REGENERATION OF TWO TROPICAL MAIZE GENOTYPES (*Zea mays* L.) FROM MATURE EMBRYOS THROUGH CALLUS INITIATION USING SPLIT SEED TECHNIQUE

By

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Regeneration of two maize genotypes (Zea

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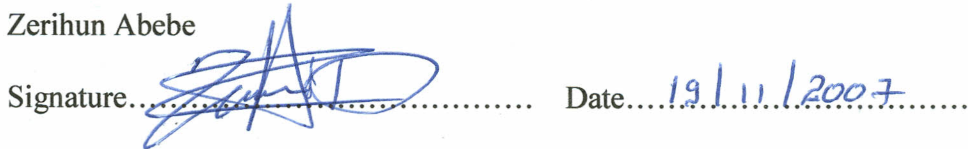
DECLARATION

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



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DEDICATION

I dedicate this thesis for those who fight our battle, pray our prayers and live our misery in different monasteries across the world. I would also like to dedicate this thesis to my father the late Abebe Demissie, whom I never had the chance to see and my grandmother the late Gete Bissa, who passed away without seeing the fruit of her prayers and hard work.

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TABLE OF CONTENTS

DECLARATION ii

DEDICATION iii

ACKNOWLEDGEMENT iv

TABLE OF CONTENTS vi

LIST OF FIGURES x

LIST OF TABLES xi

ACRONYMS and ABBREVIATIONS xii

ABSTRACT xiii

CHAPTER ONE 1

INTRODUCTION 1

1.1 Background 1

1.2 Problem statement and justification 2

1.3 Null Hypothesis 3

1.4 Objectives 3

1.4.1 Broad objective 3

1.4.2 Specific objectives 4

CHAPTER TWO 5

LITERATURE REVIEW 5

2.1 Center of origin of maize 5

2.2 Center of domestication 6

2.3 Ecological requirements........ 6

2.4 Conventional maize improvement 7

2.4.1 Maize improvement 7

2.4.2 Maize production 7

2.4.3 Conventional breeding pitfalls 8

2.4.4 Other production constraints 10

2.5 Tissue culture 11

2.5.1 Introduction 11

2.5.2 Organogenesis and embryogenesis 11

2.5.3 Tissue culture and genetic engineering 12

2.6 Maize tissue culture 12

2.6.1 History 12

2.6.2 Explants 13

2.6.2.1 Immature Embryo 13

2.6.2.2 Mature Embryos 14

2.6.2.3 Other explant sources 14

2.6.3 Maize Callus 15

2.6.4 Plant growth regulators in maize tissue culture 16

2.6.5 Role of silver nitrate in maize tissue culture 17

2.6.6 Application of maize tissue culture 18

2.7 Maize embryo physiology in relation to spitting 19

CHAPTER THREE **21**

MATERIAL AND METHODS **21**

3.1 Plant materials 21

3.2 Determination of germination potential of seeds 21

3.3 Mature seed sterilization 22

3.4 Soaking and germination 23

3.5 Callus induction on split seeds 24

3.6 Callus maintenance 25

3.7 Embryo Maturation 25

3.8 Regeneration 25

3.8.1 Plant regeneration from somatic embryos 25

3.8.2 Shooting and rooting of organogenic callus 27

3.9 Acclimatization 27

3.11 Data analysis 27

CHAPTER FOUR **28**

RESULTS **28**

4.1 Explant source preparation 28

4.2 Germination test 28

4.3 Sterilization protocol optimization 31

4.4 Callus induction 34

4.4.1 Effect of 2,4-D on callus induction from maize mature embryos 36

4.4.2 Effect of plumule size on callus induction from mature maize zygotic embryos 38

4.4.3 Effect of selected 2,4-D levels on callus induction from mature maize embryos 40

4.4.4 Effect of kinetin on callus induction from mature maize zygotic embryos . 43

4.5 Type I and Type II callus induction 45

4.6 Plant Regeneration 50

4.7 Acclimatization 50

CHAPTER FIVE 54

DISCUSSION 54

RECOMMENDATIONS 60

REFERENCES 61

Appendix 1 69

MEDIA A 69

Appendix 2 70

MEDIA B 70

LIST OF FIGURES

Figure 1: Ten year maize production in East and Central African countries 9

Figure 2: Ten year maize consumption in East and Central African countries 9

Figure 3: A longitudinal section of a mature maize seed 20

Figure 4: Size of germinated seeds ready for splitting 24

Figure 5: Schematic representation of plant regeneration protocol from mature seeds/embryos 26

Figure 6: Explant preparation 29

Figure 7: Germination test. 30

Figure 8 Sterilization of mature seeds.. 33

Figure 9: Callus inductions from split seeds.. 35

Figure 10: Callus induction from mature embryos of Katumani using 2,4 D 37

Figure 11: Effect of plumule size on morphogenic calli induction 39

Figure 12: Effect of 2,4-D levels on callus induction from mature seeds 42

Figure 13: Effect of kinetin combined with 2,4-D on callus induction from mature maize zygotic embryos 44

Figure 14: Organogenic callus induction from mature maize zygotic embryos............. 47

Figure 15: Embryogenic callus induction from mature maize zygotic embryos 47

Figure 16: Embryogenic callus 48

Figure 17: Organogenic calli 49

Figure 18: Plant regeneration from organogenic calli 52

Figure 19: Embryos cultured on regeneration media... 53

Figure 20: Plants in acclimatization.. 53

LIST OF TABLES

Table 1: Number of regenerated calli and number of shoots per callus........................... 51

ACRONYMS and ABBREVIATIONS

ASARECA..................Association for Strengthening Agricultural Research in East and Central Africa

BA............................Benzyl aminopurine

BAP..........................6-benzyl aminopurine

CIM..........................Callus induction media

CMM.........................Callus maintenance media

CML 216.................. .CYMMYT line 216

CRD..........................Completely randomized design

DAP..........................Days after pollination

2,4-D....................... .2,4-dichloro Acetic Acid

EARO........................Ethiopian Agricultural Research Organization

EIM...........................Embryo induction media

EMM.........................Embryo maturation media

FAO...........................Food and Agriculture Organization

GM............................Germination media

Kin............................6-Furfurylaminopurine

MS ....Murashige and Skoog media (1962)

NaOCl....................... Sodium hypochlorite

RM............................Regeneration media

SAS...........................Statistical Analysis System

dH_2O .... distilled water

ABSTRACT

Plant regeneration from single or few cells is a prerequisite for effective selection of transformed cells and to minimize the event of chimeras during transformation. This can only be achieved if plants are regenerated through callus initiation. To date immature embryos have been widely used as explants for maize (*Zea mays* L.) plant regeneration through callus initiation and transformation work. However, the utilization of immature embryos has been hampered by their strictly limited suitable stage for culture, 12-17 days after pollination. In contrast mature seeds are ubiquitous. Therefore, use of mature embryos as an explant can significantly reduce the time required to generate immature embryos and hence the overall time required to regenerate maize plant. However, tropical maize genotypes and mature embryos have been considered as the most recalcitrant for tissue culture work. Consequently tropical maize line regeneration using mature embryos has not been reported so far. The purpose of this study was to regenerate two tropical maize lines, CML 216 and Katumani, from mature embryo. Splitting maize seeds longitudinally exposes three different tissues of the embryo simultaneously: scutellum, coleoptile-ring and shoot apical meristem. In the present study up to 92.6% germination and 0% contamination rate was attained for mature embryos harvested directly from open field or screen house by soaking sterilized seeds in 1% NaOCl solution for 2-3 hours. Seeds were germinated on MS media supplemented with 2 mg l^{-1} 2,4-D. Both the amount and frequency of callus produced by splitting mature seeds early (one day after germination) was found to be low, 43.3 % and 57.4 % for Katumani and CML 216 genotypes respectively, as compared to 66.3 % and 75.7% for Katumani and CML 216 respectively when splitting was done late (3-5 days after germination). The maximum average callus induction recorded was 90% for CML 216, 80% for Katumani and 34.3% for A188. When 2,4-D was combined with lower levels of Kinetin (cytokinin) both the amount and frequency of callus induction was reduced to 52.5% for CML 216 and Katumani and to 34.3% for A188. The media used was LS salts and B5 vitamins supplemented with 900 mg l^{-1}, 250 mg l^{-1} and 3-4 mg l^{-1} of 2,4-D. The average production of Type II and Type I callus was 75.6% and 62.3% respectively. The media used was LS salts and B5 vitamins supplemented with 900 mg l^{-1}, 250 mg l^{-1} and 2 mg l^{-1} of 2,4-D. The frequency of regenerable calli produced was 21.14% for CML 216 and 16.51% for Katumani. The number of shoots regenerated per callus induced from single split seed ranged from 1-5. The media used was LS salts and B5 vitamins supplemented with 900 mg l^{-1}, 250 mg l^{-1} and 4 mg l^{-1} of BAP and 2 mg l^{-1} of Kinetin. Plants were acclimatized in pots contained pit moss. This regeneration protocol gives an alternative explant source for maize researchers of the tropics in transgenic maize production to tackle different production constraints.

CHAPTER ONE

INTRODUCTION

1.1 Background

The genus *Zea, Z. mays* (q.v.) as the only cultivated species, belongs to the tribe Maydeae (Goodman, 1998; Purseglove, 1975). Maize is the third most important cereal in the world after wheat and rice and is also the most widely distributed crop around the world. It is mainly used for staple human food, particularly in the tropics; livestock feed, in temperate and developed countries; and raw material for many industrial products (Pollak and White, 1995). It is Africa's second most important food crop, after cassava, and grown in a wide range of environments ranging from Niger's northern Sahel to Ethiopia's highlands and converted forest lands of Sierra Leone (Miracle, 1966). The availability of different cultivars for different agro-ecological zones and ease of production coupled with its ability to give the highest yield per human power spent, make it more popular across Africa. Hence, it has replaced the most traditionally known starchy foodstuffs such as sorghum and millets in eastern and southern Africa (Purseglove, 1975).

The productivity of this important crop has been hampered in the tropics by biotic and abiotic factors. Recurrent drought and high incidence of many disease causing organisms and pests are major characteristics of maize growing regions in tropics. These areas are also characterized by steady population growth and very high degradation of environmental resources. All this has led to the deterioration of maize productivity. Therefore, recent technological advancements in the area of agriculture should be embraced to address these problems as a matter of urgency if food self sufficiency has to

be realized in tropical and sub-tropical areas of the globe (DeVries and Toenniessen, 2001; FAO, 2006; Machuka, 2001; Pingali and Pandy, 2001).

Genetic engineering or transformation has proven its advantage over conventional breeding in combating crop production constraints. It enables introduction of genes that can be inherited in Mendelian fashion, same as conventional breeding. However, it is more accurate and faster than conventional breeding. It also widens the narrow genetic pool to enhance crop productivity (Machuka, 2003; Machuka, 2004).

1.2 Problem statement and justification

Plant regeneration has proven to be the most challenging aspect of plant transformation. An efficient regeneration system is a prerequisite for production of normal and fertile transgenic maize plants and other biotechnological goals like creating genetic variability through somaclonal variation (Al-Abed *et al.,* 2006; Huang and Wei, 2004; Oduor *et al.*, 2006). The key to success in transformation, however, depends on how quick (to avoid too many deleterious effects from somaclonal variation) and efficient the regeneration system is (Slater *et al.*, 2004).

So far most tissue culture and transformation works utilize immature embryos as an explant source for maize regeneration (Al-Abed *et al.*, 2006; Armstrong and Green, 1985; Duncan *et al.*, 1985; Oduor *et al.*, 2006; Pareddy and Petolino, 1990; Shohael *et al.*, 2003). However; immature embryos are only available for a short period of time, 14-17DAP (Al-Abed *et al.*, 2006; Huang and Wei, 2004). This imposes tedious routine tissue culture activities within the specified time frame and continuous planting for continuous supply of the immature embryos. In contrast, recent reports indicated that

plant regeneration from mature embryos is possible, which is year round available in large quantity. Moreover, use of mature seeds as an explant source reduces the cost associated with glass house and/or screen house maintenance of explant source (Al-Abed *et al.*, 2006; Huang and Wei, 2004).

In comparison to other regeneration reports from mature embryos of maize, Al-Abed *et al.* (2006), recently reported up to 92% callus induction and 76% regeneration frequency using split seeds technique. The mean number of regenerated shoots via callus was 11 per callus clump and 28 shoots per explant at first subculture. Since the ultimate objective of maize regeneration is transformation, regeneration of plants through callus initiation is imperative. Therefore, this study proposed to explore the possibility of producing a regeneration protocol from mature embryos of two tropical maize lines using split seed technique. The following were the expected outcomes of the study is to develop an alternative regeneration protocol utilizing mature embryos of Katumani and CML 216 as an explant source.

1.3 Null Hypothesis

It is impossible to regenerate maize plantlet from mature embryos

1.4 Objectives

1.4.1 Broad objective

To optimize a regeneration protocol for Katumani and CML216 maize genotypes from mature zygotic embryos using split seed technique

1.4.2 Specific objectives

i. To evaluate the effect of different levels of 2,4-D alone and in combination with kinetin to induce regenerable calli for Katumani and CML216 maize genotypes

ii. To evaluate the effect of splitting time on induction of regenerable calli

iii. To evaluate the regeneration efficiency of Katumani and CML216 from mature zygotic embryos using split seed technique

CHAPTER TWO

LITERATURE REVIEW

2.1 Center of origin of maize

Four main hypotheses dominate the controversial origin of maze, the decent from teosinte, the tripartite, the common origin and the catastrophic sexual transmutation hypothesis (Bennetzen *et al*., 2001). The decent from teosinte hypothesis is the oldest and most accepted, which proposes that maize was domesticated from teosinte (*Zea diploperennis*) by human selection (Beadle, 1986; Doebley, 1990; Doebley *et al.*, 1990; Doebley and Stec, 1991; Galinat, 1977; Goodman, 1988; Wang *et al.*, 1999). The main assumption of the tripartite hypothesis is that there existed wild maize in the past, which is now extinct. This wild maize gave origin to the annual teosinte by crossing with *Tripsacum*. Further crossing of teosinte with wild maize gave rise to the modern races of maize (Eubanks, 1995; Mangelsdorf, 1974; Mangelsdorf *et al.*, 1981).

The common origin hypothesis proposes that maize, teosinte and *Tripsacum* originated by ordinary divergent evolution from a common ancestor. Consequently, it is conceived that there existed a wild maize plant that was further transformed to a cultivated crop by the selection and care of man (Randolph, 1955; Randolph, 1959; Weatherwax, 1955). The catastrophic sexual transmutation hypothesis proposes that the maize ear evolved from the terminal male inflorescence of teosinte lateral branch by a sudden epigenetic sexual transmutation. This evolved condensation of primary branches and further genetic assimilation under human selection of an abnormality, perhaps environmentally triggered (Doebley, 1990; Doebley *et al.*, 1990; Doebley and Stec, 1991; Iltis, 1983).

2.2 Center of domestication

So far there is no agreement about the domestication of maize. Randolph (1955) proposed that maize was domesticated independently in southwestern United States, Mexico and Central America. Whereas Mangelsdorf (1974) proposed that maize had several origins including Mexico and Central America, a suggestion supported by other researchers (Kato, 1984; McClintock, 1978).

