which did not pick up the gene showed no amplification (Plate 5.3 no 4 and 11)

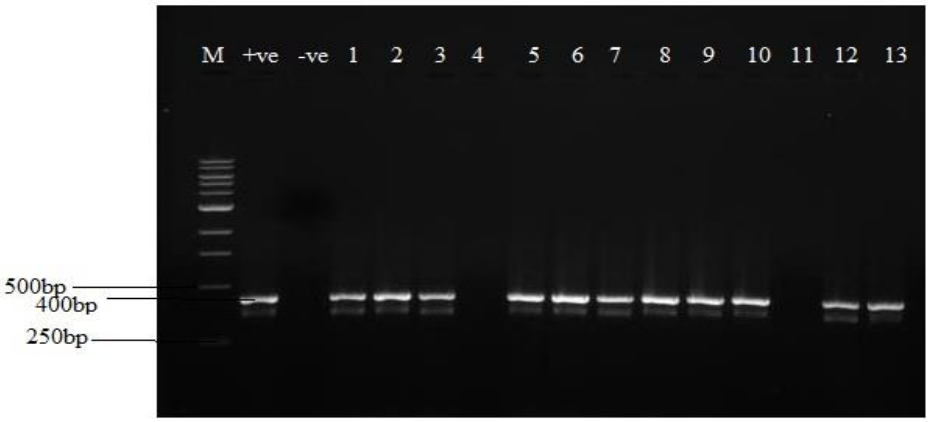

Plate 5.2 PCR amplification of GUS encoding gene in transgenic cassava. Lane 1: 1kb ladder, Lane 2; positive control, lane 3; Negative control, number 1-6, Albert, Number 7-13, TMS60444

5.5 Discussion

The transformability of two selected cassava varieties was determined. The induction of somatic embryos from immature leaf lobe explants of Albert and TMS60444 for production of FEC (which were targeted for transformation) was 76% and 52%, respectively. The highest percentage of somatic embryos was observed in TMS60444, but also Albert showed a high percentage in production of somatic embryos which is more than 50%. Generation and proliferation of FEC from the formed somatic embryos

were important for efficient transformation. Studies have been done to optimize production of FEC from different local cultivars as FEC production is cultivar-dependent, thus the previous protocols were working only for TMS60444 (Taylor *et al.* 2012; Nyaboga *et al.* 2013; Nyaboga *et al.* 2015). The genotypic dependency on production of FEC was also observed in this study which is in agreement with other previous studies (Hankoua *et al.,* 2005; Liu *et a.,l* 2011; Nyabonga *et al.,* 2013). Thus following the modified protocol by Zainuddin et al., (2012); Nyabonga et al., (2015), somatic embryos from Albert was crushed which increased the chance for FEC production. The production of FEC form Albert was almost similar to control cultivar TMS60444. It was also observed that TMS6044 produced more quality FEC compared with Albert. This was also observed by Ubalua and Mbanaso, (2014) who produced FEC from Nigerian local cultivar and TMS60444.

In this study, the transformation of the two varieties was successful following the modified protocols. Sufficient amount of FECs produced was the central to success as FECs are the target tissue for transformation. 80% and 69% of the transformed FECs for TMS60444 and Albert respectively were positive for GUS (Table5.2), this is in agreement with the studies done by Zainudinn *et al.* (2012); Nyaboga *et al.* (2013); Nyaboga *et al.* (2015) who transformed several cassava varieties from East African countries. Transformed FECs started developing somatic embryos after six cycles on MS medium supplemented with NAA with weekly replenishment of medium. The FECs on MSN 55% of TMS60444 and 44% of Albert formed

mature somatic embryos. Transformation efficiency for TMS6044 and Albert were 86% and 83.3% respectively. TMS6044 had higher efficiency than Albert. This is in conjunction with other studies reported, which showed that TMS60444 in all transformation done had higher efficiency (Bull *et al.*, 2009; Zainuddin *et al.*, 2012; Taylor *et al.*, 2012). Comparing transformation efficiency in cassava is complicated because of differences in tissue culture system, transformation method, genotypes and regeneration capacity among FEC lines (Nyabonga *et al.*, 2013). The presence of the GUS gene in transformed materials was confirmed through GUS assay and PCR. Bands corresponding to transgene integration were observed for the selected materials which confirm the integration into plant genome.

