

**REGENERATION POTENTIAL AND TRANSIENT  
EXPRESSION OF  $\beta$ -GLUCURONIDASE MEDIATED VIA  
*Agrobacterium tumefaciens* IN IMMATURE EMBRYOS OF  
KENYAN MAIZE GENOTYPES //**

**JAYNE JEBICHII BINOTT (B.Sc Hons, UoN)**

A thesis submitted in partial fulfillment of the requirements for the award of the degree of Master of Science (Biotechnology) in the School of Pure and Applied Sciences, Kenyatta University

**March, 2007**

Binott, Jayne  
*Regeneration  
potential and*



2008/322636

## DECLARATION

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

**Jayne Jebichii Binott**

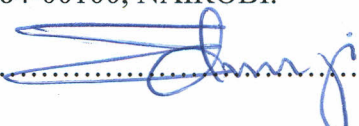
Signature.....  .....Date..... 15-07-2007 .....

We confirm that the work reported in this thesis was carried out by the candidate under our supervision.

**Prof. Jesse S. Machuka,**  
Department of Biochemistry and Biotechnology  
Kenyatta University,  
P.O.Box 43884-00100, NAIROBI

Signature.....  .....Date..... 15/5/2007 .....

**Prof. Eliud N.M. Njagi,**  
Department of Biochemistry and Biotechnology,  
Kenyatta University,  
P.O.Box 43884-00100, NAIROBI.

Signature.....  .....Date..... 15/05/07 .....

**Dr. Josephine M. Songa,**  
Kenya Agricultural research institute (KARI),  
Biotechnology Center at NARL,  
P.O.Box 14733-00800, NAIROBI.

Signature.....  .....Date..... 11-5-07 .....

## DEDICATION

To

*My son Kimaru, dear mum Jesaimo, dad Petero Kipkwe, sisters; Sally, Daisy and brother Reuben James and nephew Franco for patiently standing with me in prayers, perseverance and encouragement during my studies and preparation of this thesis. You remain truly my anchors. Godbless you abundantly.*

## ACKNOWLEDGEMENT

My deepest gratitude to my dedicated supervisors: Prof. Jesse Machuka for his unconditional pursuit for world class scientific research, his indispensable intellectual guidance and moral support, Prof. Eliud Njagi for his constructive comments encouragement, unswerving support and commitment and Dr. Josephine Songa for laying the foundation of the work by ensuring the establishment of tissue culture laboratory, timely purchase and delivery of all laboratory equipment and reagents, her availability, encouragement and support both professionally and morally throughout the entire period of study.

I wish to thank Director KARI for granting me study leave, my sponsor USAID for providing all the financial support for my studies, Plant Transformation Laboratory of Kenyatta University for providing all the facilities that were required for the transformation research work while the KARI Biotechnology Molecular Biology Laboratory was under going construction and renovations.

My appreciation and gratitude goes to Dr. Jane Ininda for her kind & generous donation of seed material, giving valuable information on their traits, KARI Muguga technical staff who assisted in seed selection particularly Florence Wambui. Catherine Taracha and Penina Mwisa for their technical advice, Mr. Eliud Thurania for his assistance with statistical analysis, Douglas Miano and Bramwel Wanjala for taking their valuable time in photographing of materials. I also wish to thank my colleagues at Biotechnology Centre and Plant Transformation Laboratory especially Allan Jalemba and Omwoyo Ombori for their persistent encouragement. Am grateful to Mary Nafubwa and Donn Otieno for taking care of the plant materials both in the field and greenhouse. Am indebted to the committed staff of Department of Biochemistry and Biotechnology for providing enabling environment for academic growth.

Above all, I thank God for the gift of life, for his guidance and successful completion of my work.

## ABSTRACT

Production of maize is constrained by both abiotic and biotic stress factors in the field and by post-harvest pest problems; the most important being the larger grain borer, *Prostephanus truncatus* (Horn) and maize weevil, *Sitophilus zeamais*. Developing insect resistance crop varieties through conventional plant breeding is elusive, expensive and time consuming due to the limited genetic variation within the maize genotypes as well as difficulty in maintaining quantitatively controlled traits such as insect resistant. However, by using genetic engineering tools, modified novel genes (e.g. from *Bt* or plant proteins) can be introduced into maize to produce transgenic maize that confer resistance to these insects pests. The present study investigated regeneration potential of Kenyan maize genotypes as a prerequisite to genetic transformation. Twelve parental inbred lines and their respective single cross hybrids were planted in Kiboko and Kabete and evaluated for callus induction, somatic embryo formation and subsequent plant regeneration. Embryos were excised from surface sterilized kernels harvested at different physiological stages namely 10, 15, 18, 21 and 24 days after pollination (DAP). They were used as explants to initiate callus on N6 induction media with varying levels of 2,4-D (0-20mg/L) supplemented with 2.87g/L proline, 0.1g/L casein hydrolysate, 2g/L glycine, 30g/L sucrose and 3g/L gelrite. The pH was adjusted to 5.8 before autoclaving. The induction frequency of primary calli at 2mg/L 2, 4 D was genotype dependent. Callus induction ranged from 80-90% for hybrids and 50-80% inbred lines. Following two biweekly subculture, the embryogenic calli formation was initiated. Three types of calli were initiated: Type I accounted for 17.6%, type II 52.9% while the remaining 29.4% made up type 0. Using this system, somatic embryo competence was demonstrated in 6 inbred lines and 4 hybrids. However, plant regeneration was only achieved in 4 inbred lines and 3 hybrids. The frequency of shoot formation ranged between 4-40%. The development of this efficient and reproducible regeneration system sets a basis for genetic transformation via *Agrobacterium tumefaciens*. Six *Agrobacterium* strains carrying two types of plasmids (pBECK2000.4) and (pCAMBIA2301) were used to introduce Gus A (*Gus*) reporter gene encoding  $\beta$ -glucuronidase to maize embryogenic tissues. The expression of *gus* activity on transformed embryogenic tissues was evaluated by histochemical staining with X-Gluc. *Gus* staining revealed variation in both intensity and pattern of blue staining in embryos transformed with the same plasmid and bacterial strain. EHA105(pCAMBIA2301) AGL1(pBECK) revealed high infectivity across various genotypes. Fifteen days (15 DAP) was optimal embryo stage for *gus* expression while incubation for 24 hrs was appropriate for co cultivation stage. There was no *gus* activity in bacterial strains that were devoid of plasmid.

## TABLE OF CONTENTS

TITLE PAGE	i
DECLARATION	ii
DEDICATION	iii
ACKNOWLEDGEMENT	iv
ABSTRACT	v
TABLE OF CONTENTS	vi
LIST OF TABLES	ix
LIST OF FIGURES	x
LIST OF PLATES	xi
ACRONYMS	xii

### CHAPTER ONE: INTRODUCTION AND LITERATURE REVIEW

1.1	Background and classification of maize	1
1.2.1	Maize as an important food crop in Africa	2
1.2.2	Constraints to maize production	2
1.2.2.1	Infestation of maize by insect pests	3
1.2.2.2	Drought as a constrain to maize production	3
1.2.3	Strategies to curb abiotic and biotic constrains	4
1.3	Advances of maize breeding using conventional methods	5
1.3.1	Maize breeding in Kenya	6
1.3.2	Shortcomings of conventional breeding	6
1.3.3	Biotechnology in plant breeding	7
1.3.4	Tissue culture as a prerequisite to genetic engineering	8
1.3.5	Callus cultures	8
1.3.5.1	Type I callus (Friable)	9
1.3.5.2	Friable Type II callus	9
1.4	Plant regeneration	10
1.4.1	Organogenesis	10
1.4.2	Somatic embryogenesis	10
1.4.3	The process of somatic embryogenesis	11
1.4.4	Advantages of somatic embryogenesis technique	14
1.4.5	Somatic embryogenesis development	14
1.4.6	Embryo development, maturation and regeneration	14
1.4.7	Factors affecting somatic embryogenesis	16
1.4.7.1	Structural factors	16
1.4.7.2	Physiological factors	17
1.4.7.3	Genetic factors	18
1.4.8	Somaclonal variations and <i>in vitro</i> selection	19
1.5	Methods for genetic transformation	20
1.5.1	Biolistic /particle bombardment transformation	20
1.5.2	Protoplast mediated transformation	21
1.5.3	<i>Agrobacterium tumefaciens</i> mediated transformation	22

1.5.3.1	Advantages of using <i>A. tumefaciens</i> in plant genetic improvement	23
1.5.4	<i>Agrobacterium</i> classification and host range	24
1.5.5	Ti-plasmid and virulence region	25
1.5.6	Molecular mechanism of T-DNA transfer	26
1.5.7	Factors influencing <i>A. tumefaciens</i> mediated genetic transformation	29
1.5.7.1	Genotype	29
1.5.7.2	Explants	30
1.5.7.3	<i>Agrobacterium</i> strains and plasmids	31
1.5.8	Reporter genes and transient expression in plants	32
1.5.8.1	$\beta$ -glucuronidase (GUS A gene)	33
1.5.8.2	Histochemical staining of $\beta$ -glucuronidase activity	34
1.6	Selectable marker genes	34
1.6.1	<i>Bar</i> and <i>pat</i> (Glufosinate resistance)	35
1.6.2	Gene sequence promoters	36
1.6.2.1	Cauliflower mosaic virus (CaMV) 35S promoter-p35S mRNA sub unit	36
1.6.3	Confirmation of stable integration and inheritance of transgene	36
1.7	Justification	37
1.7.1	Null hypothesis	38
1.7.2	Broad objective	38
1.7.2.1	Specific objectives	39

## CHAPTER TWO: MATERIALS AND METHODS

2.1	Source of planting material	40
2.2	Choice of target maize lines	40
2.3	Sample size	40
2.4	Planting site selection	41
2.5	Planting design	41
2.6	Germination viability	43
2.7	Crop management practices	43
2.8	Flowering stage / pollination	43
2.8.1	Generation of hybrids by cross pollination	44
2.8.2	Generation of inbred lines by self pollination	44
2.9	Explants harvesting, surface sterilizations and embryo excision	45
2.10	Evaluation for <i>in vitro</i> tissue	45
2.10.1	Callus initiation (induction)-CIM	45
2.10.2	Callus scores	46
2.10.3	Callus maintenance (CMM)	46
2.10.4	Somatic embryo maturation medium (Maturation medium-MaM)	47
2.10.5	Shoot formation	47
2.10.6	Rooting of plantlets	48

2.10.7	Acclimatization of regenerants	48
2.10.8	Transfer maize plants into the soil and maturation	48
2.11	Transformation protocol	49
2.11.1	<i>Agrobacterium</i> strains and plasmid culture initiation	49
2.11.2	Agroinoculations and infection	52
2.11.3	Co cultivation	53
2.11.4	Histochemical analysis of transient and <i>gus</i> expression	53
2.11.5	Levels of <i>gus</i> staining	54
2.11.6	Estimation of transformation frequencies	54
3.11.7	Statistical analysis	54

### CHAPTER THREE: RESULTS

3.1	Seed germination viability	56
3.2	Effects of genotype and ecological zone on days to flowering	56
3.3	Effect of embryo physiological stages on callus induction efficiency	58
3.4	Effect of maize genotype on callus induction efficiency	62
3.5	Effect of 2, 4-D concentration on % mean callus induction efficiency	63
3.6	Types of primary callus generated	65
3.7	Somatic embryo formation potential across maize genotypes	68
3.8	Plant regeneration	70
3.9	Evaluation of transient <i>gus</i> activity on regenerable maize lines	79
3.9.1	Effect of embryo physiological stage on $\beta$ -glucuronidase expression	79
3.9.2	Determination of optimal incubation period for transient <i>gus</i> expression	81
3.9.3	Infectivity of different <i>A. tumefaciens</i> strains carrying two types of plasmids	83

### CHAPTER FOUR: DISCUSSION, CONCLUSION AND RECOMMENDATION

4.1	Discussion	86
4.2	Conclusion	95
4.3	Recommendations	96

<b>REFERENCES</b>	98
<b>APPENDICES</b>	113
<b>APPENDIX I</b>	113
<b>APPENDIX II- CULTURE MEDIA PREPARATION</b>	114
<b>APPENDIX III-ANOVA</b>	118

## LIST OF TABLES

<b>Table No.</b>		<b>Page No.</b>
Table 1	Estimated annual losses of maize due to insect pests and diseases in Africa	5
Table 2	List of parental inbred lines and their respective hybrids, the history and special attributes	41
Table 3	Climatic and geographical distribution of experimental Site	42
Table 4	Seed germination viability of inbred lines and hybrids in Kiboko and Kabete	56
Table 5	Average days to flower of inbred lines and hybrids in Kiboko and Kabete	57
Table 6a	Callus induction efficiency for both parental inbred lines and their hybrids	63
Table 6b	Average of callus induction between inbred lines and hybrids	63
Table 7	Embryogenic potential of parents and their single cross hybrids	67
Table 8	Somatic embryo formation in parental inbred lines and their single cross hybrids	69
Table 9	Number of regenerated maize genotypes, morphological traits and their R <sub>1</sub> seed viability	79

## LIST OF PLATES

Plate No.		Page No.
Plate 1	Pollinated maize crop in the field	44
Plate 2	<i>Agrobacterium</i> strains streaked on LB agar for Maintenance at +4 °C for 2 weeks	52
Plate 3	Callus induced on N6 media	61
Plate 4	Callus morphotypes produced 28 days upon induction on N6 media	66
Plate 5	Somatic embryos embryogenic callus on maturation medium	72
Plate 6	Plant regeneration	73
Plate 7	Plant on shooting and rooting media	74
Plate 8	Plantlets in small pot containing sterile vermiculate and sand	75
Plate 9	Maize genotypes planted in 5litre plastic	76
Plate 10	Pollination of the regenerants	77
Plate 11	Confirmation of germination viability of R <sub>1</sub> seed	78
Plate 12	<i>Gus</i> activity on maize tissues (TL 17 and TL 21) stained on X-Gluc	82
Plate 13	Highly stained embryogenic tissue showing <i>gus</i> activity	85

## LIST OF FIGURES

Figure No.		Page No.
Figure 1	Comparison of somatic and zygotic embryogenesis	13
Figure 2	The process of plant cell transformation by <i>Agrobacterium</i>	28
Figure 3	Linear map of pBECK plasmid	50
Figure 4	Linear map of pCAMBIA plasmid	51
Figure 5	Estimation of level of <i>gus</i> activity following on X-Gluc	54
Figure 6	The effect of Agroecological zone on average flowering time of maize in both Kiboko and Kabete	57
Figure 7	Effect of embryo physiological stage on callus induction in hybrids	59
Figure 8	Effect of embryo physiological stage on callus induction in inbred lines	60
Figure 9	Effect of different levels of 2,4-D on percentage callus induction efficiency	65
Figure 10	Types of calli generated <i>in vitro</i> by maize	67
Figure 11	Effect of embryo physiological stage on $\beta$ -glucuronidase ( <i>gus</i> ) expression	80
Figure 12	Determination of optimal incubation period for transient <i>gus</i> expression	82
Figure 13	Infectivity of different <i>A. tumefaciens</i> strains carrying types of two plasmids	84

## LIST OF ABBREVIATIONS

½ MSO	half strength MSO
2,4-D	2,4-dichlorophenoxyacetic acid
ABA	abscisic acid
AEZ	agro ecological zone
ANOVA	analysis of variance
ASAL	arid and semi arid lands
BAP	benzyl adenine purine
CIM	callus induction media
CIMMYT	nternational maize and wheat improvement center
CMM	callus maintenance media
CO <sub>2</sub>	carbon dioxide
DAP	day after pollination
dH <sub>2</sub> O	distilled water
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
<i>Gus/UidA</i>	gene encoding β-glucuronidase enzyme activity
g	gram
HCl	hydrochloric acid
IAA	indole acetic acid, the naturally occurring auxin
IBA	indole butyric acid
KARI	Kenya Agricultural Research Institute
LB	left border of T-DNA
M	molar
MaM	maturation media
MES	2-N-morpholinoethane sulphonic acid
Mg	milligram
mg/L	milligram per litre
MS	medium of Murashige and Skoog (1962)
MSO	medium of MS full 4.43g/L
MSV	maize streak virus
N6	media based on Chu <i>et al.</i> (1975)
NAA	naphthalene acetic acid, a synthetic auxin
NaOH	sodium hydroxide
OPV	open pollinated varieties
R <sub>0</sub>	original plant/seed

R <sub>1</sub>	I <sup>st</sup> generation plant /seed
RB	right border of T-DNA
RCBD	randomized complete block design
SNK	Student Newmann- Keuls test
TH	hybrid
TL	Inbred line
USAID	United States Agency for International Development
v/v	volume in volume
v/w	volume in weight
X-Gluc	5 -bromo- 4-chloro -3 -β D glucuronide
FS	filter sterilize
DAP	days after pollination
NARL	National Agricultural Research Laboratories

## CHAPTER ONE

### INTRODUCTION and LITERATURE REVIEW

#### 1.1 Background and Classification of maize (*Zea mays L.*)

Maize (*Zea mays L.*) is one of the oldest food grains. It belongs to the grass family Poaceae (Graminae), tribe Maydeae. It is the only cultivated species of great economic importance in this genus. The other species of *Zea*, teosinte and *tripsacum* commonly called gama grass are very important wild relatives of *Zea mays*. Both teosinte and *tripsacum* are important as possible sources of desirable traits for continued improvement of maize (Wilkes, 1985; Gurney et al., 2003). *Tripsacum* has no direct economic value, while teosinte has some economic value as a source of fodder. The genus *Coix*, an oriental Maydaea is of some economic significance in South and South East Asia (Jugenheimer, 1985; Kumar and Sachan, 1991). It is used as forage crop and its seeds are popped and used as snack food. Cultivated maize is a fully domesticated plant. Humans and maize have lived and evolved together since ancient times. It does not grow in the wild, cannot survive in nature and is completely dependent on human husbandry (Galinat, 1988; Dowsell et al., 1996). Maize is one of the most productive species of food plants. It is a C4 plant with a high photosynthetic activity. It has the highest potential for carbohydrate production per unit area per day (Brown, 1999; Sage, 2004). The diversity of the environment under which maize is grown is unmatched by any other crop.

Maize is classified into two distinct types depending on the latitude and environment in which it is grown. Maize grown in warmer environments falling between the equator and 30<sup>0</sup>N and 30<sup>0</sup>S is referred to as tropical maize, while that

grown in cooler climates beyond 34<sup>0</sup>N and 34<sup>0</sup>S is classified as temperate maize (Karim *et al.*, 2000).

### **1.2.1 Maize as an important food crop in Africa**

Maize was introduced into Africa by Portuguese at the beginning of the 16<sup>th</sup> century. It has since become Africa's second most important food crop after cassava. It is Kenya's staple food planted by over 90% rural population for subsistence and as cash crop (Ayaga, 2003). Maize is used in many ways: the immature embryos (cob) are either consumed roasted or boiled while the stover is used as animal feed. It is also a source of large number of industrial products. It is nutritionally superior to most other cereals in many ways except in protein value. Maize compares well with rice and wheat. It is high in fat, iron and fiber content. (Truswell and Brock, 1964)

### **1.2.2 Constraints to maize production in Kenya**

Abiotic and biotic stress factors are major constraints to maize production in Kenya. Priority biotic factors include many diseases and pests that plague maize during different growth stages including the storage stages. The major insect pests of maize include stem borers and storage pests (Ajanga and Hillocks, 2000). Diseases taking the greatest toll in many African countries include maize streak virus, *turcicum*, leaf blight, gray leaf spot, smuts, downy mildew and *striga*. *Striga* (witch weed) *S. asiatica* and *S. hermonthica* are two parasitic weeds common in Kenya. *Striga* may cause yield losses of up to 100% on farmers' fields (Hassan and

Kenya. *Striga* may cause yield losses of up to 100% on farmers' fields (Hassan and Ransom, 1998; Odhiambo, 1998; Kanampiu *et al.*, 2001, Machuka, 2001).

Maize streak virus disease (MSV) is an economically important disease in sub-Saharan Africa. It is caused by a virus transmitted through *Cicadulina* species of leafhoppers. In Kenya, the disease is prevalent in central highland causing upto 100% yield losses especially when the crop suffers from drought ( Njuguna, 1996, Ininda *et al.*, 2002).

#### **1.2.2.1 Infestation of maize by insect pests**

Maize undergoes yield losses in the field and store. The most important field insect pests of maize in Kenya are the lepidopteran stemborers, which lead to losses in the range of 18-50% (Songa *et al.*, 2001; De Groote, *et al.*, 2002). Among the storage pests of maize the most notorious ones are the weevil (*Sitophilus zeamais*) and larger grain borer (*Prostephanus truncatus*). They contribute up to 15-60% post harvest losses. The former occurs in all maize growing areas of Kenya and is responsible for losses ranging from 10-20%. The later has been in the country for the last 20 years where it entered via cross border trade in Tanzania and has so far spread inland into maize growing central Kenyan highlands. It's the most serious pest of maize causing losses up to 60% in grain during the storage season (Nangayo, 1996). A summary of estimated losses due to pest and diseases in Africa is described in Table 1.

### **1.2.2.2 Drought as constraint to maize production**

Among other factors that constrain maize production, water has been found to be a critical factor in the growth and productivity of all crops other than nutrient required for yield production. Continuous and persistent drought is threatening particularly the African continent to feeds its own people (Machuka, 2004). In 2006, about 70% of people in Kenya were threatened by starvation due to lack of clean drinking water and food and so are the livestock they thrive in (REP of Kenya 2006). Over  $\frac{3}{4}$  of Kenyan land falls under arid and semi arid lands (ASAL) and is hardly supported by rain fed agriculture. It is estimated that  $\frac{1}{3}$  of world population will live under arid areas by 2025 if appropriate conservation measures are not undertaken (GEN, 2004).

### **1.2.3 Strategies to curb production constraints**

There is dire need to reduce losses of crops due to diseases insect pests, drought and weeds using biotechnological means to complement conventional strategies in place (Zhang *et al.*, 2000). In Kenya, most hybrids and open pollinated varieties (OPVs) in commercial production have been developed through conventional plant breeding based on backcrossing and subsequent selection of progenies for traits of interest (Lamkey, 2004).

**Table 1:** Estimated annual losses of maize due to insects' pests and diseases in Africa

Pest/disease	Area affected (million ha)	Estimated yield loss (%)	Estimate of total annual loss of production (million t)
Striga	4.33	40	2.07
Blights	14.0	20	3.36
Rusts	10.5	35	4.41
MSV	12.36	37	5.48
Stem borers	16.46	20	3.9
Totals			19.22

Source: DeVries and Toenniessen, (2001)

### 1.3 Advances in Maize breeding using conventional approaches

Maize in the tropics is cultivated in a great diversity of environments; a diversity that is much more pronounced than that of temperate maize growing environments. Maize cropping systems include wide variety of farming methods from intensively managed monoculture systems with mechanized and high levels of inputs, to subsistence maize farming on small plots with no or very low levels of inputs, multicropped with other crop species and using only hand labour. Consequently, maize varieties used in the tropics range from high tech and highly tailored single cross hybrids to open pollinated improved varieties to farmers' local varieties and landraces (Smith and Paliwal, 1996).

Low productivity of maize in the tropics is also a reflection of the intensity of maize breeding efforts in the tropics in general and of difficult and marginal environments in particular (Smith and Piliwal, 1996). Reports from Pandey and Garner (1992) indicated that 96 percent of maize breeders are involved in the improvement of yield while 67 percent are involved in improvement for disease

resistance. Other traits included but of low priority are logging resistance, insect resistance, cold tolerance, tassel size and photosynthetic ability and protein quality.

### **1.3.1 Maize breeding in Kenya**

Currently the breeding efforts are geared towards production of high yielding, locally adapted with tolerance or resistance to major diseases, insect pests' invasive weeds and drought (KARI, 2005). The varieties that have been released have been tested for adaptability to different agroecological zones namely lowlands (dry and low rainfall areas) highlands (high rainfall) and mid altitude (higher than lowland but lower than highland rainfall). *Striga* and MSV tolerant maize varieties have been developed and evaluated for tolerance by Ininda *et al.* (2002). However varieties resistant to storage pests, weevils and to some extent drought have not been developed through conventional breeding methods.

### **1.3.2 Shortcomings of conventional breeding**

The advancements in maize breeding made so far using conventional approaches cannot be under estimated as they has paved way to adoption and utilization of modern biotechnology. The method however has limited use because it is elusive, time consuming (up to 15yrs), expensive, the fact that it is difficult to modify single traits which are quantitatively controlled and that there limited genetic variability within maize genotypes (Holmberg and Bulow, 1998).

Genetic engineering therefore holds a lot of promise in complementing conventional breeding. Such techniques include the use of molecular markers linked

to quantitative trait (QTL) of interest then cloning that gene into maize genome via transformation techniques such as *Agrobacterium mediated* or gene gun systems.

### **1.3.3 Biotechnology and plant breeding**

Modern biotechnology has provided a novel means for crop improvement through the integration and expression of defined foreign genes into plant cells, which can then be grown *in vitro* and regenerated into whole plants (Zhang *et al.*, 2002). Genetic engineering has been utilized as a relatively quick (about 8yrs) and precise means of obtaining plants with desired characteristics. Traits which have been targeted for plant genetic improvement includes: agronomic traits (resistances to diseases, pests, parasites, weeds , nematodes as well as tolerance to stress, salinity, acidity, water logging), out-put traits (content and quality of starch, protein, oil and nutritional elements ( Mazur *et al.*, 1999). Traits that have been engineered into maize are resistance to herbicides (Sawahel, 2002) and incorporation of a gene that codes for the *Bacillus thuringiensis* (*Bt*) toxin, protecting plants from insect pests (Mark *et al.*, 2001; Zhu *et al.*, 2000, Bohorova *et al.*, 1999 ). Hybrids with both herbicide and pest resistance are currently grown commercially in the United States, Asia countries and South Africa. Maize has been engineered to express 1,3-glucanases and chitinases in the kernels with the aim to prevent the growth of *Aspergillus flavus* and aflatoxin production (Wu *et al.*, 1994 ). It has been established that transgenic maize will serve as bioreactors for production of biomolecules with applications in food and pharmaceutical industry (Nikilov, 1999). CIMMYT has developed maize with twice as much lysine and tryptophan levels and

also 10% greater yield as the ordinary maize (Segal *et al.*, 2003). This has served to address malnutrition in countries facing starvation. In Kenya, the first transgenic stem borer resistant maize has been grown in confinement field (Mugo *et al.*, 2005).

#### **1.3.4 Tissue culture as a prerequisite to genetic transformation**

Tissue culture and plant regeneration are an integral part of most plant transformation strategies and are the most challenging aspect of plant transformation (Dodds and Roberts, 1995). Key to success in integrating plant tissue culture into plant transformation is the realization that a quick and efficient regeneration system must be developed (Dahleen and Bregitzer, 2002). However, this system must allow high transformation efficiencies from whichever transformation technique is adopted.

Cultures are usually initiated from sterile pieces of whole plant. These pieces are termed explants, and may consist of pieces of organs, such as leaves or roots, or may be specific cell types, such as pollen or endosperm. Many features of the explants are known to affect the efficiency of culture initiation. Generally, younger and more rapidly growing tissues are the most effective targets (Hansen and Wright, 1999).

#### **1.3.5 Callus cultures**

When explants are cultured on appropriate medium, usually with both an auxin and cytokinin, can give rise to an unorganized, growing and dividing mass of unspecialized parenchyma cells (Jimenez, 2001). In culture, this proliferation can be

maintained more or less indefinitely, provided the callus is subcultured periodically. During callus formation there is some degree of differentiation resulting to lack of photosynthetic ability in cells. Callus induction is usually performed in the dark as light can encourage photosynthesis (Gamborg, 2002).

Callus cultures are extremely important in plant biotechnology. Manipulation of auxin to cytokinin ratio in the medium can lead to development of shoots, roots or somatic embryos from which whole plants can subsequently be produced (Lits and Gray, 1995). Maize embryogenic callus cultures can be classified into type I or type II (Armstrong and Green, 1985).

#### **1.3.5.1 Type I callus**

It is a compact mass of densely aggregated cells and slow growing callus. They are typically white or creamy and coral like. They can easily be distinguished using a stereo type microscope. They are easily obtained from immature zygotic embryos (Carvalho *et al.*, 1987).