2.3 Ecological requirements

Maize has a large number of cultivars with different maturity periods adapted to a wide range of environmental conditions. However, it is essentially grown in warm countries with availability of adequate moisture. The bulk of the crop is grown in the warmer parts of the temperate region and in the humid tropics. It is suited neither to semi-arid climate nor to the ever wet tropical evergreen rain forests. It is mainly grown in regions with isotherms at tasseling of 21-30°C. Rainfall during the growing period is usually above 200mm. Seasonal rainfall of 450-600mm is preferred, with periods of clear warm weather between the rain seasons. In the tropics maize performs well with 600-900mm of rain during the growing season. Maize can grow on a wide variety of soils but it performs best on well-drained, well-aerated, deep, warm loams and silt loams containing adequate organic matter and well supplied with available nutrients with a pH from 5.0-8.0, but 6.0-7.0 is optimum (Purseglove, 1975).

2.4 Conventional maize improvement

2.4.1 Maize improvement

Maize is a cross pollinated crop and this has made the exploitation of available genetic variability through recombination comparatively easy. Based on the strategy followed to develop the improved varieties three different types of cultivars are available; inbreds, open pollinated varieties and hybrids. Inbreed lines are homozygous maize varieties developed through recurrent self pollination followed by successive selections. Composite or open Pollinated varieties result from uncontrolled cross among inbred lines that possess general combining ability. They are developed through a simple selection. There is no need for manual selfing to produce seeds of such varieties. Hybrids are a result of homologous recombination of genetic material between heterotic inbreds resulting in hybrid vigour or heterosis. Heterosis is a tendency where the progeny of a specific cross outperform both parents. Varieties developed through controlled crossing between two heterotic inbreed lines are called hybrids. Maize was the first species where heterosis was widely used to produce hybrids (Wikipedia, 2007).

2.4.2 Maize production

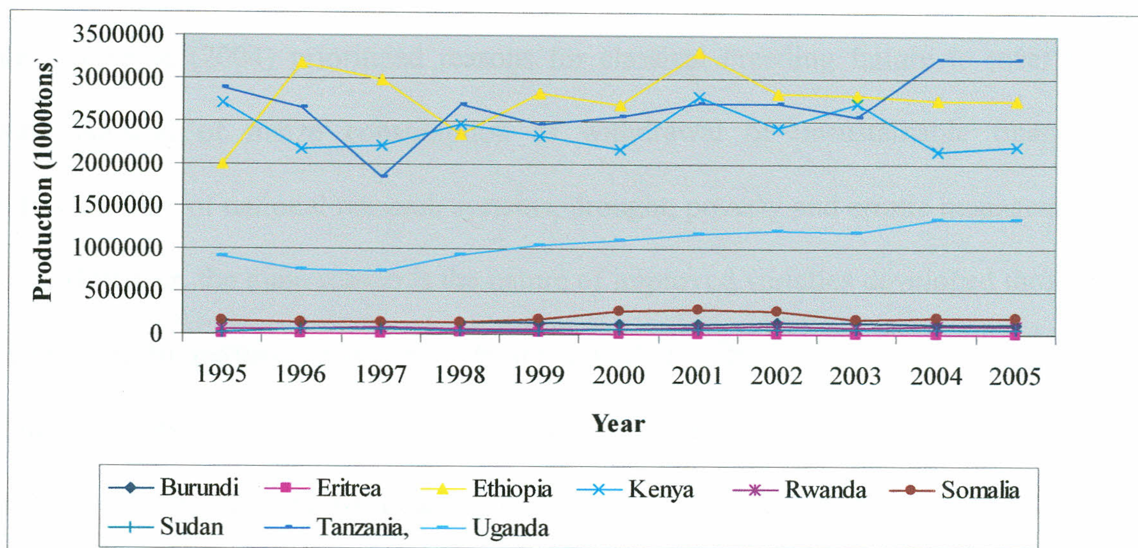
In South and Central Africa area coverage of improved cultivars through conventional breeding has reached up to 58% of total area cultivated with maize. Moreover, the productivity of the released varieties estimated to be 40% more than the local land races under cultivation (Smale and Jayne, 2004). Similar patterns have been observed in other parts of the continent (Morris, 1998). However, according to the FAO (2006) report, maize production has been declining over the past decade (Fig1). In contrast, the

consumption is increasing due to steady population growth and enhanced utilization of maize for various industrial purposes (Fig 2).

2.4.3 Conventional breeding pitfalls

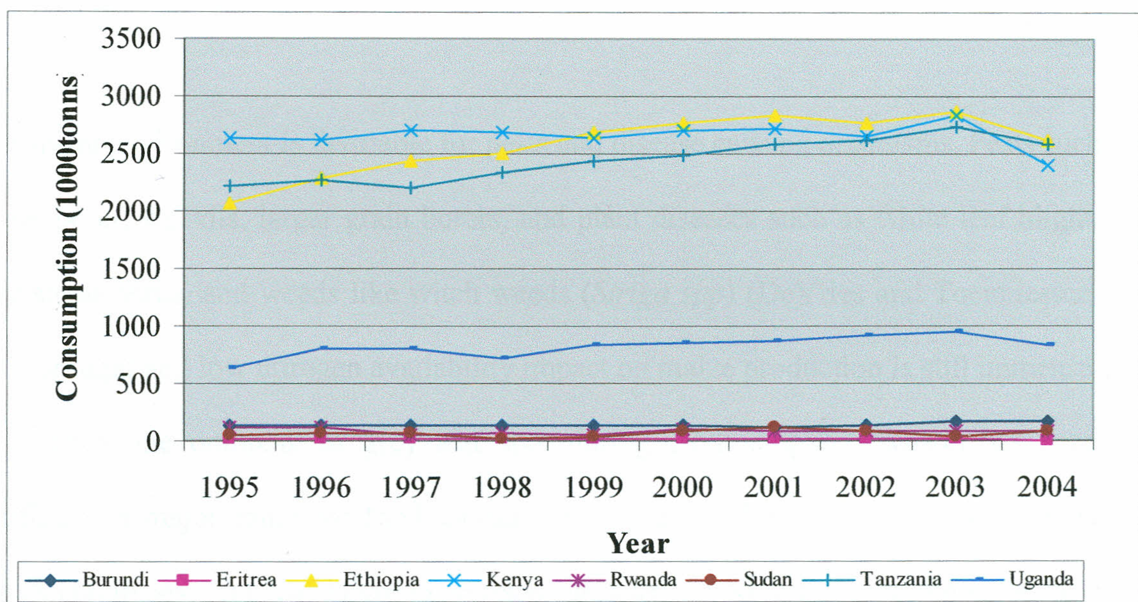
Breeders' create new gene recombination and useful variability among genotypes by intercrossing parents that posses' desirable characteristics or by introducing new germplasm from other breeding programs. This variability is then narrowed by selection of the few genotypes that perform best in the target environment. According to Falconer (1989) a breeder makes the most selection when; differences (i.e. genetic variance) among genotypes are large, selection intensity is high, i.e. only a small portion of genotypes is selected, and heritability is high. i.e. if traits can be precisely assessed in the genotypes evaluated and are transmitted to the offspring of those genotypes

Breeding, as a technique is time consuming, labor intensive, difficult to modify single traits and heavily rely on the narrow genetic pool available; which has been exploited exhaustively. Further improvement through conventional breeding is, therefore, unlikely. Moreover, high management practices are a prerequisite for better expression of heterosis in hybrids. This has been proved to be the major obstacle for a better dissemination of hybrids among the resource poor farmers of tropical regions (DeVries and Toenniessen, 2001; Morris, 1998).



Source: FAOSTAT, 16 November 2006

Figure 1: Ten year maize production in East and Central African countries



Source: FAOSTAT, 16 November 2006

Figure 2: Ten year maize consumption in East and Central African countries

2.4.4 Other production constraints

Smale and Jayne (2004) prioritized reasons for classical breeding failure to meet the growing demand of maize consumption as withdrawal of unsustainable financial subsidies, atrophy of national research systems, drought, poverty and erratic management policies. However, the main reason is the nature of improved varieties developed through this technique to perform better in a better condition. They require higher level of agricultural inputs and adequate natural resources to express their potential (DeVries and Toenniessen, 2001). In contrast, degradation of natural resources and recurrent drought has continued as the major identification of the maize growing areas. The estimated average annual loss of maize production due to moisture stress in Eastern and Southern Africa stands at 13% of the total production, or 1.8 million tones per year.

The tropical environment is favorable for pests and disease causing organisms. Pests such as stalk borer, weevils, larger grain borers, and plant diseases such as white leaf blight, maize streak virus, and weeds like witch weeds (*Striga spp*) (DeVries and Toenniessen, 2001), drought and low nitrogen availability impact on maize production is still immense. For instance, the outbreak of grey leaf spot during 1998 cropping season has been identified as a major cause for food shortage in Tanzania. Losses due to maize streak virus (Metaviruses) and leaf blight (*Turcicum turcicum)* in Kenya during the same year were also high. The spread and increased level of infestation by *Striga spp* in areas of low soil fertility has been well documented in both Kenya and Malawi (DeVries and Toenniessen, 2001). Thus significant yield loss due to various abiotic and biotic stresses is yet to be addressed.

World population is expected to double by the year 2015. This will exert more pressure on the alarmingly shrinking arable land. The consequence of global climate change further complicates the issue of feeding the future generation. Therefore, fast, effective and reliable techniques to address various maize production constraints should be embraced (Machuka, 2001; Machuka, 2003; Machuka, 2004; Pingali and Pandy, 2001).

2.5 Tissue culture

2.5.1 Introduction

All normal living cells posses the potential to regenerate into an entire organism, a natural phenomena known as totipotency. This potential has been exploited through *in vitro* culture of plant protoplasts, cells, tissues and organs (Thorpe, 1994). These living cells express their totipotency through different pathways. The two different pathways for plant regeneration through callus initiation are, organogenesis and embryogenesis.

2.5.2 Organogenesis and embryogenesis

Organogenesis is the process by which cells and tissues are forced to undergo changes that lead to the production of a unipolar structure, namely a root or shoot primordium, which is connected to the parent tissue. Somatic embryogenesis leads to the production of a bipolar structure containing a root or shoot axis, with a closed independent vascular system. Plant regeneration via somatic embryogenesis for many species can be divided into two phases: the selection or induction of cells with embryogenic competence and the development of these cells into embryos. Both of these can occur directly on the explant, called direct somatic embryogenesis or indirectly via callus, also called indirect somatic embryogenesis (Bohorova *et al.,* 1999).

2.5.3 Tissue culture and genetic engineering

Integration of genetic material that can confer important trait is only possible at cellular level. For effective transformation, it is imperative to recover and regenerate single or few cells containing the gene of interest. This can only be achieved through the application of tissue culture techniques. Among the different techniques of tissue culture, plant regeneration through callus initiation is the most preferred techniques for plant transformation work. This is because a tissue culture technique that allows the regeneration of whole plants from single cell or few cells is preferred to minimize events of chimeras. Somatic embryogenesis, where embryos arise from single or few cells, is the best to recover transformants.

2.6 Maize tissue culture

2.6.1 History

The history of maize tissue culture probably begun with the attempts to continuously culture maize endosperm by La Rue (1947) who, eventually established long-term culture from black Mexican Sweet and to a lesser degree other sweet corns became the standard for use in different types of studies (Straus and La Rue, 1954; Straus, 1958). Attempts to produce starchy endosperm cultures first succeeded in 1970 when Shannon and Batey (1973) obtained cultures of the dent inbreeds A636 and R168. These successes indicate genetic variation for tissue culture response in maize. Mascarenhas *et al.* (1975a) and Mascarenhas *et al.* (1975b) reasoned out the unsuccessful results obtained as the presence of only proliferating roots and non-morphogenic calli in cultures. It was, therefore, not possible to obtain suspension cultures by simply immersing the initial culture in liquid medium. There were differences in the growth and behavior of tissue cultures from

different cereals with maize being the least satisfactory. Shoots did not appear from cultures unless the initial explant contained the shoot apex (King and Shimamoto, 1984). The first maize regenerant was obtained from immature embryo of a specific age. However, particular genotypes (like the inbred A188) out of several tested produced morphogenic cultures (Green *et al.* 1975).

2.6.2 Explants

2.6.2.1 Immature Embryo

After the successful maize regeneration report of Green *et al.* (1975) and subsequent works by different scientists (Oduor *et al*., 2006; Shillito *et al.*, 1989) immature embryos have been considered as the ideal explant for regeneration of maize plants. Maize genotypes have shown the potential to produce embryogenic calli from immature embryos by using 2,4-D (Duncan *et al.*, 1985). For example, Emmons and Kieft (1991) observed that maize genotype 4C1 efficiently produces friable embryogenic callus from which plants regenerated via the somatic embryogenesis pathway. The use of cytokinins in combination with auxins induces somatic embryogenesis from callus cultures in cereals was demonstrated by Bhaskaran and Smith (1990). Recently, Oduor *et al.* (2006) has reported regeneration of dry-land Kenyan maize genotypes through somatic embryogenesis from immature zygotic embryos. Immature embryo derived callus is considered as the most efficient for plant regeneration and recovery of transgenic maize lines (Huang and Wei, 2004). However; low output, their strictly limited suitable stage for culture, 14-19DAP (Oduor *et al.*, 2006) and highly genotype dependent response of immature embryos (Huang and Wei, 2004) are major drawbacks.

2.6.2.2 Mature Embryos

Successful plant regeneration has been reported by different authors from mature embryos of maize and closely related cereal crops (Al-Abed *et al.*, 2006; Akula *et al.*, 1999; Carvalho *et al.*, 1997; Green and Phillips, 1974; Huang and Wei, 2004; Ozgen *et al.*, 1998; Rueb *et al.*, 1994; Wang, 1987; Ward and Jordan, 2001). The use of mature zygotic embryo from dry seeds has several advantages over immature, mature embryos are easy to handle, available year around and in large quantity. Green *et al.* (1974) first reported that mature embryos of maize could be used to induce callus but no plantlets were regenerated. Wang (1987) successfully regenerated plants from mature embryos of two maize inbreeds, B73 and Mo17, but the regeneration was genotype dependent and the frequency was only 4 to 5%. In contrast, Huang and Wei (2004) reported plant regeneration frequency from mature embryos of maize ranging from 19.85 to 32.4%. Most recently Al-Abed *et al.* (2006) reported more efficient regeneration system for two hybrid and two inbred lines using split mature seeds as an explant.

2.6.2.3 Other explant sources

Successful plant regeneration has also been reported from calli initiated from anthers (Ting *et al.*, 1981), glumes (Suprasanna *et al.*, 1986), immature inflorescence (Pareddy and Petolino, 1990), immature tassels (Rhodes *et al.*, 1986; Songstad *et al.*, 1992), leaf segments (Conger *et al.*, 1987; Ray and Ghosh, 1990), seedling segments (Santos *et al.*, 1984), shoot tips (O'Connor-Sanchez *et al.*, 2002; Zhong *et al.*, 1992) and shoot apical meristems (Zhang *et al.*, 2002). In the reported attempts of regeneration of suspension cells of maize via somatic embryogenesis, regeneration was first via callus aggregates (

Emmons and Kieft, 1991; Rhodes *et al.*, 1988; Vasil and Vasil, 1986), which was also the case from protoplasts (Prioloi and Sondahl, 1989; Shillito *et al.*, 1989).