In conclusion, the present study provides a versatile stable transformation protocol for farmer preferred cassava landrace in Tanzania using FEC. The improvement in tissue culture and regeneration procedure will enable efficient production of transgenic cassava landraces with any gene of interest so as to provide enough clean planting materials for farmers.

CHAPTER SIX

GENERAL DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

6.1 General Discussion

This research aimed at evaluating *in vitro* cost effective micropropagation, virus elimination and transformation protocol for production of virus-free Tanzanian cassava landraces. Findings from this study showed that use of locally available nutrients for mass propagation of two farmer preferred cassava varieties (namely Kibandameno and Paja la mzee) reduced the cost of production by 93%. The cost-effective medium was found to work as the conventional tissue culture medium, which was analyzed by considering plant height, number of leaves, number of nodes and number of roots. The two varieties had different responses on cost-effective medium and conventional medium. Kibandameno showed high performance on cost-effective medium compared to Paja la mzee by having a higher mean plant height, number of leaves, number of nodes and number of roots.

The acclimatization procedure for the two varieties was successful, where 85% of Kibandameno plantlets and 79% of Paja la mzee from cost-effective medium survived while 81% of Kibandameno and 75% of Paja la mzee from conventional medium survived.

Chemotherapy and thermotherapy as a means of producing virus-free plantlets, was used in this study to eliminate EACMV from farmer-preferred cassava landraces. Results showed that survival of shoots was

decreasing with the increase in the antiviral concentration. Also, the number of virus-free plantlets was observed to increase with the increase in antiviral concentrations for both Ribavirin and Salicylic acid . Ribavirin at 10mg/l had a higher survival compared to the other concentrations but the highest virus elimination was observed at the concentration of 20 mg/l (85% virus-free plantlets). Salicylic acid at 30 mg/l resulted in the highest survival rate (88.9%) and when the concentration was increased to 40 mg/l no plantlet survived.

Plants subjected to thermotherapy at different temperature regimes had different responses to treatment. It was observed that the higher the temperature the higher the rate of virus elimination but low survival rate of the *in vitro* plantlets. Survival rate was high at low ositive s but the virus elimination rate was observed to be low. When the temperature was raised to 40°C most of the plants dried up and others did not grow at all. In this study, the optimal temperature for elimination of EACMV was observed to be 35°C with 79.5% virus-free plantlets.

In this study cassava varieties were transformed with GUS and regenerated. Many studies have been done on the model cultivar (TMS6044) which is no longer used by famers (Nyaboga *et al.*, 2015). Generation and proliferation of FEC from two cassava varieties was important since FEC form target tissues for transformation. The presence of the GUS gene was confirmed by histochemical assay and molecular analysis (PCR). Transformation efficiency for the two cassava varieties was 86% and 83.3% for TMS60444

and Albert, respectively. This paves the way for other studies that will involve improvement of other traits in cassava.

6. 2 General Conclusions

Production of virus-free plantlets relies on the sound tissue culture procedures. The evaluated low-cost tissue culture protocol will help in the mass production of virus-free cassava plantlets is cost-effective due to the use of alternative nutrients in the media. Through chemotherapy and thermotherapy virus-free plantlets can be produced *in vitro* from diseased plants thereby enhancing clean seed multiplication and conservation of valuable germplasm

Through genetic engineering cassava can be altered in order to have desired traits that will favor production. Hence, having high-throughput cost-effective platform for cleaning cassava materials and genetic engineering for key traits will greatly enhance availability of cassava planting materials to farmers at an affordable cost which will boost production.

