

**DEVELOPMENT OF A REGENERATION AND
AGROBACTERIUM TRANSFORMATION SYSTEM FOR
SELECTED ELITE UGANDAN SORGHUM GENOTYPES**

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DECLARATION

I MUHUMUZA JOHN BOSCO solemnly declare that the work presented in this thesis is from my own research and has never been submitted for any award in any institution/university.

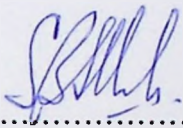
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This thesis has been submitted with my approval as university supervisor.

for. 

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Date

DEDICATION

To my children Nimrod and Viola.

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SUMMARY

Sorghum is one of the five most important staple cereals for humans being a source of food, animal feed and industrial raw material. However, the crop suffers significantly from many biotic and abiotic stresses. To improve sorghum for these traits, for the most part reliance has been on both inter- and intra species hybridization also called traditional plant breeding. Traditional plant breeding can only exploit variation present in species that can be crossed with sorghum. Genetic transformation offers a direct access outside primary gene pool of sorghum. Sorghum genetic transformation has been lagging behind other cereals due to its recalcitrance to tissue culture and genotype dependent regeneration. Therefore, the aim of this study was two-fold: to develop an *in vitro* culture system for Ugandan sorghum adapted genotypes and screen Ugandan elite sorghum genotypes for their amenability to tissue culture under different test conditions, as well as develop an efficient *Agrobacterium* mediated transformation system for genotypes with better response to *in vitro* culture.

Callus induction medium composition was optimised with emphasis on the levels of 2, 4-Dichlorophenoxyacetic acid (2, 4-D) and kinetin hormones. Twenty elite sorghum genotypes were screened for the amenability to tissue culture on two medium types; Murashige and Skoog basal medium and L3 medium. The genotypes amenable to tissue culture were advanced to *Agrobacterium* mediated transformation in which two bacterium strains EHA105 and AGL1 were used.

The results showed that the optimum sorghum regeneration parameters (frequency of somatic embryo formation, callus growth and number of shoots) were achieved at a level of 2, 4-D of either 2.0 mg/l or 2.5 mg/l supplemented with 0.5 mg/l of kinetin hormone. Inclusion of kinetin hormone in callus induction medium at low levels of 2, 4-D gradually reduced the callus induction frequency and higher levels of 2, 4-D exhibited inhibitory effects on callus induction as well as somatic embryogenesis. The best age of immature zygotic embryo for *in vitro* culture was between 16-18 days post anthesis. However, the physiological state of the embryo of different genotypes at the same age varied. This indicates that panicle harvesting decisions for *in vitro* culture should be based on the embryo size rather than age. Strong genotype by medium type influenced *in vitro* culture responses rather than the genetic control

only. The genotypes MUC007/194 and MUC007/193 had a short regeneration time on MS basal medium than on L3 medium. However, L3 medium was the best medium for most of the sorghum genotypes included in the study. Among the genotypes advanced for *Agrobacterium* mediated transformation, MUC007/193 and MUC007/124 were susceptible to *Agrobacterium* infection and no significant sorghum genotype by *Agrobacterium* strain was found. Generally, EHA105 *Agrobacterium* infected sorghum *explants* exhibited highest frequency of putatively transformed material with the high level of *gus plus* gene expression. Overall, this study has demonstrated that indeed Ugandan local sorghum genotypes can be amenable to *in vitro* processes. The model Ugandan sorghum genotypes for *in vitro* culture are the genotypes MUC007/193 and MUC007/194. Genotypes MUC007/193 and MUC007/124 had highest transformation frequencies and thus can be used for further stable transformation studies. The high recalcitrance of sorghum, however, implies that candidate genotypes amenable to *in vitro* processes can be used as systems to introgress foreign genes into non recalcitrant but commercially viable genotypes.

CHAPTER ONE

INTRODUCTION

1.1 Biogeography and taxonomy of sorghum

Sorghum (*Sorghum bicolor* (L) Moench) belongs to the family Poaceae and genus *Sorghum* which is taxonomically separated into five subgenera; *Eu-sorghum*, *Chaetosorghum*, *Heterosorghum*, *para-sorghum* and *Stiposorghum* (Garber, 1950). All cultivated sorghum races and varieties belong to the *Eu-sorghum* section as *Sorghum bicolor* subsp. *bicolor* (Dogget, 1988). The genus sorghum consists of twenty five species comprising all the five sub genera (Lazarides *et al.*, 1991). Cultivated races are believed to have evolved from an early progenitor of *bicolor* through processes of genetic adaptation and intercrossing followed by selection with the exception of Kafir race that is closely related to the wild grass sorghum *bicolor* race *verticilliform* (Schechter and de Wet, 1975; Smith and Frederisen, 2000). The crop is believed to have originated from North East Africa comprising of the present day countries of Ethiopia, Sudan, Kenya, Uganda, Tanzania, Rwanda and Burundi where agro ecological conditions are predominantly dry (Dogget, 1988).

Sorghum is thus widely found in the dry areas of Africa, Asia, America and Australia (Dicko *et al.*, 2006). It was distributed along the trade and shipping routes around the African continent and through the Middle East to India and later into China along silk route. Sorghum was first taken to North America in the 1700-1800's through the slave trade from West Africa. It was re-introduced in Africa in the late 19th century for commercial cultivation and spread to South America and Australia. This crop is widely distributed all over the world with United States of America, Nigeria, India, Mexico, Sudan, Australia, Argentina, China, Ethiopia, Brazil, Burkina Faso, and Niger as the leading producers (FAOSTAT, 2010).

1.2 Economic importance

Sorghum is the most important crop in the semi-arid tropical and sub-tropical Africa, and parts of Asia where persistent drought, high temperatures and poor soil nutrient availability are common (Dicko *et al.*, 2006; Chakauya *et al.*, 2006; O'Kennedy *et al.*, 2006). The crop is

adapted to drought prone areas and therefore grown on marginalised areas where other cereals can barely grow. Sorghum is the world's fifth most important cereal crop after wheat (*Triticum aestivum* L), rice (*Oryza sativa* L), maize (*Zea mays* L) and barley (*Hordeum vulgare* L) in terms of production. Sorghum is, however, largely a developing country crop with about 90% of the world's area cultivated to sorghum in Africa and Asia (FAO and ICRISAT, 1996). The total world annual sorghum production is estimated at over 65 million tonnes from a cultivated area of 44 million hectares with 27 million tonnes from Africa (FAOSTAT, 2010). In Uganda, sorghum production is gaining momentum. However, annual production is still low estimated at less than 0.5 million tonnes from a total cultivated area of 0.3 million hectares (FAOSTAT, 2008).

Sorghum has several end uses. In developing countries especially in Africa, Asia and Middle East, the crop is a staple food for millions of poor people (ICRISAT, 2004). It is made into unleavened breads, boiled porridge or gruel, malted beverages including beer, and specialty foods such as popped grain and syrup from sweet sorghum (ICRISAT: <http://www.icrisat.org>). In the U.S., the world's leading producer of sorghum, the crop is largely used for animal feed and industrial raw material.

Sorghum based diet has been reported to have potential benefits on human health for example overcoming cardiovascular diseases, obesity and cancer (Awika and Rooney, 2004). The crop has a great potential for being used in baking of bread after fermenting the flour and this will possibly enable the harnessing of the crop's health benefits (Hugo *et al.*, 2003). The use of properly processed sorghum can substitute maize bran in animal feed reducing competition on maize for human food and animal feed (Kyarisima *et al.*, 2004). Although sorghum is the world's important cereal, the potential of the crop is constrained by abiotic and biotic stresses as well as low grain content of lysine amino acids and calcium.

1.3 Sorghum improvement opportunities and challenges

Sorghum species within the sorghum genus are genetically separated into two lineages with *Eu-sorghum*, *Chaetosorghum* and *Heterosorghum* species forming one and *Para-sorghum* and *Stiposorghum* species another (Ng'uni *et al.*, 2010). Potential resistance genes to insect

pests and diseases attacking sorghum have been found in species of the *Chaetosorghum*, *Heterosorghum*, *Para-sorghum* and *Stiposorghum* sections (Sharma and Fransmann, 2001; Kamala *et al.*, 2002). Utilisation of these genes necessitates having successful hybridisation and backcrossing. However, reproductive barriers at either pre-zygotic or post-zygotic levels have been found to limit formation of inter-specific hybrids (Hodnett *et al.*, 2005).

The species in monotypic sections; *Heterosorghum* and *Chaetosorghum* are polyploids ($2n=40$) but are closely related to species in *Eusorghum* based on phylogenetic analysis (Dillon *et al.*, 2004; Ng'uni *et al.*, 2010), genome size (Price *et al.*, 2005a) and karyotypic similarity (Wu *et al.*, 1993). *Eu-sorghum* species have the basic chromosome number $x=10$ whereas those in *Para-sorghum* and *Stiposorghum* sections have the basic chromosome number $x=5$, with genomes that are 2.5 times larger than the sorghum genome (Price *et al.*, 2005a). The differences in ploidy level and genome size among the two lineages of sorghum are likely to pose reproductive barriers for interspecific hybridisation through conventional breeding. The interspecific hybrids from *Eu-sorghum* x (*Para-sorghum* and *Stiposorghum*) species are likely sterile due to meiotic failures involving chromosome members of $x=5$ and $x=10$ (Hodnett *et al.*, 2005). Thus species belonging to sections outside *Eu-sorghum* make up the untapped tertiary gene pool for improvement of sorghums.

1.4 Sorghum genetic improvement approaches

Throughout the world, the primary focus of sorghum breeders are on yield, adaptation and quality and most breeding programmes consistently select for tolerance to abiotic and biotic stress (Rooney, 2004). Initially, the improvement of cereals and sorghum in particular, was achieved by conventional breeding approaches, with hybridization playing a crucial role in breeding cultivars with improved agronomic performance (Jauhar and Peterson, 2001). However, conventional methods of crop improvement are slow, time consuming and limited by the size of the potential gene pool. With recent advances in molecular biology and technology, genetic transformation has become a vital tool in circumventing limitations of conventional breeding methods in cultivar development of major crop species with desirable traits (Bajaj and Mohanty, 2005; Jones, 2005). In the developing countries sorghum improvement through genetic transformation is the latest in a long series of technologies that

have been applied to create superior genotypes (Maqbool *et al.*, 2001). Genetic transformation is underpinned by tissue culture. However, sorghum response to *in vitro* culture is genotype dependent and Ugandan sorghums may not be exceptional (Godwin and Gray, 2000; Jogeswar *et al.*, 2007).

1.4 Justification of the study

Sorghum has a great potential as human food, animal feed and industrial raw material owing to its adaptability to drought and water logged conditions. However, sorghum as a crop suffers significantly from an array biotic and abiotic stresses. Sorghum also has poor nutritive quality due to low calcium content, lysine deficiency and reduced protein digestibility. Sorghum improvement has relied on traditional breeding approaches for many years, but these avenues can only exploit variation present in species that can be crossed with sorghum (O'Kennedy *et al.*, 2006). Important genes (for example for *striga* resistance, stem borer resistance, drought tolerance, better nutritive quality), for further sorghum improvement exist in species outside *Eu-sorghum* section (Sharma and Franzmann, 2001; Kamala *et al.*, 2002). These genes have not been used because of hybridisation failures (Price *et al.*, 2005b; Hodnett *et al.*, 2005). Genetic transformation offers a direct access to this vast pool of useful genes. Sorghum transformation has been lagging behind other cereals due to its recalcitrance to tissue culture and genotype dependent regeneration (Godwin and Gray, 2000; Jogeswar *et al.*, 2007). To date, major cereal crops have been successfully transformed in various laboratories using either biolistic or *Agrobacterium*-mediated transformation techniques (Repellin *et al.*, 2001). Genetic transformation is a relatively new technology in sorghum improvement, especially in the developing countries. Accordingly, this study was conducted to screen selected Ugandan elite sorghum genotypes for their amenability to tissue culture under different test conditions and develop an efficient *Agrobacterium* mediated transformation system for genotypes with better response to *in vitro* culture.