2.6.3 Maize Callus

Two types of embryogenic calli are distinguished in maize, type I and type II (Armstrong and Green, 1985). Type I is compact and nodular with many scutellum-like bodies, grows at rapid rate, with short term plant regeneration. Type II callus is fast growing, friable and creamy in color, contains well-organized somatic embryos at the coleorhizal end of the scutellum, and with long term plant regeneration (Bohorova *et al.*, 1999; Emmons and Kieft, 1991; Emmons and De Does, 1993; Emmons and Kieft, 1995; Fransz and Schel, 1991).

The primary growth region of the cultured embryos is the scutellum, near the basal end of the embryo, where callus proliferate in 4 days and grows rapidly. The scutellar surface becomes irregular, the size of embryo doubles and is transformed into dedifferentiated tissue, that is visible to the naked eyes within one week. In two weeks, callus tissue will grow and develop, hastening differentiation of small embryonic tissue. These tissues finally develop into embryoids that can be discerned by the naked eye (Bohorova *et al.*, 1999; Emmons and Kieft, 1995).

Type II embryogenic callus is formed either directly on the scutellum of the cultured immature zygotic embryos or appears later as very small sections during the subculture of Type I callus. Once formed, the Type II callus can be maintained for a long time in darkness by routine subculture every two weeks, with care to exclude the non-embryogenic segments at the time of each subculture (Bohorova et al., 1999).

2.6.4 Plant growth regulators in maize tissue culture

In general, auxins, usually 2,4-D in the range of 1-3 mg l^{-1}, are essential for the formation of callus from cereal embryos (Bi *et al.*, 2007). Maize genotypes have shown the potential to produce embryogenic calli from immature embryos by using 2,4-D alone (Duncan *et al.*, 1985). Somatic embryos have been successfully produced by using only 2,4-D both at induction and maintenance media from immature embryo (Danson, 2006; El-Itriby *et al.*, 2003; Oduor *et al.*, 2006) and from mature embryo (Al-Abed *et al.*, 2006; Huang and Wei, 2004). However, the use of cytokinins in combination with auxins to induce somatic embryogenesis in callus culture has been reported for cereals (Bhaskaran and Smith, 1990; Gaspar *et al.*, 1996). In the turf type Bermuda grass, inclusion of a low concentration of cytokinin in the callus induction medium containing 2,4-D promoted the induction of embryogenic callus (Chaudhury and Qu, 2000). Addition of 0.1 mg l^{-1} benzyl aminopurin (BA) to the subculture medium was essential for barley embryogenic callus maintenance (Cho *et al.,* 1998). Huang and Wei (2004) has also confirmed that the addition of BA to the subculture medium containing 2,4-D significantly increased the frequency of embryogenic callus induction from mature embryos of maize. It was observed by Delporte *et al.* (2001) that somatic embryo induction increased when Kinetin (0.5 mg l^{-1}) was combined with 2,4-D (2.5 mg l^{-1}) during the first weeks of callus induction from intact or half immature zygotic embryos.

The addition of cytokinins into regeneration medium had a little effect on germination rates of embryos. A possible explanation for this is that the somatic embryos capable of germinating to give rise to a new plantlet have already formed and the initiation media may predetermine their fate. Other studies, however, have shown that cytokinins may

promote development and germination of somatic embryos. According to Bohorova *et al.* (1995) a regeneration medium that contains 0.5 mg l^{-1} indole acetic acid and 1 mg l^{-1} BA was more efficient than N6 medium (Chu *et al.,* 1975) devoid of hormones for maize plant regeneration. An effective regeneration protocol for maize callus cultures grown on medium consisted of subculturing the callus onto media containing 3.5 mg l^{-1} BA for 3-6 days and then transferring the callus onto hormone free media for 15-21 days was reported by Duncan and Widholm (1988). Addition of 0.1 mg l^{-1} BA to maintenance medium resulted in an increase in barley regeneration frequency (Dahleen and Bregitzer, 2002). Chang *et al.* (2003) found that subcultured embryogenic callus of barley required more BA or Kin in the regeneration medium to induce shoot regeneration, and BA was more effective than Kin at the same levels. It is also reported that the addition of 0.5 mg l^{-1} BA into regeneration medium was more efficient than regeneration medium free of plant growth regulator for plant regeneration from mature embryos (Huang and Wei, 2004).

2.6.5 Role of silver nitrate in maize tissue culture

Silver nitrate ($AgNO_3$) has been shown to promote regeneration and somatic embryogenesis in tobacco (Purnhauser *et al.*, 1987), and several monocotyledons such as wheat (Purnhauser *et al.*, 1987) and maize (Vain *et al.*, 1989). Silver nitrate did not modify the frequency of embryos producing Type I callus. However, various $AgNO_3$ concentrations increased the production of Type II callus 4-6 folds. For all experiments 5-10 mg l^{-1} appeared to be the optimal level of $AgNO_3$ concentration for the initiation of friable embryogenic callus (Vain *et al.*, 1989). $AgNO_3$ has been shown to affect ethylene action by competing for its binding site (Beyer, 1976), thus promoting an increase in

Type II callus and plant regeneration (Siriwardana and Nabors, 1983; Songstad *et al.*, 1991).

2.6.6 Application of maize tissue culture

The main application of maize tissue culture is in production of transgenic plants. For this purpose different explant sources have been utilized. Until recently it was believed that immature embryos were the ideal explant for regeneration of transformed maize plants. Different authors have reported efficient recovery of transgenic maize plants from immature embryos using different techniques (Frame *et al.*, 2002; Frame *et al.*, 2006; Shou *et al.*, 2004).

Transgenic maize plants of tropical and sub-tropical genotypes were obtained via micro-projectile bombardment of calli containing organogenic and embryogenic-like structures derived from shoot tips of germinated mature embryos (O'Connor-Sanchez *et al.* 2002). Zhang *et al.* (2002) has also transformed recalcitrant maize elite inbreeds via micro-projectile bombardment of *in vitro* shoot meristematic cultures induced from germinated seedlings. *Agrobacterium* mediated transformation of shoot meristems yielded transgenic maize plants through both callus initiation and direct organogenesis. In other cereals, Delporte *et al.* (2001) and Özgen *et al.* (1998) have reported regeneration of complete wheat plants through callus induction from mature embryos of wheat. Mature seed embryo-derived calli have also been used for transformation of oats using micro-projectile transformation (Torbert *et al.*, 1998).

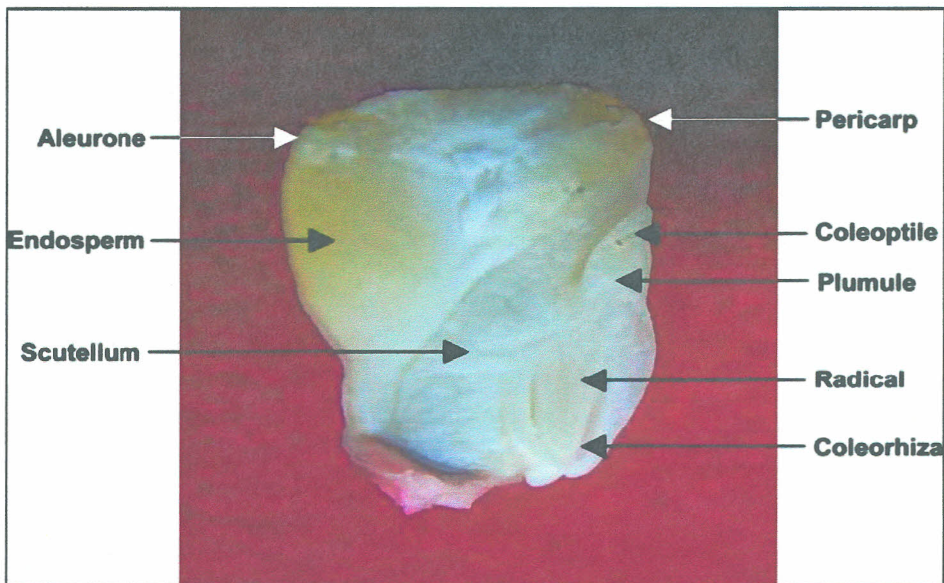
2.7 Maize embryo physiology in relation to spitting

Fertilization of maize seeds results in diploid embryo and triploid endosperm. After undergoing repeated mitotic division within the embryo sac, the endosperm develops fully and eventually cell wall formation occurs around the cells producing the multicellular food storage tissue (triploid endosperm). At the same time the diploid embryo also undergoes repeated mitotic division and organized into scutellum, coleoptile, plumule, radical and coleorhiza (Weston, 1995).

The embryo of maize lies embedded in the endosperm at one side, toward the base of the caryopsis, with the primary root directed towards the attached end. The scutellum is large, lies directly against the endosperm, and partially encloses the embryonic axis. The primary root is enclosed by coleorhiza. Upon germination the plumule develop into shoot while the radical grows into root. The scutellum, the focal point for embryogenic calli induction, will remain attached to the endosperm (Weston, 1995).

Maize, being a monocot plant, has a single cotyledon in its seed. In monocots the endosperm is stored around the embryo rather than in the single seed leaf. Therefore maize seed does not separate in two halves when the seed coat is removed. Dicots, on the other hand, do separate into two pieces. The two halves are the seed leaves or food storage areas. The main idea of using split seeds is to keep the seed contents intact at the time of culture, since natural nutrients and growth hormones are stored within the endosperm. Furthermore, the maize seed is handled as a dicotyledonous seed and split symmetrically and longitudinally into two halves to expose the scutellum, the coleoptilar ring and shoot apical meristem simultaneously (Fig 3). In comparison with all

regeneration protocols reported in maize using mature embryos, the number of shoots and callus regeneration frequency from splits is significantly higher than that of meristem culture (Sairam *et al.*, 2003). The number of multiple shoots directly regenerated from split seeds via organogenesis was 28 shoots per explant in 2-3 weeks. Moreover, the time needed to produce fertile plants was significantly reduced to 4 months from the time of initial explanting with seed being harvested 42 days later (Al-Abed *et al.*, 2006).



Source: Al-Abed *et al.*, (2006)

Figure 3: A longitudinal section of a mature maize seed

CHAPTER THREE

MATERIAL AND METHODS

3.1 Plant materials

One inbred line, CMMYT maize line (CML) 216 and a Kenyan open pollinated or composite variety, Katumani, adaptable to tropical climate were used in this study. A188 was used as a control. At least 50 pots of each variety were grown at Kenyatta University, plant transformation laboratory screen house. Two seeds were planted per pot. All agronomic practices necessary for vigorous and healthy maize plant production, fertilization, thinning, mulching, top dressing using Ridomil MZ (Syngenta, Switzerland), and watering, were applied according to maize requirements.

To avoid cross pollination from plants grown in the field, sprouting ears were covered with 12" X 4" brown paper bag just before flowering. Before the tassels started releasing pollen, they were covered with brown paper bag to collect pollens grains, which were then dusted on the silks of the same plant. Selfing was carried out 7-10 days after the silks appeared. After dusting the ears were covered with brown paper bag to prevent further cross pollination. After 12-16 weeks, fully matured cobs were removed and seeds were dried under the sun for two weeks. These mature seeds were then used as an explant source for regeneration studies.

3.2 Determination of germination potential of seeds

To determine germination potential of the mature seeds, fifty seeds of CML 216 and Katumani were placed in a petridish containing moist filter paper. The filter paper was kept moist by spraying water on it every day. The number of germinated seeds were

counted and scored daily for a maximum of 10 days. The germination media consisted media B (appendix 2) supplemented with 2 mg l^{-1} of 2,4-D.

3.3 Mature seed sterilization

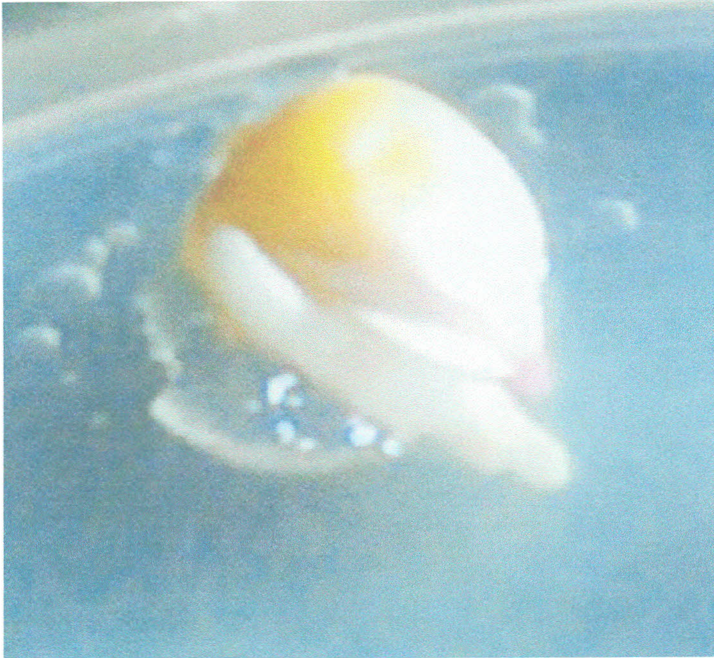
Vigorous and healthy seeds were carefully selected for sterilization and regeneration experiments. Glumes surrounding the pedicle of a dry seed were removed with a maximum precaution not to damage or expose embryos to the bleaching agent. Seeds were then washed with local liquid detergent and rinsed three to four times under running water. The washed seeds were immersed in 70% ethanol solution for three minutes and rinsed with distilled water three to four times. Seeds were then soaked in 85% commercial bleach Jik (Reclkit and Colman, Kenya) or 3% sodium hypochlorite solution twice for 15 minutes, each time using fresh solution followed by rinsing seeds three to four times in sterile distilled water (dH_2O) to completely remove remnants of Jik. The following modifications were introduced to the sterilization protocol outlined by Sairam *et al* (2003) and Al-Abed *et al* (2006) to sterilize seeds harvested from field grown plants. Then seeds were subjected to one of the following soaking schemes A) soaking in sterile dH_2O overnight B) soaking in 28% commercial bleach overnight C) soaking in 28% commercial bleach for 6 hours followed by soaking in sterile dH_2O for 18 hours D) soaking in 28% commercial bleach for 2-3 hours followed by soaking in sterile dH_2O for 21-22 hours and E) soaking in sterile dH_2O overnight followed sterilization for 20 minutes in 28% commercial bleach. To evaluate the effect of glume removal on sterilization, seeds with pedicle surrounded by glumes were also sterilized and soaked in sterile dH_2O overnight. Media, water and culture bottles used in the experiment were steam sterilized at 121 °C at a pressure of 15 pounds per square inch (psi) for 21 minutes. p^H of the media was adjusted to 5.8 using HCl/NaOH prior to sterilization. The

sterilization process was carried out under sterile condition using pre sterilized soaking bottles.

Media, water and culture bottles used in the experiment were steam sterilized at 121 °C and at a pressure of 15 pounds per square inch (psi) for 21 minutes. Instruments like forceps, blade and blade handle were flame sterilized.

3.4 Soaking and germination

Soaking was done in sterile dH_2O for 18-20 hours in 1% NaOCl and then liquid media A (appendix 1) supplemented with 3 mg l^{-1} of 2,4-D. For each variety, ten seeds were cultured per plate on solid germination media with embryo axis facing up. The germination media consisted of media A supplemented with 2 mg l^{-1} 2,4-D. The appropriate size of germinated seed for splitting was determined based previous works (Sairam *et al.*, 2003; Al-Abed *et al.*, 2006). Seeds that attained right size of plumule (Fig 4) were transferred to sterile Petri-dishes for splitting.