6.3 Recommendations

The following are the recommendations from this study

- Further research is needed to optimize the media and ensure consistent production of tissue culture plants of all crops.
- There is a need for more research for combined effect of chemotherapy and thermotherapy for the elimination of cassava mosaic viruses

- This study lays a foundation on genetic engineering with other genes of interest (For instance; resistance to diseases and drought tolerant)

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APPENDICES

Appendix I: Cassava landraces found in different places of Tanzania (Elibariki et al., 2013)

Cultivar	Region
Kaniki	Ruvuma
Kiroba	Mtwara
Machui	Zanzibar
Mahonda	Zanzibar
Sepide	Zanzibar
Shija	Tabora
Katakya	Kagera
Lyongonyeupe	Mwanza
Albert	Kibaha
Rangimbili	Mwanza
Rushura	Kagera
Gago	Tanga
Karatasi	Tanga
Kibandameno	Tanga
Kibangili	Tanga
Mkongomwaya	Kibaha
Mnazi	Bagamoyo
Nachinyaya	Kibaha
Paja la mzee	Tanga
Sagalatu	Tanga

Appendix II: CETYL TRIMETHYL AMMONIUM BROMIDE (CTAB) METHOD (Lodhi *et al.*, 1994; Xu *et al.*. 2010).

- Leaves of approximately 100 mg were measured and put into a mortar and 1ml of CTAB extraction buffer containing (0.2% (V/V) 2-mercaptoethanol was added prior to extraction.
- The samples were then ground into a fine paste. From the extract produced 750µl was then transferred into a 1.5 ml micro-centrifuge tube and incubated at 60°C for 30min.
- The extract was then mixed with an equal volume (750µl) of chloroform, isoamyl alcohol (24,1), vortexed briefly and centrifuged at 14,000 revolutions per minute for 10 min.
- The top aqueous solution (500µl) was then transferred into a new 1.5ml tube. Nucleic acids were precipitated by adding 0.6 volumes (300µl) of ice cold isopropanol and the tubes incubated at -20°C for 30 minutes.
- After incubation, the samples were centrifuged at 14,000 revolutions per minute at 4°C for 10 min and the supernatant discarded. The pellets were then resuspended in 500µl of 70% ethanol by vortexing and incubation at -20°C for 10 min. This was centrifuged for 5 min at 14,000 revolutions per minute.
- Ethanol was poured off and pellets dried. The pellets were then resuspended in 100 µl of 1x TE buffer with low salt on ice for about 30 min and stored at -20°C.

Appendix III: Objective I

i. Plant Height

Time (weeks)	Variety		t	df	Sig. (2-tailed)	Mean	Std. Error Difference
2	Kibandameno	Equal variances assumed	0.913	22	0.371	1.65	0.1278
2	Paja la mzee	Equal variances assumed	-2.769	22	0.011	1.258	0.1234
4	Kibandameno	Equal variances assumed	-1.762	22	0.092	2.192	0.2223
4	Paja la mzee	Equal variances assumed	-4.85	22	0	1.867	0.1804
6	Kibandameno	Equal variances assumed	-2.796	22	0.011	2.625	0.2265
6	Paja la mzee	Equal variances assumed	-5.116	22	0	2.3667	0.19221

ii. Number of leaves

Time (weeks)	Variety		t	df	Sig. (2-tailed)	Mean	Std. Error Difference
2	Kibandameno	Equal variances assumed	-1.076	22	0.294	1.08	0.155
2	Paja la mzee	Equal variances assumed	-1.899	22	0.071	0.42	0.614
4	Kibandameno	Equal variances assumed	-5.745	22	0	2	0.261
4	Paja la mzee	Equal variances assumed	-3.853	22	0.001	1.83	0.627
6	Kibandameno	Equal variances assumed	-6.934	22	0	2.5	0.3
6	Paja la mzee	Equal variances assumed	-4.474	22	0	2.5	0.82

iii. Number of Roots

Time (weeks)	Variety		t	Sig. (2-tailed)	Mean	Std. Error Difference
2	Kibandamenyo	Equal variances assumed	-0.928	0.364	1.83	0.539
2	Paja la mzee	Equal variances assumed	0.883	0.387	1.83	0.283
4	Kibandamenyo	Equal variances assumed	-0.777	0.445	2.5	0.536
4	Paja la mzee	Equal variances assumed	-4.902	0	2.58	0.255
6	Kibandamenyo	Equal variances assumed	-1.895	0.071	3.33	0.704
6	Paja la mzee	Equal variances assumed	-6.18	0	3.17	0.283

iv. Number of Nodes

Time(weeks)	Variety		t	Sig. (2-tailed)	Mean	Std. Error Difference
2	Kibandameno	Equal variances assumed	-0.684	0.501	1.42	0.244
2	Paja la mzee	Equal variances assumed	1.915	0.069	1	0.131
4	Kibandameno	Equal variances assumed	-5.597	0	2.17	0.223
4	Paja la mzee	Equal variances assumed	-4.757	0	1.58	0.21
6	Kibandameno	Equal variances assumed	-4.248	0	3.08	0.255
6	Paja la mzee	Equal variances assumed	-3.273	0.003	2.5	0.382