#### **1.3.5.2 Friable Type II callus**

Type II callus is soft, friable and fast growing callus characterized by high regeneration capacity. The cells are loosely associated with each other. They are a watery mass of tissues which are usually soft and break easily. Due to its friable nature, type II callus allows the establishment of cell suspension and protoplast cultures and is preferred for some form of *in vitro* manipulation. Unfortunately type II callus tends to be initiated at lower frequency than type I in embryos and has been

obtained from fewer genotypes (Armstrong and Green, 1985). Few genotypes adapted to tropical areas have shown the capacity to produce type II callus and regeneration from tissue culture (Priolo and Silva, 1989; Bohovora *et al.*, 1995). Silver nitrate ( $\text{AgNO}_3$ ) has been shown to enhance the production of type II callus and promotion of maize regeneration (Vain *et al.*, 1989a, b; Songstad *et al.*, 1992).

#### **1.4 Plant regeneration**

Two methods of plant regenerations are used in transformation studies namely organogenesis and somatic embryogenesis.

##### **1.4.1 Organogenesis**

This is the generation of organs from shoots, roots, stems and nodes. Usually embryonic calli are induced using solid Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with 1.5mg/litre of 2, 4-D. The advantage of this system is that shoots can easily form roots. However, establishment and maintenance of embryonic cultures as well as recovery of plants is extremely labor intensive

##### **1.4.2 Somatic embryogenesis**

This involves the generation of embryos from somatic tissues such as embryos microspores or leaves. It has been described as the process by which haploid or diploid somatic cells (non sexual cells) develop into zygotic embryo like structures through an orderly series of characteristic embryological stages without

fusion of gametes (Raemakers *et al.*, 1995). The process displays a characteristic formation of somatic embryos without developmental arrest (Faure *et al.*, 1998). The proliferating somatic embryos are excellent targets for transformation (Hansen and Wright, 1999). This is why somatic embryogenesis is the method for most genetic transformation protocols for recalcitrant legumes and monocots.

Maize can form two different types of somatic embryos, type I and II. Type I and II callus can be utilized depending on the transformation method used (Kozziel *et al.*, 1993). There is limited knowledge of the factors which control the mechanism of embryogenesis and hence more knowledge is required of the physiological process underlying somatic embryogenesis before large scale production of plants from somatic embryogenesis becomes routine in monocotyledonous plants (Tomes and Smith, 1985)

Tomes and Smith (1985) and Hodges *et al.* (1986) indicated that the regeneration is genetically controlled by nuclear genes in maize. Moreover, Willman *et al.* (1989) suggested that at least one or a block of genes controlled expression of somatic embryogenesis in maize tissue cultures. This was demonstrated by the fact that when a regenerable inbred plant was crossed with a recalcitrant inbred, the resulting hybrid would regenerate whole plants from single cells.

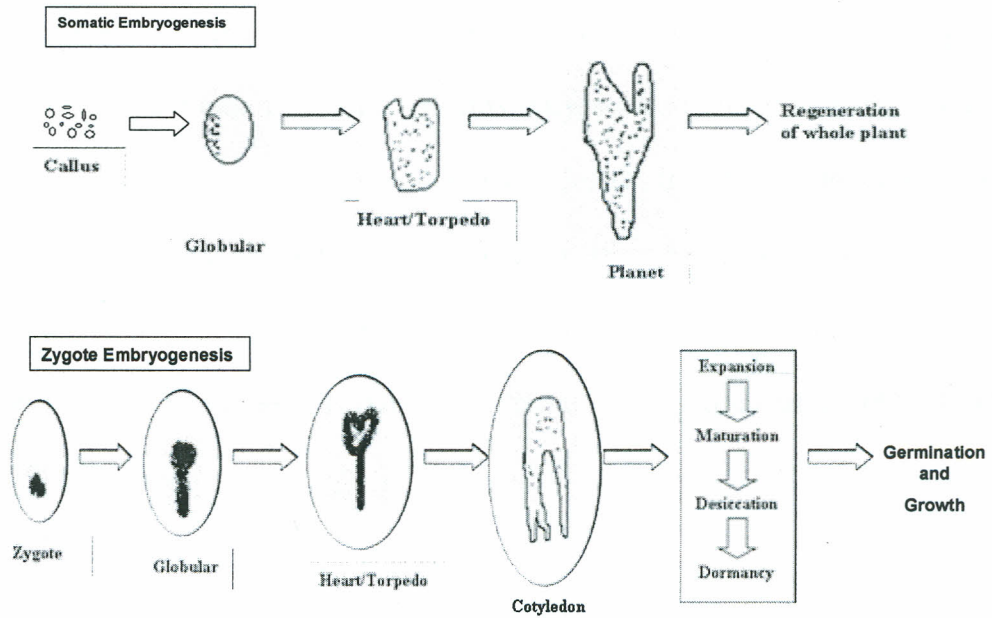
#### **1.4.3 The process of somatic embryogenesis**

Somatic embryos are morphologically and structurally identical to zygotic embryos found in seeds (Ammirato, 1987). In both zygotic and somatic

embryogenesis, there is a similar pattern of globular, heart-shaped and torpedo stages prior to plantlet development (Fig 1). Development begins with proembryogenic structures which are small clusters of meristematic cells. The globular larger cells without defined embryoid shape, appears next and after 2-3 days an oblong stage, the beginning of the heart shaped, develops (Schiavone and Cook,1985). The heart- shaped stage is the beginning of the cotyledonary stage which has one cotyledon, a radicle and an elongated hypocotyls. After 2-3 weeks, plantlets which are small immature seedlings with well defined shoots and roots can be identified (Zimmerman, 1993) concluded that the resemblance of zygotic and somatic embryogenesis demonstrates that all genetic material for embryogenesis is in fact contained within a single cell.

Major differences which exist between zygotic embryos and somatic embryos are in the nutritional uptake (Fig. 1). Nutrients are transported through suspensor from the endosperm in zygotic embryos and it is an active uptake site for young developing embryos. Unlike zygotic embryos, there is no endosperm in somatic embryos and the role of the suspensor, is not yet known although it is present in some plant species (Lindsey and Topping, 1993). Other differences include the absence of a seed coat which apart from having an important protective function also creates a barrier to regulate gaseous exchange (Gray and Purohit, 1991).

### Somatic vs. zygotic embryogenesis



**Figure 1:** Comparison of somatic embryogenesis and Zygotic embryogenesis (Karanja, 1997)

It is the control of embryo development and germination which presents the major challenge in plant production from somatic embryogenesis. Due to the differences which exist between the zygotic embryos and somatic embryos, several anomalies have been noted in somatic embryogenesis such as asynchronous development, precocious germination, extra cotyledons and poorly developed cotyledon (Gray and Purohit, 1991).

#### **1.4.4 Advantages of somatic embryogenesis technique**

The mass propagation of plants through multiplication of embryogenic propagules is the most commercially attractive application of somatic embryogenesis. The techniques have several advantages over organogenesis, namely: (a) the mode of culture permits easy scale up transfer with low labour inputs since embryos can be grown individually and freely floating in liquid medium (b) plants derived from somatic embryos are less variable than those derived from organogenesis. This characteristic allows specific and desirable genes to be introduced into the cells (Gray and Purohit, 1991; Ammirato, 1987; Merkle *et al.*, 1995).

#### **1.4.5 Somatic embryogenesis development**

Somatic embryogenesis has been divided into two main stages; one where differentiated somatic cells proliferate as embryogenic cells and the other where the embryogenic cells differentiate into somatic embryos. Both processes are independent of each other and thus influenced by different factors. The first induction stage was designated determination phase and phase two as induction phase (Dodeman *et al.*, 1997).

#### **1.4.6 Embryo development, maturation and regeneration**

Once the induction of embryogenic the state is complete, the mechanisms of pattern formation that lead to the zygotic embryo are common to all forms of embryogenesis. Thus somatic and zygotic embryos share similar gross ontogenies,

with both typically passing through globular, heart shaped and torpedo-shaped stages in dicots, or globular, scutellar and coleoptilar stages in monocots (Gray *et al.*, 1995 ).

In zygotic embryos, maturation is a stage of accumulation of storage products and acquisition of quiescent state, a process which enhances subsequent germination of the mature embryos (Kermode, 1990). Whereas in zygotic embryos, the embryo development and maturation is influenced by the presence of endosperm and testa coat; the absence of these structures in somatic embryos alters the pattern of the embryo development. Therefore precocious germination has been reported due to differences in anatomy and biochemical composition of the developing somatic embryos (Finkelstein *et al.*, 1985).

Before the onset of germination, proliferation of secondary embryos must be stopped (Raemaker *et al.*, 1995) through removal of the auxin from the medium. The use of abscisic acid (ABA) supplemented medium has been shown to be effective in stopping precocious germination and improving maturation (Eastman *et al.*, 1991). In maize, studies have shown that use of 2,4-D initiates somatic embryo with high regeneration efficiency (Bohorova and Hoisington, 1992). However in cytokinin induced embryogenesis, germination has been reported to be difficult (Raemaker *et al.*, 1995). Cytokinin increases the frequency of shoot germination in auxin induced embryogenesis.

### **1.4.7 Factors affecting somatic embryogenesis**

Expression of somatic embryogenesis might be triggered by different factors, depending on species, cultivar, and physiological conditions of the donor plant. However, the most common procedure is the exclusion or reduction of the auxin (2,4-D) concentration in the culture medium of embryogenic cultures induced with this plant growth regulator. It is equally important to understand the mechanism underlying the transition from somatic cells or gametophytic cells to embryogenic cells and subsequent regeneration of plantlets (Jimenez, 2001). It is desirable to extend these induction and expression capabilities to those species and cultivars that are recalcitrant to somatic embryogenesis and also varieties of agronomic importance. Factors that affect embryogenesis in the induction phase are regulated independently from those of the expression stage (Ermakov and Matveeva, 1994).

#### **1.4.7.1 Structural factors**

The initiation of polarity in the embryo is often regarded as the first step in embryogenesis. In carrot and *Medicago*, it appears that the first division has to be asymmetric, producing two cells of different sizes in order to confer embryogenic competence to the individual cells (Dudits *et al.*, 1991). In most species showing embryogenic capacity, the asymmetric division does not form embryos directly, but forms a pro embryogenic mass (PEM), in which only one or a few cells can subsequently develop into an embryo (Komamine *et al.*, 1992). The rest of PEM cells are probably eliminated through a cycle of programmed death, as observed in

Norway spruce (Filonova *et al.*, 2000). There are some other structural factors that could influence the capacity for embryogenic induction in certain cell: microtubule organization and cell wall size appears to play an important role in somatic embryogenesis.

#### **1.4.7.2 Physiological factors**

The embryogenic capability of the explant to induce somatic embryogenesis in most monocotyledonous plant species is influenced by various factors such as genotype, type and age of explant at the time of excision from the stock plant.

The regeneration from tissue cultures of maize was first reported by Green and Philips (1975) utilizing immature zygotic embryos as explants. Successful regeneration has been observed from calli initiated from anthers (Ting *et al.*, 1981), glumes (Suprasanna *et al.*, 1981), immature inflorescences (Pareddy and Petolino, 1990), immature tassels (Rhodes *et al.*, 1986; Songstad *et al.*, 1992), leaf segment (Conger *et al.*, 1987), seedling segments (Santos *et al.*, 1984) shoot tips (Zhong *et al.*, 1992) and shoot apical meristems (Zhang *et al.*, 2002). Immature embryos derived calli is more efficient for plant regeneration than calli derived from other explants.

The regeneration of plants *in vitro* is influenced by the physiological age of the plant part used. The ability of plants to induce callus, from somatic embryos and subsequent regeneration of shoots is purely dependent on the type of cultivar i.e. genotype (Zhang *et al.*, 2000). In plant tissue cultures, a desirable genotype is expected to possess high callus induction and plant regeneration capability.

However numerous studies have shown the absence of such a relationship between callus induction and plant regeneration (Birsin *et al.*, 2001).

Embryogenic capability can be extended to non embryogenic inbred line through crosses to develop an embryogenic hybrid line. A plant structure needs an array of nutrients for proliferation and development of cells to maturity. In zygotic embryo development and germination, the surrounding endosperm provides all the basic nutritional requirements such as carbohydrates, lipids and proteins (Gray and Purohit, 1991). Since the endosperm tissue is absent in somatic embryos both inorganic and organic essential nutrients for callus induction, development, maturation and germination of embryos have to be supplied exogenously (Murashige and Skoog, 1962).

Light and darkness have both promoting and inhibitory effects on somatic embryogenesis. Abnormal leaf cotyledons and embryo development in *Daucus carota* (Michler and Lineberger, 1987) and fused, woody and difficult to separate embryos of peanut (Baker *et al.*, 1994) were observed under light, while species such as *Pisum sativum* L. cultured in the dark were found to yield fewer somatic embryos than when cultured in the light (Doorne *et al.*, 1995).

#### **1.4.7.3 Genetic factors**

Plant development and differentiation are regulated directly or indirectly by changes of gene expression especially during embryogenesis (Dong and Dunstan, 2000). Initially zygotic embryogenesis were generally designed to estimate the number of different RNAs present in developing seeds to examine the spatial and

temporal distribution of distinct RNA species to isolate and characterize genes that code for abundant proteins (Merkle *et al.*, 1995). However, the experimental strategy for molecular analysis of somatic embryogenesis has mostly relied on comparing genes and proteins being expressed in embryogenic and non embryogenic cells as well as different stages of this process. Dodeman and Ducreux (1997) demonstrated that changes in hormonal levels in tissue culture may modify the synthesis of some somatic embryogenesis specific-proteins.

Most genes expressed differentially during somatic embryogenesis belong to the (*lea*) genes (Zhang *et al.*, 2000). The proposed functions for the products of this family of genes are the protection of the cellular structures in mature embryos during seed desiccation and prevention of precocious germination of zygotic embryos during seed development (Dong and Dunstan, 2000).

#### **1.4.8 Somaclonal variation and *in vitro* selection**

The regeneration of plants from somatic cell cultures in *in vitro* is a basic tool in biotechnology. Maize is not an easy plant to regenerate using tissue culture or cell culture. *In vitro* selection is possible due to the fact that plant cells in culture are genetically variable. The variation in the cultured cells and tissues is called somaclonal variation. Such variation is not limited to first generation regenerants. Cell culture is seen as means of preferentially selecting cell lines with mutation. Successful tissue culture selection depends on evaluating and understanding the effects of selective agents on cultures (Somers and Hibbert, 1994). Plants regenerated from selected lines in many cases express the new trait at the whole

plant level, giving it enhanced agronomic value for that specific trait. The use of specific selective agent to specifically screen specific trait reduces the unwanted phenotypes (Smith *et al.*, 1993).

## **1.5 Methods for genetic transformation**

The prerequisites for the transfer of foreign DNA into any plant species requires, target tissues competent for propagation and regeneration, an efficient DNA delivery system, agents to select for transgenic tissue, expression of transgenes on target organ, a simple, efficient, reproducible genotype independent and cost effective process and tight time frame in culture to avoid somaclonal variation. Techniques that meet these requirements include protoplast fusion, biolistic/microprojectile bombardment and *Agrobacterium*- mediated transformation systems.

### **1.5.1 Biolistic /Particle bombardment transformation**

Biolistic is the delivery of microprojectiles, usually of tungsten or gold coated with DNA and propelled into target cells by acceleration. The acceleration can be provided by gunpowder, by gases such as helium or by electric discharge (Gordon-Kamm *et al.*, 1990). The technique is used for the transformation of species recalcitrant to conventional *Agrobacterium* and protoplast methods (Songstad *et al.*, 1992). It was first used to transform onions (Klein *et al.*, 1989). Transgenic maize plants have been produced by bombardment of type I callus of immature zygotic embryos (Songstad *et al.*, 1992; Walter *et al.*, 1992 ). Transgenic barley plant has

also been regenerated using particle bombardment (Hagio *et al.*, 1995; Wan and Lemaux, 1995)

The advantage of particle bombardment is that DNA can be delivered virtually into any tissue and the transformation is genotype independent (Fromm *et al.*, 1990). However it has a lower transformation frequency than protoplasts transformation method. Biolistics is expensive and takes a great deal of skill and results in gene copy number of up to thirty leading to post translational gene silencing.

### **1.5.2 Protoplast mediated transformation**

Plant regeneration via protoplasts is the epitome of plant cells totipotency because they (protoplast) can be cultured as single cells that produce multicellular colonies from which plants develop. Protoplasts are isolated from plant cells either by mechanical or by enzymatic process to remove the cell wall. This results in the production of a suspension containing millions of individual cells and therefore offering the advantage of probable single cell targets. Protoplasts are frequently obtained from a suspension of cell line of callus initiated from immature embryos, immature leaf bases and anthers (Maheshwari *et al.*, 1993).

Protoplast mediated transformation was the first method of direct gene transfer in plants and the first transgenic cereal plants were produced via direct DNA uptake into protoplast (Toriyama *et al.*, 1988). Protoplast transformation methods do not call for highly specialized equipments and allow for the production of large number of independent transformants and selection of transformants is

usually efficient. However, totipotent protoplasts are usually obtained from cell suspensions that are difficult to establish, accumulate genetic aberration over time and rapidly lose their regeneration capability (Lazzeri and Shrewry, 1993).

### **1.5.3 *Agrobacterium tumefaciens* mediated transformation**

*Agrobacterium* has played a major role in the development of plant genetic engineering and the basic research in molecular biology. It accounts for 80% transgenic plants produced so far (1998, Gelvin, 2005). *A. tumefaciens*, natural plant pathogen has been widely used for genetic transformation of dicotyledonous species but not for monocotyledonous species (Bytebier *et al.*, 1987). Because monocotyledonous plants such as maize are rarely natural hosts of *Agrobacterium*, they are not expected to be susceptible to genetic transfer mediated by *A. tumefaciens* (Potrykus, 1990). However a study showed that *Agrobacterium* - mediated DNA transfer is possible amongst the recalcitrant species not included in its host range (Chan *et al.*, 1993; Bundock *et al.*, 1995).

Competence for *A. tumefaciens* in infecting maize was first indicated in the study of agro inoculation in which the cDNA of maize streak virus was delivered to maize plants and became systemically infected ( Grimsley *et al.*, 1987) Gould *et al.* (1991) inoculated maize shoot apex with *A. tumefaciens* and obtained a few transgenic plants. Several researchers (Shen *et al.*, 1993; Ishida *et al.*, 1996; Zhao *et al.*, 1998) observed the expression of  $\beta$ -glucuronidase (*gus*) gene delivered to maize tissues by *A. tumefaciens*. Transgenic plants and progeny from important monocotyledonous crops such as rice (Chan *et al.*, 1992, 1993; Hiei *et al.*, 1994),

wheat (Cheng *et al.*, 1997), barley (Tingay *et al.*, 1997) and sorghum (Zhao *et al.*, 2001) have been obtained using *A. tumefaciens*. High efficient transformation of rice (Hiei *et al.*, 1994) and maize (Ishida *et al.*, 1996) have been obtained with *Agrobacterium* harboring super binary vectors (Komari, 1990). *Agrobacterium*-mediated gene transfer has been extended to some liliaceous ornamental plants (Suzuki *et al.*, 2002) and forage or turf grasses (Somleva, 2000; Bettany *et al.*, 2003). Recent reports of *Agrobacterium*-mediated transformation of several filamentous fungal species (Abuodeh *et al.*, 2000) suggest that *Agrobacterium* may be useful “gene-jockeying” tool. The recent report of *Agrobacterium* mediated genetic transformation of human cells (Kunik *et al.*, 2001) suggests the exciting possibility of using *Agrobacterium*-like processes, for human and animal gene therapy.

#### **1.5.3.1. Advantages of using *A. tumefaciens* in plant genetic improvement**

The border sequences are the only sites required for T- strand formation and therefore any gene placed between these two border sequences will be transferred to plants as long as the virulence functions are provided (Draper *et al.*, 1991). This fact has been utilized widely in genetic engineering of plants by removing the genes responsible for tumour formation from T-DNA, ending up with disarmed plasmid and replacing them with genes of agronomic importance (Draper *et al.*, 1991).

Recent reports have demonstrated that *Agrobacterium*-mediated transformation may offer better mean of delivery of foreign gene to cereal crops. This gene delivery system is highly efficient and results in greater proportion of

stable, low copy number transgenic events than does the biolistic gun (Ishida *et al.*, 1996; Zhao *et al.*, 1998), offers a possibility of transferring large DNA segments into recipient cells (Hamilton *et al.*, 1996). Efficient and reproducible protocols for *A. tumefaciens* mediated transformation have used super binary vectors in which the *A. tumefaciens* strain carries extra copies of *virB*, *virC*, and *virG* (Komari, 1990) to infect immature zygotic embryos of the inbred line A188 (Ishida *et al.*, 1996; Negrotto *et al.*, 2000) or the hybrid Hi II (Zhao *et al.*, 2001).

#### **1.5.4 *Agrobacterium* classification and host range**

The genus *Agrobacterium* has been categorized into a number of species. This classification is based for some part on disease symptomology and host range. *A. radiobacter* is non virulent, *A. tumefaciens* causes crown gall disease, *A. rhizogenes* causes hairy root disease, and *A. rubi*, causes cane gall disease (Gelvin, 2003) and *A. avitis*, causes galls on grapes and a few other plant species (Gelvin, 2002). Symptoms follow for the most part, the type of tumorigenic plasmid contained within a particular strain (Gelvin, 2003). Curing a particular plasmid and replacing this plasmid with another type of tumorigenic plasmid can alter disease symptoms. For example introduction of Ri plasmids into a cured strain, converts it to rhizogenic strain (Lam *et al.*, 1984; White and Nester, 1980). They can also be classified into biovars based on growth and metabolic characteristics (Keane *et al.*, 1970). The recent completion of *A. tumefaciens* C58 genome (which is composed of linear and circular chromosome, a Ti plasmid) (Goodner *et al.*, 1999) may provide a basis of reclassifying of *A. tumefaciens* 'strains' into true 'species'.

As a genus, *Agrobacterium*- can transfer DNA to remarkably broad group of organisms including numerous dicot and monocot angiosperms. *Agrobacterium* host range can be extended by varying bacterial strains and binary vectors, manipulating host plant physiology, modifying cultivation conditions and through the use of superior selectable markers (Kuta and Tripathi, 2005; Tadeusz *et al.*, 2005).

### **1.5.5 Ti-plasmid and virulence region**

The *Agrobacterium*-plant interaction is the only known natural example of DNA transfer between kingdoms. *A. tumefaciens* has the exceptional ability to transfer a particular DNA segment (T-DNA) of the tumor inducing (Ti) plasmid (200kb) into the nucleus of infected cells where it is then stably integrated into the host genome and transcribed, causing crown gall disease as in the family Solanaceae i.e. tomato, tobacco (Binns and Bock, 1989).

Three genetic components of *A. tumefaciens* are required for plant cell interaction. The first component is the T-DNA fragment, a 25 bp repeat motif, defined by the ‘left border’ LB and ‘right border’ RB. (Zupan, and Zambryski, 1997). These borders constitute the region recognized by the transfer system of *Agrobacterium* (Zambryski, 1992). The T-DNA contains two types of genes; the oncogenes, encoding synthesis of auxins and cytokinins and responsible for tumor formation and the genes encoding for the synthesis of opines. These tumor specific compounds which are produced by condensation between amino acids and sugars or keto acids are synthesized and excreted by crown gall cells and are used as source of carbon and nitrogen. Outside the T-DNA, are located the genes for opine

catabolism involved in the process of T-DNA transfer from the bacterium to the plant cell and the genes involved in bacterium –bacterium plasmid conjugative transfer (Hooykaas and Schilperroort, 1992; Zupan and Zambryski, 1997). The second component is the 35-kb virulence (*vir*) region also located on the Ti plasmid, which is composed of seven major loci (*VirA*, *virB*, *virC*, *virD*, *virE*, *virG* and *virH*). The protein products of these genes, termed virulence (*vir*) proteins, respond to specific phenol compounds such as acetosyringone and sinapinic acid to generate a copy of the T-DNA and mediate its transfer into the host cell. The third component is the suite of chromosomal virulence (*chv*) genes, located on the *Agrobacterium* chromosome. *Chv* genes are involved in bacterial chemotaxis and attachment to the wounded plant cell (Citovsky *et al.*, 1994; Zambryski, 1992). Any foreign DNA placed between the T-DNA borders can be transferred to plant cells, no matter where it comes from. This fact allowed the construction of first vector and bacterial strain systems for plant transformation (Hooykas and Shilperroort, 1992)

### **1.5.6 Molecular mechanism of T-DNA transfer**

The infection begins at the site of wound and the injured plant cells release some small quantity of phenolic compounds such as acetosyringone (AS) acting as specific signals to bind and activate *VirA*, a membrane protein. This activation activates *VirG* by phosphorylating one of its aspartate residues. The active form of *VirG* binds specifically to the upstream of other *vir* genes called *vir* box, inducing their expression. The *VirD1* and *VirD2* are responsible for the T-strand generation, a single-stranded copy of T-DNA, by specifically recognizing and cutting the

bottom strand at the two borders of which the RB is the start site and thus more important (Fig. 2).

The VirD2/T strand complex and another ss-DNA binding protein named Vir E2 are transferred separately into plant cells through a pilus like structure made of complex set of VirB proteins, and the latter does so with the help of another chaperon protein called VirE1, which probably also prevents the VirE2 from binding T-strand within the bacterial wall (Zupan *et al.*, 1996, Veena *et al.*, 2003). Once into the plant cell, many VirE2 molecules bind cooperatively to T-strand to form ds-T-DNA complex which is then targeted to the nucleus by nuclear target signals (NLS) of its associated VirD2 and Vir E2, where the T-DNA integrates into the plant genome as single or multiple copies (Zambryski, 1992).

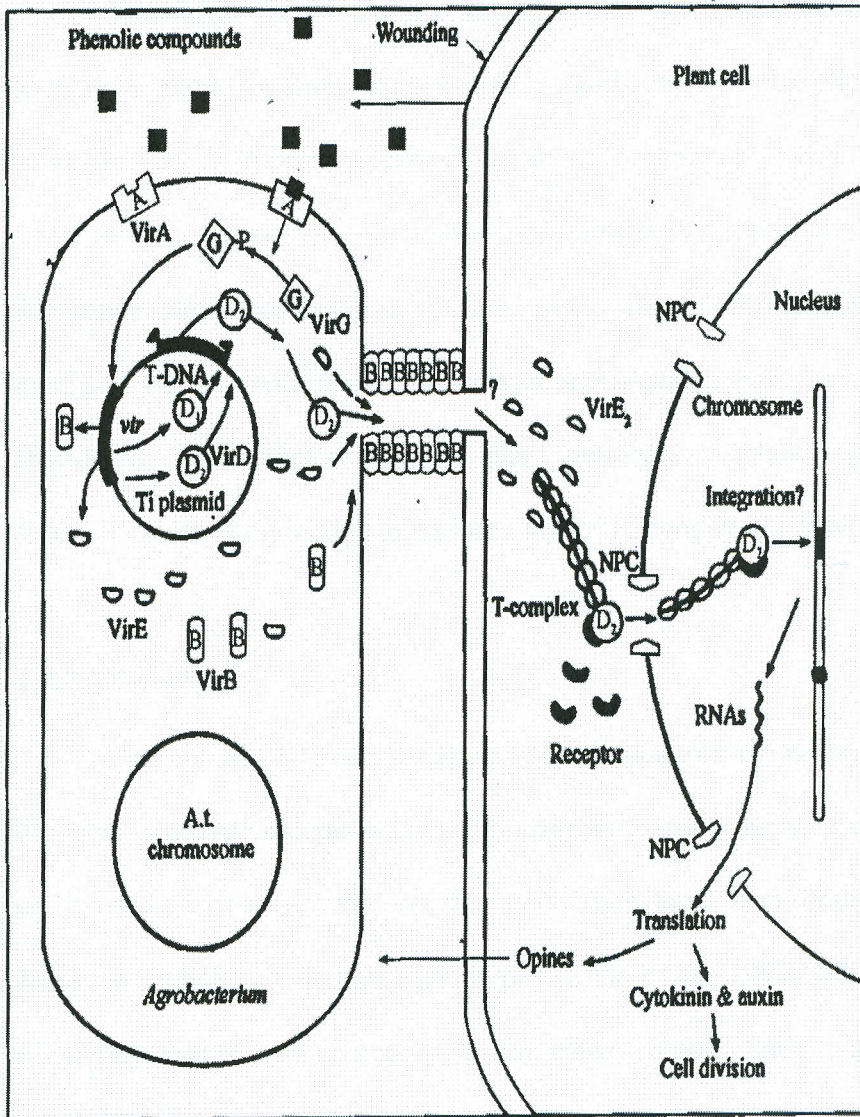


Figure 2: The process of plant cell transformation by *Agrobacterium* (Li *et al.*, 2000)

### **1.5.7 Factors influencing *Agrobacterium* mediated transformation**

Transgenic plants obtained via *Agrobacterium*-mediated transformation have been regenerated in more than a dozen monocotyledonous plant species ranging from the most important cereal crops to ornamental plant species. Efficient transformation protocols for these agronomically important crops have been developed and have become routine. Many factors influencing *Agrobacterium*-mediated transformation of monocotyledonous plants have been investigated and elucidated. These factors include plant genotype, explant type, *Agrobacterium* strain, medium composition and binary vector (Cheng *et al.*, 2004).