Source: Sairam *et al.*, 2003

Figure 4: Size of germinated seeds ready for splitting

3.5 Callus induction on split seeds

Roots, excess shoot and seed coat were removed from the germinated seed. Seeds were then split longitudinally to expose scutellum, plumule and coleoptilar ring simultaneously and transferred to callus induction media with the split side facing the media. Splitting was done one day after germination and three days after germination. The callus induction media A (appendix 1) was supplemented with 900 mg l^{-1} proline, 250 mg l^{-1} casein hydrolysate. A range of alone 2,4-D levels (0,1,2,3,4,5&6 mg l^{-1}), 3 mg l^{-1} of 2,4-D combined with kinetin in the range of 0,0.5,1.0,1.5 &2 mg l^{-1} and 10 mg l^{-1} of filter sterilized $AgNO_3$ were added to the media. Ten split seeds per plate and five plates per genotype were cultured and incubated in the dark at 26 ± 2°C. Two days later any growing radicle and excessive plumule were removed to encourage callus initiation and returned back to growth room for 2-3 weeks of incubation.

3.6 Callus maintenance

Highly proliferating callus was transferred to maintenance Media A (appendix 1) supplemented with 900 mg l^{-1} proline, 250 mg l^{-1} casein hydrolysate and 10 mg l^{-1} of filter sterilized $AgNO_3$. The callus was subcultured every two weeks during the 4-6 weeks incubation period in the dark at 26 ± 2°C. Necrotic tissues were discarded during every subculture. Calli induced using induction media supplemented with 2,4-D alone was maintained on maintenance media supplemented with 2 mg l^{-1} of 2,4-D. Calli induced on induction media supplemented with a combination 2,4-D with kinetin was maintained on maintenance media supplemented with 2.0 mg l^{-1} of 2,4-D and 0.5 mg l^{-1} kinetin.

3.7 Embryo Maturation

Upon differentiation of the induced callus on maintenance media both Type I and Type II calli were produced. Organogenic calli was directly transferred to shooting media. However, embryogenic calli was transferred to Media A supplemented with additional 30 g l^{-1} of sucrose and incubated at 26 ± 2°C in the dark for maturation of embryos.

3.8 Regeneration

Two pathways were followed to regenerate plants from the two different calli induced from split seeds as illustrated in Fig 5.

3.8.1 Plant regeneration from somatic embryos

Embryos were germinated on Media A devoid of any kind of growth regulators. The embryogenic calli was incubated at 26 ± 2°C under 16/8 photoperiod.

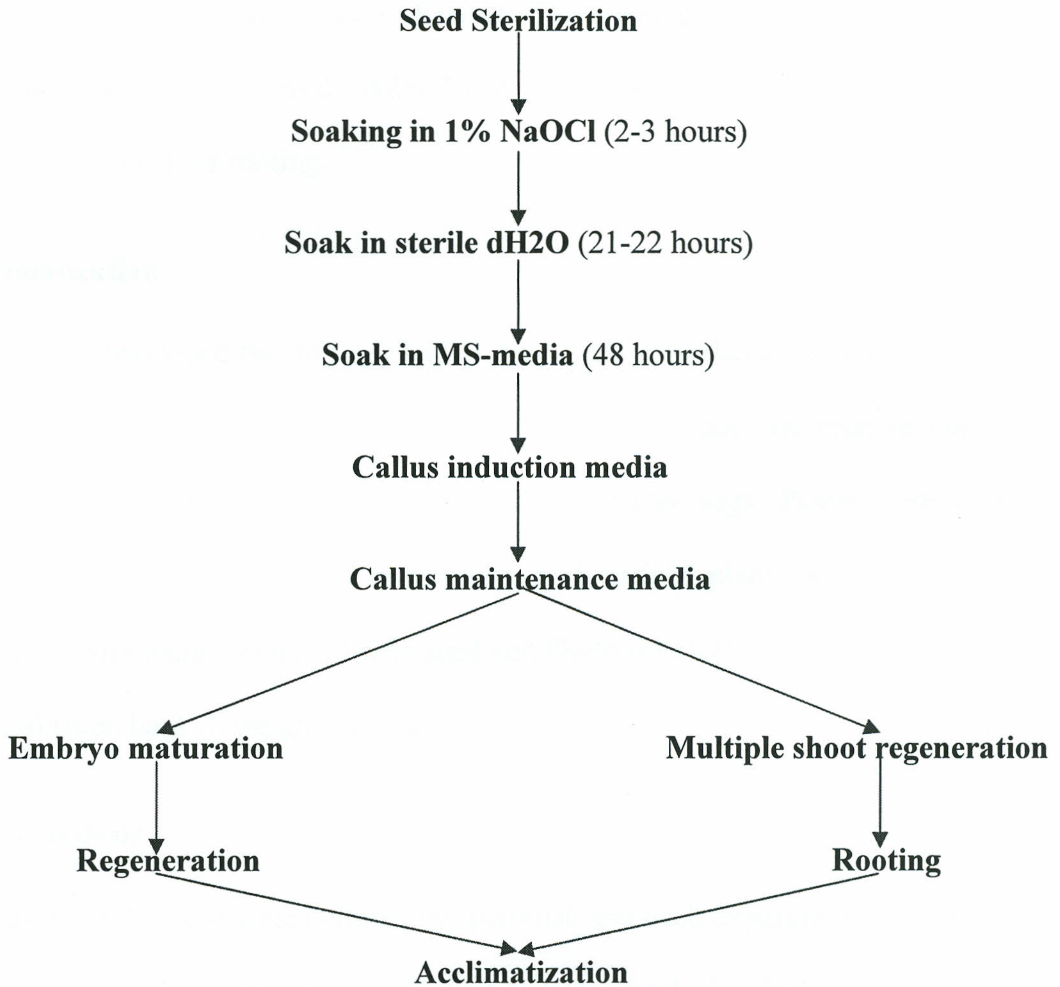


Figure 5: Schematic representation of plant regeneration protocol from mature seeds/embryos

3.8.2 Shooting and rooting of organogenic callus

The organogenic calli was transferred to shooting media A supplemented with 4 mg l^{-1} 6-benzyl amonipurin (BAP) and 2 mg l^{-1} Kinetin. The organogenic calli were incubated at 26 ± 2°C under 16/8 photoperiod. After 3-4 weeks, shoots were transferred to media B (devoid of hormones) for rooting.

3.9 Acclimatization

When plantlets developed two to three leaves, they were transplanted to plastic pot, filled with moist pit moss (Kekkilä Oyj, Tussula, Finland). To raise the relative humidity, plants were sprayed and covered with polyethylene paper bags. Plants were watered regularly in the glass house. After two weeks, acclimatized plants were transferred to buckets filled with loam soil mixed with sand and Phytomix (Kenya Seed Co. Ltd, Kitale, Kenya) and taken back to the glass house.

3.11 Data analysis

Ten explants (split seeds) were cultured per petridish and each experiment was replicated five times in Completely Randomize Design (CRD). Analysis of Variance (ANOVA) was carried out for different parameters collected (% callus induction and % Type I and II callus induction) using GenStat software (http://discovery.genstat.co.uk.) at 95% confidence interval. Error bars for graphical presentations were calculated using Microsoft excel. The standard error calculated was used to calculate error bars (both upper and lower deviations).

CHAPTER FOUR

RESULTS

4.1 Explant source preparation

All the necessary agronomic practices were applied in the screen house to produce healthy and vigorous planting materials. This resulted in true-to-type plants from each variety after 12 -16 weeks (Fig 6a). After the selfed cobs reached maturation they were removed and dried under sunlight (Fig 6b). Healthy dry mature seeds were obtained after two weeks.

4.2 Germination test

The germination test of the two varieties under investigation was done to determine the time required for seed germination and to assess the effect of sterilization on germination capability of the varieties during establishment of the *in vitro* culture. The first Katumani germinated seed appeared on the 3rd day and 100% germination was attained after 9 days culture on moist filter paper. However it reached the 50% germination mark after 5 days on culture media. The germination potential of CML 216 was inferior tando the control A188 and Katumani. It took four days for the first germinated seed to appear and ten days to attain the maximum germination percentage, which was 92%. CML 216 attained 50% germination after 6 day's culture on moist filter paper. A188 was the first to germinate; after only two days on moist filter paper; and 100% germination percentage was achieved on the 7th day. The 50% germination was attained after 4 days (Fig 7).

Figure 6: Explant preparation a) Plants maintained in the screen house b) Mature seeds dried under the sun

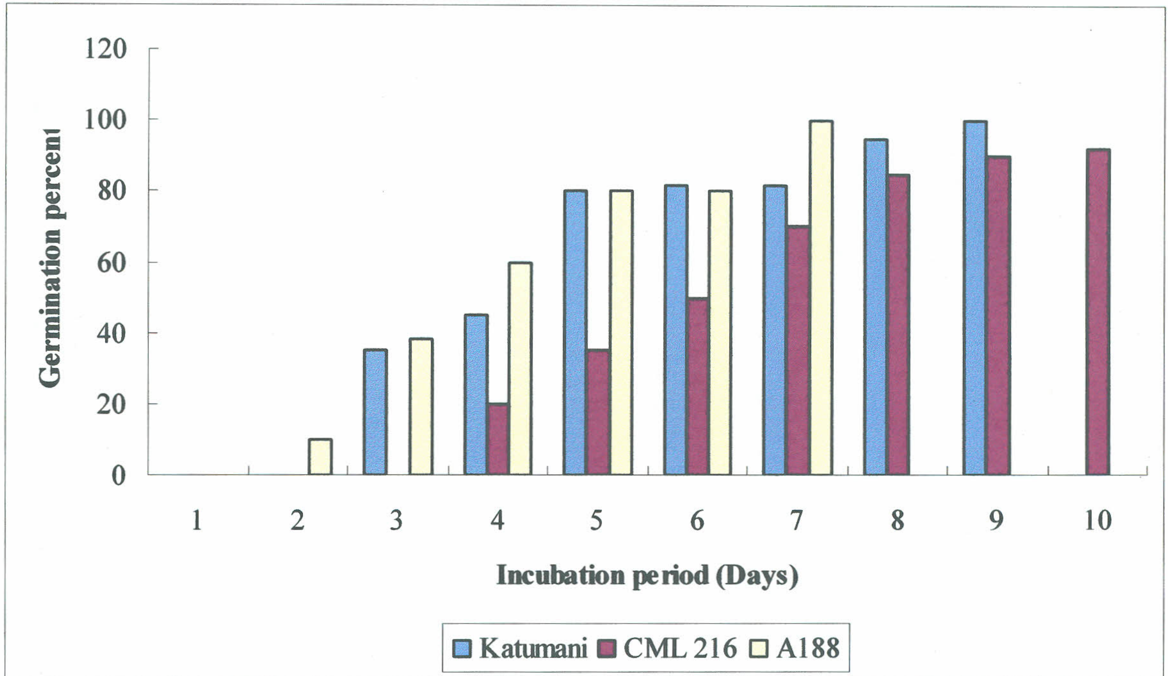


Figure 7: Germination test: Percent germination of Katumani, CML 216 and A188.

4.3 Sterilization protocol optimization

Six different modified sterilization techniques, sterilization followed by five different soaking schemes and the sterilization protocol outlined by Sairam *et al.* (2006), were studied for the purpose of developing a procedure that reduces contamination and increases percent germination of Katumani and CML 216. The different sterilization techniques displayed differential results in reducing contamination and enhancing germination. The sterilization technique outlined by Sairam *et al.* (2003) and Al-Abel *et al.* (2006) gave the highest germination percentage of 95.4% and highest percent contamination, 81.58%. The modification involved the removal of the glumes surrounding the pedicle prior to washing followed by soaking seeds in sterile dH_2O overnight gave the second highest germination, 95.12% and second highest percent contamination of 22.56%. Removal of the glume surrounding the pedicle prior to washing followed by soaking seeds in sterile dH_2O overnight and then re-sterilizing them in 2% sodium hypochlorite for 20 minutes reduced the level of percent contamination to 18.75 at the expense of germination percentage (46.9%). This was the lowest germination rate observed. When removing the skin surrounding the pedicle prior to washing was followed by soaking seeds in 1%NaOCl overnight, contamination was completely eradicated. However, the 57.3% germination obtained was the second lowest level recorded. But, this experience was used to design the next modifications. It was predicted that reducing the duration of soaking seeds in 1%NaOCl could maximize the percent germination while maintaining the contamination rate as low as 0%. Therefore, duration of soaking was reduced in series till the maximum percent germination and lowest percent contamination was attained. The first of this series was soaking the seeds in 1%NaHClO for 6 hours followed by soaking seeds in sterile dH_2O for the remaining 24

hours. This further improved the percent germination to 89.9% and kept the contamination level at 0%. Further reduction of soaking time in 1%NaOCl to 2-3 hours enhanced the percent germination to 92.6% and kept the contamination level to 0%. The above results were obtained using maize variety A188 bulked in the screen house (Fig 8).

When CML 216 and Katumani seeds were sterilized using the modified technique, it was observed that Katumani seeds still germinate earlier than CML 216. The germination percentage was reduced to 96% and 87%, as compared to 100% and 90% of seeds germinated without sterilization for KAT and CML 216 respectively. Germination was also delayed by one or two days in both varieties. Three to four and five to seven days were required for Katumani and CML 216, respectively, to reach the right size recommended for splitting (Fig 4).