1.6 Study Objectives

1.6.1 General Objective

To develop a highly efficient regeneration system and *Agrobacterium* mediated transformation system for selected elite Ugandan sorghum genotypes.

1.6.2 Specific Objectives

1. To determine appropriate age of immature zygotic embryo, culture medium composition and type for efficient regeneration of selected Ugandan sorghum genotypes.
2. To identify Ugandan elite sorghum genotypes with high potential of embryogenic callus formation and regeneration.
3. To determine the transformation efficiency of *Agrobacterium* mediated transformation systems in transformation of selected Ugandan sorghum genotypes.

1.7 Hypotheses

1. Age of immature zygotic embryo, medium type and composition greatly affects the regeneration of sorghum genotypes.
2. The frequency of embryogenic callus formation and regeneration potential among selected sorghum genotypes is genotype dependent.
3. The efficiency of *Agrobacterium* mediated transformation systems is affected by sorghum genotype by strain interaction.

CHAPTER LITERATURE

2.1 Introduction

Successful application of the new methods of genetic engineering for crop improvement requires highly efficient and effective plant regeneration. *In vitro* culture techniques provide a wide range of options for plant breeders are able to use potential genes outside the primary germplasm. This can be done through embryo rescue, protoplast fusion, and fertilization, mass propagation, somaclonal variation, and genetic transformation. However, cereals have been regarded as the most recalcitrant species to *in vitro* culture. Successful transformation of cereals requires high regeneration frequency of explants. However, cereals regeneration seems to be genotype dependent. An exception to this genetic control. Successful genetic transformation of cereals has been achieved through biolistic and *Agrobacterium* mediated approaches. Although the biolistic approach has been preferred in cereal crop transformation, plant transformation mediated by *Agrobacterium* strain interaction has been reported and compounds the process (Gamborg *et al.*, 2002). Accordingly, the purpose of this study was two-fold; to develop an efficient *in vitro* culture system and to screen regeneration potential of selected elite Ugandan sorghum genotypes. The study also aimed to develop an efficient *Agrobacterium* mediated transformation system for genotype responsive to *in vitro* culture.

2.2 Cereal *in vitro* culture

Cereals were classified as highly recalcitrant group to *in vitro* manipulation and tissue culture. Cereals were not regenerated until 1982 (Barto *et al.*, 2000). Although callus cultures of rice were first achieved in 1964 (Oorika *et al.*, 1983), the first reproducible cereal embryogenic *in vitro* culture systems for sorghum were only established in early 1980's (Gamborg *et al.*, 1977). Several factors have been reported to influence *in vitro* cereal regeneration frequency. These include explant type, explant size, explant position, culturing and regeneration conditions, medium composition, and explant age. Explant age is prerequisite for successful regeneration. Frequency of plant regeneration is prerequisite for successful transformation.

crops (Rachmawati and Anzai, 2006). Sorghum *in vitro* culture system development has lagged behind other cereals possibly because of the extremely high levels and diversity of phenolic compounds present in sorghum compared to other cereals (Dicko *et al.*, 2006). This challenge underpins research and development strategies for sorghum and the focus of this study was to develop *in vitro* techniques for African sorghum genotypes.

2.3 Sorghum *in vitro* culture

Sorghum has been classified as one of the difficult plant species to manipulate through tissue culture and transformation (Zhao *et al.*, 2000; Gao *et al.*, 2005a). Sorghum tissue culture and regeneration protocols mostly follow either organogenesis (Maheswari *et al.*, 2006) or direct somatic embryogenesis (Harshavardhan *et al.*, 2002). *In vitro* culture of cereals shows strong genotype dependence and there is strong evidence suggesting that sorghum is no exception to this genetic control (Jogeswar *et al.*, 2007). Sorghum proliferates to produce two types of calli; white and soft non-embryogenic callus and a yellow harder embryogenic callus (Pola *et al.*, 2007; Girijashankar *et al.*, 2007; Grootboom *et al.*, 2008). The occurrence of both callus types on the same *explant* has been reported (Girijashankar *et al.*, 2007). Pola *et al.* (2007) reported that only compact calli are capable of regeneration whilst the white and soft callus never develop into embryos but continue to remain as a cell mass (Girijashankar *et al.*, 2007). In this thesis the aim of the study was to develop protocols for Ugandan sorghum genotype regeneration, as well as identify genotypes amenable to regeneration *in vitro* culture.

2.3.1 Sorghum *explants*

The choice of *explant* for *in vitro* cereal regeneration is one of the most important factors that determine regeneration ability, together with physiological and developmental stage of the *explants*. Procedures for regeneration of sorghum plants via somatic embryogenesis and organogenesis have been described for immature zygotic embryos (Zhong *et al.*, 1998; Girijashankar *et al.*, 2005; Grootboom *et al.*, 2008), mature embryos (Cai *et al.*, 1987), shoot tips (SaiKishore *et al.*, 2006), germinating seeds (Zhao *et al.*, 2010) leaf fragments (Wernicke and Brettell, 1980) and anthers (Rose *et al.*, 1986) used as *explants*. However, immature zygotic embryos have been the *explant* of choice for production of sorghum transgenic plants (Zhao *et al.*, 2000; Tadesse *et al.*, 2003; Gao *et al.*, 2005a; 2005b; Nguyen *et al.*, 2007). In

this study the different developmental stages of immature zygotic embryo were tested (from three selected sorghum accessions) to identify the best source of *explants* for regeneration and transformation.

2.3.2 Callus induction and regeneration medium

The type and composition of culture media to a great extent influence the response of sorghum *explant* to callus induction and regeneration. Zhao *et al.* (2000) reported successful sorghum regeneration on Murashige and Skoog-based medium. The N6 medium decreased callus response from immature zygotic embryos of sorghum and conversely, increased production of phenolic pigments (Zhao *et al.*, 2000). In many sorghum genotype *in vitro* culture and transformation studies, MS medium has always been a medium of choice with varying modifications. Nevertheless, a strong interaction between media type and sorghum genotype influence sorghum callus induction and regeneration (Grootboom *et al.*, 2008). Sorghum exhibit different callusing ability at different concentrations of plant growth regulators and callus induction medium with 2.0 mg/L 2, 4-D and 0.5 mg/l kinetin induce outstanding callusing (Pola, 2005).

The amount of cytokinins used for plant regeneration is critical in producing vigorous number of shoots. A maximum shoot response occurs at a concentration of 2.0 mg/l BAP and a combination of cytokinins and auxins proved to be effective for multiple shoot formation in sorghum (Pola *et al.*, 2007). Furthermore, addition of amino acids such as L-proline, L-glutamine, L-asparagine and serine into shooting medium enhances shoot formation in sorghum (Hagio, 2002; Pola *et al.*, 2007). Similarly addition of polyvinylpyrrolidone together with L-proline at an optimum level of 1 % (w/v) in callus induction medium improves sorghum regeneration (Hagio, 2002). Half strength MS medium supplemented with low levels of sucrose and 1.0 mg/l of NAA is better for rooting regenerated plantlets (Sai Kishore *et al.*, 2006; Maheswari *et al.*, 2006; Pola *et al.*, 2007). In this study callus initiation medium was optimised with major emphasis on plant growth regulator concentrations.

2.4 Genetic transformation of cereals

Recombinant DNA technology has significantly augmented classical crop improvement and offers great promise to plant breeders to meet increased demand of food predicted for the 21st century (Sharma *et al.*, 2002). Various methods have been used in genetic manipulation of cereals and these include silicon carbide fibers (Frame *et al.*, 1994), electroporation (D'Halluin *et al.*, 1992; Arencibia *et al.*, 1995), microinjection (De la Pena *et al.*, 1987), and polyethelene glycol treatment method-PEG (Golovkin *et al.*, 1993). *Agrobacterium* and biolistic mediated transformation approaches have been the novel approaches lately applied in cereal genetic transformation.

Agrobacterium is a soil borne bacterium with natural capacity of transferring its T-DNA to plant cells of dicots. Although monocots have been regarded as non hosts of *Agrobacterium*, the natural ability of delivering discrete DNA segments into recipient genome has been exploited in genetic transformation of many cereal crops such as maize (Ishida *et al.*, 1996; Zhao *et al.*, 2002; Frame *et al.*, 2002), wheat (Cheng *et al.*, 1997), rice (Hiei *et al.*, 1994; Enrique-Ebregon, 1999), and sorghum (Zhao *et al.*, 2000; Gao *et al.*, 2005a, 2005b; Nguyen *et al.*, 2007). *Agrobacterium* mediated transformation in cereals was first demonstrated in rice in which acetosyringone and a super binary vector were used in a co-culture medium (Hiei *et al.*, 1994). Several factors influencing the efficiency of *Agrobacterium* mediated transformation of monocots including cereals have been extensively investigated and elucidated (Cheng *et al.*, 2004). These factors include plant genotype, *explants*, *Agrobacterium* strain, culture medium composition, vector plasmids: selectable marker genes and promoters, addition of *vir*-gene inducing synthetic phenolic compounds, tissue damage, and inoculation and co-culture conditions (Opabode, 2006). The difference in the competence of *Agrobacterium* strains to infect specific tissue, genotype and species remains a major drawback to utilising *Agrobacterium* for routine transformation of cereals (Shrawat and Ioriz, 2006). However, *Agrobacterium* mediated transformation system facilitates the precise integration of a small number of gene copies into the plant genome and shows a greater degree of transgene stability (Dia *et al.*, 2001).

Biolistic transformation approach is one of the different strategies for direct gene transfer among the many that have been developed over years (Slater *et al.*, 2003). Crops which are typically difficult to transform using *Agrobacterium* mediated approach such as monocots are better suited for transformation through biolistic approach (Finer *et al.*, 1990). Nonetheless, this approach has a major drawback of considerable variation observed in stability, integration and expression of the introduced transgene (Kohli, 1999).

2.4.1 Sorghum transformation

Genetic engineering is the latest in a long series of technologies that have been applied to create superior genotypes (Maqbool *et al.*, 2001). Unlike other cereals, no commercial transgenic sorghum has reached the market despite the fact that most of the ground work for cereal transformation was made in sorghum (O'Kennedy *et al.*, 2006).

Both biolistic and *Agrobacterium* mediated transformation have been employed in the past years to produce transgenic sorghum. Though both systems of transformation (*Agrobacterium*-mediated and particle bombardment) are successful in sorghum, the most effective method to date is *Agrobacterium* mediated transformation, with a higher transformation efficiency that ranges from 2.1%– 5% (Zhao *et al.*, 2000; Nguyen *et al.*, 2007). Zhao *et al.* (2000) reported the first production of transgenic sorghum plants by *Agrobacterium*-mediated transformation using immature embryos. Biolistic mediated transformation of sorghum has lower transformation efficiencies than *Agrobacterium* mediated system ranging from 0.33%-1.5% (Casas *et al.*, 1997; Zhu *et al.*, 1998; Tadesse *et al.*, 2003; Girijashankar *et al.*, 2005). It should be noted that these comparisons have been made based on results from independent experiments in which different *explants*, sorghum genotypes, promoters, reporter and selectable genes were used. It thus appears that various factors indeed influence transformation efficiency as reviewed below

2.4.2 Factors affecting transformation efficiency of *Agrobacterium* mediated system

The type and developmental stage of infected tissues, the concentration of *Agrobacterium tumefaciens*, the composition of media used for co-cultivation, infection, and tissue culture, the selectable marker genes used, the type of vector, and the plant genotype influence

Agrobacterium-mediated transformation efficiency (Opabode, 2006). The production of transgenic sorghum plants with an average transformation frequency of 2.1% after co-cultivation of immature embryos with *Agrobacterium* carrying a super binary vector has been achieved (Zhao *et al.*, 2000). Several factors that can influence sorghum transformation by *Agrobacterium* such as medium composition, inclusion of polyvinylpyrrolidone, frequent sub-culture in selection medium and optimised conditions for co-cultivation have been reported (Zhao *et al.*, 2000). The source of the embryos affects the transformation efficiency with field grown embryos with higher transformation frequency recorded from field obtained embryos than those obtained from glass house grown sorghum (Zhao *et al.*, 2000). In this study, embryos used were obtained from potted plants growing in open environments.