#### **1.5.7.1 Genotype**

Among all monocotyledonous species transformed so far, rice appears to be the least genotype independent. Using primarily embryogenic calluses or immature embryos as explants, over 40 rice genotypes have been transformed including Japonica, Indica and Javanica genotypes (Chan *et al.*, 1992, 1993). Transformation efficiency varies among genotypes. In wheat, maize, barley, and sugarcane, the model genotypes used in micro-projectile-mediated transformations have worked well for *Agrobacterium*-mediated transformation (Gordon-Kamm *et al.*, 1990). Transgenic plants have recently been produced from elite cultivars or lines of several cereal crops such as sorghum, barley and maize. (Zhao *et al.*, 2001; Wang *et al.*, 2001).

### 1.5.7.2 Explants

Initially Hiei *et al.* (1994) reported that embryogenic callus derived from mature seeds of japonica rice was the best target explant for *Agrobacterium*-mediated transformation due to its active cell division. Later transformation of embryogenic callus was extended to other genotypes (Rashid *et al.*, 1996) or species such as wheat (Cheng *et al.*, 1997) and asparagus (Limanton-Gevet and Jullien, 2001).

Freshly isolated immature embryos were later found to be the best explants for rice genotypes (Aldemita and Hodges, 1996), maize (Ishida *et al.*, 1996; Zhao *et al.*, 2001), and barley (Tingay *et al.*, 1997). To date, freshly isolated immature zygotic embryos are still the best explant reported to be highly competent for *Agrobacterium* infection in maize (Frame *et al.*, 2002; Gordon-Kamm *et al.*, 1990; Negrotto *et al.*, 2000; Zhang *et al.*, 2002; Zhao *et al.*, 2001).

Suspension culture cells were initially reported to be suboptimal explant choice for rice transformation (Hiei *et al.*, 1994). However they were initially shown to be an excellent target not only for rice (Urushibara *et al.*, 2001), but also for other species such as maize (Rout *et al.*, 1996), barley (Wu *et al.*, 1998), wheat (Cheng *et al.*, 1997), banana (Ganapathi *et al.*, 2001), sugarcane (Arencibia *et al.*, 1998) and forage grasses (Bettany *et al.*, 2003).

Rice seedling sections and inflorescence were transformed efficiently when explants were preincubated with necrotic reduction medium. Meristemic tissues that were not preincubated with necrotic reducing agents showed low transformation efficiency (May *et al.*, 1995; Kisaka and Kameya, 1998).

Zhao *et al.* (2001) reported that the source of explant had significant effect on transformation frequency. Immature embryos from field grown stock plants could be transformed more efficiently than immature embryos from glass-house grown stock plants of sorghum (Binott *et al.*, 2006).

### **1.5.7.3 *Agrobacterium* strains and plasmids**

Following the success of transformation of rice using the combination of standard binary in super virulent strain and super binary vector in a regular strain such as pTOK233i LBA4404, many subsequent reports were performed using identical or similar combination in crops such as rice (Rashid *et al.*, 1996) and maize (Ishida *et al.* 1996; Negrotto *et al.*, 2000; Zhao *et al.*, 2001). Although these combinations were not necessary for efficient transformation in some cases (Enriquez-Obregon *et al.*, 1999; Cheng *et al.*, 1997), the wide application of these strains and vector combinations in many plant species indicate that it may influence transformation of monocot species.

In maize and sorghum efficient transformation systems were established only with super binary vectors in LBA4404, while a standard binary vector in super virulent strain showed low transformation frequency even with improved co-culture conditions in maize (Frame *et al.*, 2002).

To date, published reports of successful monocot transformation via *Agrobacterium* described only three different strains namely: LBA4404, disarmed C58, and EHA101; and their derivatives. The use of various other strains and the combination with super binary vectors or binary vectors with constitutively active

*virG* may further improve transformation efficiency in many or all monocots species (Hansen. *et al.*, 1994).

### **1.5.8 Reporter genes and transient expression in plants**

Most of the DNA introduced into cells by direct gene transfer is degraded and a small fraction becomes stably integrated into the host genome. The level of the gene expressed in the cell forms the basis of transient assays. They are used for rapid preliminary evaluations of foreign gene expression (*Li et al.*, 1995; Register, 1997). Therefore transient expression can be defined as the expression of introduced genes that have not been integrated into host genome. The presence of a transgene can be demonstrated by means of transient assays. These assays are based on the expression of a gene called reporter gene. This is fused to the upstream region of the gene under study replacing the original cloned gene. When cloned into the host organism, the expression pattern of the reporter gene will exactly mimic that of the original gene as the reporter is under the same influence. The reporter gene must be chosen with care. The first criterion is that the reporter gene must code for a phenotype not already displayed by host plant (Galun and Breiman, 1997). The phenotype of the reporter gene must be relatively easy to detect and quantify.

A variety of different reporter genes have been used in studies of gene expression and regulation. By using a reporter gene that encodes an enzyme activity not found in the organism under study, the sensitivity with which chimeric gene activity can be measured is limited only by the properties of the reporter enzyme and the quality of the available enzyme assays (Jones *et al.*, 1987).

The reporter genes that are most often used include genes encoding enzymes with distinct substrate specificities that can be monitored radiochemically, histochemically, fluorometrically and spectrophotometrically. The most commonly used reporter genes are derived from bacteria, insects or jellyfish.

More than six reporter genes have been used in the studies of gene expressions in higher plants. These includes the *E.coli*  $\beta$ -galactosidase (*LacZ*), Chloromphenical acetyl transferase (CAT), neomycin phosphotransferase (APH3"11 NPT11), nopaline synthase (NOS), octopine synthase (OCS), firefly luciferase (LUC) (De wet *et al.*, 1985) green fluorescent protein (GFP) (Ow *et al.*, 1986) and  $\beta$ - glucuronidase (GUS) (Jefferson, 1987).

#### **1.5.8.1 $\beta$ -glucuronidase or GUS A gene**

Following the limitations of the use of other reporter genes, *E.coli*  $\beta$ -glucuronidase reporter gene was constructed. The *gus* gene has a monomer molecular weight 68.2kd (Kuroyama *et al.*, 2001). The behaviour of native enzyme on gel filtration columns indicates that it is probably a tetramer. The *gus* gene is very stable and will tolerate many detergents, widely varying ionic conditions and general abuse. It is most stable in the presence of thiol reducing agents such as DTT and  $\beta$ -mercaptoethanol.  $\beta$ -glucuronidase has no cofactors, nor any ionic requirements (Jefferson, 1985). Because *gus* is inhibited by heavy divalent metal cations such as  $\text{Cu}^{2+}$  and  $\text{Zn}^{2+}$ , it is important to include EDTA when carrying out the assays (Stoeber, 1961).  $\beta$ -glucuronidase can be assayed at any physiological pH with optimum 5.5 and 8.0. Even within lower pH of 4.4, the enzyme is 50% active.

It is resistant to thermal inactivation with a half life at 55<sup>0</sup> C of two hr. The  $\beta$ -glucuronidase has been purified, cloned and sequenced, and encodes a stable enzyme that has desirable properties for construction and analysis of gene fusions. The coding region of the  $\beta$ -glucuronidase was isolated from *E.coli* (Jefferson *et al.*, 1987).

#### **1.5.8.2 Histochemical staining for $\beta$ -glucuronidase activity**

A method for histological and microscopic manipulation of plants, including sectioning of tissues by hand has been described by O'Brien and McCully (1981) and Jefferson (1987). The best substrate currently available for histochemical localization of  $\beta$ -glucuronidase in tissues and in cells is 5-bromo-4 chloro-3 indoyl  $\beta$ -D glucuronide (X-Gluc). This enzyme works well giving a deep indigo/ blue precipitate at the site of the enzyme activity. The  $\beta$ -glucuronidase is suited as a reporter gene in transgenic plants because intrinsic- $\beta$ -glucuronidase is absent from most if not all higher plants. The enzyme catalyzes hydrolytic reactions of miscellaneous substrates resulting in disruption of glycosyl bonds. The quality of histochemical localization is affected by all aspects of tissue preparation and fixation as well as the reaction itself. The product of glucuronidase reaction is not coloured. Instead, the indoyl derivative produced must undergo oxidative dimerization to form the insoluble and highly colored indigo dye. This dimerization is catalyzed by atmospheric oxygen and can be enhanced by using an oxidation catalyst such as K<sup>+</sup> ferricyanide/ferrocyanide.

An alternative and cheaper histochemical assay for *gus* uses naphthol ASBI-glucuronide, which is then cleaved to liberate the free naphthol ASBI, then coupled to diazo dye (Jefferson *et al.*, 1988). Fixative conditions vary with tissue and its permeability. Glutaraldehyde does not easily penetrate the leaf cuticle but it is readily available to stem cross sections. Formaldehyde seems to be a better and gentle fixative than glutaraldehyde and can be used over along time. After staining, clearing the tissues with 70% ethanol seems to improve contrast in many cases (Jefferson, 1987).

### **1.6 Selectable marker genes**

Selectable genes encode for proteins that render transformed plants resistant to phototoxic agents (Galun and Breiman, 1997). They are added into culture media converting it into selective media. Tissues and organs are transferred to selective media after transformation. The phytotoxic agent is added in all tissue culture stages. Usually the plant to be transformed must be very sensitive to phytotoxic agent and the presence of selective gene must eliminate the sensitivity completely. There are several categories of selective genes. Those that confer resistances to antibiotics, herbicides and high nitrate and high amino acids levels. The most commonly used antibiotic selectable marker is neomycin phosphotransferase (NPT II), which detoxifies many aminoglycoside antibiotics such as kanamycin, paromomycin vistamycin and neomycin. A plant differs in response to phytotoxins. The other one is hygromycin phosphotransferase (HPT). An example of a herbicide selectable marker is phosphinotricin acetyltransferase (PAT) which is encoded for

by two bacterial gene *bar* and *pat*. PAT is an inhibitor of glutamine synthetase and is widely used in plant transformations as an herbicide.

### **1.6.1 *Bar* and *pat* (Glufosinate resistance)**

The *bar* gene from *Streptomyces hygroscopicus* and *pat* gene from *S. viridochromogenes* both encodes for the enzyme. phosphinothricin acetyltransferase (PAT) *Streptomyces* species (Eubacteria) are members of Actinomycete family and are found in the soil. PAT inactivates phosphinothricin, (PPT) or bialaphos the active component in glufosinate. Bialaphos is potent inhibitor of the key enzyme in the nitrogen assimilation pathway, glutamine synthetase (GS) (Wehrmann *et al.*, 1996; De Block *et al.*, 1987). There are no other reported substrates for PAT, nor has it demonstrated any activity towards acetyl transferase substrate (Bell and Charlwood, 1980). Expression of this enzyme allows for selection of transformed plant cell as well as whole plant tolerant to glufosinate.

### **1.6.2 Gene sequence promoters**

#### **1.6.2.1 Cauliflower mosaic virus (CaMV) 35S promoter -p35SmRNA subunit**

Cauliflower mosaic virus is a double stranded, circular DNA virus of approximately 8000bp that infects a number of plants in cruciferae family (Assad and Signer. 1990; Pringle, 1999). The viral segments employed in the plant expression vectors are very well characterized (Odell *et al.*, 1985). It involves the duplication of enhancer region of the promoter leading to an elevated level of expression of most genes driven by it.

### **1.6.3 Confirmation of stable integration and inheritance of transgene**

To obtain stable transformation, a functional copy of the introduced gene must be incorporated into the plant genome so that it is replicated and transmitted in subsequent progenies i.e. expressed (Rathus and Birch, 1992; Walden and Wingender, 1995). Mendelian inheritance and expression pattern of foreign genes is the ultimate evidence needed to confirm stable integration of functional genes into host plants. The aim of any transformation method is to produce normal transgenic and fertile plants. Southern blot analysis and polymerase chain reaction (PCR) are used to demonstrate the presence of foreign DNA in plant genome (Morrish *et al.*, 1993).

### **1.7 Justification**

Maize remains Kenya's staple food and food security crop. Despite its importance, its production is on the decline (Ayaga, 2003). It is increasingly becoming evident that Kenya, just like the rest of African countries did not benefit much from the green revolution. The reasons are many and complex. Among them are biotic (diseases, pests, nematodes, fungi) and abiotic factors (drought, high salinity). The current methods of controlling the factors contributing to low production are either expensive, have environmental or health concerns. Breeding for improved varieties using conventional approaches has limitations such as the difficulty in transferring quantitatively controlled genes and that the process takes a lot of time (more than 15yrs). Using biotechnological tools such as genetic engineering to breed for resistance against the factors contributing to reduction in production can contribute

significantly to improved livelihood and increased food security in Kenya's households. Identifying suitable genes that confer resistance to abiotic and biotic stress factors can be introduced to the maize genome via genetic engineering tools such as *Agrobacterium tumefaciens* or gene gun system. However, a reliable *in vitro* plant regeneration protocol is a prerequisite for application of biotechnological methods in crop improvement. Extensive regeneration and transformation studies have been undertaken on elite temperate maize lines such as A188, B73 and B79, and hybrids derived from them and no attention has been focused on tropical maize varieties (Bohorova *et al.*, 1995, Prioli and Silva, 1989). There is dire need to develop a regeneration system for tropical maize genotypes and preferably the locally adapted Kenyan maize genotypes. The current study is pegged on the central role maize plays in enhancing food security and improved livelihoods in this country and it is aimed at extending regeneration and *Agrobacterium*-mediated transformation of Kenyan maize genotypes currently used in breeding programs, commercial production and open pollinated varieties (OPVs).

### **1.7.1 Null hypothesis**

- It is impossible to regenerate Kenyan maize genotypes
- It is impossible to transform Kenyan maize genotypes using *A. tumefaciens*

### **1.7.2 Broad objective**

To explore the regeneration potential and transformation of Kenyan maize genotypes using *A. tumefaciens* system.

### **1.7.2.1 Specific objectives**

(i) To establish the effect of genotype and agroecological zone on the days to flowering of selected Kenya maize genotypes.

(ii) To determine the effect of physiological age of embryo on callus induction efficiency.

(iii) To evaluate significant difference if any in embryogenic callus formation between parental inbred lines and their respective single cross hybrids

(iv) To evaluate the potential of the formation of somatic embryos to transform into plantlets.

(v) To optimize factors that enhance transient *Gus* expression in immature zygotic embryos derived from selected Kenyan maize genotypes.

## CHAPTER TWO

### MATERIALS AND METHODS

#### 2.1. Source of planting material

Twelve parental maize inbred lines and their single cross hybrids seeds were used as genetic material in this study to generate embryos that were used to assay *in vitro* tissue response and subsequent transformation. The seeds were kindly donated by Dr. Jane Ininda, a KARI breeder based at KARI-Muguga Research Station, Nairobi.

#### 2.2 Choice of target maize lines

The choice of maize lines was based on their good agronomic traits such as high yields, drought tolerance, resistance to maize streak virus; *turcicum* leaf blight resistance and *Striga* weed (Table 2). The inbred lines were identified as parents of major hybrids in commercial production or currently used in breeding programs in Kenya

#### 2.3 Sample size

A total of eighteen genotypes used in this study: Twelve were parental inbred line combinations that gave rise to six single cross hybrids. Each line was sampled in triplicates.

**Table 2.** List of pedigree- parental inbred lines and their respective hybrids, the history and their special attributes

<b>Pedigree inbred lines (TL)</b>	<b>History and agronomic importance</b>	<b>Pedigree-hybrids</b>	<b>History and agronomic importance</b>
TL08	YS, EM, MY,MSr	TH21 (TL08 x TL09)	PS, LM, HY
TL09	PS, EM, HY		
TL18	YS, LM, HY,MSr	TH23 (TL18 x TL19)	PS, LM, HY MSr
TL19	PS, EM, HY		
TL20	PS, LM,LY	TH24 (TL20 x TL21)	YS,LM, HY
TL21	YS, LM, MY		
TL22	YS, MM, LY,TBr	TH25 (TL22 x TL23)	YS, EM,HY, MSr
TL23	YS, LM, LY,MSr		
TL24	PS, MM, LY TBs	TH26 (TL24 x TL23)	PS, LM, HY,
TL23	YS, LM, LY		
TL26	PS, LM, HY	TH27 (TL26 x TL27)	PS,LM, LY,TBr
TL27	YS, LM, LY,TBr		

**Abbreviations:**

YS	yellow stem
PS	purple stem
EM	early maturing
MM	medium maturity
LM	late maturity
HY	high yielding
LY	low yielding
MY	moderate yielding
MSr	maize streak virus resistant
MSs	maize streak virus susceptible
TBr	<i>turcicum blight</i> resistant
STr	<i>Striga</i> resistant

**2.4 Planting site selection**

The maize genotypes were planted in three different geographical sites namely: Kiboko Research Station, Kabete-NARL Station and Muguga Research Station (Table 3). Kiboko was chosen because it is a hot area and resulting in early maturity of the embryos and many generations per year. Muguga was selected as a

site for multiplying the seed material, while Kabete chosen because of proximity and easy availability of the embryos incase there were problems with accessing the lines in Kiboko. Drip irrigation was used in Kabete while overhead irrigation system supported planting in both Muguga and Kiboko station. The effect of maize genotype and agroecological zone (AEZ) on days to flowering between Kiboko and Kabete was evaluated. Muguga was not evaluated because it fell under the same AEZ as Kabete Station (Table 3).

**Table 3:** Climatic and geographical distribution of experimental sites

Site selection	Mean temp in °C	Mean annual rainfall (mm)	Altitude (m)	Agroecological zone(AEZ)
Kabete	18	1200	1900	LH
Kiboko	26	595	975	LM5
Muguga	16	1500	2200	LH

Source: Ralph and Helmut. (1983)

### 2.5 Planting design

All the maize genotypes were planted in randomized complete block design (RCBD). The experiment was replicated three times. One seed was planted per heel in a total of ten heels. Each seed was planted at a spacing of 25cm from each other and an intra row spacing of 75cm. During planting, animal manure was used to supplement DAP<sup>TM</sup> fertilizer. Furadan<sup>TM</sup> was applied on top of the seed and then the furrows were covered with soil. Planting was carried out in staggered manner at an interval of three weeks to ensure the availability of embryos at all times. In all the three sites, over head irrigation system was applied to take care of rainfall unreliability in some season.

## **2.6 Germination viability**

Viable seeds stored at +4<sup>0</sup> C were obtained from KARI-Muguga. After two weeks of planting, the number of seeds that germinated from each genotype was noted.

$\frac{\text{Maize seed germinated per genotype (MG)}}{\text{Total number of seed planted per genotype (MP)}} \times 100 = \text{Germination viability (GV)}$

## **2.7 Crop management practices**

Weeding was done periodically during the plant growth regime. Watering by irrigation was done optimally 4 hrs per day at one day interval or none at all if there was enough rainfall. CAN<sup>TM</sup> fertilizer about (10gm) was applied near the bases of the maize plants when the plants were about knee high while ensuring the soil was moist enough to avoid the corrosive effects of the fertilizer. Bulldock<sup>TM</sup> was also applied on the tassels to prevent infestation of insect pest.

## **2.8 Flowering stage / pollination**

The experimental maize genotypes were planted on the same day in both Kiboko and Kabete-NARL with the aim of establishing which zone could allow early or late maturity of the cultivars. Maize ears were covered with transparent silking bags (8x 6) to avoid the unwanted pollen from surrounding plants (Once the pollen was 80% mature the tassels were covered with brown manila pollination bags for 24hrs then tapped when it was hot and transferred from a desired male to the recipient female silk within the same plant (plate 1a) (self pollination) and then clipped to keep away foreign pollen (Plate1b). In the case of cross pollination, pollen was transferred from the desired male from one genotype to the silk of

desired male pollinator to recipient female from a different genotype or same plant. Hand /directed pollinations were necessary to ensure genetic identity.

After 24hrs, any foreign pollen that could have been transferred to the tassel by wind or any other vector died. Pollen can be viable for 10-30 minutes but at most 8hrs (Coe *et al.*, 1988) depending on environmental factors.eg under refrigerated conditions.



**Plate 1.** Pollinated maize crop in the field. A: Tassel with mature pollen  
B: Pollinated maize ear

### **2.8.1 Generation of hybrids by cross pollination**

Hybrids were generated from crossing the parental inbred lines with well defined males as sources of pollen to silk of ears from different maize genotypes. Their embryos were then used as explants for callus induction. The  $F_1$  and  $F_2$  generation embryos were also used for callus induction.

### **2.8.2 Generation of inbred lines by self pollination**

Inbred lines were either selfed or sibbed to obtain the immature zygotic embryos which were used as sources of explants for callus induction.

## **2.9 Explants harvesting, surface sterilizations and embryo excision**

Ears from six hybrids and twelve inbred lines were harvested when they were 10, 15, 18, 21 and 24 days after pollination. They were stored at +4 °C for 3-4 days for later use. They were dehusked, chopped into small pieces and surface sterilized in 70% ethanol for 2 minutes and subsequently incubated for 15 minutes in 2.5% sodium hypochlorite with a few drops of tween 20 (Biochemie Duchefa). The surface sterilized ears were rinsed three times with sterile distilled water (H<sub>2</sub>O.)

All the procedures were carried out in laminar flow. All the apparatus used to excise were previously sterilized with 70% alcohol followed by burning with bunsen flame until they turned red hot. They were then allowed to cool.

Following sterilization, the top half of the kernels were cut off using a scalpel while holding with a forceps under aseptic conditions. The endosperm and the embryo were gently separated using a spatula with curved edge.

## **2.10 Evaluation for *in vitro* tissue response**

The medium used was based on N6 vitamins and basal salts.(Chu et al., 1975). Other media salt formulation was later used in the culture including full strength (MSO) and half strength (½MSO) from Murashige and Skoog (1962).

### **2.10.1. Callus initiation (induction)-CIM**

The immature embryos were used as explants to initiate callus induction. The media comprised of N6 microelements, macro elements and N6 vitamins. The macroelements were prepared in x 10 stock solutions while microelements were

prepared in x 100 stock solutions enough to make 1000 ml of the final concentration of N6 basal salts. Dichlorophenoxyacetic acids 2,4-D was the only used plant growth regulator (PGR) at 2mg/L (Biochemie Duchefa). This was supplemented with 25 mM proline, 0.2g/L casein hydrolysate. The vitamins used included nicotinic acid 0.5 mg/L, pyridoxine 0.5 mg/L and thiamine-HCl 1 mg/L. The pH was adjusted to 5.8 using 1 M NaOH before addition of 3 g/L gelrite (Duchefa, Biochemie). Media was sterilized by autoclaving for 15 minutes at optimal pressure of 15 psi at 115<sup>0</sup> C (All American model). Once cooled, the sterile media was poured into 100 mm x 25 mm sterile Petri plates (Greiner bio-one) in the hood. Embryos were then aseptically excised using a sterile scalpel and plated onto callus induction media with the scutellum axis facing down.

A total of twenty one (21) embryos were plated per genotype replicated three times. The plates were wrapped tightly with parafilm (American parafilm) and incubated in the dark for 2-3 weeks at 26 ± 2<sup>0</sup>C. The number of embryos induced per plate and optimal embryo stage for callus induction was noted and the % callus induction calculated.

### **2.10.2 Callus scores**

The callus induction efficiency was determined by the number embryos forming callus over the total number of embryo plated on callus induction expressed as percentage.

### **2.10.3 Callus maintenance medium (CMM)**

The media composition for this stage was the same as that for callus induction. The presence of three morphotypes of calli was evaluated: Type 0 calli comprised those calli that germinated precociously. Type I calli comprised hard translucent coral like and Type II calli comprised friable soft and loosely packed slow growing calli. Subculture and transfer into fresh media was done after every 2-3 weeks. The callus was maintained for up to 3 months. Contaminated, browned tissues and those that were broken were discarded. Type I and Type II calli were carefully selected and transferred to somatic maturation.

### **2.10.4 Somatic embryo maturation medium (-MaM)**

This media composition was the same as that for CIM but with 6 % sucrose instead of 2 % and devoid of 2, 4-D. The Type I and II callus were carefully selected and subcultured into this media and kept in the dark for 2-3 weeks until the embryos turned brittle white. The numbers of embryogenic calli per plate were noted and % somatic embryos calculated.

### **2.10.5 Shoot formation**

The white embryogenic calli were carefully selected, dissected and transferred into full MSO. The media comprised of 4.43g/L complete MS. The somatic embryos were incubated under photoperiod regimes of 16hr light and 8 hr darkness at  $26 \pm 2^{\circ}\text{C}$  before subculture to fresh media after 2-3 weeks. The white surfaces of embryos gradually turned green and shoots emerged.

### **2.10.6 Rooting of plantlets**

The regenerated shoots were transferred into rooting media. The media was composed of half strength MSO i.e. 2.215g/L complete MS. They were kept in full light for 2-3 weeks until the roots were well established.

### **2.10.7 Acclimatization of regenerants**

Plantlets with well established roots were washed with warm sterile water to get rid of gelrite, then transferred to small pots containing sterile vermiculate and sand mixed in the ratio 3:1 respectively. They were maintained for 7 days in the green house while covered with transparent polythene bags to avoid heat shock.

### **2.10.8 Transfer maize plants into the soil and maturation**

The plants were then transferred into 5L plant pots containing phymix<sup>TM</sup>, vermiculate and sand mixed in the proportions 3 : 1 : 1 and transferred to the green house to progress with growth. The regenerated lines were targeted for transformation with plasmids carrying *gus* reporter genes and *bar* selectable markers.

## 2.11 Transformation protocol

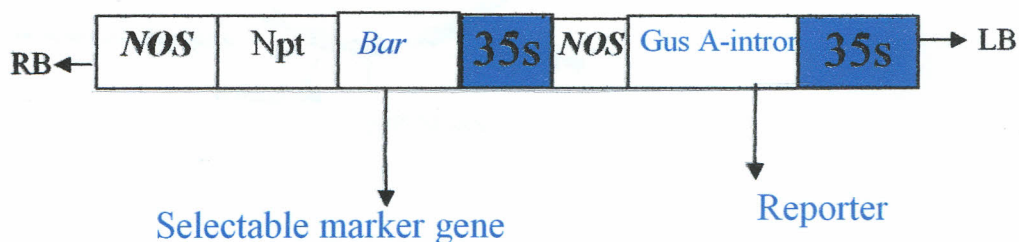
The protocol was adopted from Register *et al* 1994. With slight modification.