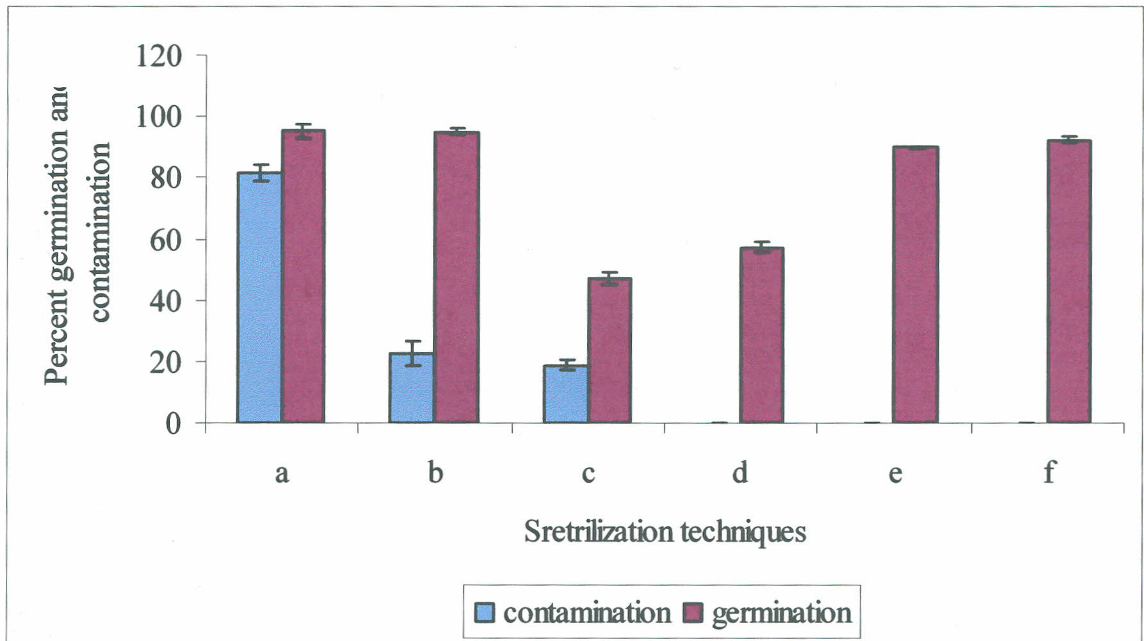


Figure 8 Sterilization of mature seeds: a) Soaking sterilized seeds in sterile dH2O overnight without removing the glumes b) Soaking sterilized seeds in sterile dH2O overnight after removing the glumes c) Sterilization of seeds in 2%NaHClO for 20 minutes after initial sterilization and overnight soaking in sterile dH2O d) Soaking sterilized seeds in 1%NaHClO overnight e) Soaking sterilized seeds in 1%NaHClO for 6 hours f) Soaking sterilized seeds in 1%NaHClO for 2-3 hours

4.4 Callus induction

A range of 2,4-D concentrations (0, 1, 2, 3, 4, 5 & 6 mg l^{-1}) and 3 mg l^{-1} 2,4-D (Al-Abed *et al.*, 2006) combined with lower levels of Kinetin (0, 0.5, 1.0, 1.5 & 2.0 mg l^{-1}) (Bhaskaran and Smith, 1990; Gasper *et al.,* 1996) were tested for callus induction from mature embryo's of the two tropical maize genotypes using split seed technique and the control A188. In addition, effect of plumule size (number of days to germinate seeds before splitting) on callus induction were explored to assess their role through exposing different tissues of the embryo for morphogenic callus induction. The surface on the split seed started to swell after two days and soft and white callus started to appear on the surface after 4-6 days depending on the variety (Fig 9). Callus appeared on Katumani seeds earlier than CML216 but Katumani was inferior with regard to callus proliferation. Two distinct types of regenerable calli were produced simultaneously on callus generated from the same seed for both varieties in all the callus induction media used.

A188 repeatedly failed to produce calli or tended to produce watery non-regenerable calli. Inconsistent callus induction response was observed in Katumani seeds collected from different cobs. However, Katumani responded for the selected 2,4-D levels and 2,4-D combined with lower levels of kinetin better than A188. CML 216 was the best in responding to split seed technique producing white friable calli in a relatively higher amount and frequency. The endosperm separates itself from the embryo where it was attached to the scutellum and embryogenic calli was produced on the surface of the scutellum. Organogenic calli was produced at the base of the plumule where it was attached to the scutellum. Callus was then maintained on media A supplemented with 2 mg l^{-1} 2,4-D (Fig 9 d).

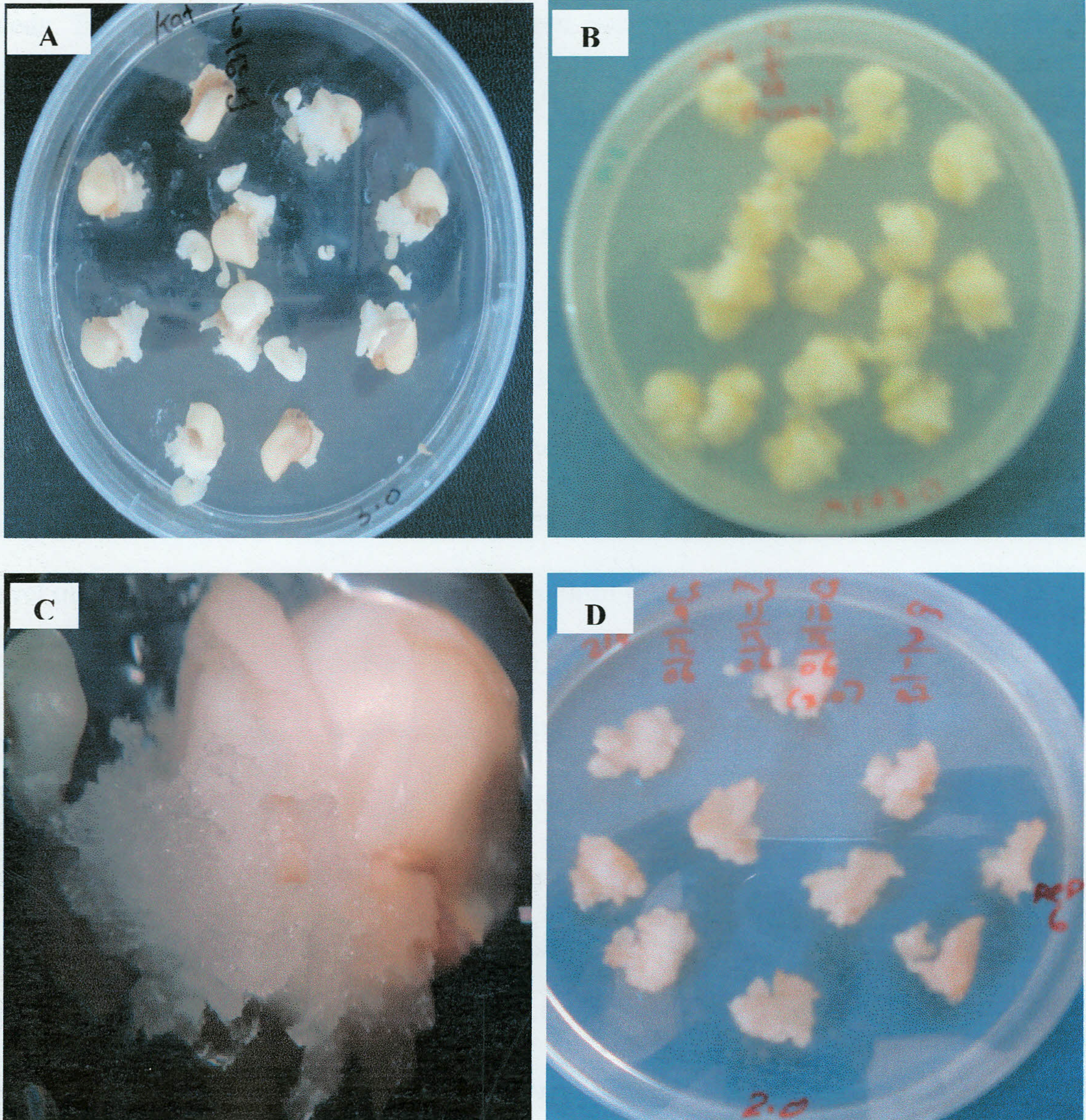


Figure 9: Callus inductions from split seeds: a)Katumani splits after 1 week b)CML 216 splits after 1 week c) Callus induced on split seeds on Katumani d)Callus maintained on maintenance media

4.4.1 Effect of 2,4-D on callus induction from maize mature embryos

Ranges of 2,4-D levels (0-6 mg l^{-1}) were tested for callus induction from Katukani mature seeds. The maximum callus induction resulted was 100% when callus induction media was supplemented with 3 mg l^{-1} and 6 mg l^{-1} of 2,4-D. Levels between them, 4 mg l^{-1} and 5 mg l^{-1} of 2,4-D, produced 95% and 95.5 % callus induction respectively. The lowest callus induction, 10.5%, was obtained when split seeds were cultured on callus induction medium devoid of growth regulators. This lowest level of callus induction was associated with the highest level of browning recorded, 94.5%. However; despite a relatively consistent result in callus induction and browning, production of morphogenic calli was highly inconsistent along with an increase in 2,4-D concentration. It reached the highest level of 55% at 3 mg l^{-1} of 2,4-D and declined until it reached 10% with 4 mg l^{-1} of 2,4-D included in the media. Then it picked up to 80% with 6 mg l^{-1} of 2,4-D (Fig 10). This result indicated the need of optimizing callus induction techniques to simultaneously expose the three different tissue of mature embryo required for morphogenic calli induction. Two alternatives were hypothesized: early splitting and inclusion or exclusion of different parts of the embryo whose presence negatively affect the contact between these tissues and the media. Increasing 2,4-D levels beyond 3 mg l^{-1} did not affect the frequency of callus induction. Based on this results, the range of 2,4-D levels used to test the effect of 2,4-D on callus induction was narrowed to four (1.5, 3.0, 4.5 and 6.0 mg l^{-1}).

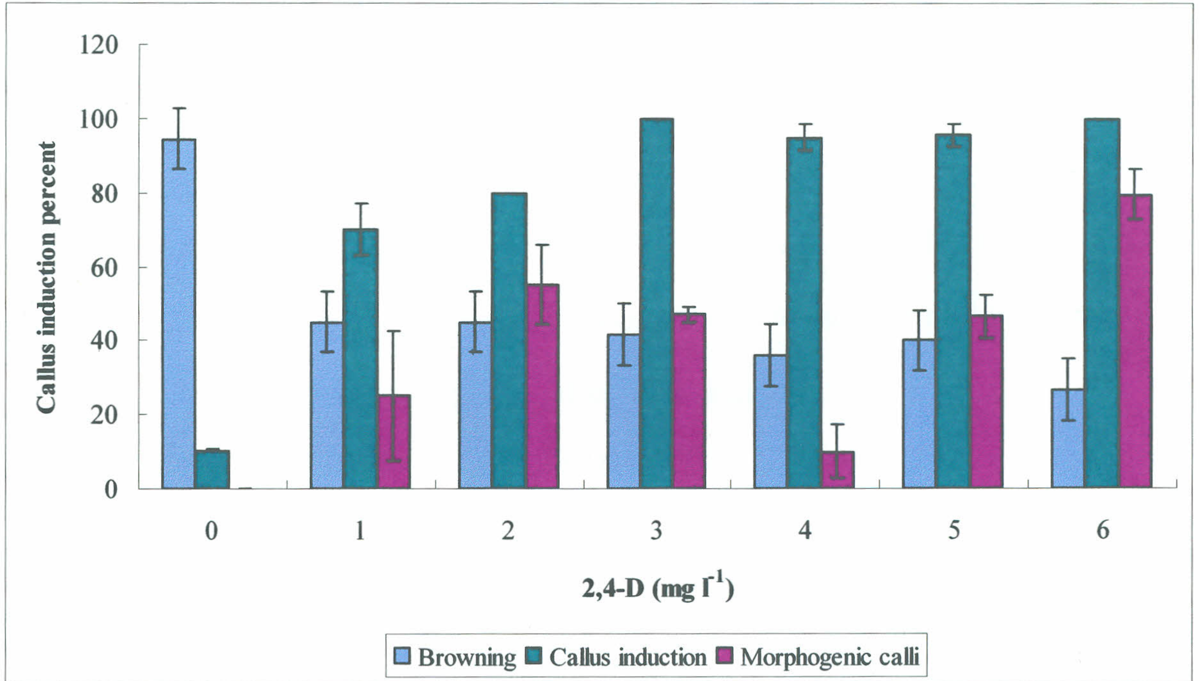


Figure 10: Callus induction from mature embryos of Katumani using 2,4 D (0-6 mg l^{-1})
Error bars = Mean ± SE

4.4.2 Effect of plumule size on callus induction from mature maize zygotic embryos

Assessing the effect of plumule size (determined by number of days after germination to split the germinate seed) on callus induction was based on the fact that embryogenic calli could be initiated from the scutellum (Al-Abed *et al.*, 2006) but the fact that the scutellum remains attached to the endosperm during germination (Weston, 1995). Therefore splitting late (three to five days after seeds germinated) was anticipated to allow the enlargement of the plumule, which would consequently push the scutellum together with the endosperm away from the media. Splitting germinated seeds late produced 66.3 % and 75.7% callus induction for Katumani and CML 216 respectively. Splitting seeds early (one day after seeds germinated) reduced both the amount of callus produced (observation) and the frequency of callus induction to 43.3 % and 57.4% for Katumani and CML 216 respectively (Fig 11). There was a significant difference between splitting early and late ($p \leq 0.003$) and among the varieties investigated ($p \leq 0.046$). 2,4-D level recommended by Al-Abed *et al* (2006), which was 3 mg l^{-1}, was used in this experiment. After 2 days any growing radicle was removed and excessive plumule was reduced. However, the significant difference among the varieties and low callus induction frequency suggested further improvement of the splitting technique.

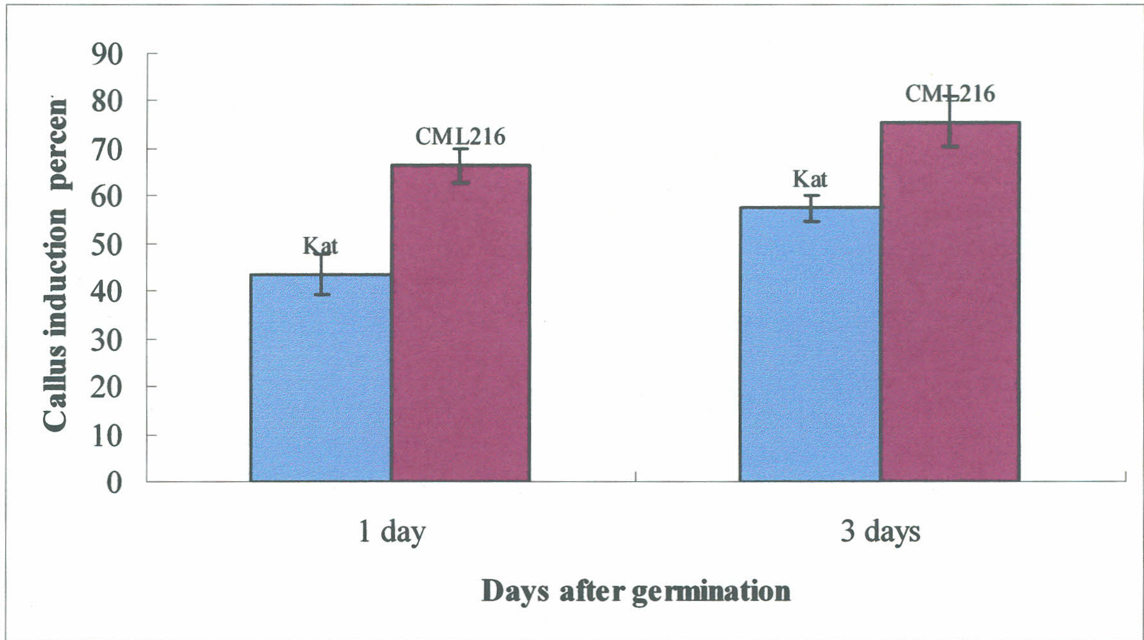


Figure 11: Effect of plumule size on morphogenic calli induction
Error bars = Mean ± SE

4.4.3 Effect of selected 2,4-D levels on callus induction from mature maize embryos

The other alternative to expose scutellum, plumule and coleoptilar ring simultaneously was exclusion or inclusion of plumule to inhibit its further growth. The plumule was removed or left loosely attached to the scutellum 2-3 days after splitting. Though removing both the plumule and radicle completely upon splitting gave consistent results. The amount of calli produced when the plumule part was left loosely attached to the split seed was found superior to that of removing plumule completely. Moreover, only embryogenic calli was produced in the absence of the plumule. In contrast, leaving the plumule loosely attached to the scutellum reduced the interference of plumule growth on callus induction by enhancing contact between the scutellum and the media. This in return improved the consistency and amount of callus produced both on the surface of scutellum and at the base of the plumule.