2.5 Sectional conclusion

An extensive research has been done to develop a reliable and efficient *in vitro* culture and *Agrobacterium* mediated transformation system of sorghum. However, strong genotype and genotype by medium interaction dependence of regeneration still exists and has compounded the use of biotechnological approaches for sorghum improvement. Although, various factors have been optimised to improve efficiency of *Agrobacterium* mediated transformation approach, strain-genotype interaction still remain and has been reported. The purpose of this study was to select elite Ugandan sorghum genotypes for their regeneration response and to develop an efficient *Agrobacterium* mediated transformation system for *in vitro* culture responsive genotypes.



Plate 1. Variation in panicle structure among the study sorghum lines (A) MUC007/124, (B) MUC007/189, (C) MUC007/194, and (D) MUC007/193

CHAPTER THREE

MATERIALS AND METHODS

3.1 Introduction

World over, sorghum genetic transformation has been lagging behind other cereals due to its recalcitrance to tissue culture and transformation (Zhu *et al.*, 1998; Sato *et al.*, 2004). The response of sorghum to *in vitro* culture is influenced by genetic factors and a number of other factors such as medium composition and choice of *explant* (Grootboom *et al.*, 2008). Thus application of genetic transformation for sorghum improvement requires a highly efficient and reliable regeneration system which inadvertently will be affected by the above endogenous and exogenous. Although relatively high transformation efficiency has been reported in *Agrobacterium* mediated transformation of sorghum (O'Kennedy *et al.*, 2006), its application in cereal transformation and sorghum in particular is hampered by the strain-genotype interaction (Jeoung *et al.*, 2002). The aim of this study was to investigate regeneration potential of selected Ugandan sorghum genotypes, under various experimental conditions such as age of *explants*, medium type and composition as well as determining the efficiency of *Agrobacterium* mediated transformation system in genetic transformation of Ugandan sorghum genotypes.

3.2 Sorghum plant materials used in the study

Twenty genetically distant Ugandan elite sorghum lines were selected among the Makerere University sorghum collections of 2007 (MUC007) based on quality characteristics (high protein and low tannin content). The selected elite sorghum lines were grown in sterile potted soil in a screen house but were later moved outside two weeks after germination. The potted seedlings were thinned to two plants per pot and poultry manure was applied into the plastic pots. The sorghum plants were sprayed against aphids and sorghum shoot fly using Dimethoate 40EC (Tufgor). At the booting stage, bagging was used to ensure controlled self-pollination.

The sorghum plants were raised at Makerere Agricultural Research Institute, Kabanyolo (MUARIK). The tissue culture experiments described hereafter were conducted at Makerere tissue culture Laboratory, Kabanyolo while transformation experiments were conducted at National Agricultural Research Laboratories, Kawanda.



Plate 2. Twenty Ugandan elite sorghum accessions used in the study established in potted soil at MUARIK

3.3 Study 1: Determining the appropriate culture medium composition and type, and age of immature zygotic embryo for efficient regeneration of selected Ugandan sorghum genotypes

3.3.1 Optimisation of regeneration medium phytohormone composition

Preliminary studies to optimise phytohormone concentrations in callus induction medium and rooting medium were conducted using an immature zygotic embryo regeneration system (Hagio, 2002). The callus induction medium was composed of MS basal salts + vitamins mg/l [0.5 nicotinic acid +0.5 pyridoxine HCl +1mg/l thiamine HCl + 0.1 g/l myo-inositol +30g/l sucrose+1%Polyvinypyrrolidone+ 3 g/l Gelrite + varying concentrations of 2,4-D (0, 1,2,2.5 and 3 mg/l) in combination with varying concentrations of kinetin (0, 0.1, 0.2 0.5mg/l) pH 5.8 was autoclaved at 121°C for 15min and dispensed into pre-autoclaved glass Petri-dishes or glass jars under sterile conditions. From the preliminary study carried out, a combination of 2-3 mg/l⁻¹ 2, 4-D and 0.5 mg/l⁻¹ kinetin was the best in inducing regenerable callus. The callus maturation medium used was of the same composition as the callus induction medium, except that it had reduced concentration of 2, 4-D of 2.0 mg/l and 0.5 mg/l kinetin. The shoot

induction medium was of the same composition as callus induction medium without 2, 4-D but with kinetin concentration maintained at 0.5 mg l^{-1} . The effect of Naphthalene Acetic Acid (NAA), Indole Butyric Acid (IBA), and Indole -3-Acetic Acid (IAA) in varying combinations of 0 mg l^{-1} growth hormones, 1 mg l^{-1} NAA, 1 mg l^{-1} IBA, 0.2 mg l^{-1} (NAA+IBA+IAA), 0.5 mg l^{-1} (IBA+NAA) and 0.5 mg l^{-1} (NAA+IAA) in rooting medium were tested on induced shoots (data not shown). The rooting medium with 1 mg l^{-1} NAA induced roots formation quickly on regenerated shoots and all shoots cultured formed roots. All the phytohormones, Vitamins, Gelrite and Polyvinylpyrrolidone and amino acids used in this study were supplied by Duchefa Biochemie, Germany. Micro and macro salt components were supplied by Sigma Aldrich, USA.

3.3.2 *Explant preparation and culture*

Immature seeds were removed from panicles of each test sorghum accession harvested 15 days post anthesis using a pair of tweezers and placed in a glass Petri dish. The seeds were surface sterilised in 70 % ethanol for 2-3 min and then immersed in a 2.5 % Sodium hypochlorite (Reckitt Benckiser, Kenya) solution in which 3-4 drops of commercial detergent has been added as described by Grootboom *et al.* (2008). After this treatment the seeds were carefully rinsed 3-4 times in sterile double distilled water. Twenty immature zygotic embryos were aseptically isolated from seeds and cultured on callus induction medium with the scutellum side facing up. Sub-culturing was done after every two weeks to medium of the same composition. After a period of 3-4 weeks, the callus was transferred to callus maturation medium for 2 weeks. The callusing frequency was determined 4 weeks post initiation of culture while frequency of embryogenesis was determined after the following two weeks. After 2 weeks, the calli with developed somatic embryos were transferred to shoot induction medium for another 3 weeks. Finally the developed shoots were transferred to a rooting medium for four weeks.

Data on callus induction frequency which was taken as a percentage of cultured immature zygotic embryos forming callus, callus growth score on a visual scale of (0=no callus growth, 1=poor growth with large dark colored parts, 2=slight growth with some darkened parts, 3=compact, white callus with some darkened and loose parts, 4=compact, hard, white callus

with some darkened parts, 5=white, compact, and hard callus without dark parts), somatic embryogenesis frequency considered as the percentage of cultured embryos showing germinated embryos, and number of shoots per 5 *explants* selected at random was recorded.

3.3.3 Optimisation of developmental stage of immature zygotic embryo

Panicles were harvested from sorghum plants of genotypes MUC007/193, MUC007/194, and MUC007/178 grown in pots at 14, 16, 18 and 20 days post anthesis to optimize the best developmental stage of Immature Zygotic Embryo for producing morphogenic callus and sorghum regeneration. The seeds were surface sterilized in 70% ethanol for 2-3 minutes and then immersed in a 2.5 % Sodium hypochlorite solution in which 3-4 drops of commercial detergent has been added as described by Grootboom *et al.* (2008). After this treatment the seeds were carefully rinsed 3-4 times in sterile double distilled water. Twenty immature zygotic embryos were aseptically isolated from seeds and cultured on callus induction medium with the scutellum side facing up. Sub-culturing was done after every two weeks or less (depending on level of phenolic compounds produced) to culture medium of the same composition. After, a period of 3-4 weeks, the callus was transferred to callus maturation medium depending on genotype for 2 weeks. The callusing frequency was determined 4 weeks post initiation of culture while frequency of embryogenesis was determined after the following two weeks. After 2 weeks, the calli with developed somatic embryos were transferred to shoot induction medium for further 3 weeks. The developed shoots were transferred to a rooting medium that was half strength Murashige and Skoog medium supplemented with 1 mg/l naphthalene acetic acid for further four weeks. Data on callus induction frequency, somatic embryogenesis frequency, and number of shoots per 5 *explants* selected at random was recorded.

3.4 Study 2: Identification of elite Ugandan sorghum genotypes with high potential of embryogenic callus formation and regeneration

Ugandan sorghum genotypes selected among the Makerere University sorghum collections of 2007 were screened for their *in vitro* response on two medium types: Murashige and Skoog (MS) basal medium (Murashige and Skoog, 1962) and L3 basal medium. L3 medium differs from Murashige and Skoog with respect to ammonium nitrate, vitamins and amino acids. L3 medium has been recommended for callus induction and regeneration of recalcitrant crops

and cultures for example microspores of cereals (Mordhorst and Lörz, 1993). The two medium types Murashige and Skoog based and L3 based medium were used with optimised phytohormone concentrations of 2.5 mg/l 2, 4-D with kinetin in callus induction medium to screen the regeneration potential of twenty sorghum genotypes selected (Table 1). The rooting medium used was comprised of half strength salts and supplemented with 1 mg/l NAA in both L3 and MS basal medium. Data on callus induction time (number of days post culture initiation to callusing), callusing frequency, somatic embryogenesis frequency, and number of regenerants formed was collected as previous described under study 1 (Chapter Three of this thesis).

Table 1. The composition of culture medium used in the screening of twenty sorghum genotypes for their *in vitro* culture response

INGREDIENTS	MS callus induction medium /mg/l	MS maturation medium/mg/l	MS Shoot induction medium/mg/l	L3 Callus induction medium /mg/l	L3 Callus maturation	L3 Shoot induction
MACROSALTS						
NH ₄ NO ₃	1650	1650	1650	200	200	200
KNO ₃	1900	1900	1900	1750	1750	1750
KH ₂ PO ₄	170	170	170	200	200	200
MgSO ₄ ·7H ₂ O	370	370	370	350	350	350
CaCl ₂ ·2 H ₂ O	440	440	440	450	450	450
MICROSALTS						
H ₃ BO ₄	6.2	6.2	6.2	5.0	5.0	5.0
MnSO ₄ ·4 H ₂ O	22.3	22.3	22.3	13.4	13.4	13.4
ZnSO ₄ ·7 H ₂ O	+	+	+	+	+	+
Na ₂ MoO ₄ ·2 H ₂ O	+	+	+	+	+	+
CuSO ₄ ·5 H ₂ O	+	+	+	+	+	+
CoCl ₂ ·6 H ₂ O	+	+	+	+	+	+
KI	+	+	+	+	+	+
IRON SOLUTION						
FeSO ₄ ·7 H ₂ O	+	+	+	+	+	+
Na ₂ EDTA·2 H ₂ O	+	+	+	+	+	+
VITAMINS SOLUTION						
Thiamine HCl	+	+	+	+	+	+
Pyridoxine HCl	+	+	+	+	+	+
Nicotinic acid	+	+	+	+	+	+
Myoinositol	+	+	+	+	+	+
Ca-pantothenate	-	-	-	+	+	+
Ascorbic acid	-	-	-	+	+	+
Folic acid	-	-	-	+	+	+
Chlorine chloride	-	-	-	+	+	+
Glycine	+	+	+	+	+	+
CARBON SOURCE						
Sucrose	+	+	+	-	-	-
Maltose	-	-	-	+	+	+
Other ingredients						
Polyvinylpyrrolidone(PVP)	+	+	+	+	+	+
Proline	+	+	+	+	+	+
Gelling agent						
Gelrite	+	+	+	+	+	+
Phytohormones						
2, 4 D	2.5	2.0	0	2.5	2.0	0
Kinetin	0.5	0.5	0.5	0.0	0.5	0.5

3.5 Study 3: Transformation efficiency of *Agrobacterium* mediated transformation of selected Ugandan sorghum genotypes

3.5.1 Vector construction and *Agrobacterium* strains

A standard binary vector pCAMBIA 1305.1 carrying *gus plus* reporter gene, Kanamycin marker gene (*kan*) for bacterial selection and hygromycin selectable marker gene (*hptII*) for plant selection obtained from National Agricultural Research Laboratories (NARL) Kawanda was used. Two *Agrobacterium* strains (AGL1 and EHA 105) used were obtained from National Agricultural Research Laboratories. The electro competent *Agrobacterium* strain cells were transformed with the vector pCAMBIA 1305.1 by electroporation as described in Bio-Rad Electroporation Instruction Manual.