### 2.11.1 *Agrobacterium* strains and plasmid culture

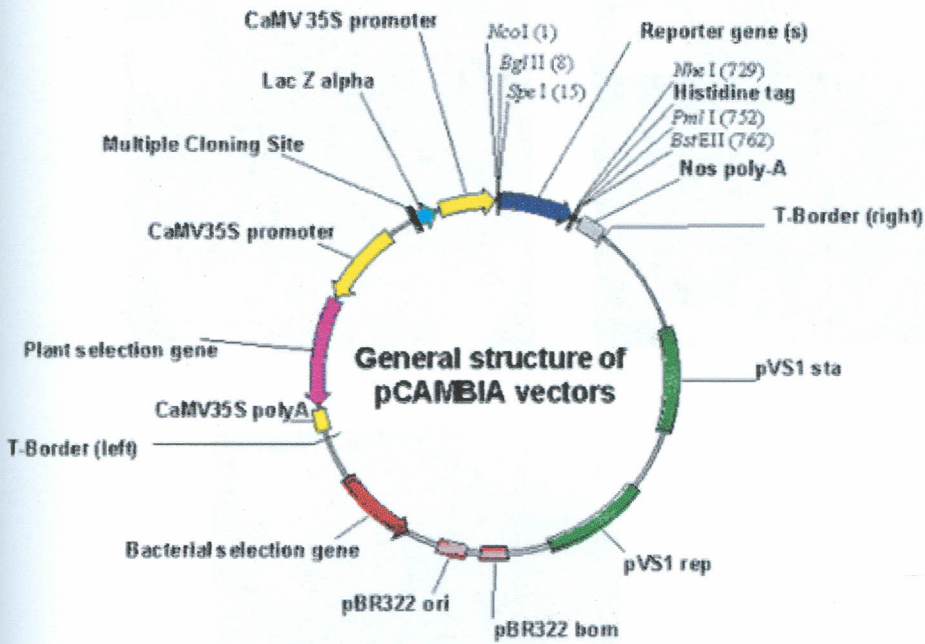
The *Agrobacterium tumefaciens* strain LBA4404, EHA101 and AGL1 carry pBECK plasmid and LBA4404, EHA105 and GV2301 carrying pCAMBIA plasmid were used to infect immature. pBECK plasmid harboured p35S *gus* intron reporter gene encoding  $\beta$ -glucuronidase. and p35S-*bar* selectable marker gene encoding phosphinothricin acetyltransferase NOS terminator gene under powerful control of cauliflower mosaic virus (CaMV35S) (Fig. 3). pCAMBIA carried p35s *gus* intron reporter gene, *Kan*<sup>R</sup> under powerful control of CaMV35S promoter (Fig. 4). The gene constructs were obtained from University of Missouri at Columbia, USA and the Donald Danforth Plant Science, Center, St, Louis, Missouri, USA.

The *Agrobacterium* strains were streaked from stocks stored at -80<sup>0</sup> C in 75% glycerol. The strains carrying pBECK were cultured on 3 % Luria Broth (LB) agar (pH 7.2) for 3 days at 26<sup>0</sup> C (Plate 2) then suspended in 15 ml 2 % LB broth (pH 5.8) containing 200 mg/l spectinomycin and incubated for one day. Similarly, pCAMBIA was cultured on 3% LB agar (pH 7.2) for 3 days at 26<sup>0</sup> C suspended in 15 ml 2 % LB broth (pH 5.8) containing 50 mg/L of rifampicin and kanamycin each. The 15 ml agrosuspension was transferred into a 50ml falcon tube and to shake at 150 rpm and incubated at 26<sup>0</sup> C for three days.

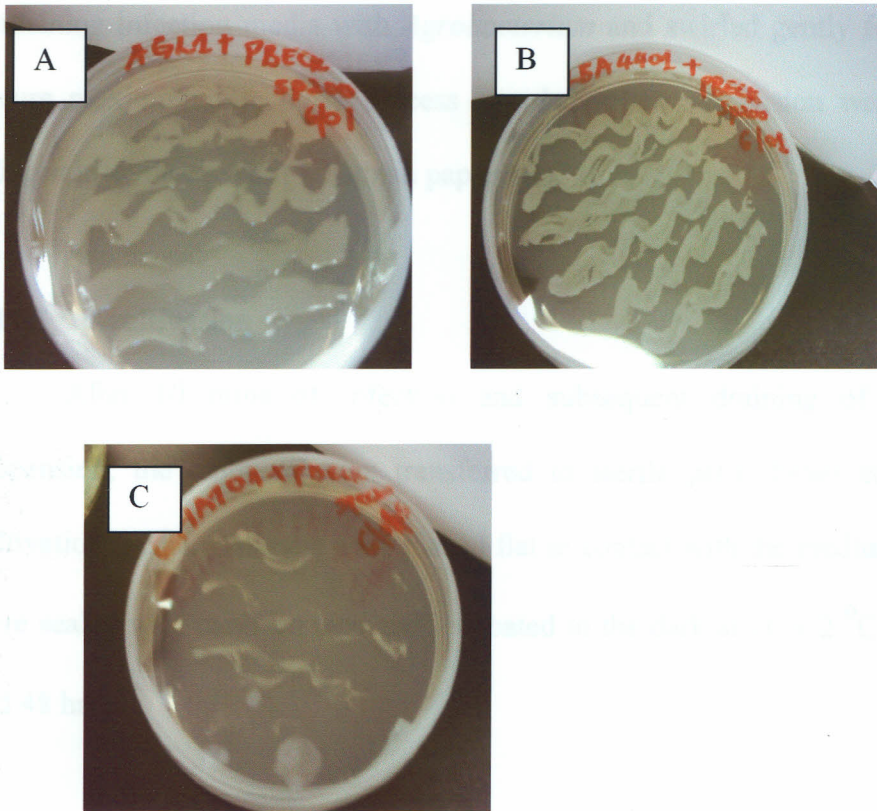
### pBECK2000.4 plasmid linear map



**Figure 3:** Linear map of pBECK plasmid carried by *Agrobacterium* strains LBA4404, AGL1 and EHA101 in the infection of immature zygotic embryos. It is a dual expression vector with *UidA* reporter gene encoded by  $\beta$ -glucuronidase enzyme and selectable marker *bar* gene encoded phosphinothricin acetyl transferase both under control of p35S promoter from Cauliflower mosaic virus NOS-nopaline synthase terminator. Adapted and modified from Deblaere *et al.* (1987)



**Figure 4:** Plasmid map of pCambia that is carried as binary vector by *Agrobacterium* strains LBA4404, EHA101 and EHA105 in the infection of immature zygotic embryos. It harboured reporter gene (*GUS*) and bacterial selection gene (*Kan<sup>R</sup>*) and plant selection gene (*bar*) under powerful control of CaMV35S promoter (<http://www.cambia.org/daisy/cambia/home.html>)



**Plate 2:** *Agrobacterium* strains streaked on 3% LB agar (p.H 7.2) for maintenance at +4<sup>0</sup> C for 2 weeks. A: AGL1-pBECK sp<sup>200</sup>. B: LBA4404-pBECK sp<sup>200</sup>. C: EHA10-pBECK sp<sup>200</sup>

### 2.11.2 Agroinoculations and infection

After three days of culture, the *Agrobacterium* suspension was removed and centrifuged at 3000 rpm for 5 mins. The supernatant was discarded and the pellet (containing agro) was reconstituted to 5 ml by addition of sterile infection media (Appendix II) and 100  $\mu$ M from (100 mM stock) acetosyringone (AS). The suspension was allowed to sit for 2 mins and shaken to make even suspension. The optical density (OD) was considered optimal between 0.35-0.45. The re-suspension tube was incubated at 28<sup>0</sup>C with continuous shaking at 150rpm for 4-5 hrs. Immature zygotic embryos were then transferred to 2 ml volume eppendorf tube

containing infection media with *Agrobacterium* and swirled gently for 10 mins to ensure maximum infectivity. Excess *Agrobacterium* suspension was drained off using sterile filter paper (Whatman paper # 1).

### **2.11.3 Co-cultivation**

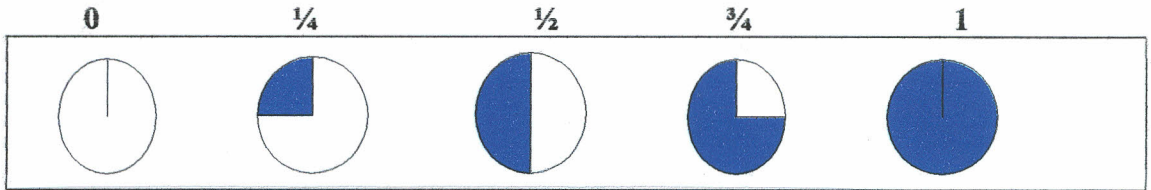
After 10 mins of infection and subsequent draining of excess agro suspension, the embryos were transferred to sterile petri dishes containing co-cultivation media. Embryos were placed flat in contact with the medium. The plates were sealed with parafilm tape and incubated in the dark at  $26 \pm 2$  °C for 6, 12, 24 and 48 hrs.

### **2.11.4 Histochemical analysis of transient *gus* expression**

Histochemical  $\beta$ -glucuronidase assays described by Jefferson (1987) were used to assess transient gene expression. The embryos were removed from co-cultivation media and transferred to 500  $\mu$ l of 0.3 % formaldehyde fixative in 0.3% mannitol and 10 mM MES (pH 5.6.) for 60 mins. The fixative was washed off by rinsing three times using 50 mM  $\text{NaH}_2\text{P}_0_4$  (pH 5.8) buffer. A minimum of 5 embryos were immersed in about 300 $\mu$ l of X-Gluc (5-bromo, 4-chloro, 3-indoyl - $\beta$ -D -glucuronide (5.0mg/L) in three replicates and incubated at 37 °C for 6, 12, 24 and 48 hrs after agro infection. The embryos were then transferred into a 500  $\mu$ l 70 % ethanol for 60 mins. The expression of  $\beta$ -glucuronidase was confirmed by presence of blue foci /pigmentation.

### 2.11.5 Levels of histochemical localization of $\beta$ -glucuronidase

Transient  $\beta$ -glucuronidase expression levels were scored as follows in the five embryos subjected to histochemical staining. It is assumed that the embryo was divided into 4 equal portions (Fig. 5).



**Figure 5:** Level of *gus* activity following incubation on X-gluc substrate

Absence of blue foci.....	0 (no stain at all)
1/4 .....	
1/2.....	
3/4 .....	
Fully stained .....	1

### 2.11.6 Estimation of transformation frequencies

The transformation frequency was estimated from the ratio of embryogenic tissues showing evidence of gene expression to the total treated or subjected to transformation. This was on the basis of *gus* activity (Pena *et al.*, 1997; Pua and Achar, 2000).

### 3.11.7 Statistical analysis

Analysis of variance (ANOVA) was performed used to evaluate the response of 18 maize genotypes to callus induction, optimal embryo stage to initiate callus, formation of somatic embryo , titration for optimal concentration for 2,4-D and

infectivity of different *A. tumefaciens* strains on different maize genotypes, at different physiological stages carrying different plasmids incubated at; 6,12, 24 and 48 hrs on X-Gluc. The ANOVA was done using SPSS soft ware (Version 9.0) with associated Student Newmanns Keuls test (SNK) at 95% level of confidence ( $p<0.05$ ). Means were separated using SNK.

## CHAPTER THREE

### RESULTS

#### 3.1 Seed germination viability.

The results obtained demonstrated that there was no significant difference between hybrids and inbred lines ( $p>0.05$ ). Maize genotypes were similar in germination viability in both Kabete and Kiboko (Table 4).

**Table 4:** Seed germination viability of inbred lines and hybrids in both Kiboko and Kabete

Maize genotype	No.of seeds	Germination viability (%)±SE	
		Kiboko	Kabete
TL(inbreds)	10 /genotype	89.3± 1.3 a	88.6± 1.8 a
TH(hybrids)	10/genotype	90.1±0.8 a	89.2 ±1.2 a

Means followed by the same letter within a location and between location are not significantly different at  $p<0.05$

#### 3.2 Effect of genotype and agroecological zone on days to flowering

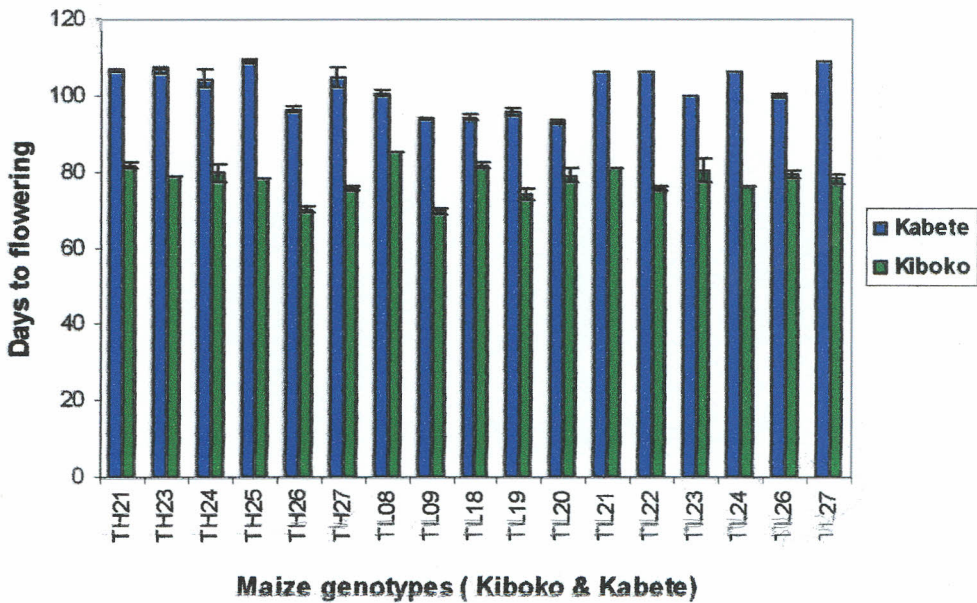
The influence of the flowering period by genotype in both Kabete and Kiboko was investigated by comparing the flowering period of both hybrids and inbred lines in Kiboko and Kabete. The results showed that the flowering period for both the inbred lines and hybrids did not differ significantly ( $p>0.06$ ) in the same site but differed significantly ( $p<0.05$ ) across the two sites. The results show that both the hybrids and inbred maize lines flowered at a significantly earlier period in

Kiboko than Kabete ( $p < 0.0001$ ). On average, the mean flowering period was 78 days in Kiboko while it was 102 days Kabete (Table 5 and Figure 6).

**Table 5:** Average Days to flower of inbred lines and hybrids in both Kiboko and Kabete

Maize genotype	No.of seeds	Average days to flower $\pm$ SE	
		Kiboko site	Kabete site
TL(inbreds)	10/genotype	78 $\pm$ 0.8a	100.3 $\pm$ 0.2b
TH(hybrids)	10/genotype	78.8 $\pm$ 1.8a	104.6 $\pm$ 1.2b

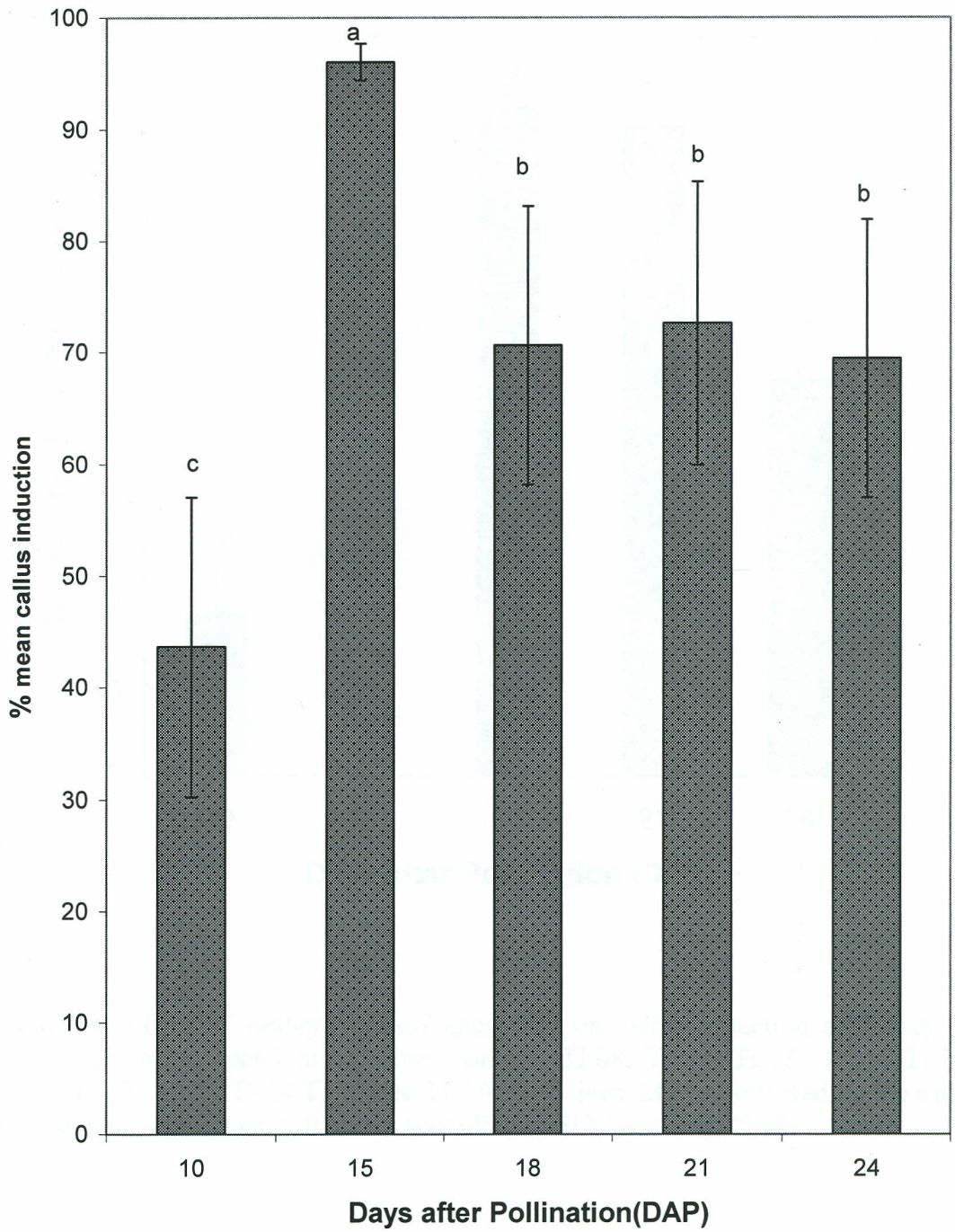
Means followed by the same letter within a location and between locations are not significantly different at  $p < 0.05$



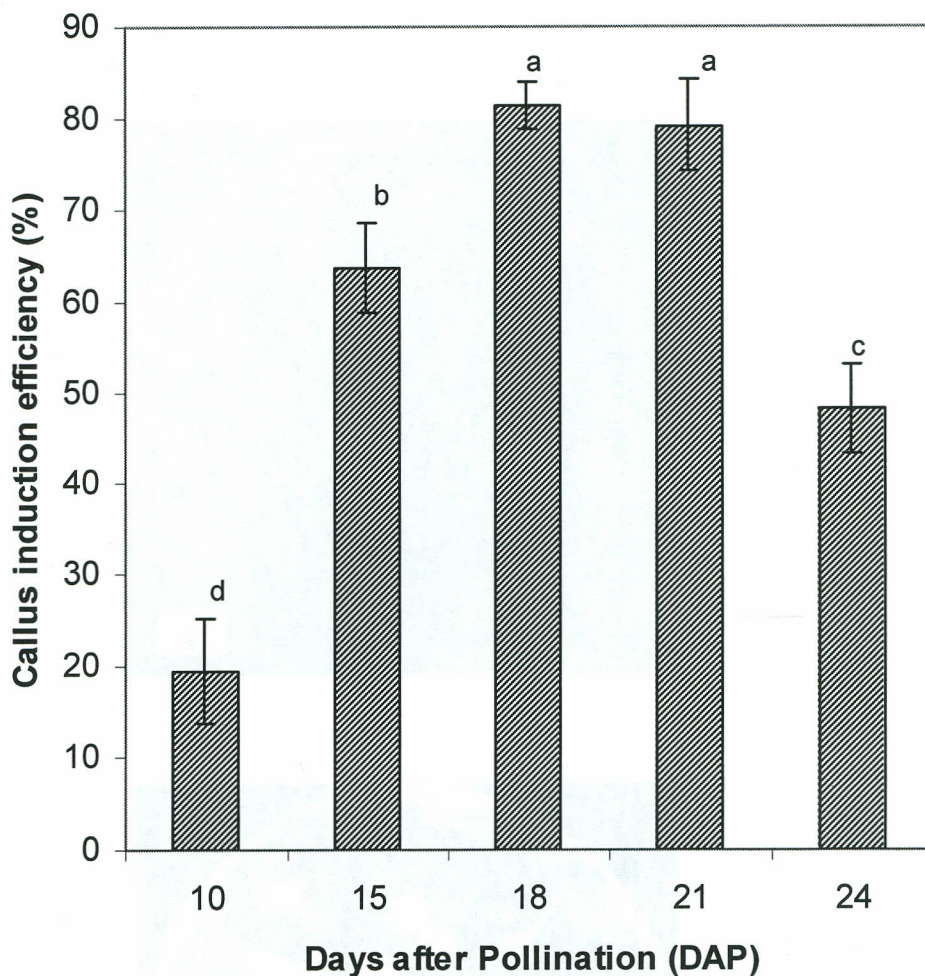
**Figure 6:** The effect of agro ecological zone on average flowering time of inbred lines and hybrids in both Kiboko (green) and Kabete (blue)

### **3.3 Effect of embryo physiological stages on callus induction efficiency**

The effect of embryo physiological stages on callus induction efficiency. Is shown on Figure 7 and 8 shows The results show that callus induction efficiency was optimal on embryos harvested 15 days after pollination for hybrids with 95.22% ( $p < 0.0005$ ) while it was 18-21 DAP for inbred lines with 82-83.9% ( $p < 0.0002$ ). For both hybrid and inbred lines, callus induction was significantly decreased below and above the optimal DAPs. Plate 3a and 3b illustrates morphological changes of embryos plated on N6 media incubated in the dark for 2-3 weeks in the dark.



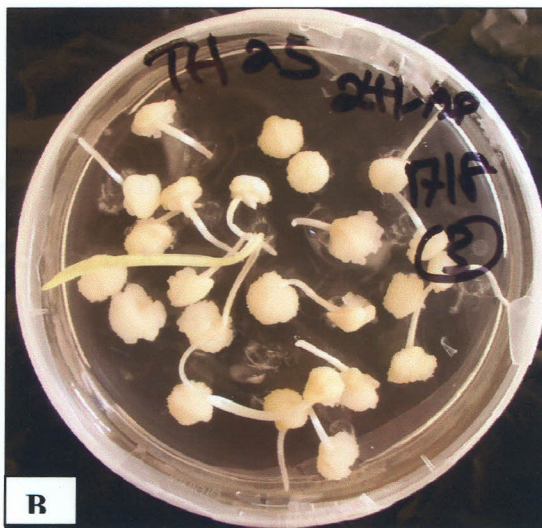
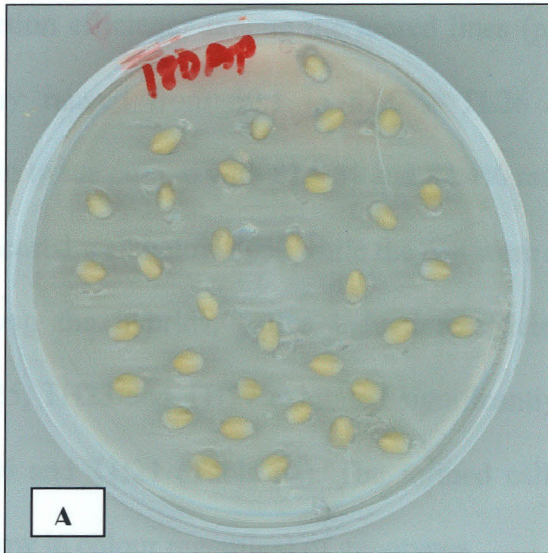
**Figure 7:** Effect of embryo physiological age on callus induction in six selected hybrids namely: TH21, TH23, TH24, TH25, TH26 and TH27. Means followed by the same letter are not significantly different according to SNK test at ( $p < 0.05$ )



**Figure 8:** Effect of embryo physiological age on callus induction efficiency in eleven selected parental inbred lines namely: TL08, TL09, TL18, TL19, TL20, TL21, TL22, TL23, TL24, TL26 and TL27 inbred lines. Means followed by the same letter are not significantly different according to SNK test at ( $p < 0.05$ )

54 Effect of water potential (salinity) on callus induction efficiency

In this study, the effect of salinity on callus induction efficiency was investigated. The results showed that the highest callus induction efficiency was observed in the control group (0% salinity) and decreased significantly with increasing salinity. The callus induction efficiency was significantly higher in the control group (71.3%) compared to the 1% salinity group (58.3%), and significantly lower in the 2% salinity group (46.7%) and 3% salinity group (33.3%). The callus induction efficiency was significantly lower in the 4% salinity group (20.0%) and 5% salinity group (13.3%). The callus induction efficiency was significantly lower in the 6% salinity group (6.7%) and 7% salinity group (3.3%). The callus induction efficiency was significantly lower in the 8% salinity group (0.0%) and 9% salinity group (0.0%). The callus induction efficiency was significantly lower in the 10% salinity group (0.0%).



**Plate 3:** Immature zygotic embryo plated on N6 media for callus induction. A: genotype TL27 at 18DAP embryos plated on day 1. B: Embryogenic callus derived from TH25 immature zygotic embryos excised 24 DAP incubated in N6 medium supplemented with 2 mg/l of 2,4 -D for 2-3wks at 26 +2<sup>0</sup> C in the dark

### **3.4 Effect of maize genotype (cultivar) on callus induction efficiency**

In the inbred category, variation in genotype caused a significant effect on callus induction efficiency among the inbred lines ( $p < 0.0000$ ). Callus induction in this category ranged from 50.1 to 82.0%. TL26 and TL18 were the highest responders in tissue culture with 81.9 % and 82.4% respectively. TL21, TL22, TL27 and TL 24 scored well below 60% while the rest were above this value (Table 6).

Among the hybrids, callus induction efficiency ranged from 77.5% to 90.7%. TH25, TH23 and TH27 exhibited significantly high induction compared to TH24, TH26 and TH21 (Table 6a). The formed calli were nodular and white to creamy-yellow in colour regardless of genotypes.

A comparison of callus induction potential between the parents and the hybrids showed that the hybrids exhibited high callus induction efficiency than either one or both of the parental inbred line except TL08 x TL09 which was not significantly different from the hybrid TH21 (Table 6). In general, hybrids had high callus induction efficiency of 83.05% compared to the inbred lines with 67.3% (Table 6b).

**Table 6a:** % Callus induction efficiency for both parental and hybrid lines

<b>Maize genotype</b>	<b>% callus induction</b>
TL08	77.40 ± 3.70b
TL09	77.80 ± 3.92b
<b>TH21</b>	<b>77.7 ± 5.2b</b>
TL18	82.40 ± 3.86a
TL19	69.87 ± 7.26b
<b>TH23</b>	<b>88.8 ± 3.3a</b>
TL20	67.00 ± 7.03 bc
TL21	54.73 ± 7.93d
<b>TH24</b>	<b>77.5 ± 5.0 b</b>
TL22	55.87 ± 5.17cd
TL23	66.60 ± 5.51bc
<b>TH25</b>	<b>90.7 ± 4.0 a</b>
TL23	66.60 ± 5.51bc
TL24	50.13 ± 5.27d
<b>TH26</b>	<b>77.7 ± 4.2b</b>
TL26	81.87 ± 4.52a
TL27	57.33 ± 5.77cd
<b>TH27</b>	<b>85.9 ± 5.2a</b>

Means followed by the same letter are not significantly different according to SNK test at (p<0.05). Bold type depicts hybrids

**Table 6b:** Average of callus induction between inbred lines and hybrids

<b>Maize genotypes</b>	<b>Average % callus induction efficiency</b>
TL(Inbreds)	67.3 %
TH(hybrids)	83.05 %

### 3.5 Effect of 2, 4-D concentration on % mean callus induction efficiency

Figure 9 shows the effect of varying 2,4-D concentrations on callus induction efficiency of the 7 selected maize genotypes. The results show that the

optimal 2,4-D concentration for callus induction was obtained with 2mg/L of 2,4-D ( $p < 0.0000$ ). Below or above the 2mg/L 2,4-D concentration, the callus induction efficiency decreased. The quality of callus significantly changed in texture and appearance. Callus observed was watery mass, fragile and translucent and subsequently failed to form compact structures upon subculture into fresh media. They were highly bruised, browned, necrosed and died gradually due to toxicity from combined endogenous and exogenous hormone concentration.