The surface of the split seed started swelling after 2-3 days on callus induction media and visible calli appeared on the surface within 4 days for CML 216 and katumani and 6 days for A188. White and soft callus was initiated on the surface of the split seeds both from scutellum and at the base of the plumule where it was attached to the scutellum. The calli that were induced on the surface of the scutellum were dominated by Type II (embryogenic) calli whereas the calli induced at the base of the plumule was mainly Type I (organogenic) calli. Watery callus was initiated on the surface of the split seed where the radicle was attached to the scutellum and eventually formed roots.

A maximum of 90% average callus induction was recorded when Media A (Appendix 1) was supplemented with 3 mg l^{-1} of 2,4-D for CML 216 . Katumani and A188 yielded a

maximum of 80% and 34.3% callus induction respectively when the same media was supplemented with 4.5 mg l^{-1} of 2,4-D. The lowest average callus induction frequency was 60% for both CML 216 and Katumani and 23.7% for A188 when callus induction media was supplemented with 1.5 mg l^{-1} of 2,4-D (Fig 12). The difference among the different levels of 2,4-D used to induce callus was significant ($p \leq 0.021$). However, no significant difference ($p \leq 0.926$) among Katumani, CML 216 and A188 was recorded for callus induction. Results showed that CML 216 was the best responder for regenerable calli initiation in terms of both amount and frequency. On the other hand, Katumani seeds sampled from seeds supplied by Kenya Seed Co Ltd produced inconsistent result for amount, frequency and type of callus across replications. Split seeds obtained from different cobs also showed inconsistent results. Therefore selected replicates that responded better or replicates from single cob were used for data collection. Callus production from A188 was generally low. Surfaces of the splits often swell and turned to brown on callus induction media.

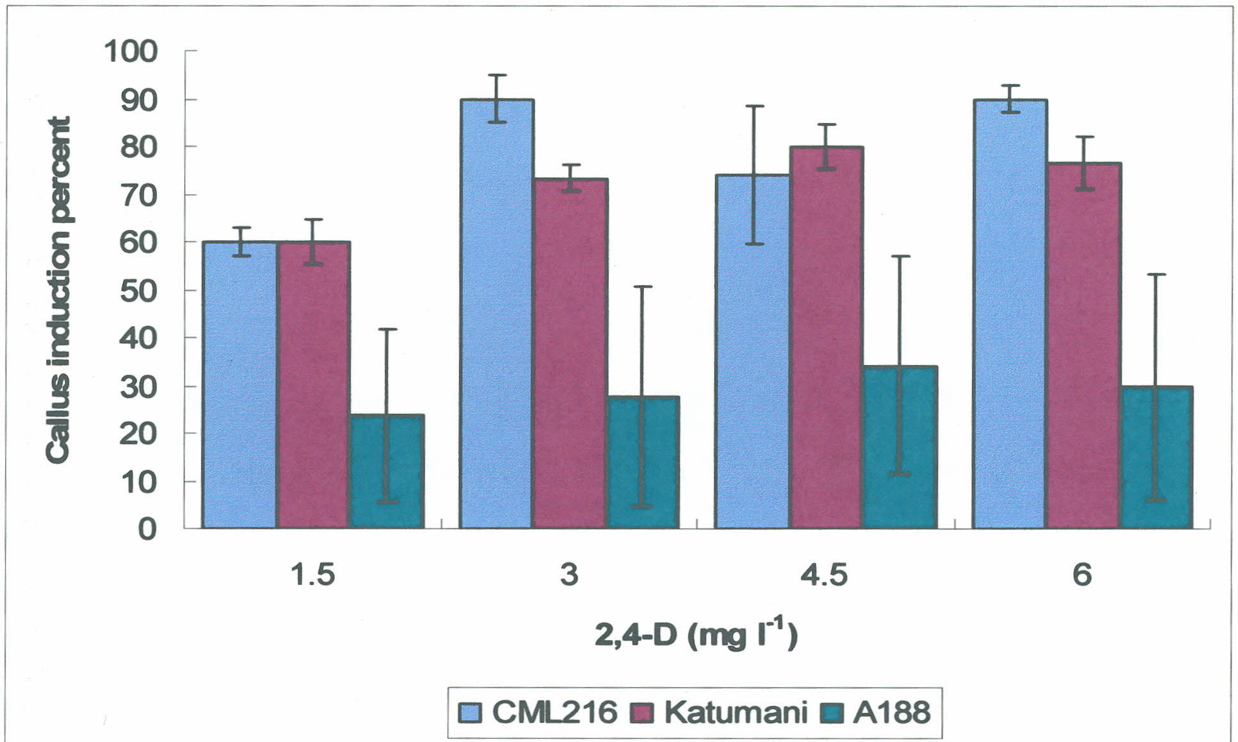


Figure 12: Effect of 2,4-D levels on callus induction from mature seeds
Error bars = Mean ± SE

4.4.4 Effect of kinetin on callus induction from mature maize zygotic embryos

The selected 2,4-D level for callus induction was combined with Kin to assess the effect of Kin on callus induction. A 52.5% mean callus induction was obtained when 3 mg l^{-1} of 2,4-D combined with 0.5 mg l^{-1} kinetin for CML 216 and 1.0 mg l^{-1} for Katumani. The highest callus induction attained for A188 was 34.3% when 3 mg l^{-1} of 2,4-D was combined with 1.5 mg l^{-1} of kinetin. Generally, it was observed that as the amount of kinetin increased, both the frequency and amount of callus production per split seed declined and necrosis increased. Among the three lines, A188 was severely affected by browning and/or necrosis. The lowest callus induction frequency attained ranged from 13.3-30% when 2.0 mg l^{-1} kinetin was used (Fig 13). The ANOVA test showed a highly significant difference among the different levels of kinetin combined with 3 mg l^{-1} of 2,4-D ($p \leq 0.001$). However, the difference among the three varieties for callus induction was found to be insignificant ($p \leq 0.565$). The control used, 3 mg l^{-1} of 2,4-D alone, responded best for frequency and amount of callus induced. Visible calli appeared after 6 days on callus induction media.

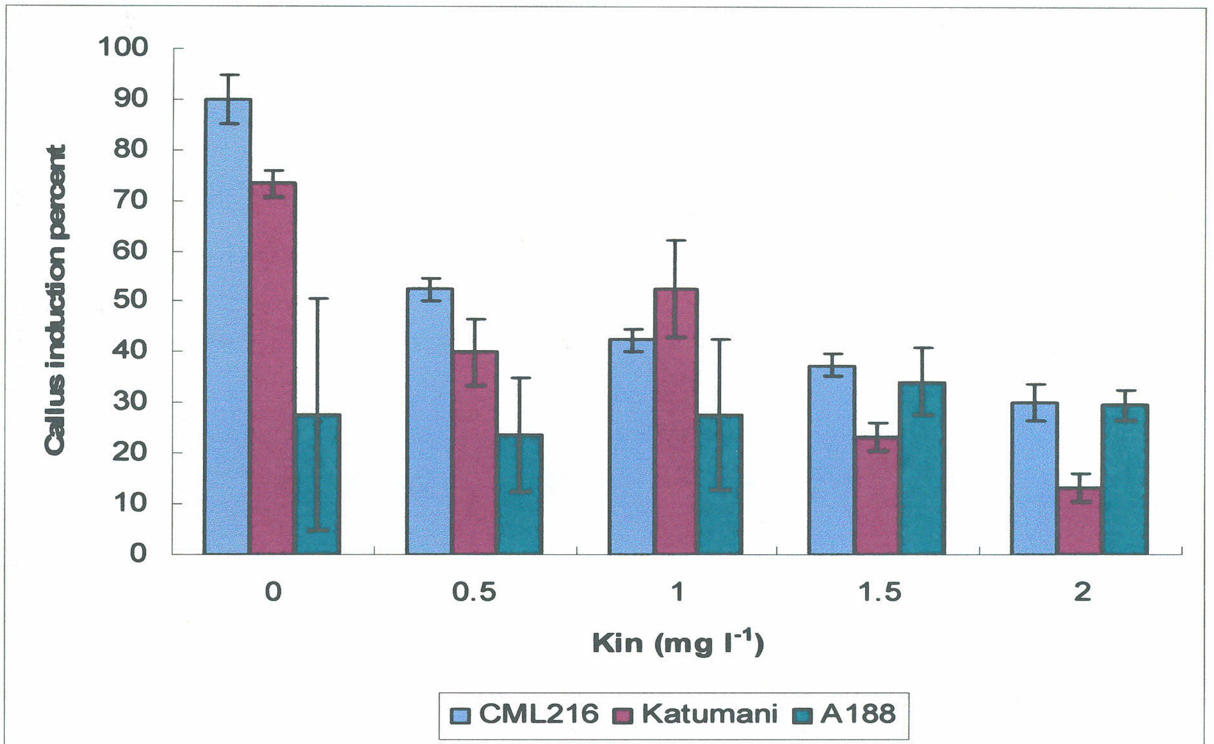


Figure 13: Effect of kinetin combined with 2,4-D on callus induction from mature maize zygotic embryos
Error bars = Mean ± SE

4.5 Type I and Type II callus induction

Three different hormone combinations, 3 mg l^{-1}2,4-D, 3 mg l^{-1}2,4-D and 0.5 mg l^{-1} Kin and 3 mg l^{-1}2,4-D and 1.0 mgl^{-1} kin, were selected from previous experiments for regenerable calli induction. The highest frequency of Type I callus, 62.3%, was obtained when calli induced on Media A (appendix 1) supplemented with 3 mg l^{-1} of 2,4-D and 1.0 mg l^{-1} of kinetin was transferred to maintenance Media A (Appendix 1) supplemented with 2 mg l^{-1} of 2,4-D and 1.0 mg l^{-1} of kinetin. The calli was subcultured every two weeks on maintenance media for two month (Fig 14). The minimum frequency of Type I callus induction was 46.7%. Highly significant difference ($p<0.004$) was obtained among the three kinds of media selected for callus induction for Type I callus.

A maximum of 75.6% embryogenic (Type II) callus induction was obtained when calli induced using Media A (appendix 1) supplemented with 3 mg l^{-1} of 2,4-D was transferred to maintenance Media A (Appendix 1) supplemented with 2 mg l^{-1} of 2,4-D. The calli was subcultured every two weeks on maintenance media for two month (Fig 15). The minimum frequency of Type II callus induction was 20.1%. There was no significant difference ($p<0.212$) among the three kinds of media selected for callus induction for Type II callus induction.

The scutellum of maize inbred line CML 216 opened up after about a week and produced white to pale yellow and translucent embryogenic calli. When this calli was subcultured on callus maintenance media, after 3-4 weeks two distinct types of calli were produced. Type II calli was induced on the surface of scutellum. Three distinct transient stages of embryogenesis were observed for embryos to develop from callus initiated on from

mature seeds (Fig 16). Creamy yellowish Type I calli was induced mainly at the base of the plumule where it was attached to the scutellum (Fig 17). Generally calli induced and subcultured on media containing kinetin produced more organogenic calli than embryogenic calli as compared to 2,4-D alone. Higher frequency of necrosis and low frequency of callus induction were observed on media supplemented with kinetin. Overall, most split seeds produced both types of calli were produced simultaneously.

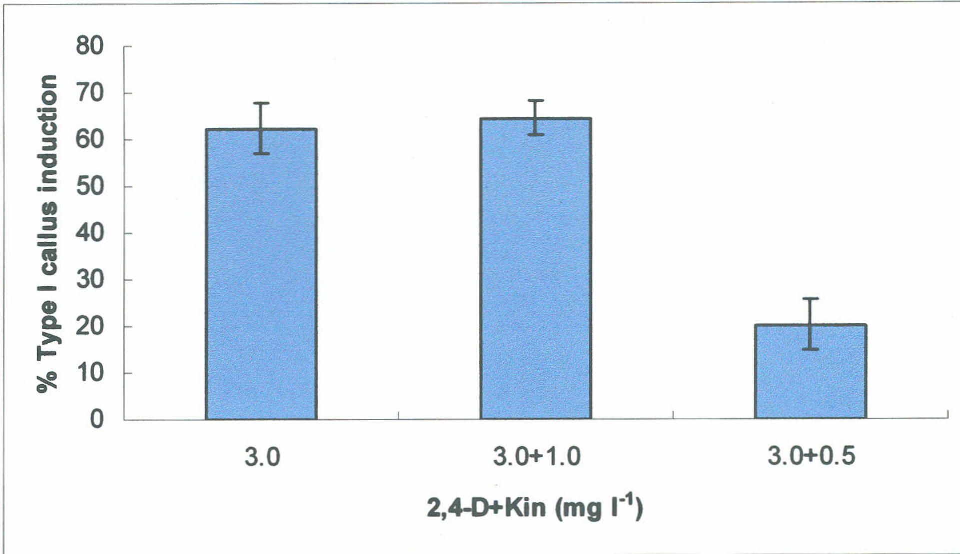


Figure 14: Organogenic callus induction from mature maize zygotic embryos: a) 3 mg l^{-1} 2,4-D b) 3 mgl^{-1} 2,4-D and 1.0 mg l^{-1}3 mgl^{-1} c)2,4-D and 0.5 mg l^{-1}
Error bars = Mean ± SE

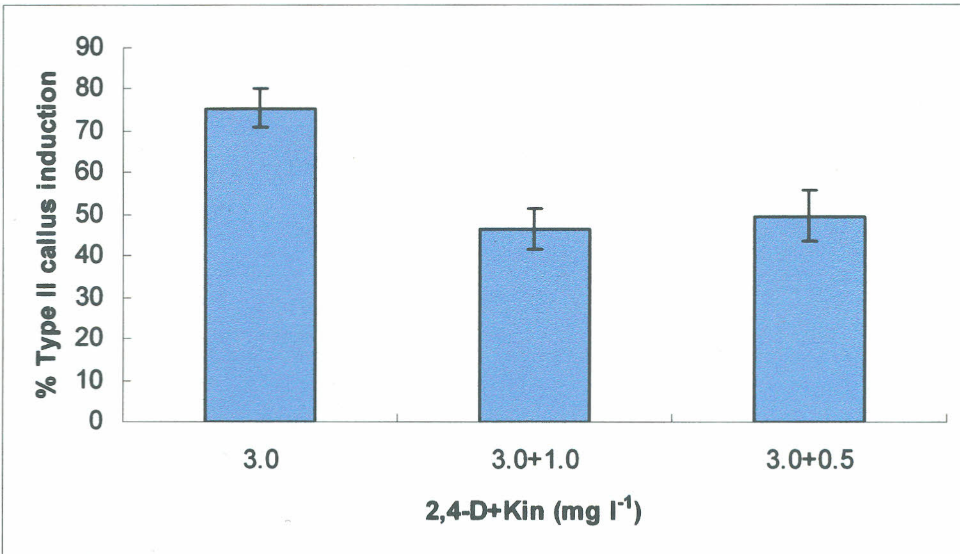


Figure 15: Embryogenic callus induction from mature maize zygotic embryos: a) 3 mg l^{-1} 2,4-D b) 3 mgl^{-1} 2,4-D and 0.5 mg l^{-1} c) 3 mgl^{-1} 2,4-D and 1.0 mg l^{-1}
Error bars = Mean ± SE