3.5.2 Inoculum preparation, infection of *explants* and co-culture

Transformed *Agrobacterium* strains were cultured on a YEP plate (yeast extract = 5 g, peptone = 10 g, sodium chloride = 5 g, Agar = 15 g/l, pH = 6.8) supplemented with 50 mg/l Kanamycin and Refampcin (for EHA 105) and 50 mg/l kanamycin, carbaneciline and Refampcin (Sigma Aldrich, USA) (for AGL1) and incubated at 28°C for 3 days. Table 2 shows culture medium used for transformation in this study. The plate was kept at 4 °C and used up to a month. A distinct single colony was picked from the YEP plate and suspended in 20 ml Luria Bertani (LB) liquid medium supplemented with appropriate antibiotics (as per strain) in a centrifuge tube and incubated for 2 days after which 20 µl of the bacterial culture was inoculated into 20 ml LB medium and grown overnight at 28 °C. The bacterial cells were pelleted at 6000 rpm for 10 minutes. The pellet was aseptically re-suspended in 20 ml of pre filter sterilised infection medium supplemented with 200 µM acetosyringone (Sigma Aldrich, USA) and incubated in a shaker at room temperature for 20 minutes. Freshly isolated immature zygotic embryos (from three genotypes identified under study 2 chapter 3 of this thesis) were suspended in bacterial suspension at OD₆₀₀=0.4-1.0 in infection medium for about 5 minutes and later transferred on to co-culture medium with a sterile filter paper overlaid. Twenty embryos were cultured per plate and each treatment replicated three times. The cultures were kept at 23 °C for 5 days. After 5 days, embryos were suspended in infection medium supplemented with 200 mg/l cefotaxime (Sigma Aldrich, USA) to arrest bacterial growth and cultured on resting medium for further 5 days.

Table 2. Composition of different culture medium used during transformation of immature zygotic embryos

Medium type	infectio n Medium	co- culture Medium	Resting Medium	CIM 1	CIM2	SEIM	SIM
Duration	5 min	3-8 days	2-5 days	-	-	-	-
MSmacrosalts	1/10th strength	1/10th strength	full strength	full strength	full strength	full strength	full strength
MSmicrosalts	1/10th strength	1/10th strength	full strength	full strength	full strength	full strength	full strength
Iron complex	1/10th strength	1/10th strength	full strength	full strength	full strength	full strength	full strength
Vitamins	1/10th strength	1/10th strength	full strength	full strength	full strength	full strength	full strength
Proline	n/A	1 g/l	1 g/l	1 g/l	1 g/l	1 g/l	1 g/l
Pvp	N/A	1 g/l	1 g/l	1 g/l	1 g/l	1 g/l	1 g/l
Acetosyringone	200µM	200µM	Nil	Nil	Nil	Nil	Nil
2,4-D	2.5 mg	2.5 mg/l	2.5 mg	2.5 mg	2.5 mg	2.0 mg	Nil
Kinetin	0.5 mg	0.5mg/l	0.5 mg/l	0.5 mg/l	0.5 mg/l	0.5 mg/l	0.5mg/l
L-cystein	N/A	400mg/l	400mg/l	400mg/l	Nil	Nil	Nil
Sugar	68.4 g	30 g	30 g	30 g	30 g	30 g	30 g
Glucose	36g/l	-	-	-	-	-	-
NAA	Nil	Nil	Nil	nil	Nil	Nil	Nil
Antibiotics	N/A	strain selection	anti-strain growth	vector selection	vector selection	Nil	Nil

3.5.3 Transient GUS assay

Transient *gus plus* gene expression was monitored in Agro-infected sorghum immature embryos after 5 days of co-culture. Six *explants* were selected at random from each treatment for GUS assay. The embryos were incubated in a pre-filter sterilized X-Gluc staining buffer as described by Jefferson *et al.* (1987) for 24 hours. The number of blue spots on embryos was counted under a compound microscope to ascertain the level of *gus plus* gene expression.

3.5.4 Selection and regeneration of transformed cells

The embryos that were not subjected to GUS assay were later transferred onto callus induction medium supplemented with 50 mg/l hygromycin (Sigma Aldrich, USA). The percentage of surviving embryos were recorded and transferred on to second medium of the same composition till embryogenesis. The number of transferred *explants* forming surviving

calli on the selection medium was recorded. The embryos developed remained dormant and no regenerants were obtained.

3.6 Data analysis

The data collected in this study as frequencies and counts was subjected to arcsine and square root transformation respectively before Analysis of variance (ANOVA) to normalize the data (Steel *et al.*, 1997; McDonald, 2009). Where significant differences were found, means were separated using Least Significant difference test at $P \leq 0.05$.

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 Optimisation of *in vitro* culture system for selected elite Ugandan sorghum genotypes

4.1.1 Effect of 2, 4-D and kinetin hormone levels in callus induction medium on *in vitro* culture responses of local Ugandan sorghum genotypes

4.1.1.1 Callus induction frequency and callus growth

The callus induction frequency of sorghum was significantly influenced by the level of kinetin hormone ($p \leq 0.05$) and 2, 4-D ($p < 0.001$) in the callus induction medium. However, there was no significant multiplicative effect between the two hormones on callus formation ability of sorghum (Table 3). A significantly high callus induction frequency was observed at a 2, 4-D level of 2.5 mg/l (75.54 %) and 0 mg/l of kinetin (47.6 %). Embryos cultured on medium supplemented with either 1 mg/l 2, 4-D or without 2, 4-D germinated to form shoots and no callus was formed. Generally, addition of kinetin hormone in the callus induction medium significantly reduced the callus induction frequency of sorghum (Figure 1).

Table 3. Mean squares for the effect of 2, 4-D and kinetin levels on sorghum callus induction frequency, callus growth, and somatic embryo formation and regeneration efficiency

Source of variation	Df	Mean squares			
		Callus induction frequency	Callus growth	Somatic embryogenic formation frequency	Shoot formation
Kinetin (Kin)		0.19 *	42.65**	0.28***	9.51***
2, 4-D	4	3.03***	2.55***	2.25***	197.08***
Kin x 2, 4-D	12	0.08ns	1.61***	0.06ns	6.47***
Error	40	0.05	0.37	0.035	0.92

*= significant at $P=0.05$, **=Significant at $P=0.01$, ***=significant at $P=0.001$, ns=Not significant

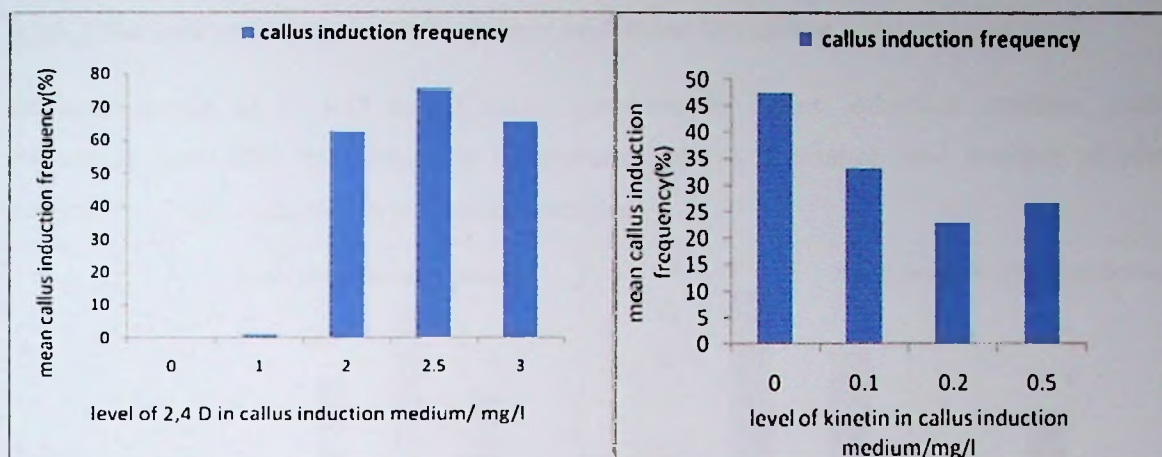


Figure 1A

Figure 1B

Figure 1. Effect of phytohormone levels in callus Induction medium on callus induction frequency. The figure on left marked 1A for 2, 4-D and figure on right marked 1B for Kinetin

The combined effects of kinetin and 2, 4-D in callus induction medium, highly significantly affected ($p < 0.001$) callus growth. Significantly good callus growth was also observed on medium supplemented with 3 mg/l 2, 4-D without kinetin, 2.0 mg/l 2, 4-D together with 0.5 mg/l kinetin or no kinetin and 2.5 mg/l 2, 4-D with, 0, 0.1 or 0.5 mg/l kinetin. A significant poor growth was observed on callus from medium supplemented with high levels of kinetin at 2, 4-D level of 3 mg/l. No callus growth was observed on callus induction medium supplemented with 1 mg/l of 2, 4-D and kinetin hormone (Figure 2). This was probably due to the fact that the effect of kinetin outweighed the 2, 4-D effect on embryos resulting into induction of shoots instead of callus.

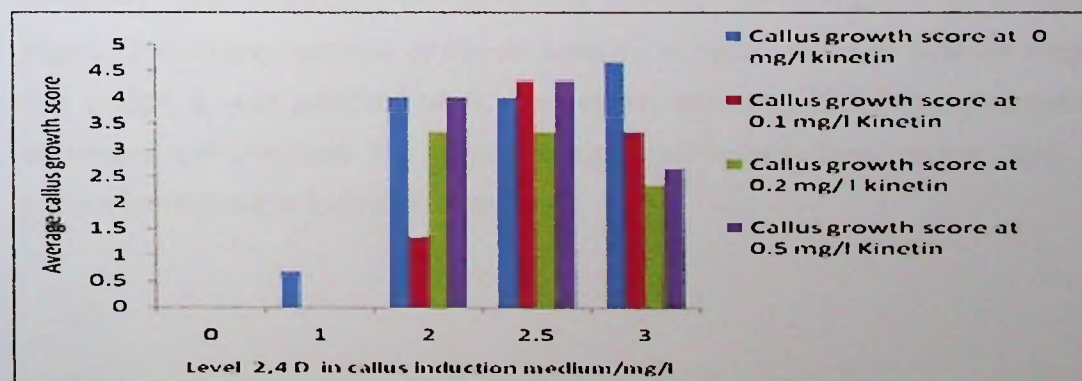


Figure 2. Effect of 2, 4-D and kinetin hormone levels in callus induction medium on callus growth

4.1.1.2 Somatic embryogenesis frequency and shoot formation

Different levels of 2, 4-D and Kinetin hormones in callus induction medium greatly influenced ($p < 0.001$) the frequency of somatic embryo formation and number of shoot formation on calli cultured on maturation medium.