However, there was no callus induction in medium that was devoid of 2,4-D and embryos germinated precociously to form shoots and root within 4-6 days on induction medium

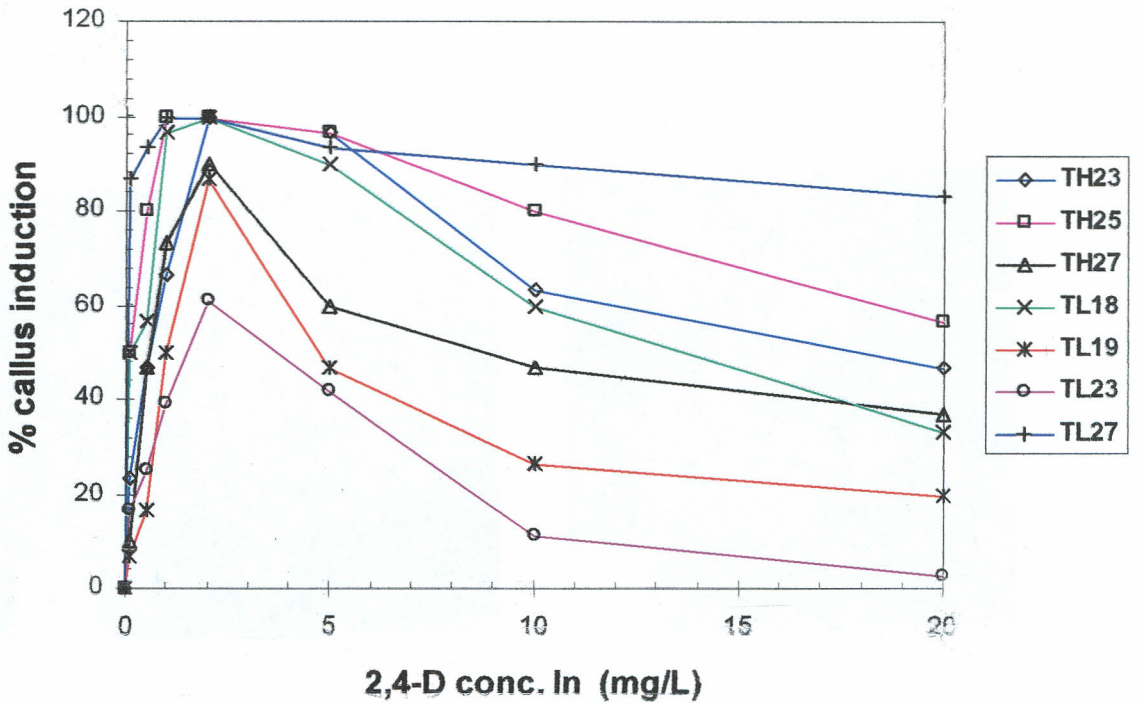
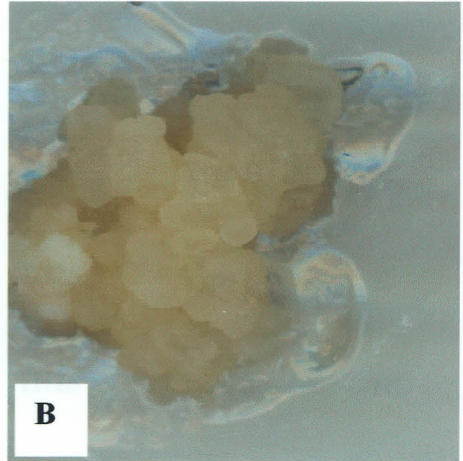
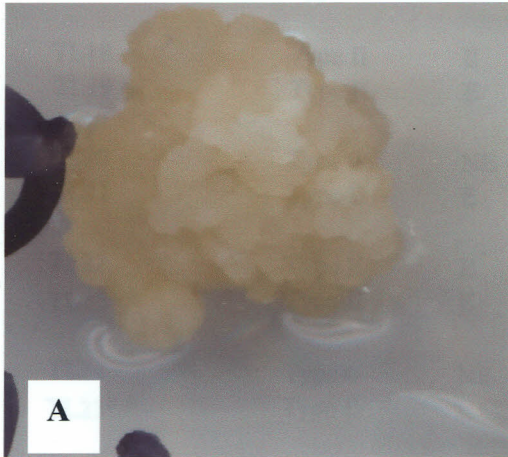


Figure 9: Effect of different levels of 2,4-D (0, 0.1, 0.5, 1.0, 2.0, 5.0, 10.0, 20.0 mg/L) on % callus induction efficiency on 7 selected maize genotypes

### 3.6 Types of primary callus generated upon induction on 2mg/L 2, 4-D

Table 7 shows the types of callus generated and their embryogenic competence by the maize genotypes on callus maintenance induction media. Results indicated that three types of calli were initiated; type I, type II and type 0 (Plate 4). Among the parental maize inbred lines, TL08, TL18, TL19, TL21, TL22, TL23, TL27 produced type II callus while TL09, TL20, TL24 and TL26 formed type 0 calli. Among the hybrid lines, TH21, TH24, TH27 produced type II while TH23 and TH25 produced type I calli and TH26 produced type 0. In general among the 17 Kenyan maize lines evaluated, 17.6 % of the lines produced type I calli. Majority of

the Kenyan maize genotypes initiated type II calli in high frequency of 53 % ( Table 7 and Figure 10).



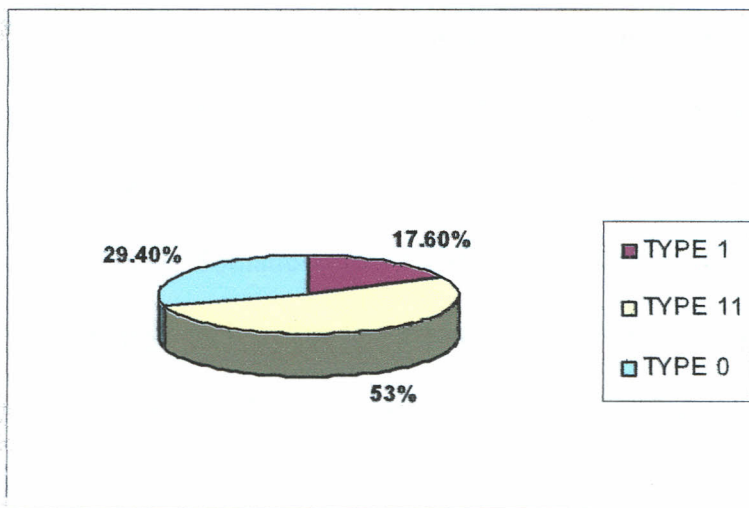
**Plate 4:** Callus morphotypes produced 28 days upon induction on N6 media A: Type I calli, (Mag.x1200) B: Type II calli, (Mag.x1200) C: Type 0 calli (mag.x200)

**Table 7:** Embryogenic callus competence of maize genotypes evaluated

Inbredlines	Calli types	Status	Hybrids	Calli types	Status
TL08	Type II	E	TH21	Type II	E
TL09	Type 0	NE			
TL18	Type II	E	TH23	Type I	E
TL19	Type II	E			
TL20	Type 0	NE	TH24	Type II	E
TL21	Type II	E			
TL22	Type II	E	TH25	Type I	E
TL23**	Type II	E			
TL24	Type 0	NE	TH26	Type 0	NE
TL23**	Type II	E			
TL26	Type 0	NE	TH27	Type I	E
TL27	Type II	E			

**Abbreviation**

NE-Non embryogenic E-embryogenic TL23\*\*-used as parent twice



**Figure 10:** Types of calli generated *in vitro* by maize genotypes evaluated (**Type I** comprised TH23, TH25, TH27; **Type II**-TL08, TL18, TL19, TL21, TL22, TL23, TL27; **Type 0**-TL09, TL20, TL24, TL26)

### **3.7 Somatic embryo formation potential across the maize genotypes**

Table 7 and 8 also shows the comparison of somatic embryo formation potential between parental inbred lines and their respective single cross. The results show that across between a either a non embryogenic parent with an embryogenic or both embryogenic parent produces an embryogenic hybrid cross except for the cross TL24 and TL23 which produced a non embryogenic hybrid TH26.

There was a significant difference across the genotypes in their somatic embryo formation potential ( $p < 0.0054$ ). Among the hybrids, the percentage somatic embryo formation ranged from 7.8% to 27.8%. The TH25 scored the highest number of somatic embryos in the entire events with a mean of 27.8 %, followed by TH23 (13.3%), TH24 and TH27 (7.8 %) each while TH21 and TH26 scored none (Table 8). Not all embryos that induced calli converted to somatic embryos efficiently. Out of the six hybrids initially evaluated for callus induction, four of them (66.7 %) formed somatic embryos (Table 7 and 8).

Among the inbred lines, there was no significant difference in their potential to form somatic embryo ( $p > 0.05$ ) (Table 8). Of the 12 parental inbred lines that were initially evaluated for callus induction, only 7 genotypes (58.33 %) converted to somatic embryos. Somatic embryogenic formation ranged from 3.3% to 9.0%. The order of embryogenic callus formation was TL23 (9.0%), TL18 (6.3%), TL08 (5.0%), TL19 (4.8%), TL27 (4.3%), TL22 (3.3%) TL21 (1.0%) and the rest were zero

Not all the genotypes that induced callus efficiently converted to somatic embryos. It was observed that although TL 22 showed low callus induction

efficiency (55.8%) it was able to convert to somatic embryos. Consequently the genotypes that gave the highest callus efficiency such as TL 26 and TL09 formed non embryogenic callus (Table 6 and Table 8).

**Table 8:** Somatic embryos formation of parental inbred lines and their cross hybrids

<b>Maize genotypes</b>	<b>Means for % somatic embryos</b>
TL08	5.0 ± 1.8b
TL09	0
<b>TH21</b>	<b>0</b>
TL18	6.3 ± 3.1b
TL19	4.8 ± 1.9b
<b>TH23</b>	<b>13.3 ± 4.1b</b>
TL20	0
TL21	1.0 ± 0.6b
<b>TH24</b>	<b>7.8 ± 2.1b</b>
TL22	3.3 ± 1.7b
TL23	9.0 ± 6.7b
<b>TH25</b>	<b>27.8 ± 6.5a</b>
TL24	0
TL23	9.0 ± 4.5b
<b>TH26</b>	<b>0</b>
TL26	0
TL27	4.3 ± 2.5b
<b>TH27</b>	<b>7.8 ± 1.7b</b>

Means followed by the same letter are not significantly different according to SNK test at (p<0.05). Bold type depicts the hybrids.

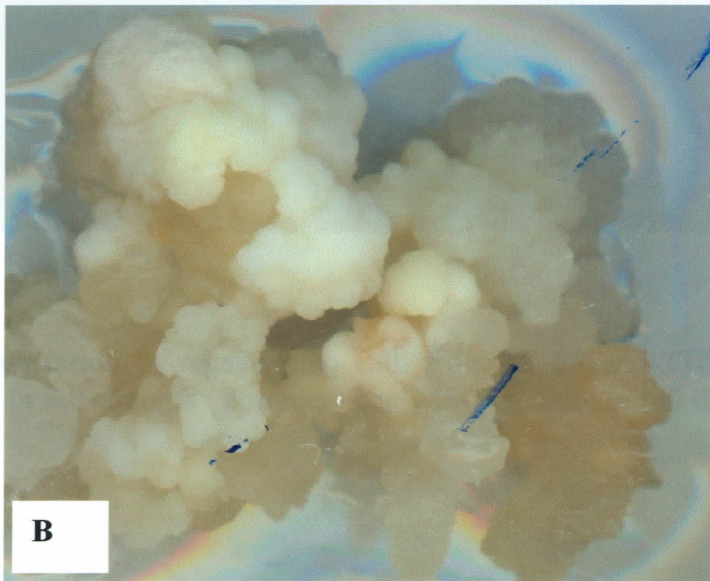
### 3.8 Plant regeneration

Once the somatic embryos matured, they were transferred to hormone free regeneration media (MSO) in full light. Their surfaces turned green within the first week and plantlet regeneration occurred within 21 days (Plate 6). Among the maize lines that formed somatic embryos only TL18, TL19, TL23, TL27, TH23, TH25 and TH27 formed shoots (Plate 8). Shooting was genotype dependent. When all the shoots were transferred to rooting media ( $\frac{1}{2}$  MSO) all formed roots within 14 days except TL08. Root system varied across the genotypes. TL 27 had a highly fibrous dendritic root system (Plate 7b). TL23 had a one central root while TL 08 was rootless even after being subjected to 1mg/L IBA for one week in full light. The classification of calli as embryogenic did not necessarily imply regenerability. TH 23 and TH 25 produced 7 and 10 plants, respectively while the remaining had one or more plantlets (Table 9).

Of the 11 inbred lines evaluated, 36.4% regenerated plantlets; of the 6 hybrids evaluated, 50% regenerated plantlets. Under the conditions described here, a total of 4 inbred lines and 3 hybrids were regenerated from the initial 17 genotypes evaluated. Out of the 25 plants regenerated. 72 % were morphologically normal with fertile males and females (Plate 10 c), 28 % manifested abnormalities with characteristic tassel seed, lacking silk, lacking tassel or were abnormally stunted in growth due to somaclonal variations ((Plate 10b and Table 9).

Experiments on germination viability of  $R_1$  seed where five seeds were planted per pot indicated that only TL18, TH23, TH25 and TL27 gave germination score in the range of 80-100% while the remaining genotypes had > 20%. TL23

seed did not germinate at all. It was observed that even abnormal seed (tassel seed) had capability to germinate to normal healthy plants (Table 9). The seed from the regenerants have been bulked in the green house for a further *in vitro* manipulation to be used in development of drought tolerant varieties.

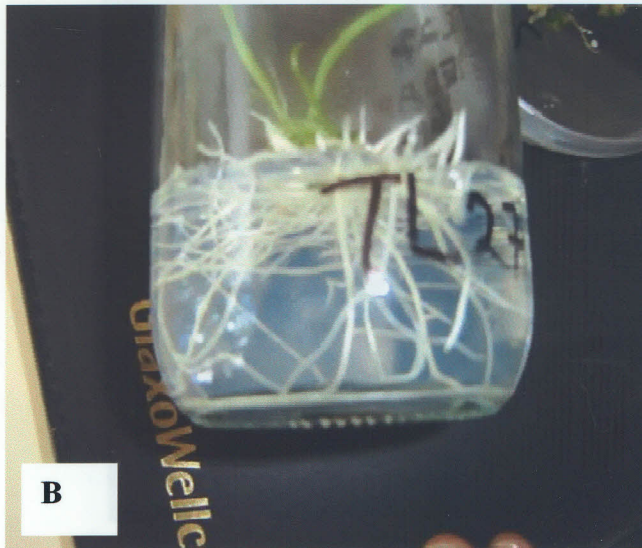


**Plate 5:** Somatic embryos: A somatic embryos from TL18 on maturation medium devoid of 2, 4-D incubated in the dark for 2-3 weeks at  $26^{\circ}\text{C} \pm 2$  (Mg.x400). B: Somatic embryos derived from TL18 (Mg. x1200)



**Plate 6:** Plant regeneration. A regenerating calli from TL 27 and TH25 derived embryos incubated in full strength hormone-free, MSO medium in full light for 2-3 weeks

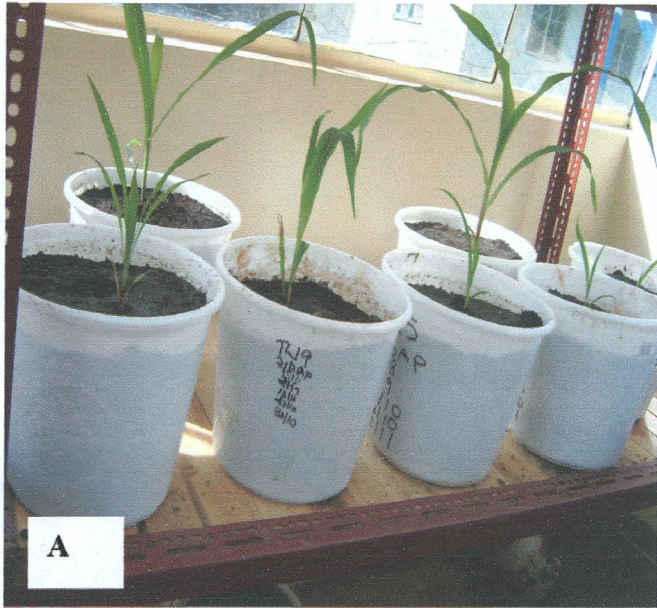
Plate 7: Plantlets on shooting and rooting media. A: maize plantlets in baby jars growing on half strength hormone-free MSO medium in full light for 2-3 weeks under 16hrs light and 8hrs darkness. B: highly fibrous and dendritic root system from TL27



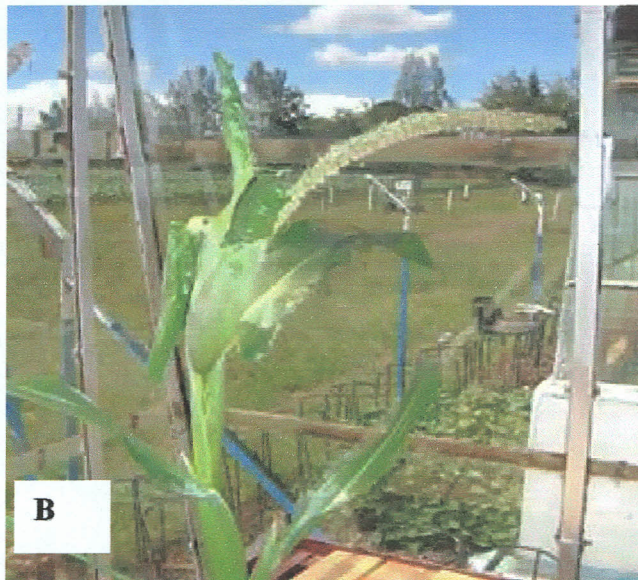
**Plate 7:** Plantlets on shooting and rooting media. A: maize plantlets in baby jars growing on half strength hormone- free MSO medium in full light for 2-3 weeks under 16hrs light and 8hrs darkness. B: highly fibrous and dendritic root system from TL27



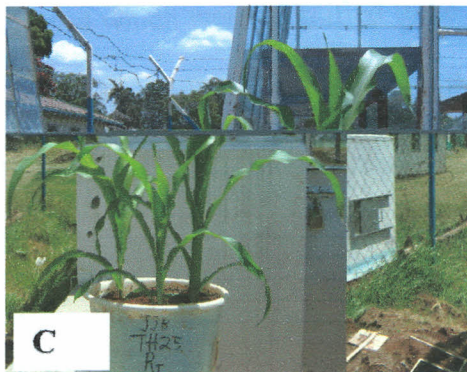
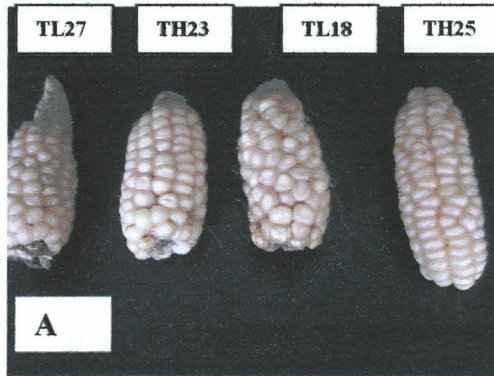
**Plate 8:** Plantlets in small pot containing sterile vermiculate and sand mixed in the ratio 3:1 derived from A: 14 days old TL27, TH23, TH25, TL18, TL19, TL23 and TH27 maize genotypes B: 21 days old TH27 genotype



**Plate 9:** Maize genotypes planted in 5 litre plastic pots in glasshouse.  
A: at 3 weeks. B: 5 weeks old



**Plate 10:** Pollination of the regenerants. A: TL18 plant (normal morphology and fertile) B: A tassel-seed derived from TL19 due to somaclonal variations



**Plate 11:** R<sub>1</sub> seeds A: derived from TL27, TH23, TL18 and TH25 B: germination viability of R<sub>1</sub> seeds C: a healthy TH25 R<sub>1</sub> plant in the green house

**Table 9:** Number of regenerated maize genotypes, morphological traits and their R<sub>1</sub> seed germination viability

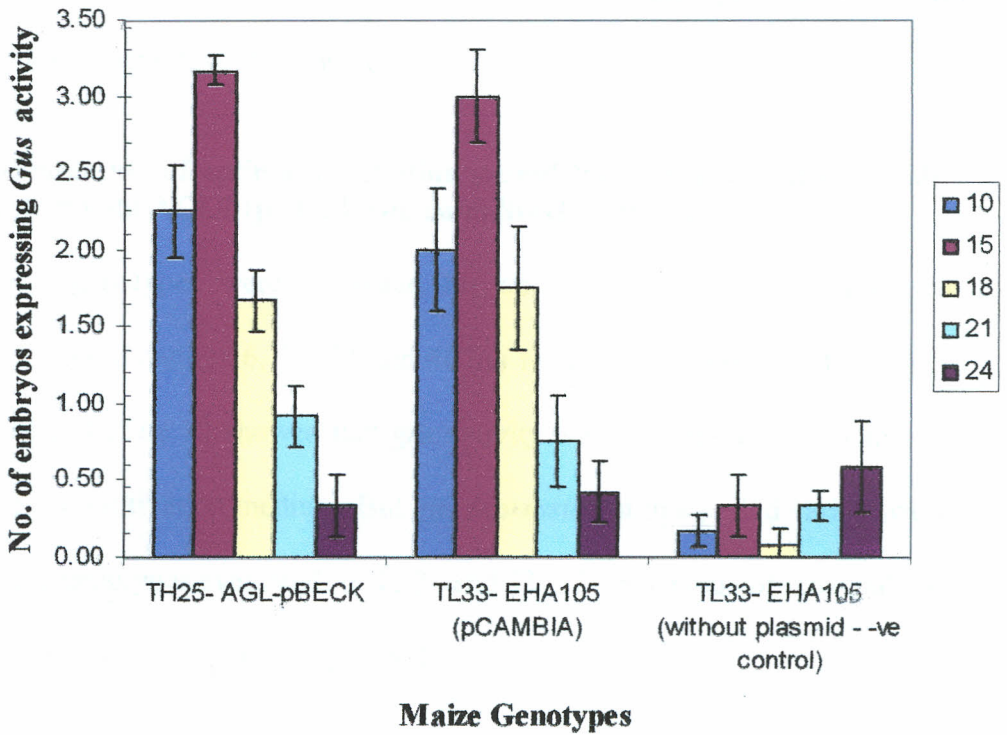
Maize genotype	No of plants in each genotype	No. of normal tassel/ear morphology	No. abnormal tassel seed	% regeneration efficiency	% viability of R <sub>1</sub> seed (5 seeds each)
TL18	1	1	0	4	80
TL19	1	0	1	4	20
TH23	7	5	2	28	100
TL23	2	1	1	8	10
TL27	2	1	1	8	80
TH25	10	8	2	40	100
TH27	2	2	0	8	20
Total Plants regenerated	25(100%)	18(72%)	7(28%)		

### 3.9 Evaluation of transient *gus* activity of regenerable maize lines

Five Maize genotypes which were identified to be amenable to tissue culture namely TL17, TL21, TL23, TL33, TH25 were used in this study. TL 17 and TL33 were lines that have been identified to be very responsive in tissue culture in the KARI-Biotechnology laboratories while the rest were evaluated during regeneration studies in this project.

#### 3.9.1 Effect of embryo physiological stage on $\beta$ -glucuronidase expression

The effect of embryo physiological stage on transient *Gus* expression was evaluated for two maize genotypes carrying two different plasmids TH25-(pBECK-AGL1), TL33-(pCAMBIA-EHA105) and TL33-EHA105 (without plasmid). Results shown in Figure 11 indicate a significant difference in *Gus* expression across the various embryo physiological stages as well as the different genotypes ( $P < 0.0000$ ).



**Figure 11:** Effect of embryo physiological stage on  $\beta$ -glucuronidase expression upon infection with pBECK and pCAMBIA plasmids

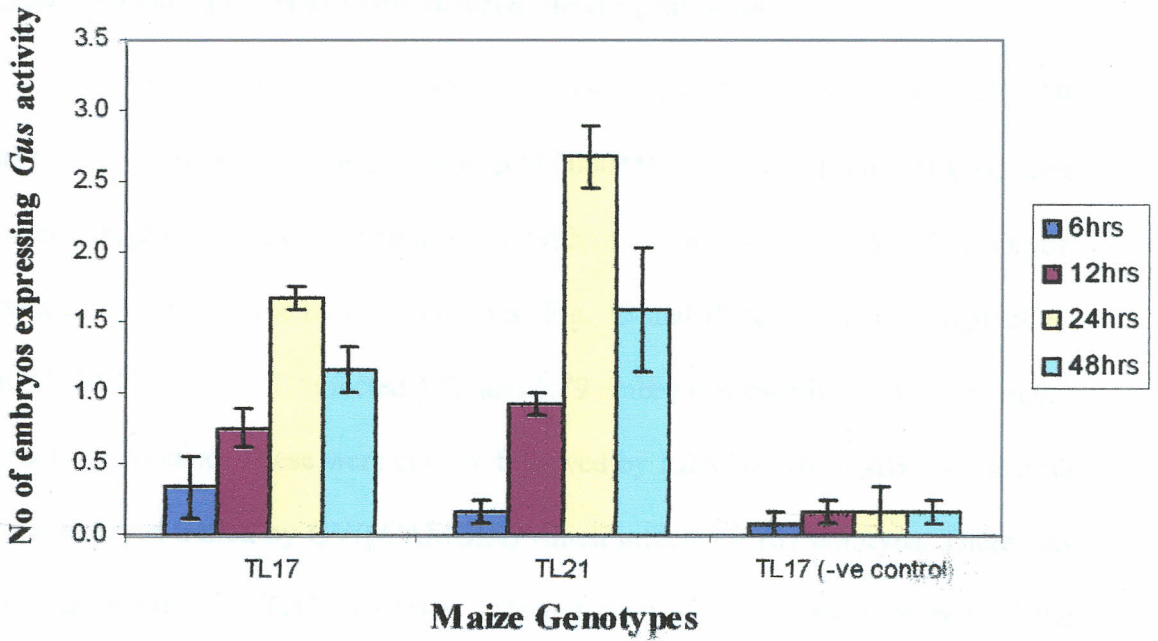
At 15 DAP *gus* expression was optimal for TH25 and TL33 with a mean of 3.0 and 3.2 embryos expressing *gus* activity respectively. There was decreased *gus* activity in very young and older immature zygotic embryos of 10, 21 and 24 DAP. However, in TL33 there was very little or no *gus* activity on tissues of different embryo stages infected with *Agrobacterium* devoid of plasmid (Figure 11) ( $p < 0.0000$ ). *Gus* activity declined in older embryos comparable to those embryos infected with *Agrobacterium* devoid of plasmids.

At 15 DAP transient *gus* assays were expressed highly in the embryogenic tissues while there was no significant difference in *gus* activity between embryos

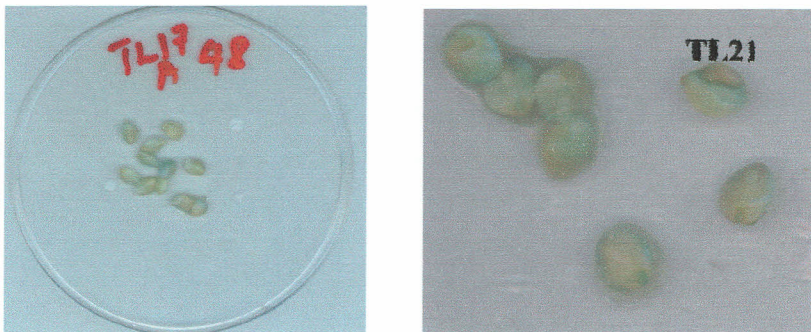
infected at 10 DAP and 18 DAP respectively ( $p>0.05$ ). *Gus* expression was negligible at 21 and 24DAP and was comparable with genotypes infected with *Agrobacterium* strains devoid of the plasmid.

### **3.9.2 Determination of optimal incubation period for transient *Gus* expression upon infection with AGL1 (pBECK) on co-cultivation media**

Maize genotypes were evaluated for optimal incubation period on co-cultivation medium namely 6, 12, 24 and 48 hrs for enhanced *gus* activity. Results in Figure 12 and Plate 12 showed that *gus* activity was maximum after incubating for 24 hrs on co cultivation medium. But *gus expression* at this period varied within the two maize genotypes evaluated. In TL21 and TL 17, an average of 2.67 and 1.67 embryos expressed *gus* activity, respectively.