Appendix IV: Objective 2; Analysis of variance, table of means and least significant differences (5%)

i. RIBAVIRIN

Analysis of variance

Variate, survival

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Conc	4	29.0500	7.2625	12.84	<.001
Residual	75	42.4375	0.5658		
Total	79	71.4875			

Tables of means

Variate, survival

Grand mean 2.138

conc	0	5	10	15	20
	3.000	2.500	2.188	1.750	1.250

Least significant differences of means (5% level)

Table	conc
rep.	16
d.f.	75
l.s.d.	0.5298

Analysis of variance

Variate, ositive

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Conc	4	90.0500	22.5125	67.71	<.001
Residual	75	24.9375	0.3325		
Total	79	114.9875			

Tables of means

Variate, ositive

Grand mean 0.988

conc	0	5	10	15	20
	3.000	1.125	0.375	0.250	0.187

Least significant differences of means (5% level)

Table	conc
rep.	16
d.f.	75
l.s.d.	0.4061

Analysis of variance

Variate, negative

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Conc	4	33.9250	8.4813	10.67	<.001
Residual	75	59.6250	0.7950		
Total	79	93.5500			

Tables of means

Variate, negative

Grand mean 1.175

conc	0	5	10	15	20
	0.000	1.375	1.937	1.500	1.062

Least significant differences of means (5% level)

Table	conc
rep.	16
d.f.	75
l.s.d.	0.6280

ii. SALICYLIC ACID

Analysis of variance

Variate, survival

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Conc	4	85.8750	21.4688	46.17	<.001
Residual	75	34.8750	0.4650		
Total	79	120.7500			

Tables of means

Variate, survival

Grand mean 1.375

conc	0	10	20	30	40
	3.000	1.750	1.562	0.562	0.000

Least significant differences of means (5% level)

Table	conc
rep.	16
d.f.	75
l.s.d.	0.4803

Analysis of variance

Variate, ositive

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Conc	4	90.0500	22.5125	67.71	<.001
Residual	75	24.9375	0.3325		
Total	79	114.9875			

Tables of means

Variate, ositive

Grand mean 0.988

conc	0	10	20	30	40
	3.000	1.125	0.375	0.250	0.187

Least significant differences of means (5% level)

Table	conc
rep.	16
d.f.	75
l.s.d.	0.4061

Analysis of variance

Variate, negative

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Conc	4	21.4250	5.3563	11.77	<.001
Residual	75	34.1250	0.4550		
Total	79	55.5500			

Tables of means

Variate, negative

Grand mean 0.575

conc	0	10	20	30	40
	0.000	1.125	1.188	0.562	0.000

Least significant differences of means (5% level)

Table	conc
rep.	16
d.f.	75
l.s.d.	0.4751

iii. TEMPERATURE

Analysis of variance

Variate, survival

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Temp	3	26.0000	8.6667	16.25	<.001
Residual	60	32.0000	0.5333		
Total	63	58.0000			

Tables of means

Variate, survival

Grand mean 2.250

temp	28	30	35	40
	3.000	2.500	2.250	1.250

Least significant differences of means (5% level)

Table	temp
rep.	16
d.f.	60
l.s.d.	0.5165

Appendix V: PUBLICATIONS

1. **Kidulile CE, Alakony AE, Ndunguru JC, Ateka EM (2018).** Cost effective medium for in vitro propagation of Tanzanian cassava landraces Afr. J. Biotech 17(25): 787-794, **https://doi.org/10.5897/AJB2017.16368**

2. **Kidulile CE, Ateka EM, Alakony AE, Ndunguru JC.** Efficacy of chemotherapy and thermotherapy in elimination of East African cassava mosaic virus from Tanzanian cassava landraces. J. Phytopathol. 2018; 00,1-7, https://doi.org/10.1111/jph.12725