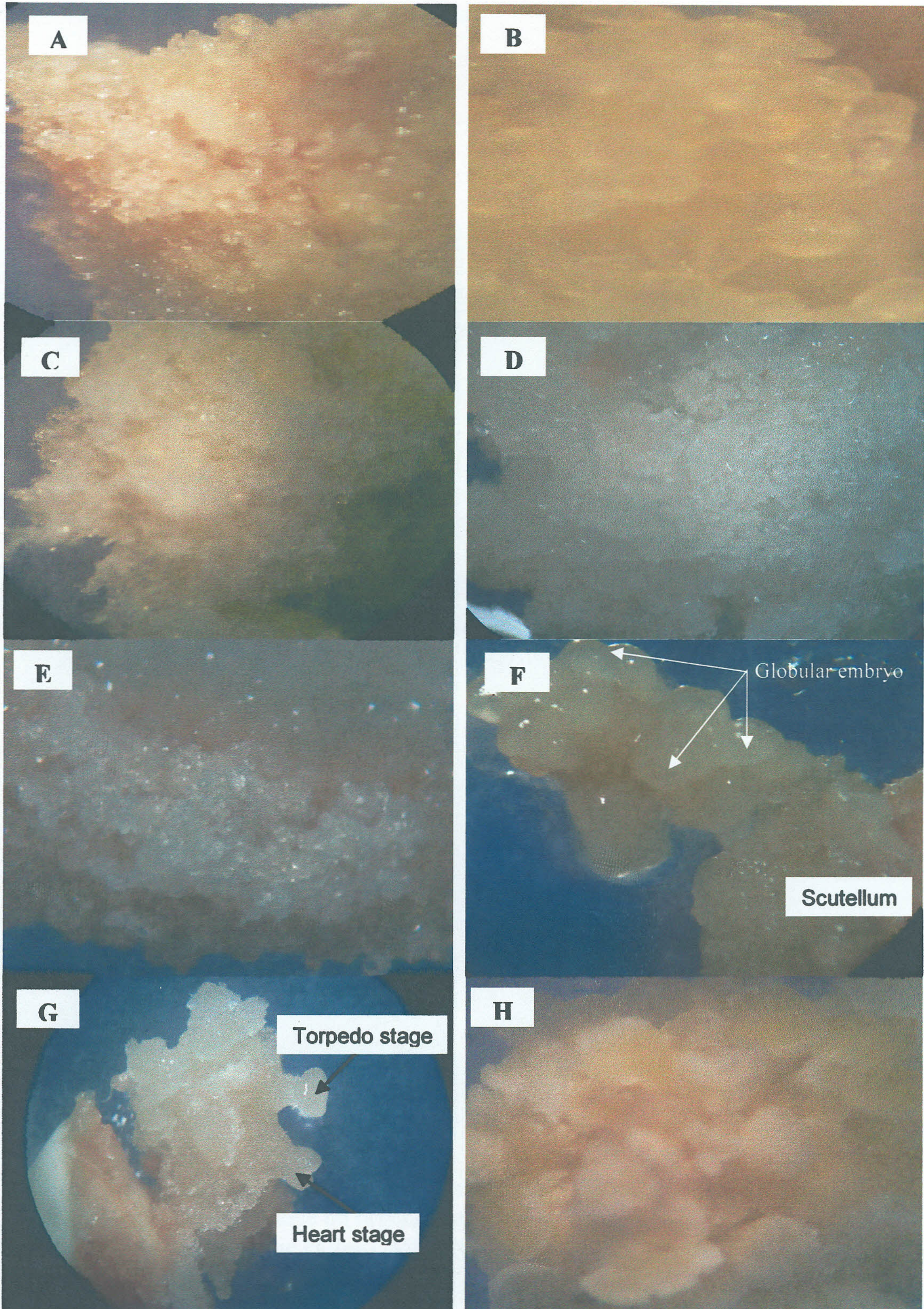


Figure 16: Embryogenic callus A) Embryogenic calli induced on split seeds after 1 month B) Embryogenic calli induced on split seeds C) Embryogenic callus proliferation D) Section of embryogenic callus proliferated E) Embryogenic callus proliferated F) Embryo at globular stage G) Split seed containing all embryo types H) embryos on maturation media

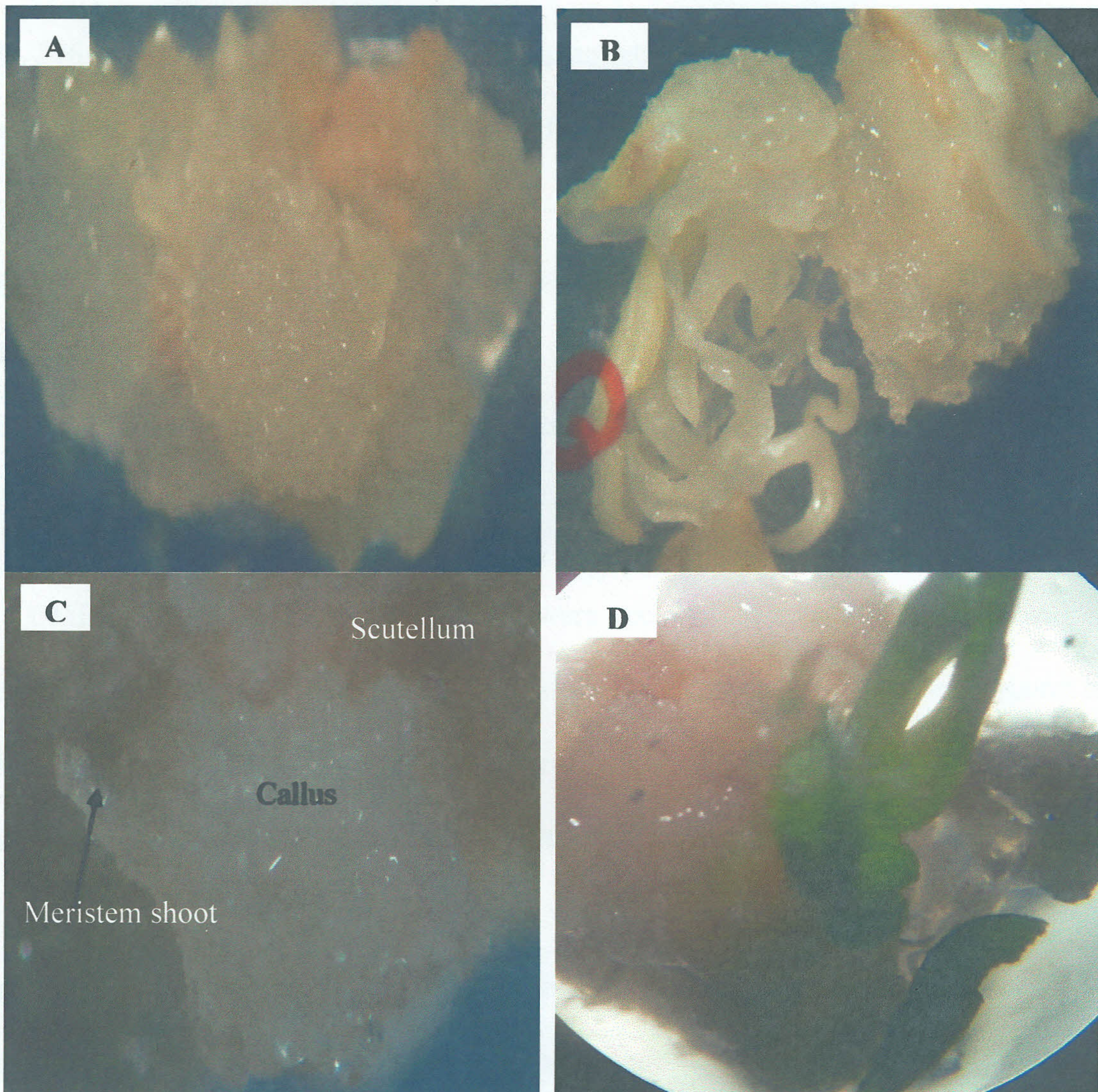


Figure 17: Organogenic calli A) Organogenic calli induced from split seeds CML 216B) Multiple shoots induce on organogenic callus CML 216 C) Meristems shoot emerging from organogenic callus CML 216 D) Multiple shoots induced on organogenic callus Katumani

4.6 Plant Regeneration

Plants were regenerated when organogenic calli were transferred into shooting media. Shoots started to appear after one week. However, due to slow *in vitro* shoot development 4-6 weeks were required to transfer shoots to rooting Media B (appendix 2). A total of 99 CML216 and 79 Katumani organogenic calli were transferred to shooting media and, 21 CML 216 and 13 Katumani calli regenerated plantlets (Fig 18). Regenerated shoots were transferred to hormone free rooting media and within a week after roots started to appear (Fig 18). The regeneration frequency was 21.2% and 16.5% for CML216 and Katumani respectively (Table 1). Number of multiple shoots emerged per callus ranged 1-5 for both CML216 and Katumani (Fig 18). Embryos produced from split seeds failed to give plants; instead they developed roots (Fig19) and A188 did not give plants.

4.7 Acclimatization

Five CML 216 and three Katumani plants were acclimatized. Prior to the removal of plants from the culture bottle, pit moss (Kekkilä Oyj, Tussula, Finland) was ground to and filed into small plastic pot (9cm X 8cm). Ground pit moss was watered using sprayers till it was well moist. Plants were then removed from the culture bottle carefully, any media left over was washed and plants were transferred into pit moss. They were then covered with a clear translucent plastic bag (8" X 4"). Relative humidity inside the plastic pot was maintained high by spraying water both on the plant body and inner part of the plastic cover (Fig 20). Plants were watered and monitored everyday. After 6 weeks survived plants were transferred to a bigger pot filled with soil and vermiculite at a ratio of 2:1, and taken to a glass house.

Table 1: Number of regenerated calli and number of shoots per callus

Line	Number of split seeds cultured	Number of regenerated calli	Regeneration Frequency (%)	Number of shoots per callus
CML 216	99	21	21.2	1-5
Katumani	79	13	16.5	1-5

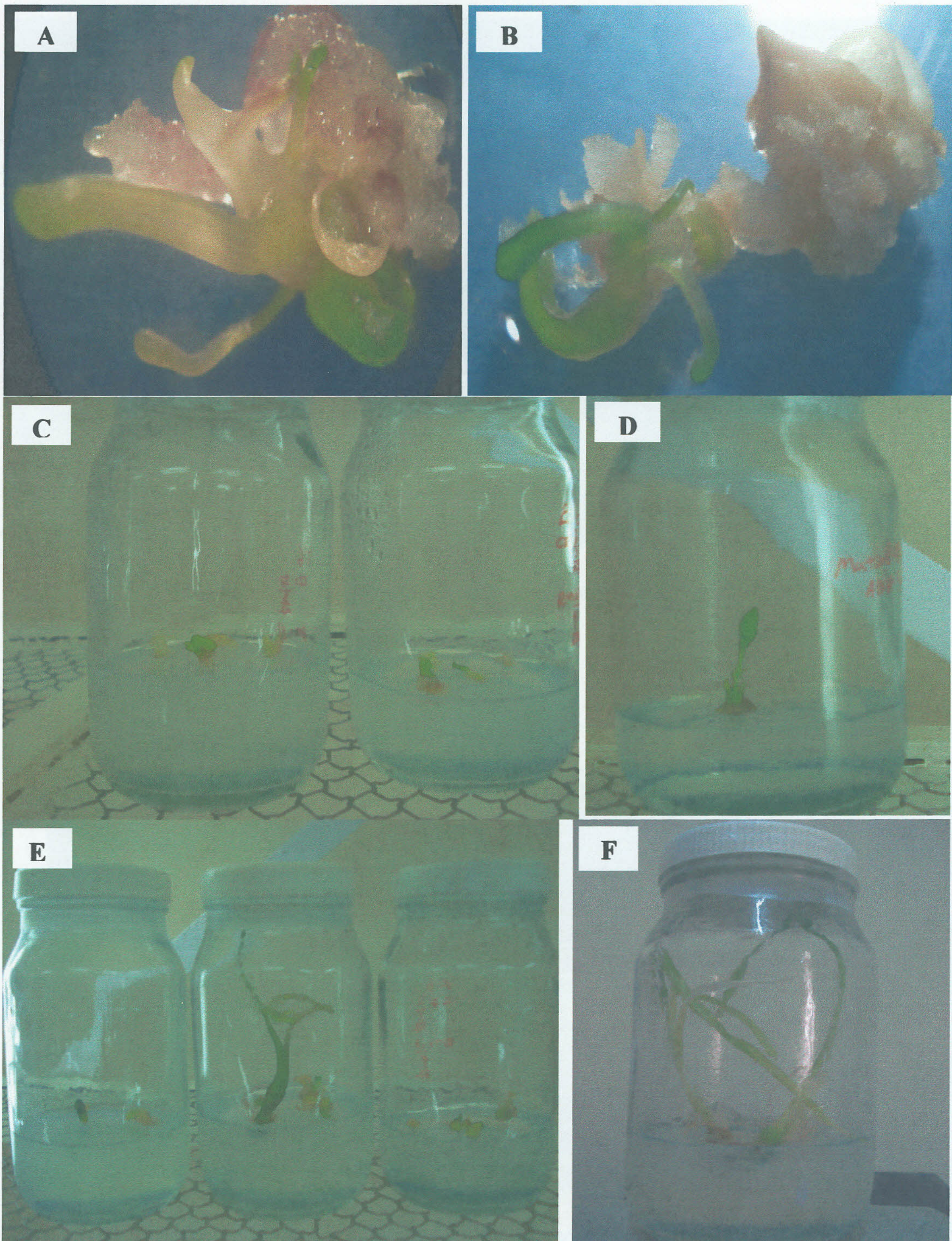


Figure 18: Plant regeneration from organogenic calli A) shoots on Katumani split seed induced on organogenic calli B) Shoots on CML 216 split seed induced on organogenic calli C) Shoots after one week on regeneration media D) Shoots after two weeks on regeneration media E) Shoots after four weeks on regeneration media F) Shoots after 6-7 weeks on regeneration media