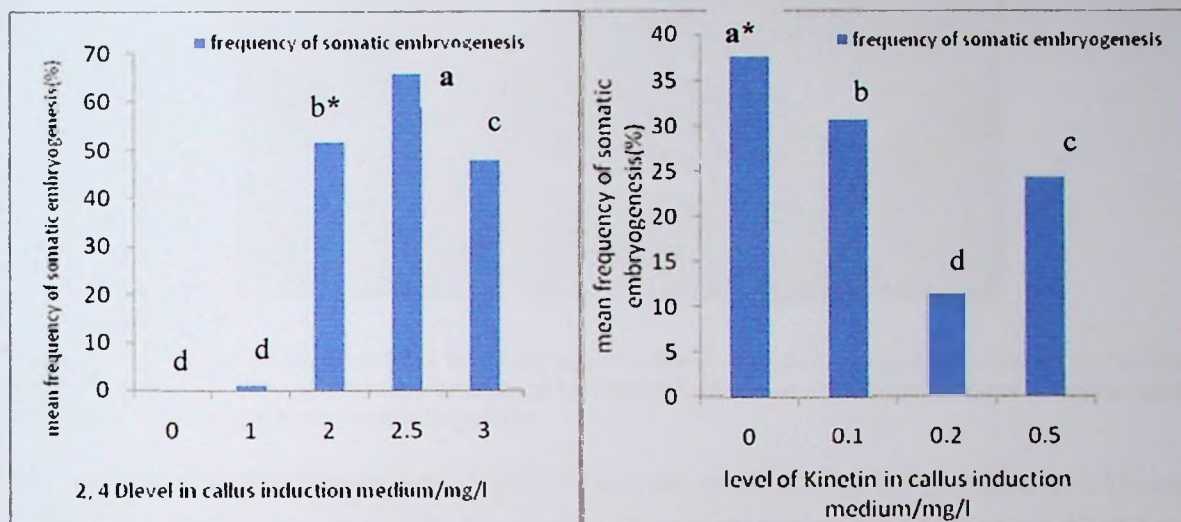


Figure 3A

Figure 3B

Figure 3. Effect of phytohormone levels in callus induction medium on somatic embryogenesis. The figure on the left marked 3A for 2, 4-D and on the right marked 3B for kinetin. *Implies that columns/bars with similar letters are not significantly different at $p > 0.05$

Significantly high frequency of somatic embryogenesis was obtained on *explants* cultured on callus induction medium supplemented with 2.5 mg/l 2, 4-D. Addition of Kinetin hormone resulted in a drastic reduction in frequency of somatic embryogenesis in an ambiguous trend (Figure 3). Similarly, addition of kinetin hormone in callus induction medium supplemented with 1 mg/l 2, 4-D inhibited plant regeneration through somatic embryogenesis and no regenerants were obtained. The immature zygotic embryos cultured on this callus induction medium germinated to form shoots and roots.

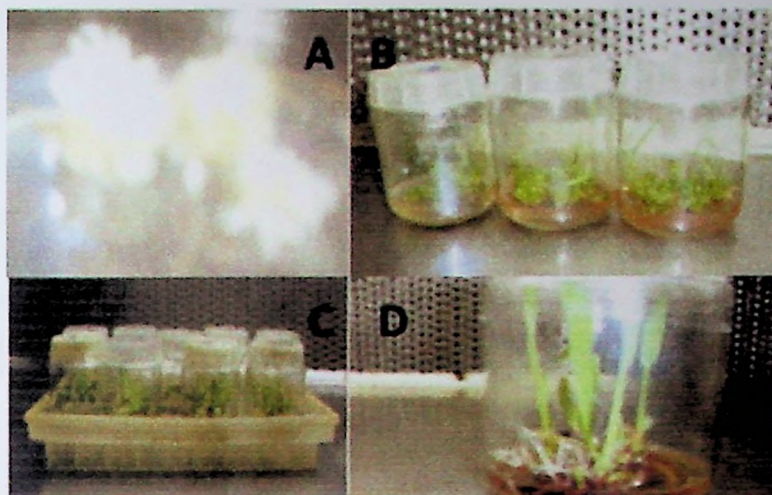


Plate 3. Regeneration of sorghum from immature zygotic embryo into fully developed plantlets (A) Somatic embryos formed on Callus, (B) Somatic embryos germinated to form shoots, (C) Shoots cultured on half strength MS rooting medium, (D) Roots formed on plantlet

The number of shoots formed were highly influenced ($p < 0.001$) by the interaction of kinetin and 2, 4-D hormones in callus induction medium (Table 3). The largest number of regenerants was achieved on calli from embryos cultured on callus induction media supplemented with 3 mg/l 2, 4-D without kinetin, and 2.5 mg/l 2, 4-D together with 0.5 mg/l kinetin hormone (Figure 4).

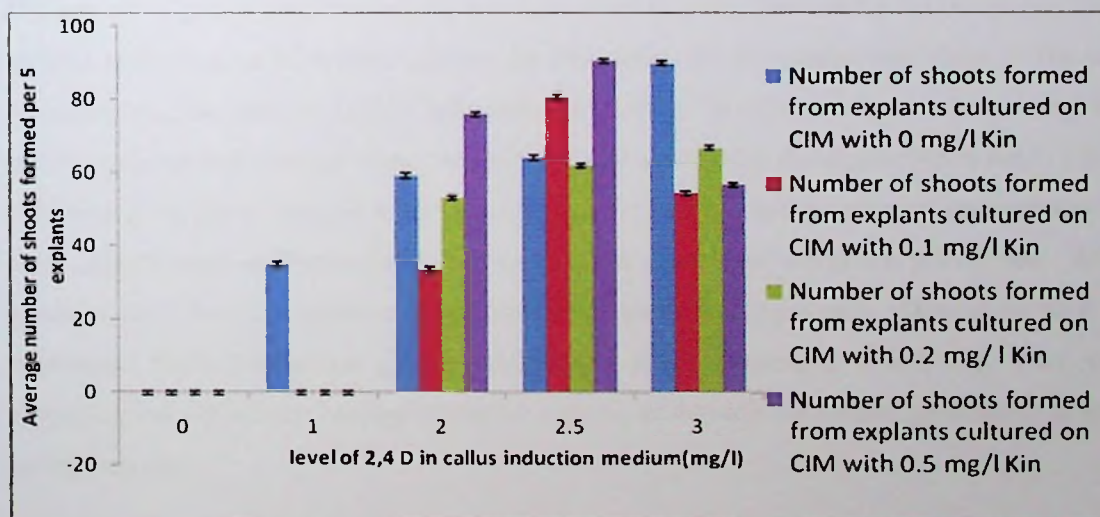


Figure 4. Effect of 2, 4-D and kinetin level in callus induction medium on number of shoots formed

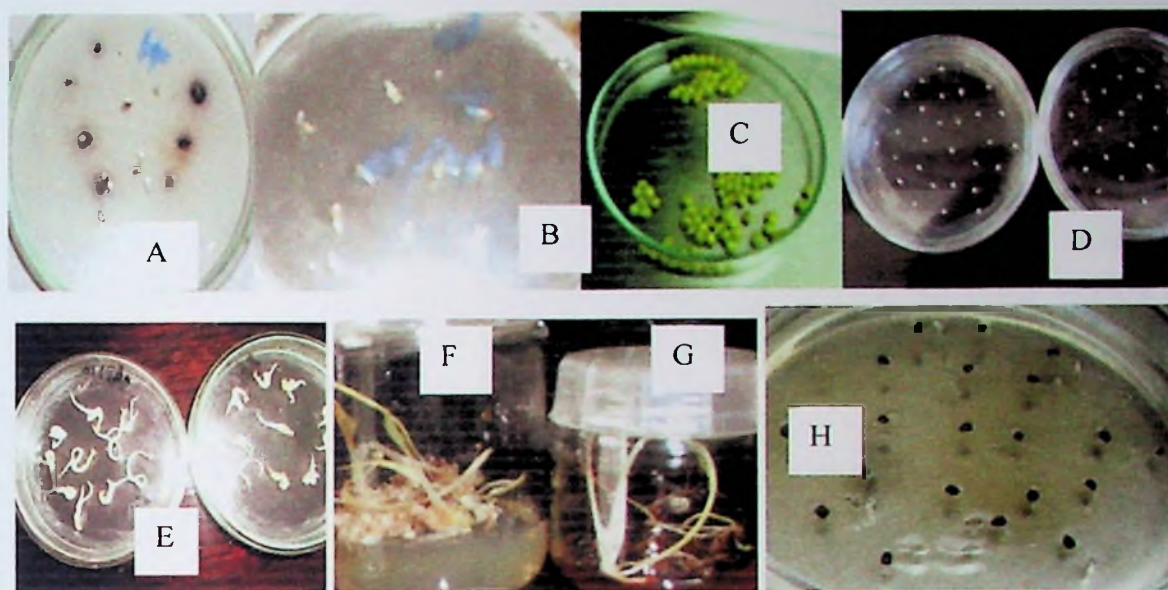


Plate 4. Sorghum explants used and response of Ugandan local sorghum genotypes to different *in vitro* conditions (A) Black phenolic compounds inhibited immature embryo, (B) Immature embryo exhibited growth response on PVP (antioxidant) supplemented callus induction medium, (C) Sorghum seeds isolated from panicle, (D) Aseptically isolated immature zygotic embryos on CIM, (E) Callus initiation 7 days post culture (F) Callus initiated on 2,4-D supplemented medium (G) Shoot initiated from Immature zygotic embryos cultured on 2,4-D free medium, and (H) Response of Immature zygotic embryo isolated 14 days post anthesis.

4.1.2 Effect of growth and developmental stage of sorghum embryos on callus induction frequency, somatic embryogenesis and number of regenerants

The age of sorghum panicles has always been used to make harvesting decisions for immature zygotic embryo used in *in vitro* culture. In this study, the developmental stage of the cultured immature zygotic embryo highly influenced ($p < 0.001$) the callus induction frequency, somatic embryogenesis and number of regenerants formed among the three genotypes used. The callus induction frequency, somatic embryo formation frequency, and number of regenerants formed were significantly different ($p < 0.001$) among the three used sorghum genotypes. Although genotype and developmental stage of embryo cultured independently affected *in vitro* culture responses, their interaction greatly influenced these responses (Table 4). This suggests physiological difference among immature zygotic embryos of different genotypes at the same harvesting age.

The embryos harvested 14 days post anthesis did not form callus among the three candidate genotypes because the embryos died before germinating (Plate 4). The genotypes

MUC007/194 and MUC007/178 had the highest callus induction frequency when their embryos cultured were harvested at 18 and 20 days post flowering while genotype MUC007/193 had the highest callus induction frequency when embryos cultured were harvested above 14 days post flowering (Figure5). Although, the callus induction frequency was highest for the three genotypes at the harvesting age of 20 days, all the calli formed were non embryogenic hence no somatic embryos were obtained. The major challenge remains on how to produce somatic embryos from mature embryos of sorghum in Uganda.

Table 4. Mean squares for the effect of developmental stage of embryo on callus induction frequency, somatic embryo formation frequency and shoot formation

Source of variation	Df	Mean squares		
		Callus induction frequency	Somatic Embryogenesis frequency	shoot formation
Days post anthesis (DPA)	3	4.31***	6.57***	113.30***
Genotype (G)	2	0.18***	0.03***	3.85***
DPA x Genotype	6	0.05**	0.03**	1.44**
Error	24	0.01	0.003	0.36

*= significant at P=0.05, **=Significant at P=0.01, ***=significant at P=0.001, ns=Not significant

Genotypes MUC007/178 and MUC007/194 had the highest formation of embryogenic calli when embryos were harvested at 18 days after flowering while genotype MUC007/193 showed formation of embryogenic calli at both 16 and 18 days (Figure 5B). The largest number of regenerants was significantly formed among the three genotypes when their embryos were harvested at 18 days post flowering and no regenerants were achieved at harvesting time of 14 and 20 days post flowering (Figure 5C).