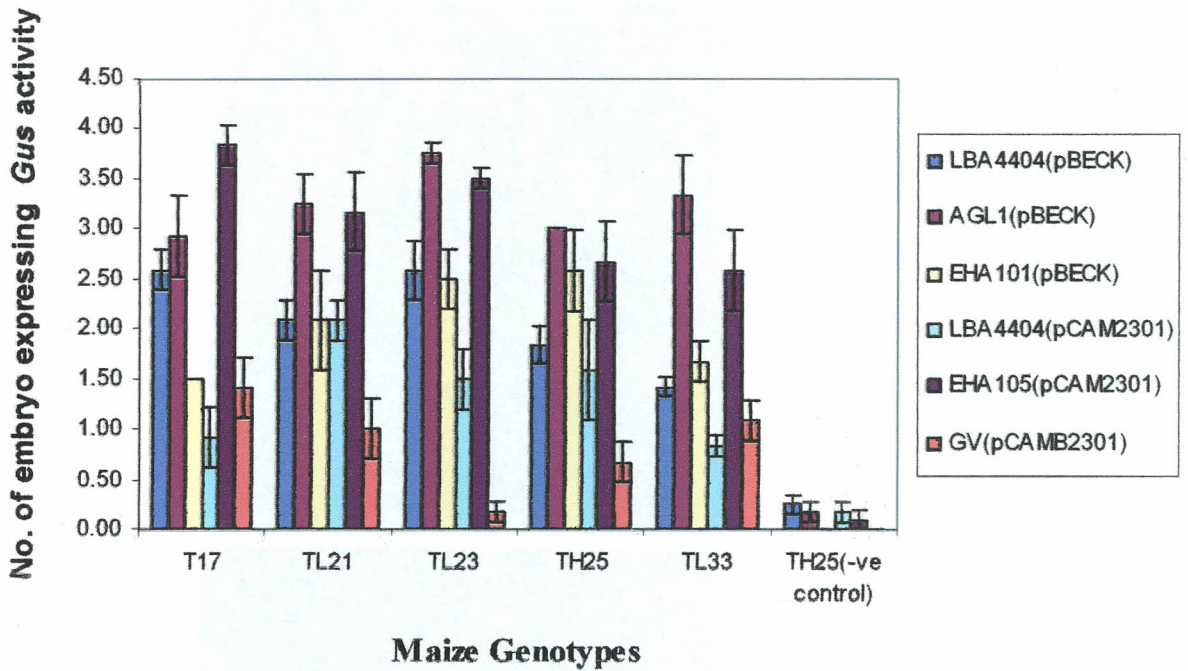
**Figure 12:** Determination of optimal incubation period for transient *gus* expression upon infection with AGL1 (pBECK) on co cultivation media



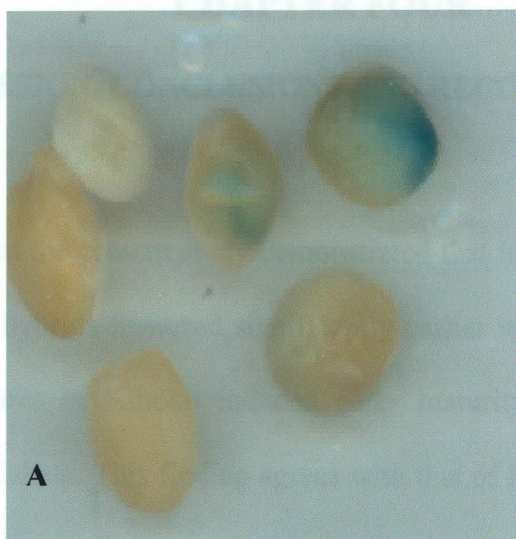
**Plate 12:** *Gus* activity on maize tissues (TL 17 and TL21) stained on X-gluc upon incubation with AGL1(pBECK) and EHA105(pCAMBIA) respectively

### **3.9.3 Infectivity of different *A.tumefaciens* strains carrying two plasmids: (pBECK) and (pCAMBIA) on different maize genotypes**

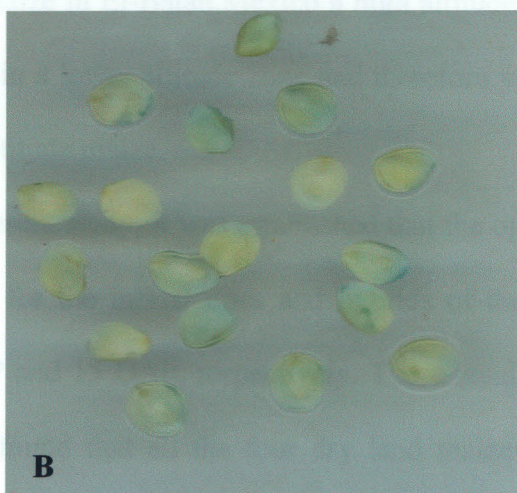
Both *A. tumefaciens* strains and maize genotypes caused a significant difference in transient *gus* expression ( $p < 0.0000^{***}$ ). The AGL1 and EHA105 were ranked highest in the infection rates with a mean of 2.9 and 2.5 embryos, respectively in the five maize genotypes (Fig. 13 and Plate 14). EHA101(pBECK) and LBA4404(pBECK) infected 1.72 and 1.79 embryos respectively in all the maize genotypes studied. These were closely followed by LBA4404(pCAMBIA) infecting 1.18 embryos trailed by GV(pCAMBIA) which infected 0.167 embryos. There was no *gus* activity on TH25 embryos infected with *Agrobacterium* devoid of the plasmid. AGL1(pBECK) infected TH25, TL21, TL23 and TL33 efficiently. EHA105(pCAMBIA) had the highest (3.88) infection compared to other strains. GV(pCAMBIA) showed reduced infectivity across all the genotypes except TL33 where it infected 1.08 embryos .



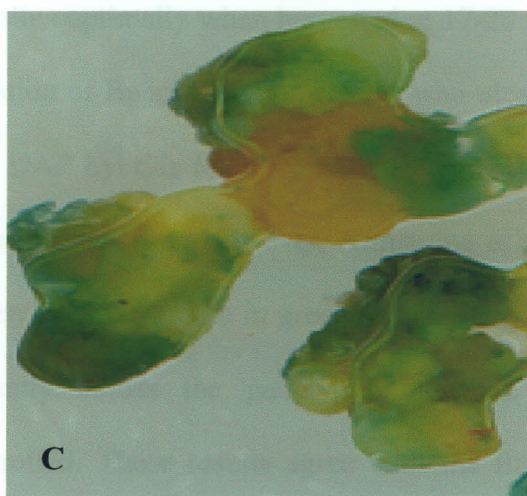
**Figure 13:** Infectivity of different *A. tumefaciens* strains carrying two plasmids (pBECK) and (pCAMBIA) on different maize genotypes at 15 DAP. Results were expressed as the mean number of embryos expressing  $\beta$ -glucuronidase activity. Mean  $\pm$  standard error 2 maize genotypes incubated at, 24, hrs on co-cultivation medium



A



B



C

**Plate 13:** Transient *gus* activity. A: TH25-EHA101 (pBECK). B & C: TL33-EHA105 (pCAMBIA2301)

## CHAPTER FOUR

### DISCUSSION, CONCLUSIONS AND RECOMMENDATION

#### 4.1 Discussion

The results of the present study demonstrated that in all the genotypes both the inbred lines and hybrids flowered significantly earlier in Kiboko than Kabete. The higher temperatures in Kiboko induced early maturity compared to the cooler temperatures at Kabete. This finding agrees with that of Bohorova *et al.* (1995) who observed that maize in El-Batan, Mexico a high altitude zone took longer to flower than that grown in a lower altitude zone and therefore yielded embryos after a later than the lower altitude maize.

In the present study, it was established that the optimal physiological age for callus induction for the inbred lines and hybrids of the tropical maize genotypes were 18-21 DAP and 15 DAP, respectively. These results concurred with Oduor *et al.* (2006) who found that all the four dry land maize hybrids evaluated in their study induced callus optimally when harvested at 17-20 DAP. This was in contrast with the observation of Bohorova *et al.* (1995) who observed 18-30 DAP in inbred lines and 13-15 DAP hybrids for tropical maize grown in high altitude zone and low altitude zone respectively. However, Armstrong *et al.* (1987) observed a DAP of 9-13 in temperate maize grown in green house. The finding that callus induction varied significantly across the genotypes indicates that callus induction is genetically controlled. These results agree with the findings of Prioli and Silva. (1989). who reported that genes controlling calli formation under culture conditions are present in high frequencies within Cateco race than Tuxpeno race. In the present

study hybrids readily induced callus after 14 days on induction media while inbred lines induction took 2-3 weeks. The callus formed were nodular white to creamy yellowish irrespective of whether it was derived from inbred lines or hybrids.

That hybrids were better callus inducers than their parental inbred lines indicates that they have better hybrid vigor than their parental inbred lines. In the current study, callus induction and plant regeneration were found to be genotype dependent just as results on regeneration of sorghum (*Sorghum bicolor* L.) by Nirwan and Kothari. (2003) and winter wheat (*Triticum aestivum* L.) by Ozgen *et al.* (1998).

Immature zygotic embryos have been frequently used as a source of explants in maize tissue culture ((Lu *et al.*, 1982; Lu and Vasil, 1983; Vasil *et al.*, 1984; Bohorova *et al.*, 1995) because they are highly prolific. However, it is usually difficult to produce immature embryos throughout the year and their suitable stage for culture. In the present study, maize genotypes were planted after every 3 weeks in order to ensure availability of embryos at any one point. This contrasts with the use of the readily available and abundant mature embryos from mature seeds as a source of explants. Mature embryos have been considered recalcitrant to regeneration compared to the highly prolific immature embryos.

Three types of calli were initiated in this study; Type I, II and 0. The callus morphotypes formed were similar to those described by Nirwan and Kothari (2003) and Carvahlo *et al.* (1987). In the current study, most of the maize genotypes evaluated formed Type II calli, in a frequency of 53 %. Type I comprised 17.6 % while Type 0 formed the remaining 29.4 %. These findings contrasted well with

results obtained by Bohorova *et al.* (1995) and Carvahlo *et al.* (1987) who found that a majority of tropical maize genotypes initiated type II calli under low frequency of 25% compared with their temperate counterparts at 97 %. This trait was genetically controlled and could be transferred to hybrids through cross pollination. Type II callus was soft, friable and was yellow or creamy in colour as described by Fransz and Schel (1991) for the genotype A188.

It was observed in this study that although TH26, TL26 and TL09 showed high callus induction efficiency, they only formed type 0 calli which turned brown upon subculture. They neither formed somatic embryos nor regenerated plantlets. These results agreed with Bohorova *et al.* (1995) which indicated that of the 44 tropical lines investigated, 75 % converted to embryogenic calli.

The auxin, 2,4-D in the range of 1 mg/L-3 mg/L, are essential for the establishment of embryogenic callus from cereal embryos (Carvalho *et al.*, 1987). In the present study, the best response for callus induction was achieved with 2 mg/l of 2,4-D concentration. This finding agrees with results obtained by Armstrong and Green (1985); Bohorova *et al.* (1995), Carvalho *et al.* (1987) and Oduor *et al.* (2006) who demonstrated that inclusion of 2 mg/L 2,4-D to induce callus in immature embryos was a critical factor. The use of cytokinin in combination with auxins to induce somatic embryogenesis has been reported for cereals (Bhaskaran and Smith, 1990; Gaspar *et al.*, 1996). Low levels of cytokinins are generally used. Cho *et al.* (1998) reported that addition of 0.1mg/L BA to the subculture medium was essential for barley. In the present study plant regeneration occurred in hormone free MSO. This observation was in line with results obtained by Lu and Vasil

(1983) and Armstrong and Green (1985) who demonstrated that somatic embryos from maize immature embryos germinated into complete plantlets on regeneration medium free from plant regulator. The addition of cytokinins into regeneration had little effect on germination rates. It has been suggested that a possible reason for this phenomena is that the somatic embryos capable of germinating to give rise to new plantlets have already formed and their fate may be predetermined by the initiation media.

In the present study somatic embryogenesis was demonstrated in 6 inbred lines and 4 hybrids. Parental genotype combination contributed significantly to Somatic embryo formation of respective hybrid cross. The somatic embryo competence can be transferred to non embryogenic inbred lines or crossing with hybrids derived from them through cross pollination. The most used maize genotypes for tissue culture are the temperate inbred line A188 and B73 and the F<sub>1</sub> hybrids of these two lines Hi II (Ishida *et al.*, 1996; Zhao *et al.*, 2001). The F<sub>1</sub> hybrids of A188 x B84 have also been cultured and used successfully for regeneration of transgenic plants (Phillips *et al.*, 1988; Gordon-Kamm *et al.*, 1990). In addition others such as B79 have been used to initiate friable callus although suspension callus has not been established (Lupotto and Lusardi, 1988). Somatic embryogenesis competence is genetically controlled and therefore can be transferred through sexual transfer (Tomes and Smith, 1985; Hodges *et al.*, 1986; Duncan *et al.*, 1988; Bohorova *et al.*, 1995; Cho *et al.*, 1998). In their studies, Hodges *et al.* (1986) reported that embryogenic potential in temperate maize inbred line A188 was controlled by 2 to 3 genes carried in the chromosomes and that somatic

embryogenesis could be transferred to non embryogenic genotypes by using the responsive genotype A188 as male pollinator. Hybrids of these elite inbred lines were also found to be embryogenic and exhibited high callus induction efficiency. However, this finding differed from Locatelli *et al.* (2003) which observed that inbred line Lo904 responded very poorly when used as female parent in the cross Lo904 x A188 while response was significantly improved when it was used as pollinator in the cross A188 x Lo904. Regeneration efficiency in some of the hybrid crosses with elite inbred lines paralleled, or even exceeded the regenerative capacity of the cross between the best responding genotypes A188 x B73. The present study concurs with Tomes and Smith, (1985) and Hodges *et al.* (1986) findings that when two parental inbred lines cross gave rise to a highly embryogenic hybrid. This was illustrated by TH23 and TH25 of which both of their parents were highly embryogenic. Similarly embryogenic calli was derived by crossing an embryogenic genotype (pollinator) with a non embryogenic (female) to give rise to highly responsive embryogenic hybrid cross. That implied that the 2-3 genes that control somatic embryogenesis were transferred. However not all calli classified as embryogenic produced plants, showing this classification does not accurately predict the regenerative capacity of callus.

Using mature embryos to initiate embryogenic callus produced low frequency regeneration efficiency of 19.9% to 32.4 % (Huang and Wei (2004) which was higher than in the present study where callus was initiated from immature zygotic embryos. Low frequency and genotype-dependent regeneration system for maize mature embryos was reported by Wang (1987). Among the three

inbred lines tested, B73 and Mo17 regenerated plantlets with low frequency (4% to 5%) while genotype A632 could not regenerate plantlets.

The ability to regenerate plants was not necessarily related to somatic embryo formation. For example TL08, TL21, TL22, TH21 and TH24 were embryogenic but did not efficiently convert to plantlets possibly due to absence of the three genes that control the pathway of somatic embryogenesis. In the present study, shoots recovered per callus was rather low compared to the multiple shoot per callus obtained by Vasil and Lu. (1984) and Duncan *et al.* (1988).

However not all the genotypes that were able to form somatic embryos were converted to whole plants. Maize genotypes showed marked differences in their root system in terms of habit, root mass and lateral root spread. In this study, the well developed and highly fibrous and dendritic root system of TH25, TL27, TH23 and TL23 for enhanced nutrient assimilation and anchorage confirms the results of Feldman. (1994) who demonstrated that, the type of root system determined the nutrient assimilation potential in various environment and such traits should be exploited to address constrains such as salinity, drought tolerance and nitrogen assimilation. A reduction of salt concentration has also been observed to provide satisfactory results in rooting in other plant species (Meney and Dixon, 1995). The positive effect of rooting on reduction in mineral salt concentration of the culture media was attributed to reduction in nitrogen levels (Driver and Suttle, 1987).

The observation of purplish stem pigmentation on regenerants in the present study was to some extent genotypic and was also attributed environment i.e. to lack of phosphorus leading to the retarded growth (Kling and Edmeades, 1997).

Although Fluminhan *et al.* (1996) suggested that changes in chromosome number and structure could occur in plant regenerated *in vitro*, they added that chromosome instability could be attributed by media components and choice of explant used. Retarded growth can be avoided by maintaining the cultures in the regeneration medium for 14-21 days before subculture to rooting media. Somaclonal variations could be induced by media components, culture age, and explant choice and even by plant genotype (Peschke and Phillips, 1992).

Most studies of regeneration have utilized genotypes adapted to temperate re climate (Vasil *et al.*, 1984; Duncan *et al.*, 1985; Tomes and Smith, 1985) and little attention has been focused on the regeneration potential of maize germplasm adapted to tropical and subtropical regions (Prioli and Silva, 1989; Bohorova and Hoisington, 1992).

The identification of elite lines that posses an enhanced embryogenic capacity provides an avenue for the direct introduction of novel traits into Kenyan maize genotypes which posses useful agronomic traits using genetic engineering such as *A tumefaciens*, with the need for relatively limited backcrossing time in breeding programs that may ultimately expedite cultivar development. Moreover, transformation protocols described for major cereal crops are generally only effective in relatively few genotypes.

Transient assays mediated by agroinfection have been increasingly employed as an alternative to the analysis of stable transformants and its utility has been reported for transgenic complementation (Tadeusz *et al.*, 2005). In the present study, factors optimal for enhanced transient *gus* expression in immature zygotic

embryos of maize as prerequisite to stable *Agrobacterium*-mediated transformation were the age of the immature zygotic embryo, the incubation period on co cultivation media, *A. tumefaciens* strains and plasmids and the maize genotypes used. The maize genotypes subjected to the *A. tumefaciens* transformation were identified to be amenable to tissue culture procedures in the regeneration studies.

*Gus* gene expression after co cultivation was over 60 % while in the optimization experiment in this study, 62% of embryos excised at 15 DAP showed  $\beta$ -glucuronidase activity upon agro infiltration with different agro strains. Ishida *et al.* (1996) successfully produced transgenic maize events via *A. tumefaciens* initiated from immature zygotic embryos at 9-13 DAP of inbred line A188 and obtained a transient expression 80 % in all the events but with a stable transformation frequency of 5.8%.

In the present study, the tropical maize genotypes were found to induce callus optimally at 15 days post pollination in the hybrids category and 18-21DAP among the inbred lines with an embryo size of 1.0-2.0mm. This contrast with temperate genotypes that are induced optimally 9-13 DAP (Armstrong and Green, 1985). This then reflect on the effect of prolonged radiations in the tropic. The results of the present study compares with that by Zhao *et al.* (2002) who found that the embryo age (1.0-1.5mm) of Hi-II hybrid which had both A188 and B 79 background was critical for transformation. Ishida *et al.* (1996) found that the *Agrobacterium* strain LBA4404 carrying super binary vector (pSB131) and (pTOKO233) expressed *gus* activity in high frequencies after co cultivation while the same *Agrobacterium* strain carrying (pIG121Hm) was relatively low on A188

embryos. Frame *et al.* (2000) found *gus* transient activity frequency of 56% on embryos of Hi-II infected with *Agrobacterium* strain EHA101 carrying a standard binary vector (pTF102).

Anderson and Moore (1979) and Cheng and Fry, (2003) found that some strains of *Agrobacterium* are more virulent than others on a particular plant species. Conversely, some plants species are more or less sensitive to particular strains of *Agrobacterium*. Similar trends were observed in the present study when the different strains manifested different degrees of infectivity with highest being in EHA105(pCAMBIA) and AGL1(pBECK) and the least seen in GV(pCAMBIA).

$\beta$ -glucuronidase activity was efficiently expressed in embryogenic tissues optimally after 24 hrs on co cultivation media. Ramesh and Gupta (2005) also found that there was maximum transient *gus* activity in callus cultures of Japonica and Indica rice (*Oryza sativa* L.) after 24hrs on co cultivation media. This contrasted with the results of Zhao *et al.* (2001) and Ishida *et al.* (1996) who observed maximum *gus* activity after 36hrs on co cultivation media. However, Tadeusz *et al.* (2005) found that Optimal *gus* activity was obtained on tissues of *Arabidopsis thaliana*, lettuce and tomato when incubated 4-5 days in the dark at 25<sup>0</sup> C on co cultivation media.

The current present study N6 media in both regeneration and transformation studies. The *gus* expression was 40- 70% which was lower than results obtained by Zhao *et al.* (2001) who obtained 80-100 % *gus* positive tissue using MS media (Murashige and Skoog, 1962) Ishida *et al.* (1996) findings compared with Zhao *et*

al. (2001) using LS media (Linsmaier and Skoog, 1965) which is superior in nitrogen content.

Regeneration of Kenyan maize genotypes initiated from immature embryos has paved way for the application of biotechnological approaches to manipulate cell cultures *in vitro* and use of powerful techniques of genetic engineering to introduce novel genes into targeted genotypes. The present study has shown that many tropical maize genotypes can induce the formation both type I and friable type II callus. Such information extends the range of genotypes that can be used in biotechniques where tissue culture is utilized, particularly when cell suspension or protoplast cultures are required.

#### **4.2 Conclusion**

The present study identified maize lines that were amenable to tissue culture procedures as well as those that were recalcitrant to regeneration. Calli was induced in the presence of 2mg/L of 2,4-D. Somatic embryogenesis pathway was found to be genetically controlled and varied significantly from one genotype to another.

The regenerable lines that were able to pick the *gus* reporter genes have been identified and it was established that infectivity of embryogenic tissues was dependent on embryo physiological stage, incubation period on co-cultivation media, choice of *Agrobacterium* strains and plasmid, as well as the maize genotype used. This study rejected the hypothesis that it is impossible to regenerate and also transform locally adapted Kenyan maize varieties. From this study, regeneration and transformation protocols were optimized successfully and

routinely used. Somatic embryogenesis is therefore a very valuable tool for achieving a wide range of objectives, from basic biochemical, physiological and morphological studies, to the development of technologies with a high degree of practical application. Some of the good agronomic traits that the genotype possesses can now be enhanced via genetic engineering.

### **4.3 Recommendations**

Possibilities of using other explants such as shoot tips, mature seeds, anthers and meristems to initiate callus and plant regenerations should be explored further in maize. It will be important to investigate the effect of other auxins i.e picloram, dicamba in regeneration efficiency of our locally adapted maize germplasm.

It will be of great significance to continue evaluating *in vitro* tissue response to a wider range of maize genotypes that are currently being used in breeding programs, major cultivars adapted to specified agro ecological zone (AEZ), and those in commercial production.

For purposes of maintaining some minimal vigour in inbred lines, they can be sibbed). When the seed is harvested, storage in cold room (+4<sup>0</sup>C) with the appropriate dusting powder is highly recommended because boring by storage pest such as weevils and larger grain borer and incomplete desiccation has contributed to reduced seed viability (germination).

Somaclonal variations can be used to identify mutants with desired characteristics such as drought tolerance. Tissue culture has been shown to contribute to variability within the regenerated maize genotypes morphologically and even

genetically. The selection of mutants from cultured plant cells has the potential for contributing new genetic variability for plant breeders. to use in selection and breeding programs.

## REFERENCES

- Abuodeh, R.O., Orbach, M.J., Mandel, M.A., Das, A. and Galgiani, J.N.** (2000). Genetic transformation of *Coccidioides immitis* facilitated by *Agrobacterium tumefaciens*. *Journal of Infectious Diseases*, 181: 2106-2110.
- Ajanga, S. and Hillocks, R.J.** (2000) Maize cob rot in Kenya and its association with stalk borer damage. *Crop Protection*, 19:297-300.
- Aldemita, R.R. and Hodges, T.K.** (1996). *Agrobacterium tumefaciens* transformation of japonica and indica rice varieties. *Planta*, 199:612-617.
- Ammirato, P.V.** (1987). Organizational events during somatic embryogenesis. In: Green, C.E., Somers, D.A., Hacket, W.P. and Biesboer, D.D (eds). *Plant Tissue and Cell culture*. New York, Alan R. Liss, pp. 57-81.
- Anderson, A.R. and More, L.W.** (1979). Host specificity of the genus *Agrobacterium*. *Phytopathology*, 69: 320-323.
- Arencibia, A.D., crmona, E.R.C., Tellez, P., Chan, M.T., Yu, S.M., Trujillo, L.E. and Oramas, P.** (1998). An efficient protocol for sugarcane (*saccharum spp L.*) transformation mediated by *Agrobacterium tumefaciens*. *Transgenic Research*, 7:2123-222.
- Armstrong, C.L. Green, C.E.** (1985). Establishment and maintenance of friable, embryogenic maize callus and the involvement of L-proline. *Planta*, 164:207-214
- Assad, F. and Signer E.R.** (1990). Cauliflower mosaic virus P35S promoter activity in *E. coli*. *Molecular Genetics*, 223, 517-520.
- Ayaga, G. O.** (2003). Maize yield trend in Kenya in the last 20 years. Paper presented at the regional workshop on declining Maize yield trend in Trans Nzoia district organized by Moi University. *Centre Consortium*. KARI Kitale and Ministry of Agriculture.
- Baker, C.M., Burns, J.A. and Wetzstein, H.Y.** (1994). Influence of photoperiod and medium formulation on peanut somatic embryogenesis. *Plant Cell, Tissue and Organ Culture*, 13:159-163.
- Bell, E.A. and Charlwood, B.V** (1980). Secondary Plant products. *Encyclopedia of Plant Physiology*, 21:95-112.
- Bettany, A.J.E., Dalton, S.J., Timms, E., Manderyck, B., Dhanoa, M.S. and Morris, P.** (2003). *Agrobacterium tumefaciens*-mediated transformation of *Fusca arundinacea* (Shreb) and *Lolium multiflorum* (LAM). *Plant Cell Reports*, 21:437-444.
- Bhaskaran, S. and Smith, A.** (1990). Regeneration in cereal tissue culture: a review. *Crop Science*, 30:1328-1336.
- Binns, A.N. and Bock, A.** (1989). Cell biology of *Agrobacterium* infection and transformation of plants. *Annual Review of Microbiology*, 42: 1-35.
- Binott, J.J., Machuka, J.S., Njagi, E.N and Songa J.M.** (2006). Plant regeneration from immature embryos of Kenyan inbred lines and their respective single

## REFERENCES

- Abuodeh, R.O., Orbach, M.J., Mandel, M.A., Das, A. and Galgiani, J.N.** (2000). Genetic transformation of *Coccidiales immitis* facilitated by *Agrobacterium tumefaciens*. *Journal of Infectious Diseases*, 181: 2106-2110.
- Ajanga, S. and Hillocks, R.J.** (2000) Maize cob rot in Kenya and its association with stalk borer damage. *Crop Protection*, 19:297-300.
- Aldemita, R.R. and Hodges, T.K.** (1996). *Agrobacterium tumefaciens* transformation of japonica and indica rice varieties. *Planta*, 199:612-617.
- Ammirato, P.V.** (1987). Organizational events during somatic embryogenesis. In: Green, C.E., Somers, D.A., Hacket, W.P. and Biesboer, D.D (eds). *Plant Tissue and Cell culture*. New York, Alan R. Liss, pp. 57-81.
- Anderson, A.R. and More, L.W.** (1979). Host specificity of the genus *Agrobacterium*. *Phytopathology*, 69: 320-323.
- Arencibia, A.D., crmona, E.R.C., Tellez, P., Chan, M.T., Yu, S.M., Trujillo, L.E. and Oramas, P.** (1998). An efficient protocol for sugarcane (*saccharum spp* L.) transformation mediated by *Agrobacterium tumefaciens*. *Transgenic Research*, 7:2123-222.
- Armstrong, C.L. Green, C.E.** (1985). Establishment and maintenance of friable, embryogenic maize callus and the involvement of L-proline. *Planta*, 164:207-214
- Assad, F. and Signer E.R.** (1990). Cauliflower mosaic virus P35S promoter activity in *E. coli*. *Molecular Genetics*, 223, 517-520.
- Ayaga, G. O. (2003).** Maize yield trend in Kenya in the last 20 years. Paper presented at the regional workshop on declining Maize yield trend in Trans Nzoia district organized by Moi University. *Centre Consortium*. KARI Kitale and Ministry of Agriculture.
- Baker, C.M., Burns, J.A. and Wetzstein, H.Y.** (1994). Influence of photoperiod and medium formulation on peanut somatic embryogenesis. *Plant Cell, Tissue and Organ Culture*, 13:159-163.
- Bell, E.A. and Charlwood, B.V** (1980). Secondary Plant products. *Encyclopedia of Plant Physiology*, 21:95-112.
- Bettany, A.J.E., Dalton, S.J., Timms, E., Manderyck, B., Dhanoa, M.S. and Morris, P.** (2003). *Agrobacterium tumefaciens*-mediated transformation of *Fusca arundinacea* (Shreb) and *Lolium multiflorum* (LAM). *Plant Cell Reports*, 21:437-444.
- Bhaskaran, S. and Smith, A.** (1990). Regeneration in cereal tissue culture: a review. *Crop Science*, 30:1328-1336.
- Binns, A.N. and Bock, A.** (1989). Cell biology of *Agrobacterium* infection and transformation of plants. *Annual Review of Microbiology*, 42: 1-35.
- Binott, J.J., Machuka, J.S., Njagi, E.N and Songa J.M.** (2006). Plant regeneration from immature embryos of Kenyan inbred lines and their respective single cross hybrids through somatic embryogenesis In Press *African Crop Science Journal*.