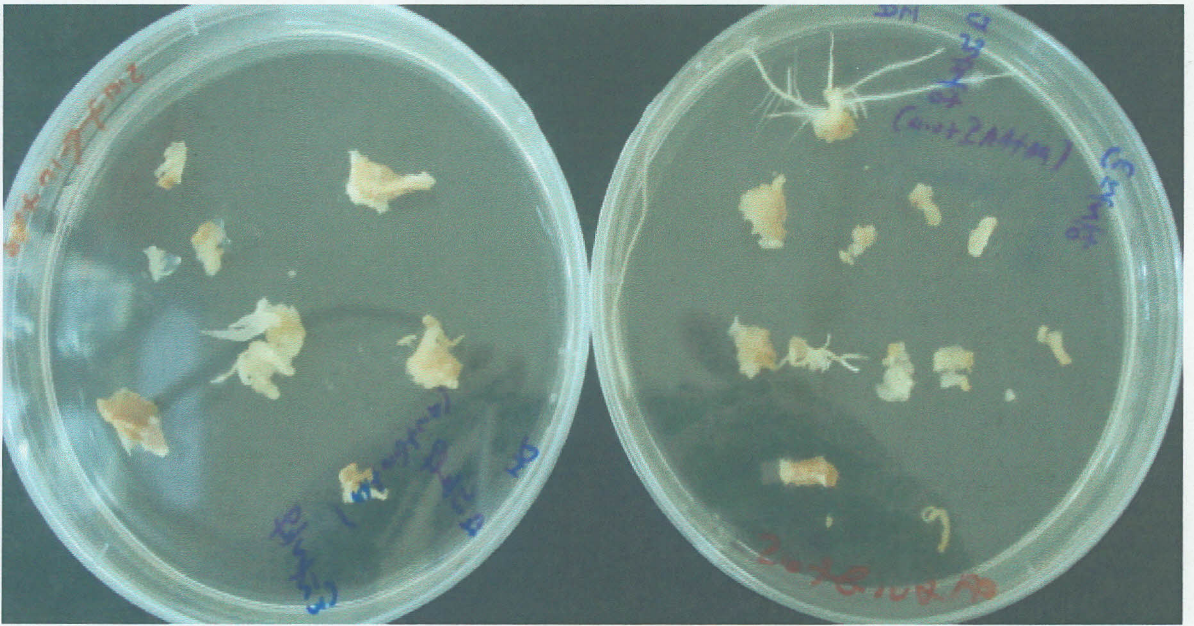


Figure 19: Embryos cultured on regeneration media

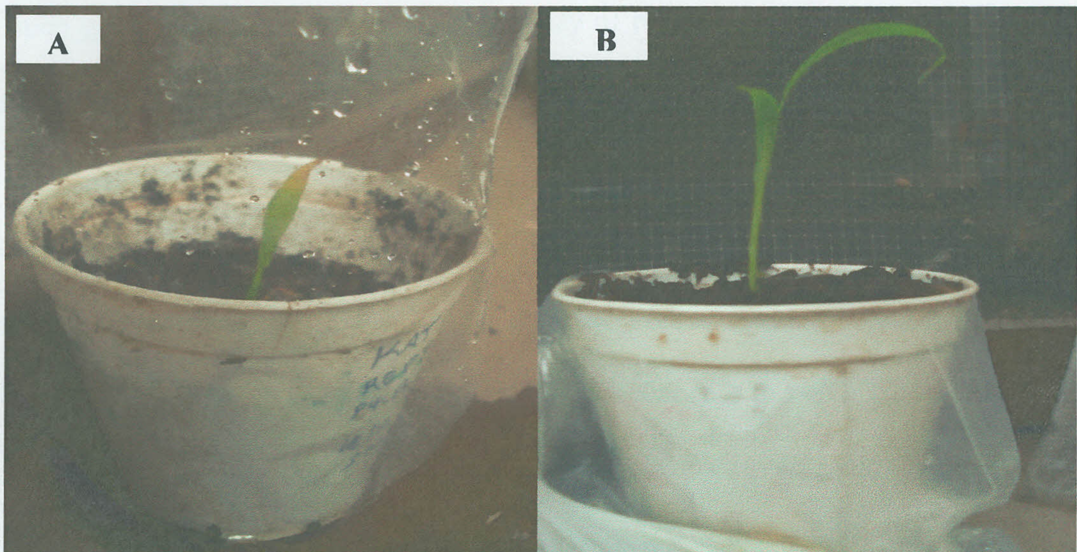


Figure 20: Plants in acclimatization A) Picture taken after three days in pith moss B) acclimatized plants for two weeks

CHAPTER FIVE

DISCUSSION

The sterilization protocol outlined by Sairam *et al.* (2003) and Al-Abed *et al.* (2006) was used to disinfect mature seeds obtained from plants grown under controlled (glass house) conditions. Similar results were obtained when Katumani seeds supplied by Kenya Seed Co Ltd (Kitale, Kenya) were sterilized using this method. However, all the plates were contaminated when seeds bulked in screen house were sterilized using this technique. This is because seeds supplied by Kenya Seed Co Ltd were treated with Dimethioate 40 EC (Chiminova Agro, Lemvin, Denmark). Maize weevil (*Sitophilus zeamais*) infestation of cobs was observed in the screen house prior to harvest. Besides, contamination was observed two days after seeds germinated and was always initiated near the pedicle, which was surrounded by glumes. This incident suggested that glumes surrounding the pedicle could be harboring microorganisms, which possibly came in contact with the media upon germination. Therefore, the possibility of removing glumes surrounding the pedicle prior to sterilization in controlling contamination was explored.

Removing glumes surrounding the pedicle prior to sterilization and following the sterilization technique outlined by Sairam *et al.* (2003) reduced contamination to 22.6%. This was probably because removing glumes improved access to NaOCl by uncovering microorganisms hiding in the glumes surrounding pedicle. However, soaking maize seeds in 1% NaOCl overnight eradicated the contamination but reduced percent germination. The most probable reason for this could be the prolonged exposure of embryos to the NaOCl. In contrast, reduced exposure, for 2-3 hours, to the same 1% NaOCl solution successfully sterilized the seeds and maintained a high germination rate of 92.6%. On the other hand re-sterilizing seeds soaked in sterile dH_2O overnight using 2% NaOCl solution

for 20 minutes resulted in the lowest percent germination. This is contrary to Atiken-Christie (1984), who disinfected mature seeds of gymnosperms by sterilizing soaked seeds under running tap water for 24 hours after the initial sterilization. This is probably because the seed coat of gymnosperms is harder than that of maize and soaking loosens it for the active ingredients to penetrate.

Inconsistent callus induction results were obtained following the culture of seeds after splitting them as stated by Al-Abed *et al.* (2006). Callus was expected to originate from the injured portion of the three embryonic tissues, i.e. scutellum, plumule and coleoptile ring. Embryogenic calli, however, was expected from the scutellar region of the mature split seed where it is attached to the coleorhiza. The scutellum remains attached to the endosperm where germination pushed it away from the media (Weston, 1995). This resulted in callus induction predominantly from the injured radicle region that remained in contact with the media. Therefore, morphogenic callus induction was inconsistent. In an attempt to expose the scutellar region, plumule and coleoptilar ring simultaneously upon splitting, two strategies were formulated. The first is splitting seeds early to expose the scutellum before the plumule grow to push it away from media and the second is injuring the plumule to slow down its growth. Leaving the plumule loosely attached to the scutellum gave consistent morphogenic callus induction. As observed by Bi *et al.* (2007) removal of radicle and slightly damaging the plumule portion of mature wheat embryos hampered root and bud formation and enhanced high quality callus induction. Continued germination of mature embryos significantly reduced callus formation in *T aestivum,* instead most germinated embryos showed no callusing response or formed low quality callus. Retaining the plumule loosely attached to the scutellum promoted the

induction of regenerable calli. As stated by different authors, inclusion of meristematic regions is a pre-requisite for regenerable callus induction. King and Shimamoto (1984) emphasized the need for the presence of the shoot apex in an initial explant source to induce shoots. The reason to split seeds was to expose the three important regions, scutellum, plumule and coleoptile ring, for regenerable calli induction (Al-Abed *et al.*, 2006). Delporte *et al.* (2001) also suggested that the origin of regenerable calli from mature embryos of wheat was epicotyl and mesocotyl regions of the embryo.

Katumani seeds derived from different cobs also gave inconsistent callus induction result. The most probable reason for this result was the mixed genetic make up of open pollinated varieties due to the uncontrolled pollination (Machuka, 1989; Machuka *et al.*, 1993).

Auxins, especially 2,4-D in the range of 1-3 mg l^{-1}, are essential for the formation of embryogenic callus from cereal embryos (Bi *et al.,* 2007; Bhaskaran and Smith, 1990; Oduor *et al.,* 2006). Al-Abed *et al.* (2006) recommended 3 mg l^{-1} of 2,4-D and Huang and Wei (2004) used 4 mg l^{-1} of 2,4-D to induce regenerable callus from mature embryos of maize. In the present study, 3-4 mg l^{-1} of 2,4-D was found to be the optimal concentration for regenerable callus induction on the surface of split seeds.

Katumani, CML216 and A188 did not show significant callus induction differences using either 2,4-D alone or in combination with kinetin. Increasing the level of 2,4-D beyond 3 mg l^{-1} did not affect the frequency of callus induction or increased the amount of calli produced. Moreover, embryogenic calli was initiated on the surface of the scutellum whereas organogenic calli was mainly obtained at the base of the plumule where it was

attached to the scutellum (Al-Abed *et al.,* 2006; Huang and Wei, 2004). Similarly, Vasil *et al.* (1985) reported that zones of meristematic cells were observed from the scutellum of maize immature embryos. The meristematic zones were initiated in close proximity to the procambial cells of the root and scutellum. The division was also found in the scutellar node.

The difference for Type II callus induction from split seeds using 3 mg l^{-1} 2,4-D alone or in combination with 0.5 mg l^{-1} and 1 mg l^{-1} of kinetin was non-significant. This result is in agreement with different authors who induced Type II calli using 2,4-D alone or in combination with lower levels of cytokinins. Type II calli production from immature embryos of maize using 2,4-D alone was reported by Danson (2006), Duncan *et al.* (1985), El-Itriby (2003) and Oduor *et al.* (2006). In contrast, somatic embryos were successfully produced from mature embryos of maize using 2,4-D in combination BAP (Al-Abed *et al.*, 2006; Huang and Wei, 2004).

Zygotic or somatic embryos contain all the necessary structures required for germination (Fransz *et al.,* 2004). Inclusion of growth regulators in regeneration media did not affect plant regeneration (Oduor *et al.,* 2006). In contrast, organogenic calli lack these necessary structures to develop in to a full plantlet. Different authors induced shoots by including cytokinins in shooting media. Chang *et al.* (2003) found that subcultured callus of barley required more BA or kinetin in the regeneration medium to induce shoot, and BA was more effective than kinetin at the same levels. In this study, shoots were induced from organogenic callus culture on regeneration media supplemented with 2 mg l^{-1} kinetin and 4 mg l^{-1} BAP.

The 16.5% and 21.2% regeneration frequency and 1-5 mean shoot number per callus reported in this study is low as compared to previous reports. However, many other workers also reported lower regeneration frequency for tropical maize genotypes. Danson *et al.* (2006) also reported significant reduction in regeneration frequency of breeder preferred mid-altitude maize lines to that of highland adapted genotypes. Oduor *et al.* (2006) reported a reduced regeneration frequency for tropical maize lines as compared to temperate lines from immature embryos. This low response is likely due to the recalcitrance nature of tropical maize genotypes to tissue culture. However, this is the first report on regeneration of tropical maize genotypes from mature embryos. Moreover, considering the availability and abundance of mature embryos from maize seeds, this technique can be useful for regeneration of tropical maize lines.

In this study, all regenerants were obtained through indirect organogenesis. Embryogenic calli turned brown soon after the second subculture on maintenance media. The few embryos that reached maturation and regeneration stage only produced roots. This is consistent with other authors who recorded similar results from tissue culture of tropical maize genotypes. Danson *et al.* (2006) reported that with the exception of CYMMYT line Pool A3-6 and msv line CMB5 with embryogenic response of 12% and 8% respectively, none of the remaining tropical maize lines advanced beyond the 45 days subculture steps for the formation of the Type II callus. Initial tissue culture works on elite tropical maize lines CML72, CML216, CML323, and CML327 also produced only Type I callus (Bohorova *et al.,* 1995). The most probable reason for this could be the role of genetic

background in the formation of the Type II callus (Armstrong and Green, 1985; Fluminah and Aguiar-Perecin, 1998).

In conclusion, an *in vitro* regeneration protocol from mature embryos of Katumani and CML 216 has been developed. A total of 34 regenerable calli was obtained using the split seed technique. A modified sterilization protocol for mature embryos developed under screen house or in open fields conditions has also been developed. The auxin 2,4-D at the level of 3-4 mg l^{-1} was found to be optimum for regenerable callus induction from mature embryos using this technique. Combining 2,4-D with lower levels of kinetin resulted in a comparable regenerable callus induction than with 2,4-D alone. However, inclusion of cytokinin (kinetin) in callus induction media enhanced the tendency of organogenic calli production. The frequency and amount of callus produced was also reduced. Different authors have already transformed maize using mature embryo derived organogenic calli. Therefore, this protocol can be useful for transformation of tropical maize genotypes.

RECOMMENDATIONS

- In this report plants were regenerated through organogenesis. But, plant regeneration through embryogenesis is more useful for transformation work. Therefore, there is a need to explore plant regeneration from embryos that developed on the surface of the scutellum. Moreover, both organogenic and embryogenic calli were induced together on the surface of the split seeds. Hence, improving conditions to regenerate plants from both type of calli can enhance regeneration frequency.
- Both *in vitro* and *ex vitro* growth rate of shoots induced from organogenic callus was slow. This resulted in prolonged *in vitro* incubation period and reduced survival of plants *ex vitro.* Therefore, there is a need to improve conditions to enhance *in vitro* growth rate and *ex vitro* survival.
- Only two tropical maize genotypes were tested for regenerability in this study. More tropical genotypes should be assessed for regenerability using split seed technique from mature embryos to confirm if the response is less genotype dependent.
- Various authors have regenerated transgenic maize plants using mature embryo derived organogenic callus. Mature embryos are ubiquitous and in the present study plants were regenerated through callus initiation. Therefore, the potential of this protocol to achieve other biotechnological goals and tropical maize transformation needs to be assessed.

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Appendix 1

Media A

Leinsiminar and Skoog Basal salts

Macro

NH_4NO_3................ 1650 mg l^{-1}

KNO_3..................1950 mg l^{-1}

$MgSO_4$ $7H_2O$...........180 mg l^{-1}

$CaCl_2$ $2H_2O$.............332 mg l^{-1}

KH_2PO_4..................170 mg l^{-1}

Micro

H_3BO_3...................6.2 mg l^{-1}

KI.........................0.8 mg l^{-1}

$MnSO_4$ H_2O............16.9 mg l^{-1}

Na_2MoO_4 $2H_2O$.........0.25 mg l^{-1}

$ZnSO_4$ $7H_2O$.............8.6 mg l^{-1}

$CoCl_2$ 6 H_2O..............025 mg l^{-1}

$CuSO_4$ $5H_2O$.............0.25 mg l^{-1}

FeNaEDTA..............36.7 mg l^{-1}

B5 vitamins

Myo-Inositol.............100

Nicotinic acid.............1.0

Pyridoxin HCl.............1.0

Thiamine HCl..............10

Sucrose ....30 g l^{-1}

Agar.....................8 g l^{-1}

Appendix 2

Media B

Murashige and Skoog media

Macro

NH_4NO_3……………. 1650 mg l^{-1}

KNO_3………………..1950 mg l^{-1}

$MgSO_4$ $7H_2O$………...180 mg l^{-1}

$CaCl_2$ $2H_2O$…………..440 mg l^{-1}

KH_2PO_4………………170 mg l^{-1}

Micro

H_3BO_3…………………6.2 mg l^{-1}

KI……………………...0.8 mg l^{-1}

$MnSO_4$ H_2O…………16.9 mg l^{-1}

Na_2MoO_4 $2H_2O$………0.25 mg l^{-1}

$ZnSO_4$ $7H_2O$…………8.60 mg l^{-1}

$CoCl_2$ 6 H_2O…………0.25 mg l^{-1}

$CuSO_4$ $5H_2O$…………..0.25 mg l^{-1}

FeNaEDTA……………36.7 mg l^{-1}

Vitamins

Myo-Inositol…………..100

Nicotinic acid…………..0.5

Pyridoxin HCl…………0.5

Thiamine HCl…………..0.1

Sucrose ……………..30 g l^{-1}

Agar…………………8 g l^{-1}