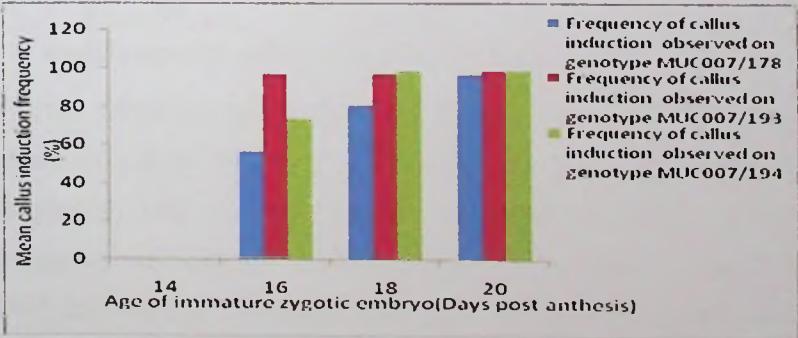


Figure 5A

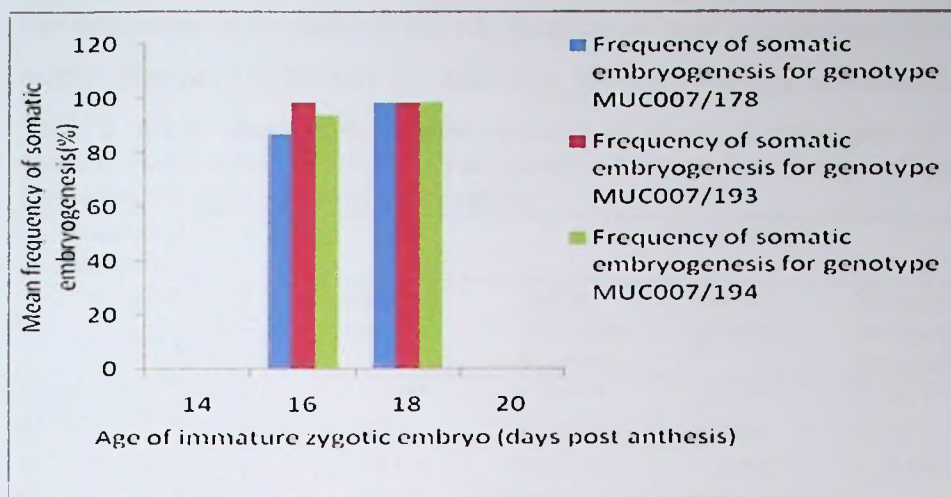


Figure 5B

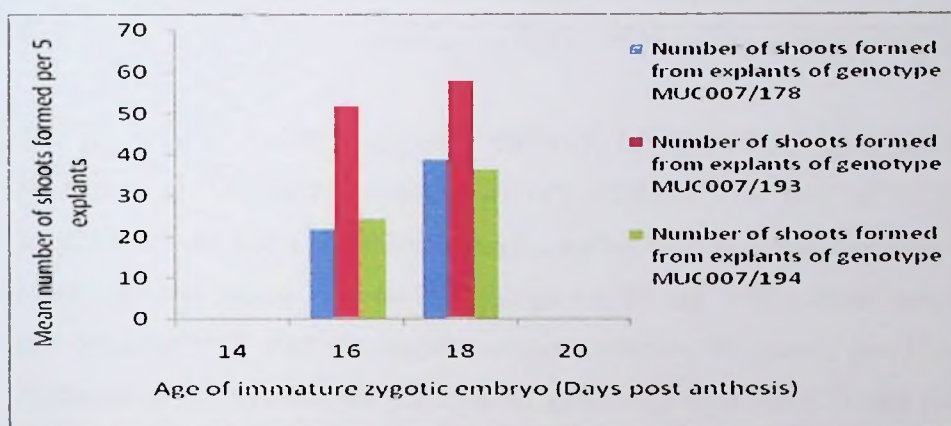


Figure 5C

Figure 5. Effect of age of zygotic embryo on callus induction and regeneration among three candidate sorghum genotypes. The figure on top marked 5A shows effect on callus induction frequency; figure in the middle marked 5B shows effect on frequency of somatic embryogenesis whereas figure marked 5C indicates the effect on number of shoots formed.

4.1.3 Identification of elite Ugandan sorghum genotypes with high potential of embryogenic callus formation and regeneration

Twenty sorghum genotypes used in this study showed significant differences ($p < 0.001$) in terms of callus induction time and frequency, callus growth, somatic embryo formation frequency, and shoot formation. Although the callus induction time and frequency, and frequency of somatic embryogenesis of sorghum genotypes was significantly different ($P \leq 0.05$) between the two media types used, growth of induced calli and shoot formation was

not influenced by medium type. All sorghum *in vitro* culture parameters considered were highly affected ($p < 0.001$) by the interaction between genotype and medium type (Table 5).

Table 5. Mean squares for *in vitro* response of sorghum genotypes on two media types in terms of callus induction time, callus induction frequency, callus growth, somatic embryo formation frequency and shoot formation

Source of variation	Df	Mean Squares				
		Callusing time	Callus induction frequency	Callus growth	Somatic embryogenesis frequency	Shoot formation
Genotype(G)	19	35.15***	0.39***	3.35***	0.38***	10.12***
Medium (M)	1	0.13*	0.49***	0.53ns	0.22*	1.37 ns
Genotype x M	19	22.98***	0.16***	1.36***	0.23***	7.27***
Error	80	0.03	0.024	0.15	0.03	1.59

*= significant at $P \leq 0.05$, **=Significant at $P \leq 0.01$, ***=significant at $P \leq 0.001$, ns=Not significant

The genotypes MUC007/102, MUC007/107, MUC007/111, MUC007/114, MUC007/127, MUC007/224, MUC007/162, MUC007/167, MUC007/178, MUC007/192, MUC007/194 and MUC007/63 showed a significantly higher callus induction frequency on L3 medium than on Murashige and Skoog medium (Murashige and Skoog, 1962) while genotypes MUC007/124 and MUC007/193 had the highest callus induction frequency on Murashige and Skoog compared to L3 medium. On L3 medium genotypes MUC007/111 and MUC007/194 had the highest callus induction frequency of 96.73 % while on MS medium, genotype MUC007/193 showed the highest callus induction frequency of 98.75 % followed by genotypes MUC007/194 (90 %) and MUC007/124 (80.69%). The sorghum genotypes MUC007/80, MUC007/88, MUC007/93, MUC007/99, and MUC007/189 showed the lowest callus induction frequency on both medium types (Table 6).

Table 6. The mean callus induction response on two *in vitro* culture medium types among twenty Ugandan sorghum genotypes

Sorghum genotype	Mean callus induction frequency	
	L3 medium	MS medium
MUC007/100	68.37	76.82
MUC007/102	86.99	70.33
MUC007/107	74.67	22.15
MUC007/111	96.73	70.33
MUC007/114	89.02	68.37
MUC007/124	46.50	80.69
MUC007/127	63.90	25.44
MUC007/224	91.32	80.69
MUC007/162	93.79	67.09
MUC007/167	77.53	46.65
MUC007/178	90.00	56.69
MUC007/189	36.10	43.31
MUC007/192	80.39	73.48
MUC007/193	39.86	98.75
MUC007/194	96.73	90.00
MUC007/63	83.64	29.67
MUC007/80	29.67	29.67
MUC007/88	23.18	29.67
MUC007/93	10.00	25.44
MUC007/99	46.65	19.31
LSD _{0.05}	6.18	6.18
Standard error of the mean	2.37	2.37
Coefficient of Variation (CV %)	18.8	18.8

LSD = Fishers protected Least significant difference test calculated at $P \leq 0.05$ (Steel *et al.*, 1997).

The callus induction time estimated as the number of days at which callus is formed post culture initiation was highly influenced by the multiplicative effect of medium and sorghum genotype (Table 5). Genotypes MUC007/194 and MUC007/193 formed callus faster on Murashige and Skoog medium (7 days post culture initiation) than on L3 medium while genotypes MUC007/162 and MUC007/192 formed callus much faster on L3 medium than on MS medium (Table 7). The genotypes MUC007/102 and MUC007/194 showed better callus growth on L3 medium while on MS medium, genotype MUC007/193 showed the best callus growth. The growth of callus from genotype MUC007/124 was better on MS than on L3 medium (Table 8).

Table 7. Average number of days to callus initiation among the 20 elite Ugandan sorghum genotypes on two different medium types

Sorghum genotype	Mean number of days to callus initiation	
	L3 medium	MS medium
MUC007/100	17	16
MUC007/102	10	14
MUC007/107	14	15
MUC007/111	14	13
MUC007/114	13	13
MUC007/124	14	16
MUC007/127	18	16
MUC007/224	10	13
MUC007/162	8	12
MUC007/167	12	14
MUC007/178	14	14
MUC007/189	14	10
MUC007/192	9	10
MUC007/193	18	7
MUC007/194	10	7
MUC007/63	12.7	16
MUC007/80	15	17
MUC007/88	10	16
MUC007/93	20	14
MUC007/99	15	16
LSD _{0.05}	0.30	0.30
Coefficient of variation (CV %)	1.4	1.4

LSD = Fishers protected Least significant difference test calculated at $P \leq 0.05$ (Steel *et al.*, 1997).

The frequency of somatic embryogenesis indirectly measures the frequency of embryogenic callus formation among sorghum genotypes. On L3 medium, the sorghum genotypes showed the somatic embryogenesis frequency ranging from 10 % up to 91.31 % with genotypes MUC007/102, MUC007/111, MUC007/114, MUC007/162, MUC007/178, and MUC007/194 showing a somatic embryogenesis frequency above 80 %. On MS medium, only eight genotypes showed a frequency of somatic embryogenesis above 60 % with the genotypes MUC007/194 (93.8 %) and MUC007/193 (83.6 %) showing the highest frequency of somatic embryogenesis. In terms of frequency of somatic embryo formation, genotypes MUC007/80, MUC007/88, MUC007/93, MUC007/99, and MUC007/189 were ranked the worst (Table 9).

Table 8. Mean callus growth scores among twenty selected elite Ugandan sorghum genotypes on two medium types

Sorghum genotype	Mean callus growth score	
	L3 medium	MS medium
MUC007/100	1.33	2
MUC007/102	3	2.33
MUC007/107	1	1
MUC007/111	1.33	2
MUC007/114	1.33	1
MUC007/124	1	2.67
MUC007/127	1	1
MUC007/224	1.67	2
MUC007/162	1.67	1
MUC007/167	1	1
MUC007/178	2	1.33
MUC007/189	1	1
MUC007/192	1.33	1
MUC007/193	1	4.33
MUC007/194	4	3.33
MUC007/63	1.67	1
MUC007/80	1	1
MUC007/88	1.33	1
MUC007/93	0.33	0.67
MUC007/99	1	1
LSD _{0.05}	0.63	0.63

LSD = Fishers protected Least significant difference test calculated at $P \leq 0.05$ (Steel *et al.*, 1997).

The genotype by medium interaction greatly affected the number of regenerants formed among selected sorghum genotypes. Genotype MUC007/193 formed the highest number of regenerants on Murashige and Skoog medium (20.9 shoots per *explant*) while sorghum genotypes MUC007/102 (19 shoots) and MUC007/194 (16.3 shoots per *explant*) showed the highest number of regenerants on L3 medium. Although genotype MUC007/99 had the lowest callus induction and somatic embryogenic frequency, it had a reasonably higher number of regenerants than most genotypes that were considered to perform better in terms of callus induction frequency and somatic embryo formation frequency (Table 10). This was possibly due to the differences in number of germinating embryos per *explant*.

Table 9. Mean somatic embryogenesis frequency on two medium types among twenty elite Ugandan sorghum genotypes

Sorghum genotype	Mean somatic embryogenesis frequency	
	L3 medium	MS medium
MUC007/100	46.65	56.69
MUC007/102	83.64	63.55
MUC007/107	27.96	13.01
MUC007/111	83.64	70.33
MUC007/114	80.39	42.80
MUC007/124	13.01	74.56
MUC007/127	56.84	22.15
MUC007/224	53.35	74.56
MUC007/162	91.32	60.14
MUC007/167	53.50	13.01
MUC007/178	90.00	39.86
MUC007/189	21.02	33.26
MUC007/192	65.21	63.90
MUC007/193	16.36	93.79
MUC007/194	83.64	83.64
MUC007/63	68.51	11.61
MUC007/80	19.31	15.72
MUC007/88	23.18	23.18
MUC007/93	10.00	16.74
MUC007/99	36.10	4.53
LSD _{0.05}	8.82	8.82
CV%	24.5	24.5

Overall, the response of selected Ugandan sorghum genotypes was highly influenced by a multiplicative effect of genotype and medium and to some degree genotype dependent. Genotypes MUC007/193, MUC007/194, and MUC007/124 were highly regenerable on Murashige and Skoog medium with MUC007/193 and MUC007/194 forming callus faster than all other genotypes. They resulted in callus induction frequency above 90 % on MS medium while on L3 medium genotypes MUC007/102, MUC007/162, MUC007/178, MUC007/99, and MUC007/194 were highly regenerable.