- Birsin, M.A., Önde, S. and Özgen, M.** (2001). Callus induction and plant regeneration from mature embryos of oats (*Avena sativa* L). *Turkey Journal of Biology*, 25:427-434.
- Bohorova, N., Zhang, W., Julstrum, P., Mclean, S., Luna, B., Brito, B. R. M., Diaz, L., Ramos, M. E., Estanol, P., Pacheco, M., Salgado M., Hoisington, D.** (1999). Production of transgenic tropical maize with CryAb and CryAc genes via microprojectile bombardment. *Theoretical and Applied Genetics*, 99: 437-444.
- Bohovora, N.E. and Hoisington, D.A.** (1992). Plant regeneration from immature embryos of tropical maize inbred lines. In: Resumenes del XIV Congreso Nacional de Fitogenetica.editor. Society Mexico de Fitogenetica.A.C. Chapingo. Mexico. pp.20-21.
- Bohovora, N.E., Luna, B., Brito, R.M., Huerta, L.D. and Hoisington, D.A.** (1995). Regeneration potential of tropical, subtropical, midaltitude, and highland maize inbred. *Maydica*, 40:275-281
- Brown, R.H.** (1999). Agronomic implications of C4 photosynthesis. In: Sage, R.F. Monson, R.K. eds. C4 Plant biology. San Diego, CA, USA: Academic Press, 473-507.
- Bundock, P.A., dan Dulk-Ras, A., Beijersbergen, A. and Hooyakas, P.J.J.** (1995). Trans kingdom T-DNA in *Saccharomyces cerevisiae*. *European Molecular Biology Organization Journal*, 14:3206-3214.
- Bytebier, B., Deboeck, F., Degrave, H., Van Montagu, M. and Hernalsteens, J.P.** (1987). T-DNA organization in tumor cultures and transgenic plants of monocotyledon *Asparagus officinalis*. *Proceedings of National Academy of Sciences*, USA.84:5345-5349.
- Carvalho, C.H.S., Bohovora, N., Bordallo, P.N., Abreu, L.L., Valicente F.H., Bressan, W. and Paiva, E.** (1987). Type II callus production and plant regeneration in tropical maize genotypes. *Plant Cell Reports*, 17: 73-76.
- Chan, M.T., Chang, H.H., Ho, S.L., Tong, W.F. and Yu, S.M.** (1993). *Agrobacterium*-mediated production of transgenic rice plants expressing chimeric  $\alpha$ -amylase promoter glucuronidase gene. *Plant Molecular Biology*, 22:491-506.
- Chan, M.T., Lee, T.M. and Chang, H.H.** (1992). Transformation of Indica rice (*Oryza sativa* L.) mediated by *Agrobacterium*. *Plant Cell Physiology*, 33:577-583.
- Cheng, M., Fry, E., Pang, S., Zhou, H., Hironaka, C.M., Duncan, D.R., Conner, T.W. and Wan, W.** (1997). Genetic transformation of wheat mediated by *Agrobacterium tumefaciens*. *Plant Physiology*, 115:971-980.
- Cheng, M., Fry, F.J.** (2000). An improved efficient *Agrobacterium* mediated plant transformation method..Int. Patent Publ. WO 00/34491.
- Cheng, M., Lowe, B.A., Spencer, M.T., Ye, X. and Armstrong, L.A.** (2004). Factors influencing *Agrobacterium*-mediated transformation of monocotyledonous species. *In Vitro Cell Development Plant*, 40:31-45.
- Cho, M.J., Jiang, W. and Lemaux, P.G.** (1998). Transformation of recalcitrant barley cultivars through improvement of regenerability and decreased albinism. *Plant Science*, 138:229-244.

- Chu, C.C., Wang C.C., Sun, C.S., Hsu,C., Yin, K.C., Chu C.Y., Bi, F.Y.** (1975). Establishment of efficient medium for anther culture of rice through comparative experiments on the nitrogen source. *Science Sinica*, 18:659-668.
- Citovsky, V., Zupan, J., Warnick, D. and Zambryski, P.** (1994). Nuclear localization of *Agrobacterium* Vir E2 protein in plant cells. *Science*, 256:1802-1805.
- Coe, E.H., Neuffer, M.G., and Hoishington, D.A.** (1988). The genetics of corn. In Sprague *et al* (eds) Corn and Corn Improvement, 3<sup>rd</sup> Edition, ASA, CSSA,SSSA.
- Conger, B.V, Novak, F.J., Afza, R and Erdelsky, K.E.** (1987). Somatic embryogenesis from cultured leaf segment of *Zea mays* L. *Plant Cell Reports*, 6:345-347.
- Dahleen, L.S. and Bregitzer, P.** (2002). An improved media system for high regeneration rates from barley immature embryos -derived callus cultures of commercial cultivars. *Crop Science*,42:934-938.
- De Block, M., Botterman, J., Vandewiele, M., Dockx, J., Thoen, C. and Gosselé, V.** (1987). Engineering herbicide resistance in plants by expression of a detoxifying enzyme. *European Molecular Biology Organization Journal*, 6: 2513-2518.
- Delaere, R.R., Hofte,H., Hernasteens, J.P., Leemans ,J. and Montagu, M.** (1987). Vectors for cloning in plant cells. *Methods in Enzymology*, 153:227-292.
- De Groote, H.** (2002). Maize yield losses from stem borers in Kenya. *Insect Science and its Applications*, 22: 89-96
- DeVries, J. and Toenniessen, G.** (2001). Securing the harvest: biotechnology, breeding and seed systems for African crops. CABI International, New York. pp. 59-60.
- DE wet, J.R., Wood, L.V., Helsinki, D.R. and Deluca, M.** (1985). Cloning of firefly luciferase DNA and the expression of active luciferase in *Escherichia coli*. *Proceedings of National Academy of Sciences*, 82:7870-7873.
- Dodds, J.H. and Roberts, L.W.** (1995). Experiments in tissue culture. Cambridge University Press, Cambridge.
- Dodeman, V.L., Ducreux, G. and Kreis, M.** (1997). Zygotic embryogenesis versus somatic embryogenesis. *Journal of Experimental Botany*, 48: 120:1493-1509.
- Dong, J.Z. and Dunstan, D.I.** (2000). Molecular biology of somatic embryogenesis in conifers. In: Jain, S.M and Minocha, S.C. (ed). Molecular Biology of woody Plants. Dordrecht, Kluwer Academic Publishers. pp 55-87.
- Doorne, L., Marshall, G. and Kirkwood, R.C.** (1995). Somatic embryogenesis in pea (*Pisum sativum* L.) Effects of explants, genotype and culture condition. *Annals of Applied Biology*, 126: 169-179
- Dowswell, C.D., Paliwal, R.L. and Cantrell, R.P.** (1996). Maize in the third world. Boulder,CO, USA, Westview Press.

- Draper, J., Scott, R. and Hamil, J.** (1991). Transformation of dicotyledonous plants cells using the Ti plasmid of *A. rhizogenes*. In: Plant genetic Transformation and Gene expression. Draper, J., Scott, R., Armitage, P. and Walden, R. (Ed) Blackwell Scientific Publications, Oxford. pp.69-160.
- Driver, J.A. and Suttle, G.R.L.** (1987). Nursery handling of propagules. In: Cell and tissue culture in forestry. Bonga, J.M and Durzan, D. J. (Ed). Martinus Nijhoff, Dordrecht, The Netherlands . pp 320-335.
- Dudits, D., Borge, I. and Gyorgyey, J.** (1991). Molecular and cellular approaches to the analysis of plant embryo development from somatic cells *in vitro*. *Journal of Cell Sciences*, 99:475-484.
- Duncan, D.R. and Widholm, J.M.** (1985). Improved plant regeneration from maize callus cultures using 6-benzylaminopurine. *Plant Cell Reports*, 7:452-455.
- Duncan, D.R., Williams, M.E., Zehr, B.E. and Widholm, J.M.** (1988). The production of callus capable of plant regeneration from immature embryos of numerous *Zea mays* genotypes. *Planta*.165:322-332.
- Eastman, P.A.K., Webster, F.B., Pitel, J.A. and Roberts, D.R.** (1991). Evaluation of somaclonal variation during somatic embryogenesis of interior spruce (*Picea glauca engelmannii* Complex) using culture morphology and isozyme analysis. *Plant Cell Reports*, 6:372-374.
- Enriquez-Obregon, G.A., Prieto-Samsonov, D.L., de la Riva, G.A., Perez, M., Selman-Housein, G. and Vazquez-Padron, R.I.** (1999). *Agrobacterium*-mediated Japonica rice transformation: a procedure assisted by anti-necrotic treatment. *Plant Cell Tissue Organ Culture*, 59:159-168.
- Ermakov, I.P. and Matveeva, N.P.** (1994). Regulation of early embryogenesis in higher plants. *Russian Journal of Plant Physiology*, 41:414-423.
- Faure, O., and Dewitt, W., Nougarede, A. and Van Onckelen, H.** (1998). Precociously germinating somatic embryos of *Vitis vinifera* have lower ABA and IAA levels than their germinating zygotic counterpart. *Physiologia Plantarum*, 102:591-595.
- Feldman, L.** (1994). The maize root. In: M.Freeling and Walbort, eds. The maize handbook, pp 29-37. New York, NY, USA, Springer-Verlag.
- Filonova, L.H., Bozhkov, P.V., Brukhin, V.B., Daniel G., Zhivotovsky, B. and Von Arnold, S.** (2000). Two waves of programmed cell death occur during the formation of somatic embryos in the gymnosperm, Norway spruce. *Journal of Cell Science*, 113:4399-4411.
- Finkelstein, R.R., Tenberge, K.M., Shumway, J.E. and Crouch M.L.** (1985). Role of abscisic acid in maturation of rapeseed embryos. *Plant Physiology*, 78:630-636.
- Fluminhan, A., De Aquir-Perencin, M.L.R. and Dos Santos, J.A.** (1996) Evidence for heterochromatin involvement in chromosome breakage in maize callus culture. *Annals of Botany*, 78:73-81.
- Frame, B., Shou, H., Chikwamba, R., Zhang, Z., Xiang, C., Fonger, T., Pegg, S.E., Li, B., Nettleton, D., Pei, D. and Wang, K.** (2002) *Agrobacterium* mediated transformation of maize embryos using standard binary vector system. *Plant physiology*, 29: 13-22.

- Fransz, P.F. and Schel, J.H.N.** (1991) Cytodifferentiation during development of friable embryogenic callus of maize (*Zea mays* L.) *Canadian Journal of Botany*, 69:26-33.
- Fromm, M.E., Morrish, F., Armstrong, C., Williams, R., Thomas, J., and Klein, T.M.** (1990) Inheritance and expression of chimeric genes in the progeny of transgenic maize. *Plant Biotechnology*, 8:833-839.
- Galinat, W.C.** (1988) The origin of corn. In: Sprague, G.F. and Dudley, J.W. (ed). Corn and corn improvement, 3rd (ed). Madison, WI, USA, *American Society of Agronomy*. Pp.1-31.
- Galun, E. and A. Breiman.** (1997). Transgenic plants. Imperial Colleges Press Singapore. P 375 .
- Gamborg, O.L.** (2002) Plant tissue culture. Biotechnology.Milestones. In: *In vitro Cellular and Developmental Biology-plant*, 38:116-124.
- Ganapathi, T.R., Higgs, N.S., Balint-Kurti, P.J., Arntzen, C.J., May, G.D. and Van Eck, J.M.** (2001). *Agrobacterium*-mediated transformation of embryogenic cell suspension of banana cultivar Rasthali (AAB). *Plant Cell Reports*, 20:157-162.
- Gasper, T., Kevers, C., Penel, C., Greppin, H., Reid, D.M. and Thorp, T.A** (1996). Plant hormones and plant growth regulators in plant tissue. In: *In vitro Cell Development Biology-Plant*, 32:272-289.
- Gelvin, S.B.** (1998). *Agrobacterium* Vir E2 proteins can form a complex T strands in the plant cytoplasm. *Journal of Bacteriology*, 180:4300-4302.
- Gelvin, S.B.** (2002). *Agrobacterium* and plant genes involved in T-DNA transfer and integration. *Annual Review of Plant Physiology and Plant Molecular Biology*, 51:223-256.
- Gelvin, S.B.** (2003). *Agrobacterium*-mediated plant transformation: the Biology behind the gene- jockeying tool. *Microbiology and Molecular Biology Reviews*, 67:16-37.
- Gelvin, S.B.** (2005). Gene exchange by design. *Nature* 433: 583- 584.
- Genetic Engineering News letter.** (2004). Transgenic drought and salt tolerant plants. Special Issue 15.
- Goodner, B.W., Merkelz, B.P., Flanagan, M.C., Crowell, C.B., Racette, J.L., Schilling, A., Halfon, L.M., Mellors, J.S. and Grabowski, G.** (1999). Combined genetic and physical map of the complex genome of *Agrobacterium tumefaciens*. *Journal of bacteriology*, 181:5160-5166.
- Gordon-Kamm, W.J., Spencer, T.M., Mangano, M. L., Adams ,T.R., Daines ,R.J., Start, W.G., O'Brien, J.V., Chambers, S.S., Adams jr, .W.R., Willets,N.G., Rice ,T.B., Mackey ,C.J., Krueger, R.W., Kausch, A.P., and Lemaux, P.G.**(1990) Transformation of maize cells and regeneration of fertile transgenic plants. *Plant Cell Reports*, 2:603-618.
- Gould, J., Devery, M., Hasegawa, O., Ulian, E.C., Peterson, G. and Smith, R. H.** (1991). Transformation of maize (*Zea mays* L.) using *Agrobacterium tumefaciens* on the shoot apex. *Plant physiology*, 95:426-434.

- Gurney, A. L., Grimanelli, D., Kanampiu, F., Hoisington, D., Scholes, J. D. and Press, M. C.** (2003). Novel sources of resistance to *Striga hermonthica* in *Tripsacum dactyloides*, a wild relative of maize. *New physiologist*, 160: 557-568.
- Gray, D.J. and Purohit, T.** (1991). Somatic embryogenesis and development of synthetic seed technology. *Critical Reviews in Plant sciences*, 10:33-61.
- Gray, D.J., Compton, M.E., Harrell, R.C and Cantliffe, D.J.** (1995). Somatic embryogenesis and the technology of synthetic seed. In Bajaj, Y.P.S (ed) Somatic embryogenesis and synthetic seed 1. Biotechnology in Agriculture and Forestry. Berlin, Springer-verlag Vol. 30 pp 126-151.
- Green, C.E and Phillips, R.L.** (1975). Plant regeneration from tissue culture of maize. *Crop science*, 15:417-421.
- Grimsley, N.H., Ramos, C., Hein, T. and Hohn, B.** (1987). Meristemic tissues of maize plants are most susceptible to Agroinfection with maize streak virus. *Biotechnology*. 6: 185-189.
- Hagio, T., Hirabayashi, T., Machii, H O., and Tomotsune, H.** (1995). Generation of transgenic barley using particle bombardment. *Plant Cell Reports*, 14:329-334.
- Hamilton, C.M., Frary, A., Lewis, C. and Tranksley, S.D.** (1996). Stable transfer of intact high molecular DNA into plant chromosomes. *Proceeding of National Academy of Science, USA*.93:9975-9979.
- Hansen, G., Das, A. and Chilton, M-D.** (1994). Constitutive expression of the virulence genes improves the efficiency of plant transformation by *Agrobacterium* *Proceedings National Academy of Science, USA* .91:7603-7605
- Hansen, G. and Wright, M.S.** (1999). Recent advances in the transformation of plants. *Trends in Plant Science*, 6:226-231.
- Hassan, R.M. and Ransom, J.K.** (1998). Determinants of the incidence and severity of *striga* infestation in maize in Kenya. In: Hassan, R.M. (ed) Maize technology Development and Transfer: a GIS Application for Research Planning in Kenya .CAB international, Wallingford, UK. pp.71-88.
- Hiei, Y.K., Ohta, S., Komari, T. and Kumashiro, T.** (1994). Efficient transformation of rice (*Oryza sativa* L.) mediated by *Agrobacterium* and sequence analysis of boundaries of the T-DNA. *Plant Journal*, 6:271-282.
- Hodges, T.K., Kamo, K.K., Imbrie, C.W. and Becwar, M.R.** (1986). Genotype specificity of somatic embryogenesis and regeneration of maize. *Biotechnology*, 4:219-229.
- Holmberg, N. and Bulow, L.** (1998). Improving stress tolerance in plants by gene transfer. *Trends in Plant Sciences*, 3: 61-66.
- Hooykaas, P.J.J. and Shilperoort, R.A.** (1992). *Agrobacterium* and plant genetic engineering. *Plant Molecular Biology*. 19: 15-38.
- [Http://www.cambia.org/daisy/cambia/home.html](http://www.cambia.org/daisy/cambia/home.html).
- Huang, X.-Q. and Wei, Z.-M.** (2004). High frequency plant generation through callus initiation from mature embryos of maize (*Zea mays* L.). *Plant Cell Reports*, 22:793-800.

- Ininda, J., Gethi, M., Odongo, O., Njuguna, J. and Ochieng, J.A.W.** (2002). Research abstract: Coordinated Ecosystem Breeding in Kenya: progress, challenges and opportunities. In: Biotechnology Breeding and Seed Systems for African Crops: Research and Product Development that Reaches Farmers. Program, Participants and Abstracts. The Rockefeller Foundation, Nairobi ([www.africancrops.net](http://www.africancrops.net))
- Ishida, Y., Saito, H., Ohta, S., Hiei, Y., Komari, T. and Kumashiro, T.** (1996). High efficiency transformation of maize (*Zea mays L*) mediated by *Agrobacterium tumefaciens*. *Nature Biotechnology*, 4:745-750.
- Jefferson, R.A., Kavanagh, T.A. and Bevan, M.W.** (1988). *Gus* fusion,  $\beta$ -glucuronidase as sensitive and versatile gene fusion marker in higher plants. *European Molecular Biology Journal*, 6:3901-3907.
- Jefferson, R.A.** (1987). Assaying the Chimeric genes in plants: the *Gus* gene fusion system. *Plant Molecular Biology Reporter*, 5:387-405.
- Jefferson, R.A.** (1985) DNA transformation of *C.Alegans*: development and application of a new gene fusion. Ph.D. Dissertation, University of Colorado.
- Jimenez, V.M.** (2001). Regulation of *in vitro* somatic embryogenesis with emphasis on the role of endogenous hormones. *Revista Brasileira de Fisiologia Vegetal*, 13:196-223.
- Jones, H., Tempelaar, M. and Jones, G.K.** (1987). Recent advances in plant electroporation. *Journal of Experimental Biology*, 44:359-374
- Jugenheimer, R.W.** (1985). Corn improvement, seed production and uses. Malabar, FL, USA, Robert E. Krieger Publishing.
- Karanja, L.S.** (1997). Somatic embryogenesis in chrysanthemum (*D. grandiflora* cv. Polaris) MSc Thesis. pp100.
- Karim, M.A., Fracheboud, Y., Stamp, P.** (2000). Effect of High Temperature on Seedling Growth and Photosynthesis of Tropical Maize Genotypes. *Journal of Agronomy and Crop Science*. 184: 217-223.
- KARI.** (2005). Agricultural Innovations for sustainable development. A Strategic plan for 2005-2015. Nairobi, Kenya. pp 40.
- Kanampiu, F., Ransom, J., Gresel, J., Jewell, D., Friedsen, D., Grimenelli, D. and Hoisington, D.A.** (2002). Appropriateness of Biotechnology to African Agriculture: *Striga* and maize paradigms. *Plant Tissue Culture and Organ Culture*, 69:105-110.
- Keane, P.J., Kerr, A. and New, R.B.** (1970). Crown gall galls of stone fruit: identification and nomenclature of *Agrobacterium* isolates. *Australian Journal of Biological Science*, 23:585-595.
- Kermode, A.R.** (1990). Regulatory mechanism involved in transition from seed development to germination. *Critical Reviews in Plant Science*, 9:155-195.
- Kermode, A.R., Dumbroff, E.B. and Bewley, J.D.** (1989). The role of maturation drying in the transition from seed development to germination: Effects of partial and complete desiccation on abscisic acid levels and sensitivity in *Ricinus communis L.* seeds. *Journal of Experimental biology*, 40: 303-313.

- Kisaka, H. and Kameya, T.** (1998). Fertile transgenic Asparagus plants produced by *Agrobacterium*-mediated transformation. *Plant Biotechnology*, 15:177-181
- Klein, T.M., Kornstein, L., Sanford, J.C. and Fromm, M.E.** (1989). Genetic transformation of maize cells by particle bombardment. *Plant Physiology*, 96:440-446.
- Kling, J.G. and Edmeades, G.** (1997). Morphology of maize. IITA/CYMMY Research Guide 9.Ibadan, Nigeria. pp 36.
- Komamine, A., Kawahara, R., Matsumoto, M., Sunabori, S., Toya, T., Fujiwara, A., Tsukuhara, M., Smith, J., Ito, M., Fukuda, H., Nomura, K. and Fujimura, T.** (1992). Mechanism of somatic embryogenesis in cell cultures: physiology, biochemistry and molecular biology. *In Vitro Cellular and Developmental Biology*, 28:11-14.
- Komari, T.** (1990). Transformation of callus cells of *Chenopodium quinoa* by binary vectors that carry a fragment of DNA from virulence region of pTiBo542. *Plant Cell Reports*, 9:303-306.
- Koziel, M.G., Beland, G.L., Bowman, C., Carozzi, N.B., Crenshaw, R., Crossland, L., Dawson, J., Desai, N., Hill, M., Kadwell, S.** (1993). Field performance of elite transgenic maize plants expressing an insecticidal protein derived from *Bacillus thuringiensis*. *Biotechnology*, 11: 194-200.
- Kumar, M. and Sachan, J.K.S.** (1991). Maize and its Asiatic relatives. In: Sarkar, K.R. Singh, N.N. and Sachan, J.K.S. (ed) Maize genetics perspectives. New Delhi, Indian Society of Genetics and Plant Breeding. pp.32-52.
- Kunik, T., Tzfira, T., Kapulnik, Y., Gafni, Y., Dingwall, C. and Citovsky, V.** (2001). Genetic transformation of Hela cells by *Agrobacterium*. *Proceedings of National Academy of Sciences, USA*. 98:1871-1876.
- Kuroyama, H., Tsutsui, N., Hashimoto, Y. and Tsumuraya Y.** (2001) Purification and characterization of a beta-glucuronidase from *Aspergillus niger*. *Carbohydrate Research*, 333: 27-39.
- Kuta, D.D. and Tripathi, L.** (2005). *Agrobacterium*-induced hypersensitive necrotic reaction in plant cells: a resistance response against *Agrobacterium tumefaciens*. *African Journal of Biotechnology*, 8: 752-757.
- Lamkey, K.** (2004). Seed production in corn and soybean. In: Mellon, M. and Rissler, J.(eds) Achieving Zero Contamination of Food Supply by Pharm Crops. Union of Concerned Scientist, Boston, Massachusetts.
- Lams, S., Lam, B., Harrison, L. and Strobel.** (1984). Genetic information of R<sub>1</sub> plasmid of *Agrobacterium rhizogenes* determines host specificity. *Plant Science letters*, 34:345-352.
- Lazzeri P.A. and shewry, P.R.** (1993). In: Tombs MP (ed) Biotechnology and genetic engineering reviews, VOL I. T.T. Intercept ltd (UK). pp 79-145.
- Li, Z., Jarret, R.L., Cheng, M. and Demski, J.W.** (1995). Improved electroporation buffer enhances transient gene expression in *Arachis hypogea* protoplasts. *Genome*, 38: 858-863.
- Li, W., Guo, G and Zheng, G.** (2000). *Agrobacterium*-mediated transformation: state of the art and future prospects. *Chinese Science Bulletin*,45:1537-1546.

- Limanton-Gevet, A. and Jullien, M.** (2001). *Agrobacterium*-mediated transformation of *Asparagus officinalis*: molecular and genetic analysis of transgenic plants. *Plant Molecular Breeding*, 7:141-150.
- Lindsey, K. and Topping, J. F.** (1993). Embryogenesis: a question of pattern. *Journal of Experimental Botany*, 44:359-374.
- Linsmaier, E. and Skoog, F.** (1965). Organic growth factor requirement of tobacco tissue culture. *Plant Physiology*, 18:100-127.
- Litz, R.E. and Gray, D.J.** (1995). Somatic embryogenesis for Agricultural improvement. *World journal of Microbiology and Biotechnology*, 11:416-425.
- Locatelli, F., Bertolini, M. and Lupotto, E.** (2003). *In vitro* response to somatic embryogenesis of Lo inbred lines of maize. In:Maize genetics Cooperation Newsletter.pp.219-132.
- Lu, C. and Vasil, I.K.** (1983). Improved efficiency of somatic embryogenesis and plant regeneration in tissue cultures in maize. *Theoretical and Applied Genetics*, 66:285-289.
- Lu, C., Vasil, I.K. and Ozias-Akins, P.** (1982). Somatic embryogenesis on *Zea mays* L.). *Theoretical and Applied Genetics*, 62: 285-289.
- Lupotto, E. and Lusardi, M.C.** (1988). Secondary somatic embryogenesis from regenerating plantlets of the inbred line B79 of maize (*Zea mays* L.) switch from type I to type II callus and effect on regenerative potential. *Maydica*, 33: 163-173
- Machuka, J.** (2001). Agricultural genomics and sustainable development. Perspective and prospects for Africa. *African Journal of Biotechnology*,3:127-137.
- Machuka, J.** (2004). Agricultural Biotechnology for Africa. African Scientist must feed their own people. *Plant physiology*, 126:16-19.
- Maheshwanri, N.** (1993). *In vitro* culture of wheat and genetic transformation.-retrospect and prospect. *Plant science*, 14:149-178.
- May, G.D., Afza, R., Mason, H.S., Wiecko, A, A., Novak, F.J. and Arntzen, C.J.** (1995). Generation of transgenic banana (*Musa acuminata*) plants via *Agrobacterium*-mediated transformation. *Biotechnology*. 13:486-492.
- Mazur, B., Kebbers, E. and Tingay, S.** (1999). Gene discovery and products development for grain quality traits. *Science*, 285: 372-375.
- Mark, K. S., Richard, L. H., Diane, E. Stanley. H., Karen, S., Oberhauser, J. M. Pleasants, H. R., Mattila, B., Siegfried, D. and Galen P. D.** (2001). "Impact of *Bt* corn pollen on monarch butterfly populations: A risk assessment". *Proceedings of the National Academy of Sciences, USA* 98:11937-11942.
- Meney, K.A. and Dixon, K.W.** (1995). *In Vitro* propagation of western Australian Rushes (Restionaceae and related families) by embryo culture. Part 2.Micropagation. *Plant Cell, Tissue and Organ Culture*, 41: 115-124.
- Merkle, S.A., Parrot, W.A. and Flinn, B.S.** (1995). Morphogenic aspects of somatic embryogenesis. In: *in vitro* embryogenesis in plants, (ed) Thorpe, T.A. The Netherlands: Kluwer Academic Publishers. pp.155-203.