Table 10. Mean number of regenerants formed per 5 explants on two medium types among 20 sorghum genotypes

Sorghum genotype	Mean number of regenerants formed per 5 explants	
	L3 medium	MS medium
MUC007/100	43.44	42.56
MUC007/102	95.01	27.07
MUC007/107	20.04	27.01
MUC007/111	34.20	25.68
MUC007/114	30.24	22.62
MUC007/124	14.98	56.42
MUC007/127	11.90	36.15
MUC007/224	21.99	37.97
MUC007/162	46.77	31.24
MUC007/167	34.52	23.20
MUC007/178	45.00	34.83
MUC007/189	37.20	21.78
MUC007/192	25.35	25.55
MUC007/193	23.49	104.23
MUC007/194	81.53	64.16
MUC007/63	19.11	41.53
MUC007/80	27.30	29.16
MUC007/88	29.62	28.57
MUC007/93	1.11	10.33
MUC007/99	54.63	27.90
LSD _{0.05}	4.19	4.19
CV%	22.1	22.1

LSD = Fishers protected Least significant difference test calculated at $P \leq 0.05$ (Steel *et al.*, 1997).

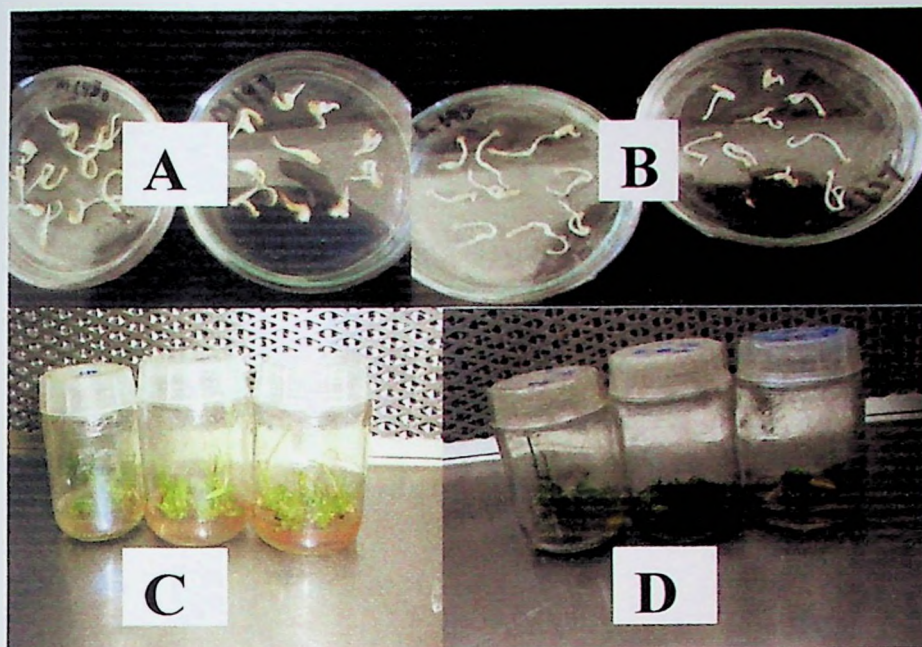


Plate 5. Response of Ugandan adapted sorghum genotypes on different types of *in vitro* culture medium (A) Response of sorghum genotype MUC007/193 on MS callus induction medium (B) Callusing Response of MUC007/193 on L3 callus induction medium, (C) Brown phenolic compounds produced in culture (D) Black phenolic compounds produced in culture.

4.1.4 Discussion

4.1.4.1 Effect of 2, 4-D and kinetin hormone levels in callus induction medium on sorghum *in vitro* culture responses

Plant growth regulators play a vital role in cereal tissue culture. Several plant growth hormones have been developed and tested. Among them auxin and cytokinin products that are also natural plant growth regulators are being used. 2, 4-Dichlorophenoxyacetic acid hormone (2, 4-D) is one of the most widely used auxins for induction of callus and somatic embryogenesis in both monocots and dicot plant species (Nguyen *et al.*, 1998; Saradamani *et al.*, 2003; Jogeswaret *et al.*, 2007). In this study, callus induction frequency of sorghum was highly influenced by level of 2, 4-D and kinetin included in the media. An optimum callus induction frequency was obtained at a 2, 4-D level of 2.5 mg/l and addition of kinetin hormone resulted into a drastic reduction in callus induction and somatic embryogenesis. Other studies have also observed that high levels of cytokinins indeed reduce callus induction (Oldach *et al.*, 2001). Embryos cultured on callus induction medium supplemented with 2, 4-D level above 2.5 mg/l resulted in a substantial decline in callus induction and somatic

embryogenesis. This suggests an inhibitory effect of increased levels of 2, 4-D on callus induction, growth, shoot induction and number of shoots per callus (Baskaran *et al.*, 2005; Gupta *et al.*, 2006). Inclusion of kinetin in callus induction medium however improves these responses. In other studies, a high frequency of somatic embryogenesis has been achieved on Murashige and Skoog medium (Murashige and Skoog, 1962) supplemented with 2 mg/l 2, 4-D and 0.5 mg/l of kinetin, which overall enhanced the formation of somatic embryos from explants (Jogeswar *et al.*, 2007).

In this study, a substantial good callus growth and high number of regenerants was achieved from explants cultured on callus induction medium supplemented with 2, 4-D ranging from 2.0 to 3.0 mg/l. It was further observed that addition of kinetin hormone to callus induction medium with 2.0 and 2.5 mg/l of 2, 4-D further improved sorghum regeneration. Therefore the use of 2, 4-D and kinetin in subsequent studies at a level of 2.5 and 0.5 mg/l respectively was opted for due to the quality of callus induced coupled with increased number of regenerants obtained.

4.1.4.2 Effect of age of sorghum embryo on *in vitro* culture responses

In this study, the age of immature zygotic embryo influenced *in vitro* culture responses. The optimum age of sorghum immature zygotic embryo varied among different sorghum genotypes investigated in this study. The embryos isolated from panicles of all genotypes harvested at 14 days after anthesis were too small (about <0.5mm) and neither did they germinate nor form callus. Similarly embryos isolated from panicles of all the genotypes harvested at 20 days post anthesis were too long (about 2.5mm long). These embryos germinated after 3 days and started forming callus however, the calli formed was non embryogenic. Embryos isolated from the panicles at 16 to 18 days after flowering, were in a range of about 0.9-1.8 mm long and produced calli that regenerated into plantlets. The physiological state of the embryos of different sorghum genotypes at the same age appeared to be different and thus judging the harvesting time of the panicles basing on the age may be erroneous. This possibly showed that other characteristics especially size of the embryo and consistence of the seed should be used in making decision of when to harvest panicles. In similar studies elsewhere immature zygotic embryos of 8-18 days post anthesis were most

suitable for *in-vitro* studies of sorghum although this was also genotype dependent (Ma *et al.*, 1984; Indra Arulselvi and Krishnaveni, 2009). In other studies the size of the immature embryo of *Sorghum sudanenses* (sudan grass) influenced callus formation and plant regeneration with embryos of 0.7-1.5 initiating callus quickly, inducing fast growing callus that frequently forms more shoots (Gupta *et al.*, 2004). In this study, it was observed that either too young or mature embryos were less responsive to *in vitro* culture. In maize, a gramineae relative of sorghum, embryo size influences callus induction with a large decline in frequency of callus induction occurring when immature embryos are harvested at 10 and 24 days post pollination (Bonett *et al.*, 2008). These studies on sorghum *in vitro* culture and other gramineae all indicate that age and size of embryo influence the responses expected *in vitro*. Indeed, various reports all confirm this position, with successful *in vitro* responses on embryos harvested 14 days after flowering (Hagio, 2002), 12- 15 days after anthesis (Tadesse *et al.*, 2003), and 12-14 days after flowering (Grootboom *et al.*, 2008; 2010). Overall, this study shows that the developmental stage of immature zygotic embryo affects the *in vitro* culture responses of sorghum. The age of sorghum immature zygotic embryo to be used should thus not only be based on number of days post flowering or pollination but also on embryo size. The best immature embryo to be cultured should neither be too small (<0.5mm long) nor big (>2mm long).

4.1.4.3 Response of different Ugandan sorghum genotypes to *in vitro* culture

Callus induction and regeneration parameters varied significantly among the selected Ugandan elite sorghum genotypes and this suggests a strong genotypic control of sorghum *in vitro* culture response among Ugandan sorghum genotype. The sorghum genotypes used showed variation in phenolic compound production on culture medium in terms of colour (brown to black) and quantity (Plate 5). Genotype dependence of sorghum in *in vitro* culture has been widely reported by many researchers (Arulselvi and Krishnaveni, 2009; Grootboom *et al.*, 2008; Hagio, 2002) and this genetic control has been reported in other cereals such as sugarcane (Gandonou *et al.*, 2005), wheat (Malik *et al.*, 2004), and maize (Binott *et al.*, 2008; Omer *et al.*, 2008). In this study, medium composition greatly influenced callus induction time, callus induction frequency, and somatic embryogenesis frequency but not shoot formation and callus growth. Callus induction and regeneration of Ugandan sorghums was

influenced by genotype by medium interaction and thus model genotypes for transformation studies were identified. On Murashige and Skoog basal medium, genotypes MUC007/193, MUC007/194 and MUC007/124 were highly amenable to *in vitro* analyses. On L3 basal medium, most of the genotypes exhibited high levels of regeneration and these included genotypes MUC007/194, MUC007/102, and MUC007/162, MUC007/99, and MUC007/100. A similar phenomenon of genotype by medium interaction in cereal *in vitro* culture was reported by Grootboom *et al.* (2008). In this study L3 culture medium proved to be the best basal medium for most of less *in vitro* culture responsive sorghum genotypes.

4.2 *Agrobacterium* mediated transformation of highly regenerable Ugandan sorghum accessions

4.2.1 Transient *gus plus* gene expression in pre-cultured embryos

The transient *gus plus* gene expression was assayed at initial stages of callus formation from cultured immature zygotic embryos (five days post culture initiation). The transient *gus plus* gene expression in assayed *explants* tissues was highly influenced ($p \leq 0.001$) by the strain of *Agrobacterium* used to infect the *explants*. Significant sorghum genotypic differences in the level of *gus plus* gene expression was also observed in this study (Table 11). The *explants* infected with EHA105 showed a significantly high level of transient *gus plus* gene expression than AGL1 infected embryos irrespective of sorghum genotype. The highest level of *gus plus* gene expression was observed in *explants* isolated from sorghum genotypes MUC007/193 and MUC007/124 thus suggesting their relatively high level of susceptibility to *Agrobacterium* infection (Figure 6). This could be linked to the differential production of phenolic compounds among sorghum genotypes. No significant multiplicative effect between sorghum genotypes and *Agrobacterium* strains was found to influence the level of *gus plus* gene expression in assayed *explants*.

Table 11. Mean squares for the transient *gus plus* gene expression in *Agrobacterium* infected embryos of selected Ugandan sorghum genotypes

Source of variation	Df	Mean squares
<i>Agrobacterium</i> strain	1	26.01 ***
sorghum genotype	2	1.79**
Genotype x <i>Agrobacterium</i> strain	2	0.72ns
Error	30	0.35

*= significant at $P=0.05$, **=Significant at $P=0.01$, ***=significant at $P=0.001$, ns=Not significant

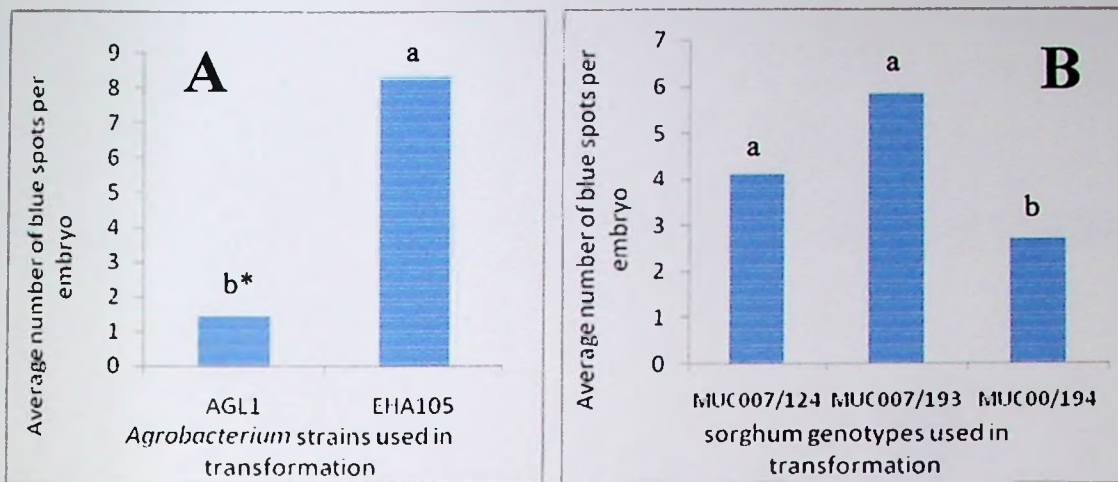


Figure 6. Influence of *Agrobacterium* strain and sorghum genotype used on transient *gus plus* gene expression in immature zygotic embryos. *Bars marked with the same letter are not significantly different at $p > 0.05$. (A) Effect of *Agrobacterium* strain used (B) Effect of sorghum genotype used

4.2.2 Putative transformation frequency among the selected sorghum accessions

The transformation frequency was recorded as the number of sorghum calli surviving on hygromycin amended media. The interaction between sorghum genotype and *Agrobacterium* strain did not significantly affect ($p \geq 0.05$) the number of hygromycin resistant calli obtained. The frequency of putatively transformed calli was greatly influenced by the strain of *Agrobacterium* used ($p < 0.001$) and was sorghum genotype dependent ($p < 0.05$). The frequency of hygromycin resistant calli obtained from EHA105 infected *explants* was significantly higher than that from AGL1 infected *explants* (Figure 7A). The largest number of sorghum genotypes MUC007/193 and MUC007/124 derived calli were hygromycin resistant compared to genotype MUC007/194 (Figure 7B). In this study, the somatic embryos derived from hygromycin resistant calli remained dormant and no transgenic plants were obtained. Generally, this study demonstrated that the efficiency of *Agrobacterium* mediated transformation system is not affected by sorghum genotype by *Agrobacterium* strain interaction.

Table 12. Mean squares for the putative transformation frequency of selected Ugandan sorghum genotypes

Source of variation	Df	Mean square
<i>Agrobacterium</i> strain	1	6.14***
Plant genotype	2	1.21*
<i>Agrobacterium</i> strain x plant genotype	2	0.07ns
Error	12	0.17

*= significant at $P=0.05$, **=Significant at $P=0.01$, ***=significant at $P=0.001$, ns=Not significant

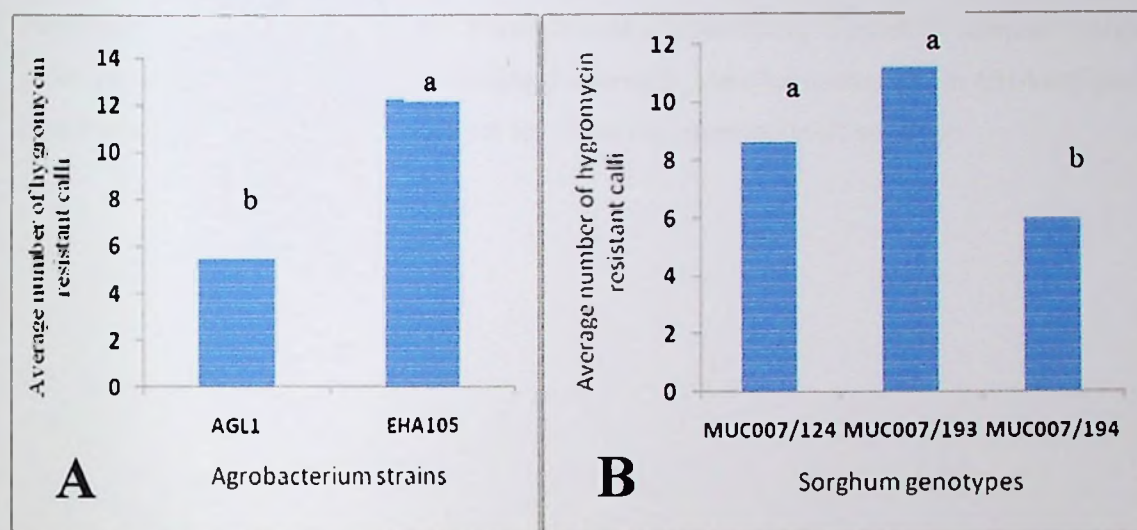


Figure 7. Effect of *Agrobacterium* strain used to infect *Explants* on the frequency of putative transformed calli. *Bars marked with the same letter are not significantly different at $p>0.05$. (A) Effect of *Agrobacterium* strain used (B) Effect of sorghum genotype used

4.2.3 Discussion

Extensive studies have been done over the last decade world over to optimise conditions for *Agrobacterium* mediated sorghum transformation (Zhao *et al.*, 2000; Gao *et al.*, 2005b; Nguyen *et al.*, 2007; Arun Kumar Pandey *et al.*, 2010). However, the most challenging part has been the influence of the plant genotype and *Agrobacterium* strain on the transformation efficiency and expression of gene of interest in the transformants (Jeoung *et al.*, 2002; Arun Kumar Pandey *et al.*, 2010). In this study, the three highly regenerable Ugandan sorghum genotypes (chapter four of this thesis) used exhibited differential expression of the *gus plus* gene and transformation frequency. Additionally, *Agrobacterium* strain EHA 105 infected immature Zygotic embryos showed a higher level of *gus plus* gene expression and

transformation frequencies than AGL1 strain infected embryos showing that EHA105 is the best strain of choice. In other studies, the *Agrobacterium* strain EHA105 had the highest transformation frequency than EHA101 and AGL1 (Jeoung *et al.*, 2002). Similar observations have been made by Arun Kumar Pandey *et al.*(2010) who show that the *Agrobacterium* strain EHA105 resulted in greater proportion of *explants* expressing *gus plus* gene in both sorghum genotypes used. In rice *Agrobacterium* EHA105 was more efficient in rice transformation than AGL1 and LBA4404 (Yi *et al.*, 2001). Although this study did not regenerate transformed plants, it showed the possibility of transforming Uganda's adapted sorghum genotypes through *Agrobacterium* mediated approach. *Agrobacterium* strain EHA105 proved to be the best carrier of vector construct for stable transformation of sorghum.

CHAPTER FIVE

GENERAL DISCUSSION

5.1 Efficient regeneration system for selected Ugandan sorghum genotypes

The first objective of this study was to determine appropriate culture medium composition and age of immature embryo for efficient regeneration of selected Ugandan sorghum genotypes. The study focused on optimising phytohormone concentrations for callus induction medium and identifying the suitable developmental stage of immature zygotic embryo for *in vitro* analyses. The results of this study show that the level of auxin compound 2,4-Dichlorophenoxyacetic acid (2, 4-D) included in callus induction medium as well as the inclusion of kinetin hormone significantly influence *in vitro* culture responses of sorghum genotypes included in the study. The auxin (2, 4-D) level of 2.0, 2.5, and 3.0 mg/l in callus induction medium induced good quality callus that resulted in higher numbers of regenerants obtained. Addition of 0.5 mg/l of kinetin into callus induction medium supplemented with 2, 4-D at either 2 or 2.5 mg/l, resulted into a significantly higher number of sorghum regenerants. Although the kinetin was observed to reduce callus induction frequency, its addition in callus induction medium resulted into higher numbers of regenerants obtained. This study therefore has developed an *in vitro* system for callus induction and regeneration of sorghum the first of its kind on Ugandan genotypes.

The study also found that the response to *in vitro* manipulation was influenced by genotypes and the age of the *explants* used. The highest frequency of somatic embryo formation and number of shoots was obtained on materials harvested 16-18 days after flowering. The genotypes used had the highest frequency of somatic embryo formation and number of shoots when their embryos cultured were harvested at 18 days post flowering. Genotype MUC007/193 was highly responsive to *in vitro* culture compared to other genotypes include in the study. However, several other genotypes were amenable to *in vitro* processes depending on the media used. Overall L3 was a better media than MS. These results are corroborated by earlier reports on sorghum regeneration (Seetharama *et al.*, 2000; Gupta *et al.*, 2004; Jogeswar *et al.*, 2007; Indra Arulselvi and Krishnaveni, 2009). This study has shown that the age of the

explant greatly influenced the *in vitro* culture responses of sorghum (callus induction frequency, frequency of somatic embryogenesis and number of regenerants formed).

5.2 Response of selected Ugandan sorghum genotypes to *in vitro* culture

An independent study was carried out to screen twenty sorghum genotypes on two medium types: Murashige and Skoog and L3 basal medium. The results show that medium and sorghum genotype independently influenced *in vitro* culture responses of sorghum leading to a strong effect of their interaction. These study findings are in agreement with earlier report of Grootboom *et al.* (2008). There was also evidence that regeneration potential of Ugandan sorghum genotypes was not only genotype dependent but also genotype by medium interaction played a big role. Overall, this study has identified the suitable media and Uganda's adapted sorghum genotypes that are most amenable to *in vitro* analysis.

5.3 Response of selected Ugandan sorghum genotypes to genetic transformation

The three highly *in vitro* culture responsive sorghum genotypes on MS basal medium identified in this study were infected with two different strains of *Agrobacterium tumefaciens* (AGL1 and EHA105) in a factorial design. The results show that the transgene expression and frequency of transformation was genotype dependent and independently influenced by the *Agrobacterium* strain. The genotypes MUC007193 and MUC007/124 were susceptible to *Agrobacterium* infection compared to sorghum genotype MUC007/194 possibly due to their low level of phenolic compound production in the co-culture medium. The interaction between sorghum genotype and *Agrobacterium* strain did not influence the transformation parameters considered in this study. Thus this study provided enough evidence that sorghum transformation efficiency is not influenced by the multiplicative effect of *Agrobacterium* strain and sorghum genotype. The Ugandan sorghum genotypes MUC007/124 and MUC007/193 appeared to be model genotypes for further development of transformation systems.

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

The main objective of this study was to develop an efficient *in vitro* culture system for selected Ugandan elite sorghum genotypes as well as demonstrate the possibility of *Agrobacterium* mediated transformation of sorghum genotypes amenable to tissue culture. This study developed an efficient *in vitro* culture system the first of its kind for Ugandan adapted sorghum genotypes. It was found that the level of 2, 4-D and kinetin hormones in callus induction medium greatly influence the *in vitro* culture responses of sorghum. A combination of 2.5 mg/l 2, 4-D and 0.5 mg/l Kinetin was outstanding for sorghum regeneration. It was also found that too immature and too old zygotic embryos did not regenerate into sorghum plants under the test conditions. However, a regeneration system using old zygotic embryos as *explants* would be desirable since mature embryos would be available throughout the year. This study found that the age of immature zygotic embryos of sorghum is not a good parameter for making harvesting decisions but this should be supplemented with other parameters such as embryo size and seed consistency.

This study found that the *in vitro* culture responses of Ugandan sorghum genotypes were not only influenced by genotypic differences but also strong genotype by medium interaction. Thus model Ugandan sorghum genotype on each medium type was identified with genotype MUC007/193 and MUC007/194 highly amenable to tissue culture on MS basal medium.

This study further demonstrated that *Agrobacterium* strain by sorghum genotype did not appreciably affect the transformation efficiency and level of *gus plus* gene expression in *explants*. The *Agrobacterium* strain and sorghum genotypic differences independently influenced the transformation parameters considered under this study. The *Agrobacterium* strain EHA105 was better than AGL1 whereas genotypes MUC007/193 and MUC007/124 showed highest transformation frequencies and can thus be used as model genotypes for further sorghum transformation studies.

6.2 Recommendations

Based on the results of this study, the *in vitro* culture system developed for Ugandan sorghum genotypes together with the basic information on *Agrobacterium* mediated transformation generated, paves way for further sorghum genetic improvement. However, there is a need to characterize the tissue culture generated sorghum plants to estimate the frequency of somaclonal variation. Additionally, the model sorghum genotype identified should be used to study the possibility of Ugandan sorghum stable transformation with transgene for agronomic traits.

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