- Michler, C. and Lineberger, D.** (1987). Effects of light on somatic embryo development and abscisic acid in carrot suspension cultures. *Plant Cell, Tissue and Organ Culture*, 13:159-163.
- Morrish, F., Songstad, D.D., Armstrong, C.L. and Fromm, L.** (1993). Microprojectile bombardment: a method for the production of transgenic cereal crop plants and the functional analysis of genes. In: *Transgenic plants: fundamentals and applications*. pp133-172.
- Mugo, S., De Groote, H., Bergvinson, D., Mulaa, M., Songa, J. and Gichuki, S.** (2005). Developing *Bt* maize for resource poor farmers: Recent advances in Insect Resistant Maize for Africa. *African Journal of Biotechnology*, 4: 1491-1504)
- Murashige, T. and Skoog, F.** (1962). A revised medium for rapid growth and bioassays with tobacco cultures. *Plant physiology*, 31:805-813
- Nangayo, F.L.O.** (1996). Ecological studies of larger grain borer in savannah woodlands of Kenya. PhD Thesis, University of London. pp 179.
- Negrotto, D., Jolley, M., Beer, S., Wench, A.R. and Hansen, G.** (2000). The use of phosphomannose-isomerase as a selectable marker to recover transgenic maize plants (*Zea mays* L.) via *Agrobacterium* transformation. *Plant Cell Reports*, 19:798-803.
- Nikilov, Z.L.** (1999). Production of recombinant proteins in transgenic plants. Department of Food Science and Human Nutrition and Department of Agricultural and Biosystems Engineering, Iowa State University, Ames IA.
- Nirwan, R.S. and Kothari S.L** (2003). Higher copper levels improve callus induction and plant regeneration in sorghum (*Sorghum bicolor* L.). *Society for In Vitro Biology*, 39: 161-164.
- Njuguna, J.G.M.** (1996). Epidemiology of maize streak virus disease in Kenya. PhD Thesis, Ohio State University. pp. 185.
- O'Brien, T.P. and McCully, M.E.** (1981). "The study of plant structure: Principles and selected methods". Termarcarphi pty. Ltd, Melbourne, Australia
- Odell, J.T., Nagy, F. and Chua, N-H.** (1985). Identification of DNA sequences required for activity of the cauliflower mosaic virus 35S promoter. *Nature*, 331:810-812
- Odhiambo, G.D.** (1998). Studies on witch weed (*Striga hermonthica*)(Del) Benth) seed longevity and maize (*Zea mays* L.) productivity under different management systems in Kenya. PhD Thesis, University of Leading, pp.198.
- Oduor, R.O., Njagi, E.N.M., Ndungu, S. and Machuka, J.S.** (2006). *In vitro* regeneration of dryland Kenyan maize genotypes through somatic embryogenesis. *International Journal of Botany*, 2: 146-151
- Ow, D., wood, K., Deluca, M., De Wet, J.R., Helinski, D.R. and Howell, S.H.** (1986). Transient and stable expression of firefly luciferase gene in plant cells and transgenic plants. *Science*. 234: 856-859.
- Ozgen, M., Tilret, M., Altmok, S. and Sancak, C.** (1998). Efficient callus culture induction and plant regeneration from mature embryo culture of winter wheat (*Triticum aestivum* L.) genotypes. *Plant Cell Reports*, 18:331-335.

- Pandey, S. and Gardner, C.O.** (1992). Recurrent selection for population, variety and hybrid improvement in tropical maize. *Advancements in Agronomy*, 48:1-87.
- Pareddy, D.R. and Petolino, J.F.** (1990). Somatic embryogenesis and plant regeneration from immature inflorescences of several elite inbreds of maize. *Plant Science*, 67:211-219.
- Pena, L., Cervera, M., Juarez, J., Navarro, A., Pina J.A. and Navarro, L.** (1997). Genetic transformation of lime: factors affecting transformation and regeneration. *Plant Cell Reports*, 16: 731-737.
- Peschke, V.M. and Philips, R.L.** (1992). Genetic implication of somaclonal variations in plants. *Advances in Genetics*, 30:30:41-75.
- Phillips, R.L., Somers, D.A and Hibberd, K.A.** (1988). Cell Tissue. In: G.F, Sprague, and Dudley, J.W. eds. Corn and corn improvement, 3<sup>rd</sup> ed., pp.345-387.
- Pius, P.K and Achar, P.N.** (2000). *Agrobacterium*-mediated transformation and plant regeneration of *Brassica oleracea* var *capitata*. *Plant Cell Reports*, 19: 888-892.
- Potrykus, I.** (1990). Gene transfer to cereals: An assessment. *Biotechnology*, 8:535-543
- Pringle, C.R.** (1999). Virus nomenclature *Archives of Virology*. 144, 1463-1466
- Prioli, L.M. and Silva, W.J.** (1989). Somatic embryogenesis and plant regeneration capacity in tropical maize inbreds. *Review Genetics*, 12:553-566.
- Raemaker, C.C.J.M., Jacobsen, E. and Visser, R.G.F.** (1995). Secondary somatic embryogenesis and application in plant breeding. *Euphytica*, 81:93-107.
- Ralph, J. and Helmut, S.** (1983). Farm management handbook of Kenya Volume II. Natural conditions and Farm Management Information part B (Central province and Rift valley) and part C (Eastern and Coast province. Published by Ministry of Agriculture in Cooperation with German Agricultural Team (GAT) of German Agency for Technical cooperation (GTZ) Pp 54-76.
- Ramesh, M. and Gupta, K.A.** (2005). Transient expression of  $\beta$ -glucuronidase gene in Indica and Japonica rice (*Oryza sativa* L.) callus cultures after different stages of co-bombardment. *African Journal of Biotechnology*, 7:596-600.
- Rashid, H., Yokoi, S., Toriyama, K. and Hinata, K.** (1996). Transgenic plant production mediated by *Agrobacterium* in indica rice. *Plant Cell Reports*, 15:727-730.
- Rathus, C. and Birch, R.G.** (1992). Electroporation for direct gene transfer into plant protoplast in: Advanced methods of plant breeding and biotechnology. (Edited by Murray, R.D.) pp 74-102.
- Register, J.C.** (1997). Approaches to evaluating transgenic status of transformed plants. *Trends in Biotechnology*, 15:141-146.
- Republic of Kenya** (2006). Agriculture sector review. Ministry of Agriculture, Nairobi.
- Rhodes, C.A., Green, C.E and Philips, R.L.** (1986). Factors affecting tissue culture initiation from maize tassel. *Plant Science*, 46: 225-232.

- Sage, F.R.** (2004) The evolution of C4 photosynthesis. *New physiologist*, 161:341-370.
- Segal, G., Song, R. and Messing, J.** (2003). A New Opaque Variant of Maize by a Single Dominant RNA-Interference-Inducing Transgene. *Genetics*, 165:387-397.
- Santos, M.A., Torne, J.M. and Blanco, J. L.** (1984). Methods of obtaining maize totipotent tissues. *In: Seedling segment culture. Plant Science Letter*, 33:309-315.
- Sawahel, A.W.** (2002). Production of herbicide-resistant transgenic maize plants using electroporation of seed derived embryos. *Plant Molecular Biology Reporter*, 20:303a-303h.
- Schiavone, F.M. and Cooke, T.J.** (1985). A geometric analysis of somatic embryo formation in carrot cell cultures. *Canadian Journal of Botany*. 63:1573-1578.
- Shen, W.H., Escudero, J., Schlappi, M., Ramos, C., Hohn, B., Koukolikova-Nicola, Z.** (1993). T-DNA transfer to maize cells: Histochemical investigation of  $\beta$ -glucuronidase activity on maize tissues. *Proceedings National Academy of Sciences*, 90:1488-1492.
- Smith, M.E. and Paliwal, R.L.** (1996). Contribution of genetic resources and biotechnology to sustainable productivity increase in maize. *In: wanatabe, K. and Pehu, E. (ed). Plant biotechnology and plant genetic resource to sustainability and productivity.* Austin, TX, USA, R.G. Landes and Academic Press.
- Smith, R.H., Duncan, R.R. and Bhaskaran, S.** (1993). *In vitro* selection and somaclonal variation for crop improvement. *International Crop Science*, 1:629-632.
- Somers, D.A and Hibbert, K.A.** (1994). *In vitro* selection. *In: Freeling M. and Walbot, V. (ed). The maize handbook.* New York, NY, USA, Springer-verdag. pp 685-689.
- Somleva, M.N., Schmidt, E.D.L. and De Vries, S.C.** (2000) Embryogenic cells in (*Dactylis glomerata* L)-Poaceae) explants identified by cell tracking and SERK expression. *Plant Cell Reports*, 19:718-708.
- Songa, J.M., Zhou Guofa and Overholt, W.A.** (2001). Relationships of stemborer damage and plant physical conditions to maize yield in a semi-arid zone of eastern Kenya. *Insect Science and its Application*, 21:243-249.
- Songstad, D.D., Armstrong, C.L. and Peterson, W.L.** (1991) Silver nitrate increases type II callus from immature embryos of maize inbred line B73 and its derivatives. *Plant Cell Reports*, 9: 699-702.
- Songstad, D.D., Peterson, W.L. and Armstrong, C.L.** (1992). Establishment of friable embryogenic callus (type II) from immature tassels of *Zea mays* L. (Poaceae). *American Journal of Botany*, 79:761-764.
- Stoeber, F.** (1961). Étude sur le glucuronidase et le glucornide permease chez *E.coli*. Theses de Docteur des Sciences, Paris
- Suprasanna, P., Rao, K.V. and Reddy, G.M.** (1981). Plantlet regeneration from glume calli of maize (*Zea mays* L.). *Theory of Applied Genetics*, 72:120-122.

- Suzuki, S., Supaibulwatana, K., Mii, M. and Nakano, M.** (2002). Production of transgenic plants of liliaceous ornamental plants *Agapanthus praecox* ssp. *Orientalis* (Leighton) via *Agrobacterium*-mediated transformation of embryogenic calli. *Plant Science*, 161: 89-97.
- Tadeusz, W., Tomczak, A. and Michelmor, R.** (2005). Optimization of *Agrobacterium*-mediated transient assays of gene expression in lettuce, tomato and Arabidopsis. *Plant Biotechnology Journal*, 3:259-273.
- Ting, Y.C., Yu, M. and Zheng, W.Z.** (1981) Improved anther culture of maize. *Plant Science Letter*, 23:139-145.
- Tingay, S., McElroy, D., Kalla, R., Fieg, S., Wang, M., Thornton, S. and Brettel, R.** (1997). *Agrobacterium*-mediated barley transformation. *Plant Journal*, 11:1369-1376.
- Tomes, D.T. and Smith O.S.** (1985). The effect of parental genotype on initiation of embryogenic callus from elite maize (*Zea mays* L.) germplasm. *Theoretical and Applied Genetics*, 70:505-509.
- Toriyama, K. and Hinata, K.** (1985). Cell suspension and protoplast culture in rice. *Plant Science*, 41:179-183.
- Toriyama, K., Y. Arimoto, Uchimiya, H. and Hinata, K.** (1988). Transgenic rice plants after direct gene transfer into protoplasts. *Bio/Technology*, 6: 1072-1074.
- Truswell, S.A and Brock, J.F** (1964) The Nutritive Value of Maize Protein for Man  
*America Journal of Clinical Nutrition*, 10:142-152
- Urushibara, S., Tozawa, Y., Kawagishi-Kobayashi, M. and Wakasa, K.** (2001). Efficient transformation of suspension –cultured rice cells mediated by *Agrobacterium tumefaciens*. *Breeding Science*, 51:33-38.
- Vain, P., Flament, P. and Soudain, P.** (1989a). Role of ethylene in embryogenic callus initiation and regeneration of (*Zea mays* L). *Journal of Plant Physiology*, 135:537-540.
- Vain, V., Yean, H., Flament, and P.** (1989b). Enhancement of production and regeneration of embryogenic type II callus in *Zea mays* L by AgNO<sub>3</sub>. *Plant Cell, Tissue and Organ culture*, 18:143-151.
- Vasil, V., Vasil, I. and Lu, C.** (1984). Somatic embryogenesis in long term callus cultures of *Zea mays* L. (Graminae) . *American Journal of Botany*, 71:153-161.
- Veena, S., Jiang, H., Doerge, R.W., and Gelvin, S. B.** ( 2003). Transfer of T-DNA and Vir proteins to plant cells by *Agrobacterium tumefaciens* induces expression of host genes involved in mediating transformation and suppresses host defense gene expression. *Plant Journal*, 35: 219-236.
- Walden, R. and Wingender, R.** (1995). Gene transfer and plant regeneration techniques. *Trends in Biotechnology*, 13:324-331.
- Walters, D.A., Vetsch, C.S., Potts, D.E. and Lindquist, R.C.** (1992). Transformation and inheritance of hygromycin phosphotransferase gene in maize plants. *Plant Molecular Biology*, 18:189-200.
- Wan, Y.C. and Lemaux, P.G.** (1995). Type II callus as bombardment target for generating fertile transgenic maize (*Zea mays* L.). *Planta*, 196:7-14.

- Wang, A.S.** (1987). Callus formation and plant regeneration from maize mature embryos. *Plant Cell Reports*, 6:360-362.
- Wang, M.B., Abbott, D.C., Upadhyaya, N.M., Jacobsen, .V. and Waterhouse, P.M.** (2001) *Agrobacterium tumefaciens*-mediated transformation of an elite Australian barley cultivar with virus resistance genes. *Australian Journal of Plant Physiology*, 28:149-156.
- Wehrmann, A., Vliet, V.A., Opsomer, C, Botterman, J. and Schulz, A.** (1996). The similarities of *bar* and *pat* gene products make them equally applicable for plant engineers. *Nature*, 14:1274-1278.
- White, F.F. and Nester, E.W.** (1980). Hairy root: plasmid encodes virulence traits in *Agrobacterium rhizogenes*. *Journal of Bacteriology*, 141:1134-1141.
- Wilkes, H.G.** (1985) Teosinte: the closest relative of maize revisited. *Maydica*, 30:209-223.
- Willman, M.R., Schroll, S.M. and Hodges, T.K.** (1989). Inheritance of somatic embryogenesis and plantlet regeneration from primary (type I) callus in maize. *In Vitro Cell Development and Biology Plant*, 25: 95-100.
- Wu, H., Sparks, C., Amoah, B. and Jones, H.D.** (1998). Factors influencing successful *Agrobacterium*-mediated genetic transformation of wheat. *Plant Cell Reports*, 21:659-668.
- Wu, S., Kriz, A.L., Wdholm, J.M.** (1994). Molecular analysis of two cDNAs clones encoding acidic class I chitinase in maize. *Plant Physiology*, 105: 1097-1105.
- Zambryski, P.C.** (1992). Chronicles from *Agrobacterium*- plant cell DNA transfer story. *Annual Review of Plant Molecular Biology and Plant Molecular Biology*, 43: 463 –490.
- Zhang, L Ohta, A., Takagi, M., and Imai, R.** (2000). Expression of Plant Group 2 and Group 3 *lea* Genes in *Saccharomyces cerevisiae* Revealed Functional Divergence among LEA Proteins. *Journal of Biochemistry*, 127: 4 611-616
- Zhang S, Williams-Carrier, R. and Lemaux, P.G.** (2002). Transformation of recalcitrant maize elite inbred lines using *in vitro* shoot meristematic cultures induced from germinated seedlings. *Plant, Cell Reports*, 21:263-270.
- Zhang, J., Klueva, N., Wang, Z., Wu, R., David-Ho, T. and Nguyen, T.H.** (2000). Genetic engineering for abiotic stress resistance in crops plants. *In vitro Development and Biology*, 36:108-114.
- Zhao, Z.Y., Gu, W., Cai T., Miller M., Wang, N., Pang, H., Rudert, M., Shroeder, S., Hondred, D.A., Seltzer, J. and Pierce, D.A.** (2000). *Agrobacterium* –mediated sorghum transformation. *Plant Molecular Biology*, 44: 789-798.
- Zhao, Z.Y., Gu, W., Cai T., Taghani, L.A., Hondred, D.A., Bond, D., Krell, S., Rudert, M. L., Bruce, W.B. and Pierce, D.A.** (1998). Molecular analysis of T<sub>0</sub> plant transformed by *Agrobacterium* and comparison of *Agrobacterium* mediated transformation with bombardment in maize. *Maize Genetic Newsletter*, 72: 34-37.

- Zhao, Z.Y., Gu, W., Cai T., Taghani, L.A., Hondred, D.A., Bond, D., Shroeder , S., Rudert, M. and Pierce , D.A.** (2001). High throughput genetic transformation mediated by *Agrobacterium tumefaciens* in maize. *Molecular Breeding*, 8: 323-333.
- Zhu, T., Mettenburg, K., Peterson, D.J., Tagliani, L. and Baszczynski, C.L.** (2000). Engineering herbicide-resistant maize using chimeric RNA/DNA oligonucleotides. *Nature Biotechnology*, 18:555-558.
- Zimmerman, L.J.** (1993). Somatic embryogenesis: a model for early development in higher plants. *Plant Cell*, 5:1411-1423.
- Zupan, J.R. and Zambryski, P.C.** (1997). The *Agrobacterium* DNA transfer complex. *Critical Review Plant Science*, 16: 279-295.

## APPENDICES

### APPENDIX I

#### 1.1 Maize genotypes under study

Ent ry	Pedigree	Code No.	Cultivar type
1	EM11-133	TL08	Inbred line
2	EM12-210	TL09	Inbred line
3	<b>EM11-133/ EM12-210</b>	<b>TH21</b>	<b>S-cross hybrid</b>
4	DC-31S2-18	TL18	Inbred line
5	DC-96-S2-8	TL19	Inbred line
6	<b>MU99221</b>	<b>TH23</b>	<b>S-cross hybrid</b>
7	340-1	TL20	Inbred line
8	CN 207	TL21	Inbred line
9	<b>EM0070</b>	<b>TH24</b>	<b>S-cross hybrid</b>
10	DC-80-S2-18	TL22	Inbred line
11	CN 244	TL23	Inbred line
12	<b>MU00-013</b>	<b>TH25</b>	<b>S-cross hybrid</b>
13	DC-80-S2-25	TL24	Inbred line
14	CN 244	TL23	Inbred line
15	<b>MU00-016</b>	<b>TH26</b>	<b>S-cross hybrid</b>
16	EM12-210/OSU-231-x-81	TL26	Inbred line
17	EM11-133/OSU-231-x-36	TL27	Inbred line
18	<b>MU00-017</b>	<b>TH27</b>	<b>S-cross hybrid</b>

Bolded types are single cross hybrids

## APPENDIX II-CULTURE MEDIUM PREPARATION

### 1.2 Culture media-N6 media composition (Chu *et al.*, 1975)

Ingredient	Amount mg/L	Stock g/L	100ml/WS	100ml/ WS
<b>Macro elements</b>		<b>X20</b>		
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	463.0	9.26	5	50
KNO <sub>3</sub>	2830.0	56.6	5	50
CaCl <sub>2</sub> .2H <sub>2</sub> O	25.33	3.32	5	50
MgSO <sub>4</sub> .7H <sub>2</sub> O	185.0	3.7	5	50
	400.0	8.0	5	50
KH <sub>2</sub> PO <sub>4</sub>		<b>x100</b>		
<b>Micro elements</b>				
H <sub>3</sub> BO <sub>3</sub>	1.6	0.16		
MnSO <sub>2</sub> .H <sub>2</sub> O	3.33	0.333	1	10
ZnSO <sub>4</sub> .7H <sub>2</sub> O	1.5	0.15		
KI	0.8	0.08		
FeNaEDTA	36.7	3.67	1	
<b>Vitamins</b>		<b>x100</b>		10
Thiamine HCl			1	
Pyridoxine HCl	1.0	0.1		1
Nicotinic acid	0.5	0.05	1	
Glycine	0.5	0.05		10
Sucrose	2.0	0.2		
	30g/L		3.0	30
Proline	2.9g/L		0.29	2.9
Casein	0.1		0.01	0.1
Gelrite	3.0g/L		0.3	3.0

**NOTE:** Store vitamins and the microelements refrigerated and FeNaEDTA in the dark WS-working solution, FS-filter sterilize

### 1.3 Preparation of stock solutions

#### 2, 4-D (2mg/L).

Add 100mg to 10ml sterile water. Add a few drops of 1 M NaOH until all 2,4-D is dissolved. Adjust volume to 1000ml and store at 4<sup>0</sup>C.

#### NAA (1mg/L)

Add 100mg to 10ml sterile water. Add a few drops of 1 M NaOH until all is dissolved. Adjust volume to 1000ml and store at 4<sup>0</sup>C.

**L-cystein (400mg/L)**

Dissolve 1.0gm of L-cysteine in 250ml of sterile distilled water. It can be heated slightly if it does not dissolve efficiently.

**AgNO<sub>3</sub> (5mg/ml)**

Dissolve 50mg in 10ml sterile distilled water. Filter sterilize through 0.22µm membrane in laminar flow hood into a sterile 10ml tube, cover with aluminum foil and store at 4<sup>0</sup>C.

**Rifampicin 50mg/ml)**

Dissolve 500mg in 10ml sterile distilled water. Filter sterilize through 0.22µm membrane in laminar flow hood. Divide into 1mL aliquots in sterile Eppendorf tube and store at -20<sup>0</sup>C.

**Kanamycin (50mg/ml)**

Dissolve 500mg in 10ml sterile distilled water. Filter sterilize through 0.22µm membrane in laminar flow hood. Divide into 1mL aliquots in sterile Eppendorf tube and store at -20<sup>0</sup>C.

**Streptomycin (100mg/ml)**

Dissolve 1.0gm in 10ml sterile distilled water. Filter sterilize through 0.22µm membrane in laminar flow hood. Divide into 1mL aliquots in sterile Eppendorf tube and store at -20<sup>0</sup>C.

**Spectinomycin 200mg/ml**

Dissolve 2.0g in 10ml sterile distilled water. Filter sterilize through 0.22µm membrane in laminar flow hood. Divide into 1mL aliquots in sterile Eppendorf tube and store at -20<sup>0</sup>C.

**Carbenicillin (250mg/ml)**

Disolve 2.5g in 10ml sterile distilled water. Filter sterilize through 0.22µm membrane in laminar flow hood. Divide into 1mL aliquots in sterile Eppendorf tube and store at -20<sup>0</sup>C.

**1.4 Growth media for *Agrobacterium* culture**

**Luria Bertani medium**

Tryptone	10g/L
Yeast Extract	5g/L
NaCl	10g/L
Agar	15g/L

**LB agar (Low salt)**

3gm in 100ml dH<sub>2</sub>O (pH 7.2)

**LB broth (High salt)**

2gm in 100ml of dH<sub>2</sub>O (pH 7.2)

**Infection media (1000ml WS)**

N6 salts and vitamins

2,4-D 2.0mg/L

Proline 0.7g L

Sucrose 68.4 g

Glucose 36.0 g

pH adjusted to 5.2

Filter sterilized (FS) and store at +4<sup>0</sup> C

100μM AS (Acetosyringone ) from 100mM stock solution

**Co cultivation media (1000mL WS)**

N6 salts

2, 4-D 2.0mg/L

Proline 0.7g L

Sucrose 30.0g

Gelrite 3.0g

pH adjusted to pH 5.8

Autoclaved

+

**FS**

AgNO<sub>3</sub> 0.85mg

AS 100μM

L-cysteine 300mg/L

N6 vitamins

**1.4 Reagents for histochemical staining with X-Gluc**

**MES fixative**

MES 10mM

Mannitol 0.3% formaldehyde

pH adjusted to pH 5.6

**Preparation of fixative**

For 500ml volume: Dissolve 0.976g of MES in 400ml of distilled water. Add 27.326g of mannitol and 3.75ml of formaldehyde. Top up volume to 500ml with dH<sub>2</sub>O and adjust pH to 5.6.

### **Fixing**

In eppendorf tubes, place 5 embryos and fix in 0.3% formaldehyde in 0.3M mannitol and 10mM MES (pH 5.6) for 60mins. Embryos washed X3 in buffer to remove fixative.

Transfer embryos to 300 $\mu$ l of X-Gluc and incubate for 24-48hrs. Remove embryos and place in 500 $\mu$ l of 70% ethanol for one hour to improve contrast.

### **X-Gluc Buffer**

500mM solution of NaH<sub>2</sub>PO<sub>4</sub> at pH 7.0 is used as X-Gluc buffer

Dissolve 2.9995g of NaH<sub>2</sub>PO<sub>4</sub> in 500ml of distilled water. Adjust pH to 7.0.

### **Preparation of X-Gluc**

5mg of X-Gluc dissolved in 10ml of 50mM NaH<sub>2</sub>PO<sub>4</sub> (pH 7.0)

Wrap in foil and keep in +4<sup>0</sup> C before use.

## APPENDICES III-ANALYSIS OF VARIANCE

### 2.1. Analysis of variance for the effect of ecological zone on days to flowering of maize genotypes

K value	Source of variance	df	SS	MS	F	P $\alpha_{0.05}$
1	Replication	2	14.137	7.069	1.8721	0.1619
2	Factor A-site	1	14712.010	14712.010	3896.5005	0.0000
4	Factor B-G	16	1127.510	70.469	18.6639	0.0100ns
6	Int AB	16	1134.490	70.906	18.7795	0.0000*
-7	Error	66	49.196	3.776		
Total		101	17237.343			

### 2.2 Analysis of variance for % callus induction in inbred lines

K value	Source of variance	df	SS	MS	F	P $\alpha_{0.05}$
1	Replication	2	682.945	341.473	2.4628	0.1329ns
2	Factor A	10	20133.782	2013.378	14.5211	0.0000***
4	Factor B	4	35865.030	8966.258	64.6675	
6	Int AB	40	21456.036	536.401	3.8687	
-7	Error	108	14974.388	138.652	198.0	
Total		164	93112.182			

### 2.3 Analysis of variance for % callus induction in single cross hybrids

K value	Source of variance	df	SS	MS	F	P $\alpha_{0.05}$
1	Replication	2	648.689	324.344	3.4628	0.1329ns
2	Factor A	5	2832.722	566.544	5.3034	0.0004***
4	Factor B	4	15016.600	3754.150	35.1423	0.0000***
6	Int AB	20	3829.000	191.451	1.7921	0.0439
-7	Error	58	6195.978	106.827		
Total		89	28522.989			

### 2.4 Analysis of variance for effect of 2, 4-D concentration on callus induction on various maize genotypes

K value	Source of variance	Df	SS	MS	F	P $\alpha_{0.05}$
1	Replication	2	1540.583	770.292	30.1672	0.0000
2	Factor A	7	66556.	9508.000	372.365	0.0000***
4	Factor B	6	0.000	0.000	0.000	
6	Int AB	42	0.000	0.000	0.000	
-7	Error	110	2808.750	25.534		
Total		167	70905.333			

### 2.5 Analysis of variance for % mean somatic embryo formation in Hybrids (TH)

K value	Source of variance	df	SS	MS	F	P $\alpha_{0.05}$
1	Replication	3	410.250	136.750	3.2668	0.0732
2	Factor A	3	1070.750	356.917	8.5262	0.0054**
3	Error	9	376.750	41.861		
Total		15	1857.750			

**2.6 Analysis of variance for % mean somatic embryo formation in inbred lines (TLs)**

K value	Source of variance	df	SS	MS	F	P $\alpha_{0.05}$
1	Replication	3	356.714	118.905	4.3476	0.0180
2	Factor A	6	147.714	24.619	0.9002	ns
3	Error	18	492.286	27.349		
Total		27	996.714			

**2.7 Analysis of variance for effect of embryo physiological stage on transient *gus* expression.**

K value	Source of variance	df	SS	MS	F	P $\alpha_{0.05}$
1	Replication	2	0.933	0.467	3.3866	.0482 ns
2	Factor A	2	17.608	8.804	63.8920	0.0000***
4	Factor B	4	16.772	4.193	30.4291	0.0000***
6	Int AB	8	11.253	1.407	10.2077	0.0000***
-7	Error	28	3.858	0.138		
Total		44	50.425			

**2.8 Analysis of variance for optimal incubation period to initiate transient *Gus* expression following agro inoculation**

K value	Source of variance	df	SS	MS	F	P $\alpha_{0.05}$
1	Replication	2	0.337	0.168	1.7463	0.1977ns
2	Factor A	2	8.920	4.460	46.2504	0.0000***
4	Factor B	3	8.285	2.762	28.6372	0.000***
6	Int AB	6	4.788	0.798	8.2755	0.0001**
-7	Error	22	2.122	0.096		
Total		35	24.451			

**2.9 Analysis of variance for effect infectivity of different agro strains on different maize genotypes on Gus expression**

K value	Source of variance	df	SS	MS	F	$P\alpha_{0,05}$
1	Replication	2	0.698	0.349	1.7645	0.1788ns
2	Factor A	5	64.554	12.911	65.2824	0.0000***
4	Factor B	5	6.366	11.273	57.0025	0.0000***
6	Int AB	25	23.970	0.959	4.8482	0.0000***
-7	Error	70	13.844	0.198		
Total		107	159.432			